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The United States Agency
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The Department of Tropical Medicine
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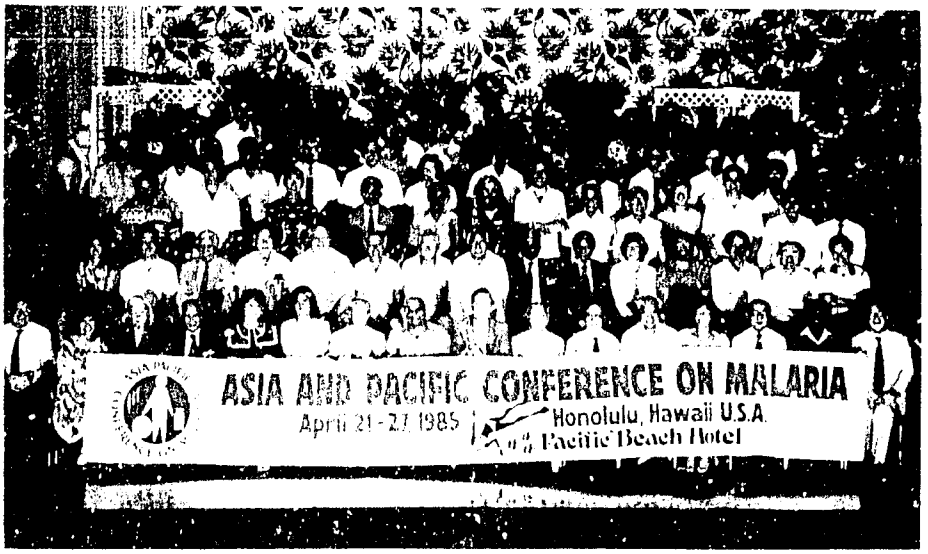
John A. Burns School of Medicine
University of Hawaii

and

The Editor

Proceedings of the Asia and Pacific Conference on Malaria



ASIA PACIFIC CONFERENCE ON MALARIA
Honolulu, Hawaii, U.S.A.
April 21-27, 1985

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Proceedings of the Asia and Pacific Conference on Malaria

Practical Considerations on Malaria Vaccines and Clinical Trials

Honolulu, Hawaii, U.S.A.
April 21–27, 1985

Edited by
WASIM A. SIDDIQUI

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Asia and Pacific Conference on Malaria
Honolulu, Hawaii, U.S.A.
April 21–27, 1985

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Preface

Under the sponsorship of the University of Hawaii and the United States Agency for International Development, the "Asia and Pacific Conference on Malaria" was convened in April 1985 in Honolulu, Hawaii. This conference was unique in that it brought together for the first time research scientists currently working on the development of malaria vaccines with scientists and health policy-makers from the nations of Asia and the Pacific Basin to discuss the current status of malaria and the current status of malaria control programs in their countries. The current development of malaria vaccines was also discussed with emphasis on an in-depth review of the basic principles, the specific outlines, and the development of strategies for future malaria vaccine trials. These topics were addressed during a week-long series of presentations, directed questionings, and general discussions which now form the basis of these proceedings.

The Asia and Pacific Conference on Malaria owes its inception to the foresight, commitment, and dedication of many people and institutions. The United States Agency for International Development supplied the vision of an international gathering of top scientific minds and world leaders and gave generously of their support and encouragement to ensure the actuality of such an event. Within this organization, I am deeply grateful to Dr. Nyle Brady and Dr. James Erickson for their tireless personal attention and devotion. The State of Hawaii has long been a supporter of inter-cultural exchanges between the East and West. I am greatly indebted to Senator Daniel K. Inouye and Senator Spark M. Matsunaga for their interest and support. The University of Hawaii, in its capacity as a leading institution for cooperation between the nations of Asia and the Pacific Basin, provided encouragement and support. I am extremely grateful to Dr. Albert Simone, President of the University of Hawaii, to Dr. Terence Rogers, Dean of the University of Hawaii John A. Burns School of Medicine, and to Dr. Jerrold Michael, Dean of

the University of Hawaii School of Public Health, for their generosity and guidance.

The key to the success of this conference was due in large part to the wise counsel of the Honorary Presidents, to the cooperation and commitment of the Organizing Committee, to the tireless efforts of the Local Organizing Committee, and to the unmatched dedication and hard work of the Conference Coordinator and her staff. I am truly indebted to all of these individuals.

The publication of the proceedings of this conference is a result of the commitment and cooperation of the participants, the rapporteurs, and the discussants of the conference. I deeply thank them and the members of the Local Organizing Committee for their efforts.

As in all endeavors, there exist individuals who devote enormous amounts of time and effort to insure the success of the goal. I am, therefore, especially thankful and personally indebted to Dr. Leslie Tam, Ms. Susan Lofton, and Ms. Carol Ahmed for their contributions toward the preparation of these proceedings.

I extend my thanks to the staff of the University of Hawaii Press for the fine quality of this publication.

Finally, I must say that presiding over this conference has given me a tremendous sense of the responsibility which we have all undertaken by our participation. It is through the atmosphere of understanding and humility which such conferences offer that we scientists and government leaders will lead the way to the future worldwide health and prosperity of mankind. To have been a part of this gathering of great and gifted people dedicated to the noble ideal of the eradication of malaria has been a true honor. I will forever cherish the exchanges of knowledge, personal interactions, and especially the creation of new and lasting friendships and collaborations which were acquired through this conference.

Wasim A. Siddiqui
Editor

OPENING SESSION

Hawaiian Blessing

LOLA AND JOSEPH HART

Aloha! On behalf of the people of Hawaii, *welcome* to our "aina"—our land! Hawaii is a special place of great beauty, mild weather, and a very unique history. We hope that you will have time to experience *"E Aloha kekahi i kekalii"*—the love of one another which allows many people of many races to live together in peace and harmony here.

We in Hawaii would like to send our best thoughts and prayers to all who have come from the East and from the West to attend this conference as you strive to *"Onipa'a"*—be steadfast, and *"Kulia i ka nu'u"*—reach your goals in the mutual quest to help mankind to live free of the disease of malaria.

May this conference provide an important step toward victory over disease worldwide. *"Imua. Imua a lanakila."*

Welcoming Remarks

WASIM A. SIDDIQUI
Professor and Chairman
Department of Tropical Medicine and Medical Microbiology
John A. Burns School of Medicine
University of Hawaii
Honolulu, Hawaii, U.S.A.

Senator Matsunaga, Dr. Brady, President Simone, distinguished guests, ladies and gentlemen:

On behalf of the Organizing Committee, it is a great honor for me to welcome you to the Asia and Pacific Conference on Malaria. This is indeed a unique conference because it has, for the first time, brought research scientists currently working on the development of malaria vaccines together with scientists and health policy-makers from the nations of Asia and the Pacific Basin to exchange ideas and recommendations for the development of a strategic plan for the evaluation and field testing of malaria vaccines when they become available for clinical trials.

It is significant that the Department of Tropical Medicine, which is deeply involved in the health problems, particularly malaria, of the countries of Asia and the Pacific Basin, has been given the honor of hosting this conference. It is also appropriate that this conference has been convened in Hawaii, which has long been a leader of cultural exchanges between the East and West.

This landmark conference would never have been realized without the generous sponsorship of the United States Agency for International Development. I am greatly indebted to Dr. Nyle Brady and Dr. James Erickson of USAID for their support. I am grateful to President Simone, Dean Rogers, and Dean Michael of the University of Hawaii for their own contributions of guidance and support. Finally, I am most appreciative and thankful for the unmatched hard work and dedication of the Local Organizing Committee.

Again, I give my warm aloha and welcome to our honored guests from the United States, Europe, and especially to those who have traveled from the countries of Asia and the Pacific Basin to be here today. This conference has been truly honored by your noble commitment to

the realization of its goals. It is with great expectation and hope that I look forward to a mutually enlightening and beneficial exchange of ideas and information culminating in the successful deliberation and conclusion of the Asia and Pacific Conference on Malaria. By working together, we can accomplish our goals and bring to the world a brighter hope for the future welfare of mankind. May God bless each of us in this endeavor.

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Welcoming Remarks

TERENCE A. ROGERS
Dean
John A. Burns School of Medicine
University of Hawaii
Honolulu, Hawaii, U.S.A.

Chairman Siddiqui, Senator Matsunaga, Dr. Brady, distinguished guests and colleagues:

Those of you who are as old (or older) than I will recall that despite our early exhilarating hope, it was with bitter disappointment that we realized that mosquitoes adapted to insecticides and this led to the failure of malaria control after World War II. Therefore, I am particularly proud, as an immigrant to this great country, of the fact that it was USAID who made the decision to finance the development of a vaccine which would be a real solution to the problem of malaria.

USAID established a network of research laboratories aimed at developing a malaria vaccine. In the last three decades, there have been very promising results which indicate the likelihood of the development of a vaccine which would handle once and for all this dreadful disease which kills more people than any other single clinical entity in the world. If next year, an epidemic of one-fifth of this virulence were to occur in the developed countries, there would be an incredible public outcry and civic unrest to force the authorities into doing something about it. The United States Congress, of course, as the collective representatives of the American people had to appropriate the funds for USAID to do these imaginative things. Therefore the United States Congress shared USAID's vision of a long-term view of malaria research. Let us also never forget that the money appropriated by Congress came from the taxes paid by ordinary Americans. The vision and imagination had to be there, but hard-working Americans paid the bill.

It is significant that Hawaii, as a crossroads between East and West, has been the focus of one of the major efforts in malaria. It gives me great pride to recognize that this focus is due to one of my own colleagues, Dr. Wasim Siddiqui, whose great contributions in malaria research have awarded us this recognition. The control of malaria opens

up what Winston Churchill once called the "sunlit uplands of the human spirit" for so many people in our world. I am most encouraged that USAID has again led the way in bringing together this group of experts to discuss what will be done when a malaria vaccine is developed, which will undoubtedly be in the near future. Thank you.

Welcoming Remarks

JERROLD M. MICHAEL
Dean
University of Hawaii
School of Public Health
Honolulu, Hawaii, U.S.A.

This is a unique occasion at which scientists, researchers, and scholars as well as policy-makers and nation builders meet together to address the issue of malaria control—a disease that should no longer be a major problem but still potentially affects 1.5 billion people.

As dean of the School of Public Health at the University of Hawaii, I am pleased to be part of these proceedings, and of course proud to be a colleague of Dr. Siddiqui, who is so well known to all of you as a scientist and a driving force in bringing this aggregation of talent together.

We in the Asia-Pacific region have a special opportunity to become a responsible part not only of finding appropriate ways to address the study, evaluation, and use of malaria vaccines but also to serve in a leadership role to advance the frontiers of public acceptance to a valid public health intervention.

We in this region have joined together in an Asia-Pacific Academic Consortium for Public Health specifically geared to support the multinational institutional efforts of such bodies as the World Health Organization and the U.S. Agency for International Development.

We have created that federation of schools of public health from Indonesia, Thailand, Nepal, Sri Lanka, the Philippines, Malaysia, Singapore, and the People's Republic of China—linked with this University, UCLA, Berkeley, and Tulane—for a reason that flows from more than the common sense of the merit of collegiality among faculties but as much for a reason that I suggest tonight deserves your attention along with the technical aspects of problems of laboratory production of vaccine and the epidemiology of clinical trials. For us, that was the problem of regional complacency in our chosen field of public health.

We can't write off the danger of staleness, complacency, growing rigidity, imprisonment by our own comfortable habits and long-held professional opinions. How many people do you know well who are already trapped in fixed attitudes and habits? A famous French writer

said, "There are people whose clocks stop at a certain point in their lives." I could without any trouble name half a dozen national figures resident in Washington, D.C., whom you would recognize and could tell you roughly the year their clocks stopped. I don't do it because I have to go back and raise resources in that town.

Just as individuals rigidify, so do organizations, universities, and societies. The web of custom tightens. An organization develops precedents, traditions, a fixed pecking order—and lots and lots of rules. The last act of a dying scientific group may be to get out a new edition of the laboratory rulebook.

Any modern organization tends to become a thicket of procedures that frustrate positive action. I've seen it at first hand in a dozen government agencies. I could tell you some stories about the University of Hawaii, but I won't.

Now I want to say a word about our attitudes as scientists, academicians, and administrators toward the future. I value heritage and the lessons of the past, but excessive preoccupation with the past is a hindrance to creative action. So is an excessive preoccupation with past mistakes, past injustices, past feuds. In the city of Washington, D.C., there are a lot of people who are proud of the fact that they never forget where the hatchet is buried. It may give them pleasure but it diminishes their capacity to deal with the future.

Anyone who studies the diaries and memoirs of the Western Movement in the United States has sensed the energetically positive view of the future that prevailed then. The people involved believed that they were moving with history, carving a better future.

Optimism is unfashionable today, particularly among scientists and intellectuals. Everyone makes fun of it. Someone said, "Pessimists got that way by financing optimists." But I'm not pessimistic about our ability to work together and I advise you not to be. As the fellow said, "I'd be a pessimist but it would never work."

To remind oneself of the sheer power of attitudes toward what's possible, one need only mention the 4-minute mile. When it was universally regarded as a physical impossibility, no runner made it. But when Roger Bannister broke the time barrier, he also destroyed the mental barrier and several others ran below 4 minutes within a matter of months. One can call to mind countless examples of such mental barriers. J. P. Morgan told Alexander Graham Bell that the latter's invention was "a very interesting novelty" but had "no commercial possibilities." Morgan's considerable powers of business judgment never had the chance to function. He was groping behind a mental barrier.

But if you are optimistic, it had better be a *toughminded* optimism. We have to believe in ourselves but we mustn't imagine the path will be easy. It's tough as hell. Life is painful and rain falls on the just, and Mr.

Churchill was not being a pessimist when he said, "I have nothing to offer but blood, sweat, and tears." He had a great deal to offer but as a good leader he was saying it wasn't going to be easy, and he was also saying something that all great leaders say constantly—that failure is simply a reason to strengthen resolve.

A negative attitude toward what is possible involves more than our usual notions of pessimism. It involves loss of confidence and fear—fear of the future, fear of risk, a fatal timidity when the moment of opportunity breaks. No one starting from that base can act decisively and lead vigorously.

In working with the U.S. Department of Education, I first observed what I have come to think of as the battered-agency syndrome. Any human organization can take just so much punishment—attacks by the media, punishment from Congress, whatever, and it begins to lose confidence. We all know about bureaucratic defensiveness in an organization and how it hampers renewal. One can easily demonstrate that as confidence wanes, bureaucratic defensiveness rises.

So we created our linkage of schools of public health to foster our creativity. Creativity within an organization is to be found among men and women who have a powerful conviction that they can affect events *in some measure*. There are innumerable factors in contemporary life that crush that belief. We as leaders need to help to keep it alive—among our staff, our associates, all the people whose lives we touch.

All of which is to say that we welcome you here in the spirit of William James who pointed out that just as our courage is so often a reflex of another's courage, so our faith is often a faith in someone else's faith. When you are in a position to lead—as you are now in this conference—let the faith begin with you!

Me ke aloha pumehana.

Welcoming Speech

ALBERT J. SIMONE
President
University of Hawaii
Honolulu, Hawaii, U.S.A.

I would like to express my warm welcome to all of you here, the researchers primarily who make things happen and who are going to make this a better world for us all; the conference organizers and the academic administrators from the University of Hawaii; and the Hawaii state leaders, such as Senator Neil Abercrombie, for their active interest. I would also like to welcome and thank our two Senators from Washington, D.C., for their support, as expressed by Senator Inouye in his letter and Senator Matsunaga by his presence here today. It is a special honor to also welcome, among other distinguished visitors from Washington, D.C., Dr. Nyle C. Brady, Senior Assistant Administrator of the United States Agency for International Development, who has given so much support and encouragement to the malaria research program. Finally, I want to thank you, Wasim, because you represent an academic researcher par excellence and will serve as the conference leader. You deserve a special warm thank you from us all in the form of applause.

I echo all of the hopes and the highest expectations that all of the previous speakers and the audience have expressed for this conference. The battle against the disease of malaria is a noble cause. We owe a debt of gratitude to all who are responsible for this conference and especially to those who will be contributing to and participating in it. From my point of view as President of the University of Hawaii, this conference is of particular importance. I have just completed a year-long process of developing a strategic plan for this University. This plan outlines the kind of university which I foresee developing in the next few years, this being a quality university which would meet state needs, possess a true international dimension, particularly in serving the Asia and Pacific areas, and be responsive and contribute to the challenge of new science and technologies. Finally, I have envisioned a university which works with its colleagues at other academic institutions both national and

international; a university which understands and acknowledges the total community of which it is a part; one which incorporates the political, educational, economic, and social communities of the nation and world. The Asia and Pacific Conference on Malaria gives life to all of these ambitions and hopes because it reflects this plan. It has given the University of Hawaii the opportunity to offer world leaders, leading scientists, and, quite possibly, future nobel laureates an international arena in which to converge and contribute their expertise and cooperation toward the eradication of one of the most dreadful diseases in the world. This conference, which has been created to address the current needs of science and to bring together these needs with the new scientific technologies, has quite suddenly brought into actuality my strategic plan and the hopes of this University. For this beginning, let me again thank Dr. Siddiqui, and all of you. It is with honor that I again welcome you to the Asia and Pacific Conference on Malaria and leave you with the best wishes from the Univesity of Hawaii for the successful achievement of your goals.

Welcoming Message

THE HONORABLE DANIEL K. INOUYE
United States Senator (Hawaii)

I am sincerely sorry that I cannot be with you and your colleagues today at the opening of the Asia and Pacific Conference on Malaria. However, I do wish to extend you my sincerest greetings and congratulations in convening, for the first time in the history of malaria research, a forum of scientists from various leading malaria laboratories throughout the world, as well as scientists, epidemiologists, and policy-makers from Asia and the Pacific Basin region to exchange views and expertise regarding how to implement the findings of rapidly advancing research breakthroughs in actual field conditions.

Without question, the control and eradication of malaria will have a most profound and beneficial impact upon the health and economy of developing nations of the world and generations to come will be well-served by your efforts here today. Over 300 million individuals worldwide are affected by malaria each year, and I understand that this is the direct cause of death of nearly one million of our fellow human beings, most of these being children under the age of five.

The University of Hawaii, your sister educational institutions, and, in particular, the Agency for International Development, should indeed be commended for your commitment to implementing a bold and decisive coordinated approach to develop an effective malaria vaccine.

On behalf of the world's future generations, I wish you the best of success.

Opening Address

THE HONORABLE SPARK M. MATSUNAGA
United States Senator (Hawaii)

On behalf of the people of Hawaii, whom I have the honor of representing in the United States Senate, I welcome you all and extend warm greetings to those who have come from abroad. The highest reward for attending this international conference here in Aloha Land will come to some of you on Judgement Day, for on that day, because of your visit here, you will at least have the satisfaction of knowing what heaven looks like, if you are directed to go the other way.

As for me, it is always a rejuvenating respite to return to my native land and a privilege to address a group of distinguished scientists such as you, who are gathered here for the Asia and Pacific Conference on Malaria. Dr. Siddiqui and his colleagues are to be congratulated for organizing this conference, which holds promise of contributing immeasurably toward world progress in overcoming mankind's ancient and lingering scourge: malaria.

Your attendance is, in part, a tribute to the notable work being performed at the University of Hawaii's Department of Tropical Medicine, under Dr. Siddiqui's leadership, toward the development of protective antibodies against malaria, a key step in the search for a vaccine against the dread disease. Many of you also have critical and valuable information to share as well, and some day in the not-too-distant future the whole human race will be grateful for your participation in this historic conference.

We are also most honored to have with us this evening representatives of an organization which has supported the fight against malaria for many years throughout the world. I refer, of course, to the Honorable M. Peter McPherson, Administrator of the United States Agency for International Development (USAID), with whom I have had the pleasure of working in Washington in many worthy causes. USAID, as it is more widely known, under Mr. M. Peter McPherson's and Dr. Nyle Brady's leadership, and Dr. James Erickson's research directorship, has

rendered invaluable assistance and great impetus to malaria vaccine research.

It is most heartening to me, as a politician (in the good sense of the term, of course) that your conference represents a good cross-section of medical researchers, public policy-makers, and administrators responsible for carrying out programs of public health. The interchange among all of you can be focused on how best to implement the findings of the laboratories, where the search for a malaria vaccine is being conducted.

Hawaii's people are proud of the role their university's school of medicine and school of public health have assumed in the treatment and conquest of a disease which afflicts islands and rim nations of the Pacific. It follows that we are proud to be a part of this global search for the eradication of malaria.

Certainly we recognize this plague to be a critical health problem, not only to the developing nations of the Pacific Basin and Asia but also to Africa and Central and South America. It is little known that nearly one-third of the world's population—some one and a half to two billion people—live under the constant threat of malaria infection. In Africa alone, approximately one million children under five years of age die of this disease every year. Morbidity and mortality due to malaria infection continue to be a major impediment toward the economic development of countries in these areas.

I am told that between 200 to 400 million people contract malaria each year, making it the most common, severe parasitic disease of all, and a disease which claims two to four million lives annually.

Although great strides have been made toward the control of the disease within the last three decades, because of the mutative development of drug-resistant strains of malaria parasites, as well as insecticide-resistant strains of mosquitos, there is currently a resurgence of malaria infection in our developing countries.

Malaria has been a problem for such a long period of time, it is surprising to a layman, such as myself, that it remains such a virulent threat. In World War II we expected DDT to bring the disease under control, not foreseeing that the carrier mosquitos would develop a resistance to the new pesticide. Serious scientific research has been underway for nearly a century and it has been approximately 90 years since medical researchers learned that the name of the disease was a misnomer. Its cause is not the decay or filth associated with "bad air," or "mala aria" in Italian, but a single-celled creature, a protozoan, carried by mosquitos, the Anopheles mosquito to be precise. This discovery seemed to present a simple solution: Eliminate the mosquito and the disease is gone.

Indeed, the World Health Organization (WHO) in 1954 and 1957

was able to launch a $6 billion attack employing DDT against the mosquitos and drugs, chloroquine, to kill the malaria parasite, *Plasmodium falciparum*. By the mid-1960s malaria was virtually eliminated from 80% of this project's target areas. Unfortunately, the mosquitos developed resistance to DDT and the protozoan became resistant to the drugs, and, as a consequence we have seen a resurgence of the disease in many tropical areas. In Sri Lanka, for example, where the WHO's effort had reduced its incidence from one million cases annually to only 17 in 1963, this little country had 600,000 reported cases in 1975.

In the early 1960s, when it was evident that the WHO program was running into trouble, USAID became the first agency to take a bold and decisive policy step by initiating a significant research program aimed at the development of malaria vaccines. The agency established a "malaria research network" to achieve this goal by assisting leading laboratories of the United States in this work. This network is a collaborative group of 17 universities and other research institutions under contract to get a vaccine ready for human testing. USAID places contracts for specific research objectives and coordinates the entire effort. With an eye toward approval by the United States Federal Drug Administration (FDA) of such a vaccine, USAID requires its contract researchers to follow FDA guidelines. One can appreciate, then, how important continuing USAID coordination is to the speedy adoption of the vaccine, once it is test-proven.

Based on some important breakthroughs in research on the development of malaria vaccines at laboratories within the USAID network here in Hawaii, at the Rockefeller University in New York, and at New York University, we hope to see the expeditious development of malaria vaccines in the very near future. In fact, I am told we can expect clinical human testings within the next two years.

Such a vaccine would, of course, be a first; there has never before been a vaccine against a parasitic disease. The development of this vaccine is far more complex than creating a vaccine against, say, smallpox or other viruses or bacteria because the malaria parasite is so much larger and has such a complex life cycle which appears to have been designed to elude its host's immune system. As you know, there are three distinct stages in this life cycle, and researchers are addressing a vaccine against each of them.

A major breakthrough is in the manufacture of a sporozoite vaccine, given the problem of obtaining the sporozoites. I am informed that the hurdle on this point is in dissecting the salivary glands of anesthetized mosquitos. This challenge has been circumvented by powerful techniques of molecular biology and genetic engineering that enable researchers to clone genes of the malaria parasite, identify how the parasite interacts with its host, and discover how to foil those interactions.

Your conference this week breaks new ground. It is the first time in the long history of malaria research that scientists from various leading malaria laboratories of the world are meeting with scientists, epidemiologists, and policy-makers from Asia and the Pacific Basin to compare notes and exchange views. You will no doubt discuss methods by which to carry out, under actual field conditions, the findings of research. You are all to be commended for this undertaking and the coordinated approach it represents.

Needless to say, much work lies ahead of you, not the least of which is the design of a vaccine delivery system. It may be extremely difficult to vaccinate in areas of Africa and India, for example, if the vaccines must be refrigerated and then injected with a sterile hypodermic needle. It is interesting to note that some of you believe it possible to deliver a malaria vaccine in a vaccinia virus--the way smallpox vaccine is delivered by scratching over skin.

Speaking as a member of the U.S. Senate, I can say that Congress has been most sympathetic to USAID's aim at achieving the goal of an effective malaria vaccine. The control and eradication of this disease represent a noble mission for the agency, and I for one pledge my support to see that funds are made available to finish this great work. In this connection, you will be happy to learn that Mr. McPherson's agency doubled funding for malaria vaccine research from the $11.9 million level of Fiscal Year 1983 to $22.7 million in Fiscal Year 1985. In addition, the National Institute of Allergy and Infectious Diseases (NIAID) is spending $5.4 million in malaria vaccine studies, which represents 20% of the $27 million it has earmarked for tropical disease research.

Let me say in concluding my remarks that Hawaii intends to play an important role as a liaison between the U.S. Agency for International Development and the nations of Asia and the Pacific Basin toward the achievement of this malaria vaccine goal for all humanity.

The control and eradication of malaria will have a profound and positive effect on the health and economy of the developing nations of Asia and the Pacific. Indeed, such a feat will contribute to the larger goal of peace and prosperity for these nations. A malaria vaccine will give the eradication effort new life and hope. Although it is possible that the parasite could become resistant to a vaccine as it became resistant to drugs, a vaccine approach—combined with better sanitation, drugs, and insecticides—offers the world a chance to quell, and perhaps rid itself of, this ancient and terrible scourge.

I wish you great success in your deliberations throughout the coming week. Thank you for this opportunity to address you.

Mahalo.

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Inaugural Address

NYLE C. BRADY
Senior Assistant Administrator
for Science and Technology
United States Agency for International Development
Washington, D.C.

Through the years, the Agency for International Development (AID) has had a significant positive effect on the world's health—not alone, but by collaborating with research institutions such as the University of Hawaii and supporting the efforts of scientists and educators, such as are represented at this conference.

During the last decade, in particular, we have learned much about what does and doesn't work in Third World health programs. We have used research to come to grips with some basic health problems, i.e., diagnosing, preventing, and curing the most common diseases. We've made progress against the various barriers to providing adequate primary health care. We've designed mechanisms to stretch our assistance dollars.

And we've combined health efforts with mass-media techniques to train people who have little or no formal education to use new technologies. It has, indeed, been rewarding to observe mass communication experiments that help mothers learn techniques that would be beyond their reach if they had to use written directions.

These efforts, conducted with the assistance of health scientists in the United States and other countries, have borne fruit. They have helped reduce infant and adult mortality. They have encouraged the use of vaccines, oral rehydration therapy, and essential drugs. They have increased the numbers of births attended by trained midwives and have provided better maternal and child health care and nutrition for millions of mothers, infants, and children in developing countries. And they have helped provide new systems of health evaluation and supervision.

This year, new initiatives in the primary health care area have been made possible by a significant increase in resources appropriated by the U.S. Congress. The total is some $85 million more than we received in 1984. Fifty million dollars is for increased primary health care, $25 million for child survival programs, and $10 million for nutrition programs. This was in addition to the $146 million that was appropriated last year.

Biomedical Research

Turning now to research, in addition to focussing on health-services delivery, we have recognized that basic and applied research is essential to solving the more tenacious health problems facing peoples of the developing world. Primary health care systems must have access to a broad array of new preventive, diagnostic, and curative technologies if they are to be effective in improving the health of developing world peoples. In the past 5 years, AID's biomedical research investment has just about doubled to develop and improve diagnostic tools, drugs, and vaccines. Allow me to first tell you about a few of our other research efforts before turning to malaria.

Oral Rehydration Therapy

In most countries, the single greatest cause of infant and early childhood death is acute dehydration from diarrhea. In cooperation with other organizations such as WHO, NIH, CDC, etc., AID has helped broaden scientific understanding of diarrheal diseases and has encouraged the development of effective means of rehydration.

For more than 25 years, AID has provided support for what was originally the Cholera Laboratory and is now the International Center for Diarrhoeal Disease Research in Bangladesh. From this laboratory and other research cooperators came a remarkable and low-priced treatment for dehydration that is associated with diarrhea. Small packets of premixed salt and sugar, costing ten to twenty-five cents, are mixed with clean water to make a rehydration solution for youngsters. This solution has markedly reduced infant mortality from diarrhea-caused dehydration.

We are now evaluating techniques that will eliminate the need for these packets: a rice, salt, and water mixture, cooked in a particular way, which can then be fed to the dehydrated child. This oral rehydration therapy is something that mothers can easily do. It is an easy-to-use technology that is having a major impact on reducing child mortality caused by dehydration.

In Health Region I in Honduras, for example, there has been a 50% decline in infant deaths from dehydration associated with diarrhea. Since the inception of ORT programs in Honduras and Gambia, over 60% of infants and young children who become dehydrated from diarrhea are treated with oral rehydration salts. In Egypt, we expect to reduce mortality by 25% in children under three years of age by the end of the project (5 years). In these countries, illiterate mothers are frequently the technology users; they have no trouble understanding how it is applied.

In 1983, we held the International Conference on Oral Rehydration

Therapy—ICORT. At that time, we challenged ourselves and the rest of the world to really make use of this low-cost means of keeping children alive. This year we have already held two regional conferences—one in Africa and one in Asia—focussing on oral rehydration therapy. The second international conference, ICORT II, will take place in Washington, D.C., in December.

Vaccine Development

We are supporting work on a number of vaccines, including one against cholera. We all know that the currently-used cholera vaccines aren't very good—only 50% protective and of short duration (3–6 months). To help eliminate this gap, in 1985 we are focussing support on the International Center for Diarrhoeal Disease Research in Bangladesh (ICDDR/B) where field tests are being carried out on two new oral cholera vaccines which look promising. The vaccines will be tested in both Asia and Africa.

We are also supporting work on a measles vaccine that will be effective in protecting infants under 9 months of age and will be easy to administer. In addition, researchers hope to incorporate heat stability into the new measles vaccine.

Many people have said that we have ample immunization technology to protect everyone in the world. And, in a general sense, that is true. However, the dispensation of the present technology in underdeveloped areas is very costly. Let me give you an example. Many of the vaccines we have today require refrigeration, which is not available in many developing country settings. This is why we can vaccinate only about 20–25% of developing world children. In fact, we cannot be certain how many of the innoculations we do administer are well-enough preserved to be effective. If these vaccines were heat-stable and did not require refrigeration, we could successfully innoculate many more children, particularly in rural areas and in regions, such as Africa, where the infrastructure to maintain the cold chain is very limited.

As you know, I'm an agriculturalist. It is very clear to me that the Green Revolution did not take place until we went beyond merely trying to apply existing agricultural technologies to environments very different from those in which the methods had worked. It was only when, through research, we gained new knowledge and came up with new tools that a massive change occured in developing country agriculture.

I think the same generation of new knowledge must occur in the field of health to create large-scale improvements. I don't mean to imply that we have to wait. As we did in the field of agriculture, we should continue to move ahead as rapidly as we can while new knowledge is being sought. But, I am convinced that research is necessary to provide new tools that really will be effective.

Through cooperation with the World Health Organization (WHO) and its Tropical Disease Research (TDR) program, AID is also working to eliminate or control other tropical diseases such as schistosomiasis, onchocerciasis, and leprosy. TDR's research program benefits from the expertise of scientists in the U.S. and abroad.

In a new project, beginning this year, we are developing and adapting modern diagnostic techniques for use in tropical settings. In developing countries, particularly in the rural areas where adequate laboratory diagnosis may not be available, patients are often subjected to inappropriate treatments based on clinical guesswork. The goal of the Diagnostic Technology Development project is to create simple and inexpensive accurate tests that can take the guesswork out of diagnosis in such settings.

Research on Malaria

While we are working on diagnostic techniques, etc., we in AID are well aware that malaria is still a major health problem in developing nations and that malaria research can give us the tools to save many lives. We know that about 59% of the world's population is exposed to malaria infection and that an estimated 200 million people are infected annually in the Pacific Basin, Asia, Africa, and Central and South America. It is estimated that in Africa alone at least 1 million children die of malaria each year. The loss of productivity and permanent damage to those who survive this disease is devastating and it continues to be a significant barrier to economic development in countries where it is prevalent.

During the last three decades, significant efforts have been made toward the control of malaria and AID has played an important role in these events. I understand that since 1954 AID has contributed more than $1 billion toward malaria-control programs worldwide. Of that $1 billion dollars, only about 0.5% has been spent for research. Now, we are becoming aware that, in response to the products of earlier malaria-related research, drug-resistant strains of malaria parasites and insecticide-resistant strains of mosquitoes have developed causing a resurgence of malaria infection in developing countries.

Recognizing the crucial need to speed up development of the vaccine, several years ago AID established a malaria vaccine research program. Some very forward-looking and optimistic individuals within the Agency have nurtured and encouraged this effort.

From 1966 to 1984, AID provided a total of more than $35 million to support malaria vaccine research being done by a network of some 17 institutions. During the last 3 years, the annual funding for this network has been greatly increased ($10.5 million in FY 1985).

In 1984, as you know, a major breakthrough was achieved toward

developing a vaccine against falciparum malaria. This work was based on research done at New York University, with comparable work done at the National Institutes of Health. For the first time, there is at least a candidate vaccine on the horizon. Network scientists and researchers from Australia, Europe, and here in Hawaii have also achieved a breakthrough in the search for a vaccine against the blood stage of the falciparum malaria. Through biotechnology, we have discovered a new method to determine which mosquitos carry the disease and we are exploring new techniques which will permit the rapid diagnosis of malaria and improve the ability to identify resistance of malaria parasites to specific drugs.

Scientists, epidemiologists, and policy-makers from Asia, the Pacific Basin, and around the world are here to initiate a unique and, hopefully, historically significant undertaking—the testing of recent research products in clinical and field conditions. We are hopeful that the recent rapid research progress toward the development of malaria vaccines will continue and that the outcome of this meeting will provide focus and impetus for subsequent field trials and the production of a vitally needed malaria vaccine.

This year, AID support for malaria vaccine research will begin to focus on field and clinical trials of a prototype anti-sporozoite vaccine and on the development of other elements of the polyvalent vaccine which ultimately is needed to protect Less Developed Countries (LDC) populations against various types of malaria.

This is, indeed, a timely meeting to mark a momentous occasion. Being here in Hawaii is quite appropriate. We recognize the importance of the research work that is being done on malaria. However, the University of Hawaii and other institutions in this state are also carrying out other important research efforts—efforts relating to the education and training of people in health areas. I wish you well in your assignment.

Opening Remarks

James M. Erickson
United States Agency for International Development
Washington, D.C.

Despite the enormous worldwide effort from 1955 to 1975 to eradicate Malaria, this disease continues to be a major public health problem and a cause of significant morbidity and mortality in many tropical and subtropical countries of the world.

The malaria eradication effort, which involved over 100 countries and over a billion dollars of U.S. funds, led to a significant decline in the total number of deaths attributable to this dreaded disease. However, over these past 15 years, the development of insecticide resistance in the mosquito vector and drug resistance in the malaria parasite has brought about a major worldwide resurgence of malaria. The latest data from the World Health Organization indicate that almost 2.6 billion people live in areas where malaria is endemic. The number of clinical cases of malaria approaches 300 million per year resulting in 2–3 million deaths, primarily in African children under the age of five.

In 1964 the Agency initiated a research program to test the feasibility of developing a vaccine against the four species of human malaria. Although the scientific community scoffed at the idea, by 1975 AID funded researchers were able to prove the technical feasibility of immunizing a human and protecting him from malaria challenge. With this knowledge, our malaria vaccine program was expanded and accelerated to bring the latest biomedical technologies to bear on the development of prototype malaria vaccines.

Several major biomedical breakthroughs have emerged from the AID funded collaborative research network, which now consists of 14 major projects, including:

1. the development of a tissue culture system to grow the malaria parasite outside a living host;
2. the first use of monoclonal antibody methodologies which are used to identify potential vaccines;

3. the first use of genetic engineering and synthetic chemistry to develop prototype malaria vaccines.

In August of last year, AID administrator Peter McPherson announced that AID-funded researchers at New York University had completed work on a malaria vaccine against the mosquito form of the most lethal species of malaria (*Plasmodium falciparum*).

As a result of this breakthrough, AID has significantly expanded the malaria vaccine program in three areas:

1. *Basic research*—building on the New York University work, the AID program has focused on the isolation of candidate vaccines from other parts of the life cycle of *P. falciparum* as well as on the other three species of human malaria. AID will soon be announcing that work has been completed on a second vaccine, against *P. vivax* (the malaria species that causes a cyclic reoccurrence in man).
2. *Human volunteer studies*—AID has recently contracted with the University of Maryland to conduct the FDA required human trials of the *P. falciparum* prototype vaccine. These trials may begin in the next 3–4 months, after FDA completes its review of the animal and toxicology studies.
3. *Field trials in malaria endemic areas*—the ultimate test of the vaccine(s) will be in areas of the world where people are at risk from malaria on a constant basis. The Agency has begun to prepare for eventual large-scale field trials of malaria vaccines, which could start within two years. This conference, which brought over 100 top scientists from countries of Asia and the Pacific Basin, will assist USAID in developing all the technical details for the actual large-scale trials. Based on the model of this conference, we plan to hold two more conferences: one for the Latin American–Caribbean region and the other for Africa.

Our long-term effort to develop a malaria vaccine could not have borne fruit at a more important time. Drug resistance by the parasite is now firmly established in 10 countries of East Asia and Oceania, 10 countries in South America, and 15 countries in Africa. The integration of a polyvalent vaccine(s) into ongoing antimalaria efforts (insecticide sprays, drug distribution, etc.) where they still work will provide us with a tremendous potential to significantly reduce the impact of this disease on the health and economic well-being of half of humanity.

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SESSION I

CURRENT STATUS OF MALARIA AND MALARIA CONTROL PROGRAMS IN COUNTRIES OF ASIA AND THE PACIFIC BASIN

Introduction

WASIM A. SIDDIQUI

Aloha and welcome to Hawaii. It is indeed a great honor for the Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, University of Hawaii, the local committee members, and myself to host the first Asia and Pacific Conference on Malaria.

Attempts to develop a vaccine against malaria were initiated half a century ago, but gave way to searches for new drugs during World War II and to the anti-mosquito programs that followed. During the late 1930s, recognition of the insecticidal effect of DDT turned the eyes of the world toward the possibility of eradicating the disease. From 1956 to 1962, a large-scale malaria eradication program was initiated in many of the countries of Asia, North Africa, Europe, and Central and South America, resulting in a significant reduction in the prevalence of malaria. The overwhelming success and impact of the control programs was so impressive that by 1961, a rapid decline in scientific research and in the training of malaria workers had resulted. The initial success and momentum, however, have since been replaced by a gradual, unrelenting battle with the disease that has now undergone a sudden resurgence in many areas of the world. Continuation of the malaria eradication program has been hampered by operational, administrative, and financial problems in many malarious countries. In addition, such underlying technical difficulties as the resistance of *Plasmodium falciparum* to 4-aminoquinolines and recently to Fansidar and mefloquine as well as certain anopheline species to insecticides have aided the resurgence of malaria.

In view of the precarious situation of malaria control, it seems obvious that neither insecticides nor antimalarial drugs are sufficient to control the vectors and parasites, and hence the disease. Consequently, there has been renewed interest in the search for an alternate or supplemental means aimed toward a solution of the malaria problem. Re-

search activities for developing an immunological solution have accelerated in recent years. Formerly, much of the pessimism concerning the use of such a vaccine was founded on the belief that since natural infection did not induce complete immunity, vaccination was unlikely to succeed. Fortunately, however, recent advances made in the light of new knowledge of the immune response and successful immunization of various animal model systems against malaria have brought about considerable revision of this viewpoint. The discovery of *Aotus* monkeys for direct experimentation with human malaria parasites and the landmark discovery of continuous *in vitro* cultivation of *P. falciparum* have given great impetus to the development of malaria vaccines. By a stroke of good fortune, this has happened at a time when two momentous biological advances, namely, the production of monoclonal antibodies and the advances of gene manipulation, have opened up heretofore undreamed of vistas for the production of vaccines. The capability of cloning and sequencing nucleic acids now means that it is possible to synthesize, either chemically or biologically, almost any relevant peptide or protein as immunogens and hopefully as effective immunogens. Within a short period of 3–4 years, the impact of molecular biology toward the development of malaria vaccines has been unprecedented. It is offering the means to convert the dream of immunoprophylaxis to a practical reality.

Objectives

With this background, let me state briefly the significance and objective of this conference. The unique feature of this conference is that it marks the first time that under one roof research scientists currently working on the development of malaria vaccines have been brought together with scientists and policy-makers from the nations of Asia and the Pacific Basin for the purposes of developing a strategic plan that will be used in evaluating and field testing malaria vaccines when they become available for human testing. In order to achieve this objective, it is necessary to evaluate the current status of the development of malaria vaccines and to identify those laboratory and field studies which are required to be completed before malaria vaccines are available for testing in human subjects.

The format of the scientific sessions will be that of a series of formal workshops followed by informal mini-workshops. On Monday there will be presentations to review the current status of malaria and malaria control programs in Asia and Pacific Countries. On Tuesday, we will review the current status of malaria vaccines with special emphasis on the identification and characterization of candidate antigen followed by a session reviewing evidence for protection. Practical considerations of malaria vaccines will be reviewed on Wednesday. Presentations and dis-

cussion will focus on three specific aspects, namely (a) Production and Plasmodia Antigens by New Biotechnology, (b) Applicability of Information on Modern Vaccines for the Production of Malaria Vaccines, and, very importantly—(c) use of Adjuvants in Malaria Vaccines. I am particularly very grateful to the leading virologists and immunologists who so graciously accepted our invitation to share their experiences here. We look forward to their participation. Workshop sessions on Thursday and Friday will be devoted to the discussion of basic principles, specific outlines, and development of strategies for future malaria vaccine trials. Based on the background information accumulated during the first three days, the deliberation of the last two days constitute the main focus of this conference.

Regarding the format of the workshops, the Organizing Committee requested the participants, as presentors or discussants, to review scientific developments in specific areas of their expertise. In most cases, these reviews will be concise and include the highlights of the subject area. I sincerely hope that these will not only include the work from the reviewer's own laboratory. In short, we are requesting the participants to give short critical summaries in specific areas. Ample time has been allotted for a general discussion at the end of each session. It is here that all participants will have the opportunity to critically evaluate the evidence. Mini-workshop sessions will provide another forum where further discussion can be carried out.

At the end of this introductory statement, let me again emphasize the uniqueness of this conference through the participation of our scientific colleagues from Asia and Pacific Countries. The Organizing Committee is extremely grateful that they accepted our invitation and that they are here. We look forward to their full participation throughout the conference.

We hope that in the next five days, we will through our work and through formal paper presentations and in-depth discussions meet the objective of this conference.

In the end, let me extend our best wishes and warmest aloha and hope that all our honored guests from abroad and mainland USA will enjoy themselves and the beauty of these islands.

Introductory Remarks

WILLIAM TRAGER

I must begin by thanking Dr. Wasim Siddiqui and the members of the Organizing Committee for having invited me to serve as an Honorary President of this first Conference on Malaria in Asia and the Pacific. It is an honor and a privilege. I look forward to an exciting and informative, and also an enjoyable, week. I am particularly glad to be serving as Moderator at this first workshop which will deal with the current status of malaria and malaria control in the different countries of Asia and the Pacific. We are going to hear at first hand from the experts on the local situation in China, Papua New Guinea, the Philippines, Malaysia, Indonesia, Thailand, India, Bangladesh, and Sri Lanka. This will set the stage. It will show the extent and magnitude of malaria in Asia and the Pacific. Of special importance, it will indicate the problems and opportunities that the various regions will present in relation to future trials of malaria vaccines.

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Current Status of Malaria in China

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Malaria is one of the major parasitic diseases in China. During the period of 1930s to 1940s, it was widely distributed nearly all over the country and provoked disaster in the community. According to the incomplete statistics in the 1950s, the annual malaria cases were estimated to be 35 million and morbidity 6.46% before 1949. Since the founding of the People's Republic, the Ministry of Public Health has paid great attention to the pursuance of the policy of "prevention first" in health and sanitary work, and antimalaria campaigns were launched since 1950. As a result of active implementation of malaria control measures for more than 30 years, the endemic areas have been diminished, the malaria hazard dramatically lessened, and the morbidity rate markedly decreased.

In recent years, the number of malaria cases in the whole country (Taiwan Province not included, the same below) is continuously decreasing to a low level, but the position is unstable as local outbreaks have often been recorded (Table 1). So at present malaria is still one of the most important parasitic diseases in China. In view of this, the Ministry of Public Health shows deep concern over the strategies to eradicate the disease, which has been listed in the national program of disease control.

Data collected in 1983 revealed that there were 1,377,647 malaria cases in China, the morbidity being 0.14%, and 40 cases succumbed to malaria. Of the total malaria cases reported, 78% (1,077,520 cases, mainly vivax malaria) occurred in Huanghuai plain and Jianghan plain, which are considered to be the major malaria endemic areas in China now. In the southern part, the former high endemic areas, including several provinces, an autonomous region, and a municipality, prevalence of malaria has been declining steadily to less than 5 per ten thousand. But there are still some high endemic areas, especially on the southwest border, and those are inaccessible mountainous areas of poor

TABLE 1. Incidence of Malaria in China (1980–1983)

Year	*No. of cases*	*Incidence %*	*Cases died*	*Mortality per hundred thousand*
1980	3,300,349	0.33783	64	0.0065
1981	3,059,635	0.30713	70	0.0070
1982	2,041,359	0.20338	61	0.0060
1983	1,377,647	0.13560	40	0.0039

socioeconomic conditions. The annual prevalence in the northern and northwestern parts has decreased to less than 1 per ten thousand (Fig. 1). Of 1,873 counties in 19 previous endemic provinces, autonomous regions, and municipalities of malaria, 367 counties with a population of 200,942 thousand were still noted to have malaria morbidity more than 10 per ten thousand (Table 2). These counties are regarded as the focal point in the malaria control programme, and have gained special concern from the People's Government.

Falciparum malaria was distributed in 8 provinces and an autonomous region in 1983 as shown by active and passive detections (Fig. 2). In areas south of 25° north latitude, including western and southern parts of Yunnan Province and southwestern part of Hainan Island of Guangdong Province, falciparum malaria accounted for 15% of the total cases, and in certain counties it may reach as high as 30–50%. While in some counties of Fujian and Guizhou Provinces and Guangxi Autonomous Region, falciparum malaria cases amounted to only less than 2.5% of the total cases. As for provinces near 33° north latitude, such as Jiangsu, Anhui, and Henan, falciparum malaria was only endemic in several counties where cases were merely found to be 2% of the total. The anopheline vectors of falciparum malaria in China are *An. minimus* in the southern part, *An. dirus* in Hainan Island, and *An. lesteri anthropophagus* in the middle latitude zone where it is deemed an important vector of *Plasmodium falciparum*.[1]

Generally speaking, morbidity may decrease, prevalence can be pre-

TABLE 2. Incidence of Malaria in Former Endemic Regions (1983)

Incidence	*No. of counties*	*Population (thousand)*
0	389	114,868
<5‰	1,024	514,536
5‰ —	93	47,226
10‰ —	315	167,756
>100‰	52	33,186

vented, and achievement consolidated in places where there are perfect health centers at the grass-root level with qualified staff and the integrated antimalarial measures have been appropriately implemented. In 1983, by the co-effort of the masses and professional organizations for antimalarial activities, out of 29 provinces, autonomous regions, and municipalities in the China mainland, 22 with 62.3% of the total population showed morbidity of malaria lower than 5 per ten thousand (Fig. 3). Among them 13 with 23.5% of the total population have reported that malaria cases were continously decreased to less than 1 per ten thousand for 4 consecutive years since 1980.

In the meantime there are three situations which hinder the progress of antimalaria activities and the consolidation of acquired achievements. The first one is in Huanghuai plain and Jianghan plain (as mentioned above), where vivax malaria is the predominant species and *An. sinensis* the only vector. As the vector has exophilic habit, indoor residual spraying measure is ineffective. Besides, the majority of inhabitants have out-door sleeping habits in summer-autumn, so man-mosquito contact is very frequent (Table 3). The second one is that in the forested hilly regions of Hainan Island where *An. dirus*, the chief vector, is also exophilic, moreover, its manner of disperse distribution will cause trouble in vector control. The residents there are used to hunting at night, and thus have abundant opportunities to be exposed to mosquito bite. And the third one is the appearance of chloroquine resistant falciparum malaria in some areas in China (Fig. 4). It has been demonstrated that the degree of resistance in the southern part is higher than that in the northern one. And it is also found that the resistant *P. falciparum* has a tendency to spread northward.[2]

Under the guidance of the malaria control policy, "active control, prevention of prevalence, and finally attain the goal of eradication," now the general task of the antimalaria programme is "to prevent outbreak of malaria, to further decrease malaria morbidity, to enlarge the under-controlled area steadily, and to give priority to the implementation of control measures in those areas where malaria prevalence is higher than 1% and where falciparum malaria is still endemic." The

TABLE 3. Incidence of Malaria in the 5 Provinces in Huanghuai and Jianghan Plains (1980–1983)

Year	*No. of cases*	*As a proportion of the total cases in whole country %*
1980	2,798,274	84.79
1981	2,637,436	86.20
1982	1,709,606	83.75
1983	1,077,520	78.21

target of malaria control programme by the year 2000 is to reduce malaria morbidity to less than 1 per ten thousand in the whole county. In other words, the programme will be synchronized with the achievement of reconstruction to reach a comparatively well-off life level of the whole country. And it will also be in parallel with the World Health Organization: "Health for all by the year 2000."

As for malaria vaccine, which serves as a means of prevention for malaria, it might acquire a worthwhile position and play a suitable role in malaria control and the protection of people's health as well. For the time being, effective malaria vaccines, if available, would prevent the constructors, troops, travellers, and children under 6 years from malaria hazard in our country.

On this account, the production of a large amount and supply of sufficient quantity of malaria vaccines are expected. Furthermore, that it is easy to preserve, transport, and use with no marked side-effect and has a reasonable price should be taken into consideration.

It is desired that the idea of developing malaria vaccines could be realized in the near future.

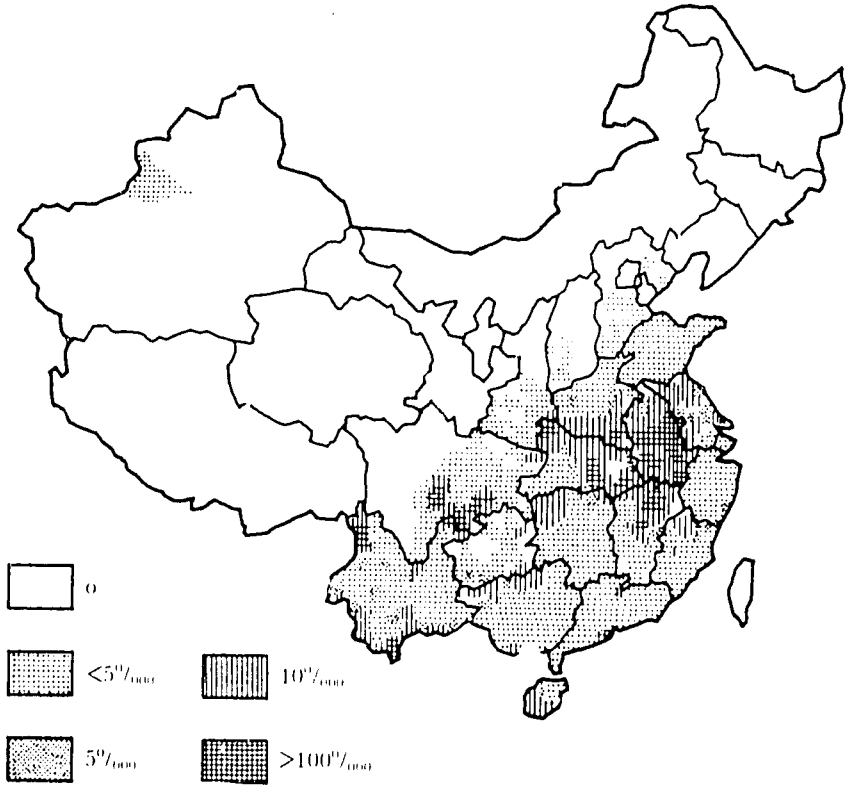

FIGURE 1. Annual Incidence of Malaria in China in 1983.

FIGURE 2. Distribution of Falciparum Malaria in China in 1983.

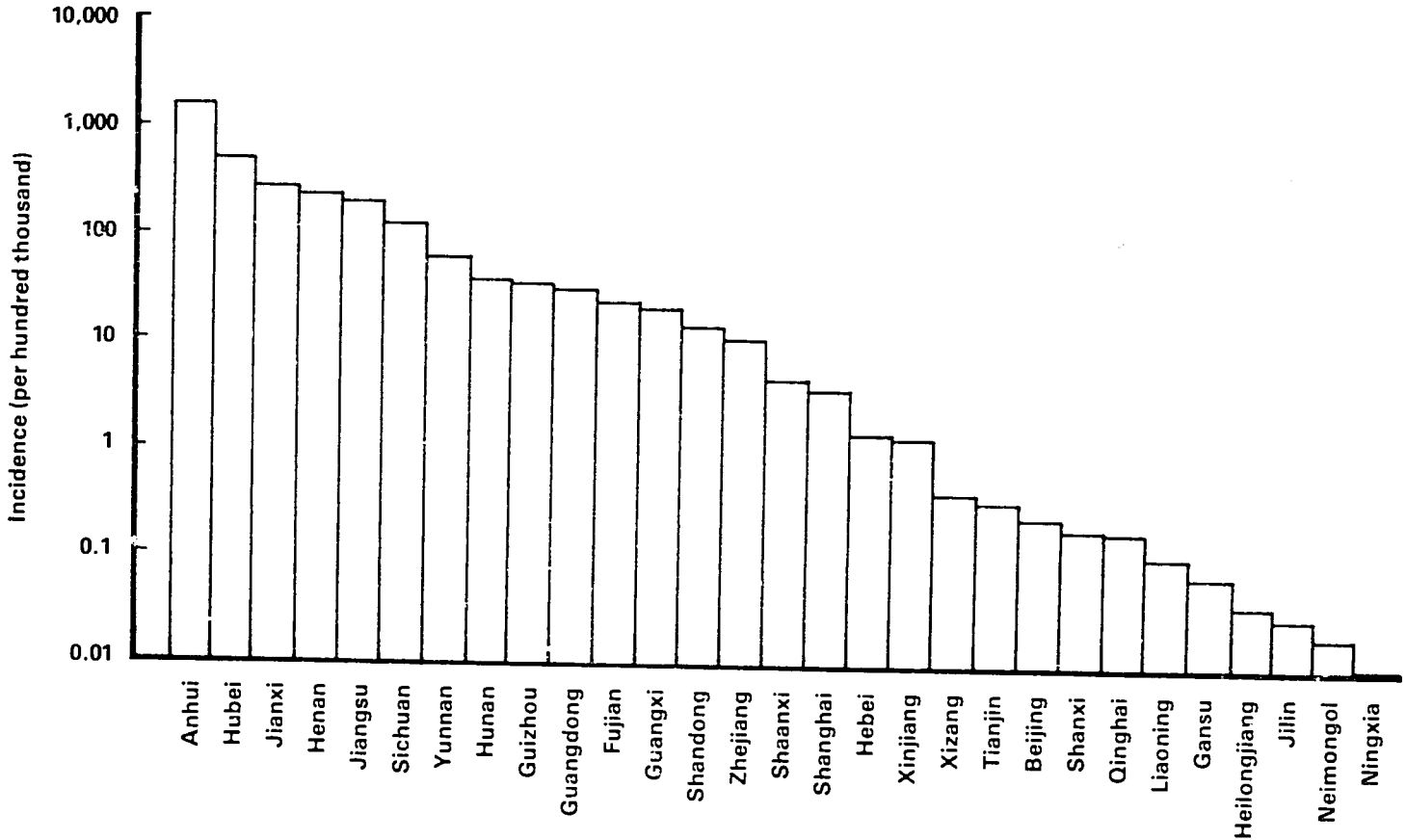

FIGURE 3. Incidence of Malaria in 29 Provinces, Autonomous Regions and Municipalities in 1983.

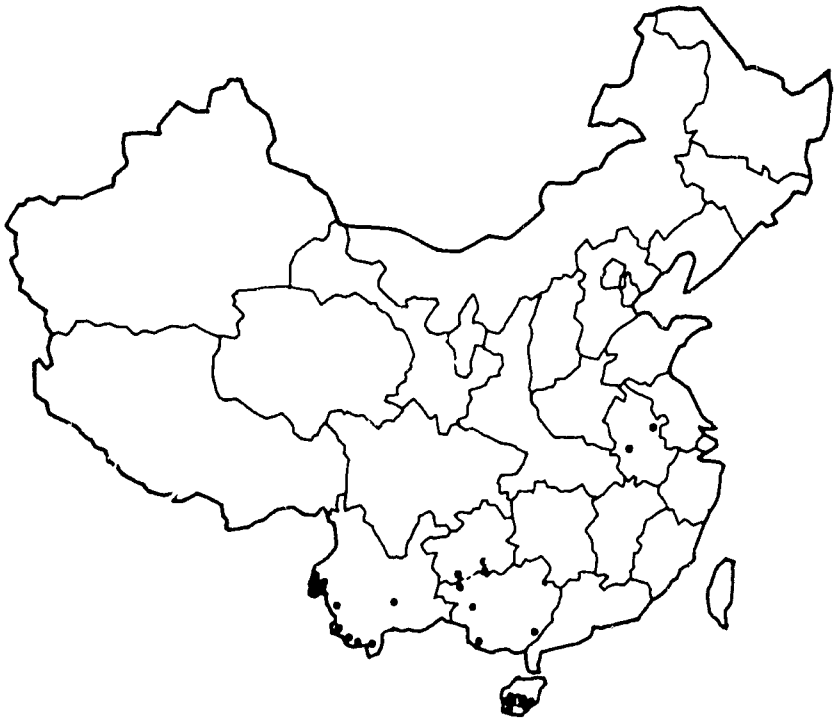

FIGURE 4. Distribution of Chloroquine Resistant *P. falciparum* in China.

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Malaria in the Southwest Pacific: A Prototype Defined Population for Vaccine Trials

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Introduction

In 1900 Robert Koch studied the effects of quinine on malaria near what is now Madang, Papua New Guinea. The results of his work were interpreted by some as proof that it is possible using quinine alone ". . . to reduce the disease to a minimum or even make it disappear altogether."[1] Half a century later, following research carried out prior to and during World War II, it was decided that the malaria problem in Papua and New Guinea could be solved by the use of residual insecticides. In 1957 Wallace Peters, malariologist to the Territory of Papua and New Guinea, commenced a pilot project using DDT residual spray at Maprik, East Sepik.[2] On the basis of the success of this project an eradication campaign was proposed, and between 1959 and 1963 interterritorial meetings of representatives from West Irian, Papua and New Guinea, Australia, and the Solomon Islands were held with the objective of coordinating antimalarial activities. A large infrastructure was created and resources commited toward the eradication of malaria. Scaled down in objective to malaria control, the program has continued over the past 25 years—with more success in some areas than in others. Reductions in resource commitment lack of adequate field supervision, poor compliance on the part of covered populations, and inattention to many of the broader aspects of malaria epidemiology are some of the problems that have confronted the malaria control program. Consequentially, it is difficult to interpret official figures, evaluate existing or proposed control measures, or make informed policy decisions on alternative courses of action. Rumors of treatment failure and drug resistance cannot always be followed up by carefully conducted trials; and reported increases in incidence or severity of acute malaria cases are often neither well-documented nor can they be evaluated against a defined baseline. In 1985 the prospect of an antimalaria vaccine again raises hopes and expectations for a solution to the malaria problem. Optimism regarding its role and effectiveness should be tempered by

reflection on the reasons for lack of success in the past. Failures have rarely been exclusively attributable to limitations of the technology itself. The objectives of this paper are to: 1) briefly summarize the current status of malaria in selected areas of the Southwest Pacific; 2) describe the experience of defining and monitoring a study population in Papua New Guinea in which a vaccine could be tested; and 3) outline practical considerations in terms of expectations, selection of target populations, and problems of large-scale administration of a vaccine in the Southwest Pacific once one becomes available.

Status of Malaria in the Southwest Pacific

Several areas in the Pacific Basin including New Zealand, Micronesia, Polynesia, and some of the island groups of Melanesia have never been malarious due to the absence of anopheline vectors. While endemic malaria has been eliminated from Australia, it remains a major public health problem in Papua New Guinea, the Solomon Islands, and Vanuatu. In the 1950s and 1960s there was general optimism that given sufficient resources malaria eradication could be achieved through residual insecticide spraying. In Papua New Guinea the spraying program was initiated following a pilot project in 1957 and was gradually expanded to cover nearly half of the country's population by 1971.[3] There was initial reduction in malaria in all provinces where DDT was applied; however, transmission was never interrupted and parasite rates rose again to original levels during the 1970s. In 1972, following the recommendations of a WHO Assessment Team, the program was reorganized with the major objectives being changed from eradication to control; but DDT remained the only control measure and no changes in methodology were considered.[4] In 1984, following further deterioration of the program, another WHO team recommended that residual spraying be discontinued in all provinces as a routine malaria control measure.[5]

Malaria endemicity varies throughout Papua New Guinea and the country can be divided into 4 regions: the highlands; the New Guinea mainland; the New Guinea Islands; and the Papua region. Figure 1 shows the pattern of geographic variation in malaria endemicity within the country. The principal vectors throughout the country are members of the *Anopheles punctulatus* complex: *An. farauti; An. punctulatus;* and *An. koliensis*. Transmission is perennial with some variation from the dry to the wet season occurring in most areas. *Plasmodium falciparum* is the predominant species. The change from a predominance of *Plasmodium vivax* to *P. falciparum* has been noted since 1972.[5] However, there is some evidence that *P. vivax* was dominant in the 1950s, prior to initiation of spraying.[2,6] Thus the change in species dominance may not be entirely related to cessation of spraying. Chloroquine resistant strains of *P. falci-*

parum, first confirmed in 1976, are now widespread.[7,8] In the area of Madang on the north coast of Papua New Guinea, 84 of the 103 infections tested *in vitro* were resistant to chloroquine, but only 48 showed resistance *in vivo*.[9] Of those resistant *in vivo*, 43 (89.6%) were at the RI level. Resistance to Fansidar (pyrimethamine 25 mg plus sulfadoxine 500 mg) has also been reported.[10]

In the Solomon Islands, optimism regarding eradication persisted longer than in Papua New Guinea. Levels of endemicity prior to initiation of spraying are also shown in Figure 1. The existing Malaria Eradication Program was more aggressive and included mass drug administration and other supplementary measures in addition to residual insecticide spraying.[11] In 1973–1976 progress was reported to be hindered mainly by persistent foci in areas of high endemicity such as north Guadalcanal.[12,13] Since 1977 the resurgence of malaria has become one of the biggest health problems in the country.[14] Epidemic in proportion, it is characterized by a predominance of *P. falciparum*, as in Papua New Guinea. Chloroquine resistant strains, first reported in 1980 are now widespread. Approximately 50% of *P. falciparum* infections are currently resistant, but an estimated 95% of these are at the RI level (personal communication from D. Parkinson, malariologist, WHO, Solomon Islands). Although pyrimethamine resistance has been shown to exist, no resistance to Fansidar has been reported.[15]

The malaria situation in Vanuatu, although previously characterized by somewhat lower levels of endemicity (Fig. 2), would appear to be facing problems similar to those encountered in Papua New Guinea and in the Solomon Islands.[16]

It is somewhat difficult to interpret data reported from official sources on the status of malaria and its control based on passive case detection, index village monitoring, and mass blood surveys. Blood slides are not always prepared from fever cases, quality control in slide preparation and microscopy is sometimes neglected, and in many areas populations have become less willing to participate in routine malariometric surveys. These data would not be adequate for use as a baseline for evaluating vaccine effectiveness at least in Papua New Guinea. Thus even for narrowly defined populations, baseline epidemiological data will need to be collected to establish prevalence, transmission dynamics, and variations in parasite populations well in advance of the advent of a vaccine if well-controlled trials are to be conducted. A major objective of this paper is to describe a multidisciplinary approach to the development of a defined population in which vaccine efficacy could be tested.

Defined Populations for Vaccine Field Trials

Three alternative populations in which a vaccine could be tested in Papua New Guinea include: 1) migrant labor or armed forces moving

from non-malarious to malarious areas; 2) community schools in endemic areas; and 3) village populations in endemic or epidemic areas. The choice of population would be contingent on preliminary assessment of the efficacy and safety of a candidate vaccine. Non-immunes from the highlands migrate to lowland areas to work on plantations, for logging companies, and for resource exploration and mining enterprises. Although there is some turnover in the work force, employment records are normally well kept and employees often live within compounds in housing provided by the company. Informed consent, enrollment, and followup would be facilitated in this type of population; but care would have to be exercised that participation was not perceived as a contingency to continuing employment. Non-immunes also migrate to towns on the coast seeking employment: since such migrant groups tend to live together in small settlements and form an identifiable community, followup of individuals is feasible. Children attending community schools in endemic areas constitute another possible population in which to evaluate efficacy of a vaccine. Again enrollment and followup would be relatively easy, but monitoring morbidity on non-school days and during vacations would pose problems. Also, the safety of the vaccine would have to be unquestionable and the ethics of informed consent resolved before considering a trial in this age group. Village populations in endemic or epidemic areas would be a third type of population in which to field test a vaccine. The Papua New Guinea Institute of Medical Research (PNG IMR) has over the past 3 years defined a study population near Madang on the north coast of Papua New Guinea in which a vaccine might be tested.

In a study population of approximately 16,500 we have integrated laboratory-based studies and an extensive field program to obtain data on the prevalence of malaria, transmission dynamics, antibody profiles of the population, antigenic variation in parasite populations, drug sensitivity of *P. falciparum*, and use of antimalarial drugs. The population has been characterized demographically, and migrations in and out are routinely monitored. Attempts to assess morbidity and document mortality were carried out over a 2-year period, and preliminary studies of genetic variation within the population have been conducted.

The study area has been described elsewhere.[9] There are 72 villages in the area which vary in size from 50 to 700 inhabitants. The larger of these villages are often divided into geographically discrete hamlets. Demographic data on the population were compiled through a house to house census conducted by the PNG IMR in 1981. Information recorded on census forms and stored on each individual includes: a unique ID number; house number; sex; actual date or estimated year of birth; names of parents; marital status: date of in- or out-migration; and date of visits by census workers. The study area was divided into 8 census units each containing approximately 2,000 persons. A resident

recorder reports births, deaths, and migrations on a monthly basis, and these data are entered on computer files. A full census is conducted annually to validate the results of the reporting system.

Cross-sectional malariometric surveys were conducted at 6-monthly intervals in both the wet and the dry seasons. Systematic random samples of 20-house clusters were designed to include 25% of the population at each survey. An index house was selected at random and the clusters were generated from complete house lists maintained by the demography unit. Sampling was with replacement. Compliance rates were highest in the first 2 surveys (>80%) and dropped to approximately 70% in the following 2 surveys. Data collection included preparation of thick and thin blood films and spleen palpation on the full sample. Whole blood was collected in microtainers from a subsample for serology and hemoglobin estimation. Parasite isolate typing was carried out in later surveys. Urine samples were collected from approximately 40% of the sample in the fourth survey for determination of presence of 4-aminoquinoline antimalarial drugs. Both *in vitro* and *in vivo* chloroquine sensitivity tests were carried out in the population during the period of the cross-sectional surveys. Laboratory methods used in processing the samples are described elsewhere.[9]

Entomological data were gathered concurrently in selected villages maintained as part of a longitudinal study within the area. A morbidity questionnaire was used to ascertain the incidence of fever and other symptoms over a 2-week recall period each 3 months in this same subset of villages. A senior member of each household was the principal respondent and interviews were conducted with those reported as having been ill by medically or paramedically qualified staff members. All reported deaths, whether occurring in hospital or in the villages, were followed up by a physician and a cause of death was assigned to each case using a protocol designed and pre-tested for this purpose.

Results of the malariometrical and morbidity surveys and the mortality followups are reported elsewhere.[9,17,18] Data on antimalarial drugs, genetic variation within the study population, and preliminary data on antigenic variation in parasite populations in the area suggest that these are factors that must be investigated, possibly in pilot studies, prior to the selection of populations in which to test a vaccine. Population mobility should also be assessed prior to initiation of trials. We found that 12.7% of urine samples tested using Dill-Glazko reagent showed strong positive reactions for 4-aminoquinolines. If a lighter color precipitate was accepted as positive the percentage was much higher. Problems of sensitivity and specificity are known to be associated with this test but queries regarding use and sources of supply of antimalarials tended to substantiate the widespread use of chloroquine and amodiaquine.[19] The need for a micro assay for serum levels of chloroquine

that could be performed in the field is clear. An extraordinarily high frequency of α globin gene deletion (>90%) was observed in this population, and variations in frequency of ovalocytosis (20.4 to 31.4%) appeared to exist between the north and south of the study area.[20-22] G-6-PD deficiency and β thalassemia were also shown to be present but at frequencies <10% (unpublished data, PNG IMR). Many of these genetic polymorphisms are also known to occur in Southeast Asia, and populations in this area should be evaluated for presence of these traits prior to their inclusion in vaccine trials. In- and out-migrations of 5.6% and 5.3%, respectively, were mainly internal to the study population, but short-term mobility (<3 months absence from the village) was relatively high. This aspect of a population must also be considered in studies in which followup will be critical.

Antigenic diversity in primary isolates of *P. falciparum* collected in Madang was demonstrated using monoclonal antibodies.[23] One antigen previously shown to be absent in one-third of isolates from different countries was present in all 108 isolates from this sample. The preliminary studies using monoclonal antibodies suggest significant genetic diversity of parasites that may be geographic-specific in nature. Characterization of populations of *P. falciparum* with monoclonal antibodies could provide information highly relevant to vaccine evaluation. Attempts to monitor malaria morbidity on a large scale at the village level would appear to be difficult from our experience. Severe episodes of malaria such as cerebral malaria are relatively infrequent in the area; therefore reduction in these episodes could not be used as an outcome measure. Even with a medically qualified observer stationed in each village, episodes of fever in dispersed hamlets would be likely to be missed, and staffing requirements would be unrealistic in areas of scattered rural populations. Mortality that can be directly attributed to malaria in this area is low;[18] therefore, demonstrating reductions in mortality would be difficult in this population.

Our experience has confirmed the importance of having good laboratory facilities available at a field site along with a complement of scientists to integrate studies of the immunology, parasitology, and epidemiology, including entomology, of malaria. Micro assays which could measure protective immunity and serum chloroquine levels in the field would also greatly enhance the ability to evaluate a vaccine.

Practical Considerations of a Malaria Vaccine

In addition to questions of the feasibility and proximity of a safe, effective vaccine, several issues will have significant impact on the success or failure of vaccination as a control measure for malaria in the Southwest Pacific as well as in other areas of the world. These issues can be some-

what arbitrarily divided into three categories: 1) expectations for a vaccine; 2) factors affecting the feasibility of administration of a vaccine to populations at risk; and 3) issues related to the longer-term implications of dependence on vaccination as a control or eradication strategy. The first of these categories encompasses differing expectations among politicians, health professionals, and affected populations. The second includes potential political, economic, and sociocultural constraints that can affect the planning and implementation of vaccination campaigns. The third covers epidemiological issues that must be considered in assessing the degree to which vaccination would be an acceptable control measure. The desirability of an integrated approach to control, combining a variety of methods, is stressed.

EXPECTATIONS FOR A MALARIA VACCINE

The Papua New Guinea Institute of Medical Research has received inquiries from members of the press and government officials asking when the malaria vaccine will be available to the people of Papua New Guinea—the implication being that a vaccine now exists and that it is a question only of timely distribution. Lack of information and unrealistic expectations on the part of decision making bodies may lead to unwarranted decreases in commitment of resources to other forms of malaria control in anticipation of an imminent solution to the malaria problem. Receiving the impression from the media that a vaccine is close to a reality, they may also begin to question commitments to malaria research in general and to on-going evaluation of other forms of control, thereby reducing the possibility of an integrated approach to malaria control for the future. In areas where village populations have become routine consumers of health care, some already ask when a "sut" (Melanesian Pidgin for injection or vaccine) will be available to protect them against malaria. The easiest but unfortunately not the most responsible answer would be that a vaccine may soon be available. In the end an answer of this nature is likely to contribute to a reduction in the credibility of the health care system in general and an increasing reluctance among villagers to support and/or participate in other forms of control. We believe that health professionals and researchers have an obligation to provide accurate information and to make an effort to temper excessive enthusiasm and perhaps misplaced confidence in a vaccine as the definitive solution to malaria. Realistic expectations should center around its use in selected target populations and as a complement not a replacement for other control measures.

POLITICAL AND ECONOMIC FACTORS

There are many examples of disease control programs that have failed owing to insufficient commitment of resources. Some of the problems

associated with conventional malaria control or eradication programs have included: lack of funds to attract and retain adequate staff; administrative complexities hindering recruitment and employment of personnel; budgetary delays affecting purchase of supplies; and bureaucratic procedures affecting the timely delivery of supplies and equipment.[24] Other difficulties have included inadequate staff training, problems of morale, and competing priorities when modest success is reached—to mention only a few. Health delivery systems are subject to many of the same problems and there is reason to expect malaria vaccination campaigns will suffer from these and other difficulties. It is unlikely, given the nature of vaccines currently under development, that a malaria vaccine would be a sufficient control measure in isolation. Vaccination will have to be combined with other existing measures, thereby increasing the already considerable burden on resources required for malaria control. The efficacy, feasibility of administration, and cost of a malaria vaccine may weigh against its acceptibility for widespread application in developing countries like Papua New Guinea with chiefly rural populations. Target populations may be apathetic or even offer resistance to receiving the vaccine. The vaccines currently under development are likely to require re-vaccination to ensure continued protection, and even with long-term vaccines such as typhoid, tetanus, polio, and yellow fever the administration, cost, and acceptance factors have greatly limited success. These are examples of the internal problems facing prospective vaccine administration.

The magnitude of resource commitment, approach (i.e., integrated with existing health delivery or administered independently), complementary control measures, selection of an appropriate target population, and effectiveness of long-term planning could increase the potential for success of vaccination as a control measure. However, it is probably not reasonable to expect the same degree of success as that achieved in eradication of smallpox. Attempts to control malaria by vaccination might be more appropriately compared with measles. In West Africa, programs in the late 1960s and early 1970s succeeded in significantly reducing measles incidence and mortality, but these declines were not sustained over time. The reasons suggested for diminishing success included the necessity for mass campaigns every six months, difficulties in storing and transporting the vaccine, and the high costs of delivery in rural areas. Once foreign assistance was withdrawn, the West African governments became unwilling or were unable to assume the costs of the vaccination campaigns, and measles incidence returned to its former levels.[25] This does not imply that a malaria vaccine could not play an important role in malaria control strategies. Given the limitations of the prospective vaccines, however, it would be unrealistic to think that they will provide a definitive solution.

SOCIOCULTURAL ISSUES

Many factors affect the response of people with different cultural identities to immunization programs. These include but are not limited to: beliefs, knowledge, perceptions about preventive medicine, and attitudes toward health workers.[26] Whether or not people in high risk areas will accept vaccination must be considered in the context of what motivates individual and group innovation. Foster and Anderson suggest that people will accept innovation if: 1) they perceive social, psychological, health, or other advantages as a result; 2) they can afford the economic costs; and 3) the social costs do not outweigh the advantages.[27] One obstacle to acceptance of vaccination against malaria may be the manner in which malaria is perceived in highly endemic areas. Adults develop a level of acquired immunity and symptomatology is usually infrequent. People are infected throughout their lives, making comparison with a malaria-free state of health virtually impossible.

Inhabitants of Papua New Guinea represent many cultures and each small community has a unique political/socioeconomic structure. The nature of the cultures and the characteristics of the communities will have major impact on the level of participation obtained in vaccination campaigns. Malaria vaccination campaigns will be faced with the same sociocultural problems that the WHO eradication campaign experienced—including ignorance and/or disregard for important biological and ecological factors. In order to ensure optimum participation rates, these aspects must be carefully considered and addressed in both planning and implementation of programs.

EPIDEMIOLOGICAL CONSIDERATIONS IN THE ROLE OF A VACCINE

The place of a vaccine in malaria control or eradication depends on both the local epidemiology of the disease and operational facilities. The epidemiology of malaria in Papua New Guinea as elsewhere varies between different areas of the country, and often within a single area over time

First we consider vaccination as a control strategy in the case of unstable malaria. Since protection of non-immunes by a high level of immunity in the population is not operative with malaria vaccination, and the expected duration of vaccines currently being developed may be relatively short, the entire population of epidemic areas would have to be vaccinated and revaccinated at regular intervals to provide adequate protection. Priorities would have to be established to determine where to focus vaccination efforts—these could be geographical, age-specific, occupation-specific, etc. Effectiveness, as with other control strategies, would depend on the regularity and thoroughness of the efforts to vaccinate and revaccinate the target populations as well as on the effective-

ness of the vaccine. Second, in areas where malaria is endemic and stable, the target groups could be more easily selected since morbidity and mortality are clearly more severe in early childhood, among pregnant women, and in migrants from non-malarious areas. Again, thoroughness and persistence would be key factors. Risk of infection would have to be evaluated as vaccine protection is allowed to fade.

Decisions regarding the extent to which vaccination might be effectively practiced will depend upon the local epidemiology of malaria, the efficacy of the vaccine, and available resources. Good epidemiological baseline data will be necessary to determine the extent of the problem, as well as to evaluate vaccine effectiveness. It must be kept in mind that nutritional status, socioeconomic conditions, and general access to health services have major impact on health status and therefore on incidence, prevalence, and intensity of malaria. Acquired immunity offers protection from severe clinical disease, but it also may tax the entire social structure through its effects on the general level of health of the population. It is possible that sterilizing immunity in the short term could lead to increased susceptibility in the long-term, with increased morbidity and mortality. On the other hand, it is possible that a vaccine developed against a novel immunogen (such as a key receptor site) which established an immunity against which the parasite had no inbuilt counter-strategies could result in a powerful long-term protection, which would be significantly better than the fragile immunity which slowly develops in people growing up in an endemic area. Though we cannot presently expect to develop a vaccine of such power —and we should not create such expectations in the people living in endemic areas—this possibility should not be lost sight of.

Effectiveness of available methods, resources, sensitivity to attitudes and preferences, and some degree of local responsibility are important issues in the success of a control program. Integration of existing and potential methods—including chemotherapy, insecticides, biological control of vectors, environmental modification, human behavioral modification, use of bed nets and other means of reducing vector-person contact, health education, and vaccination—must all be considered in the design of a balanced approach for Papua New Guinea and the rest of the Southwest Pacific.

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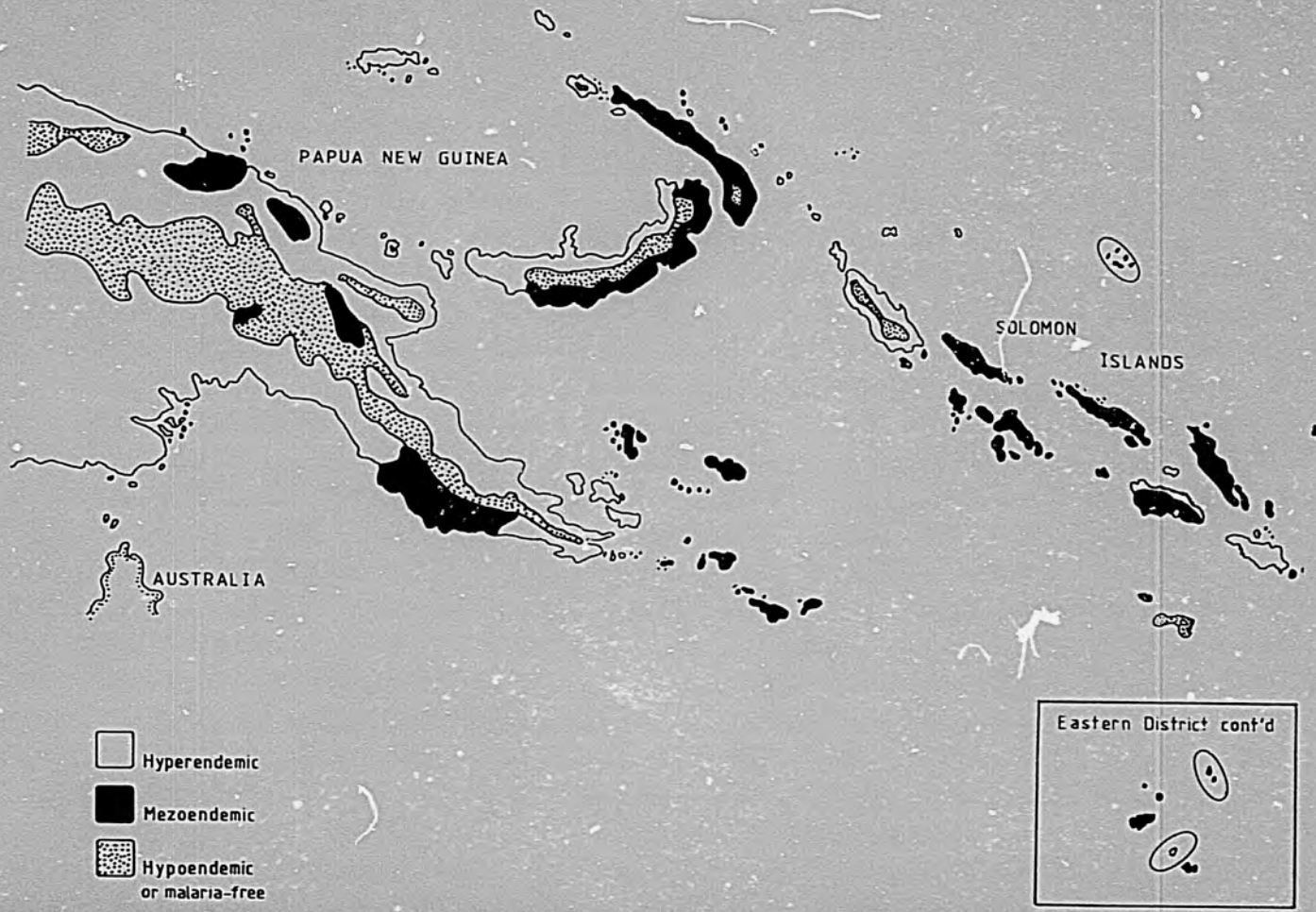

FIGURE 1 Endemicity of Malaria in Papua New Guinea and the Solomon Islands Prior to Initiation of Control Programs.[3,11]

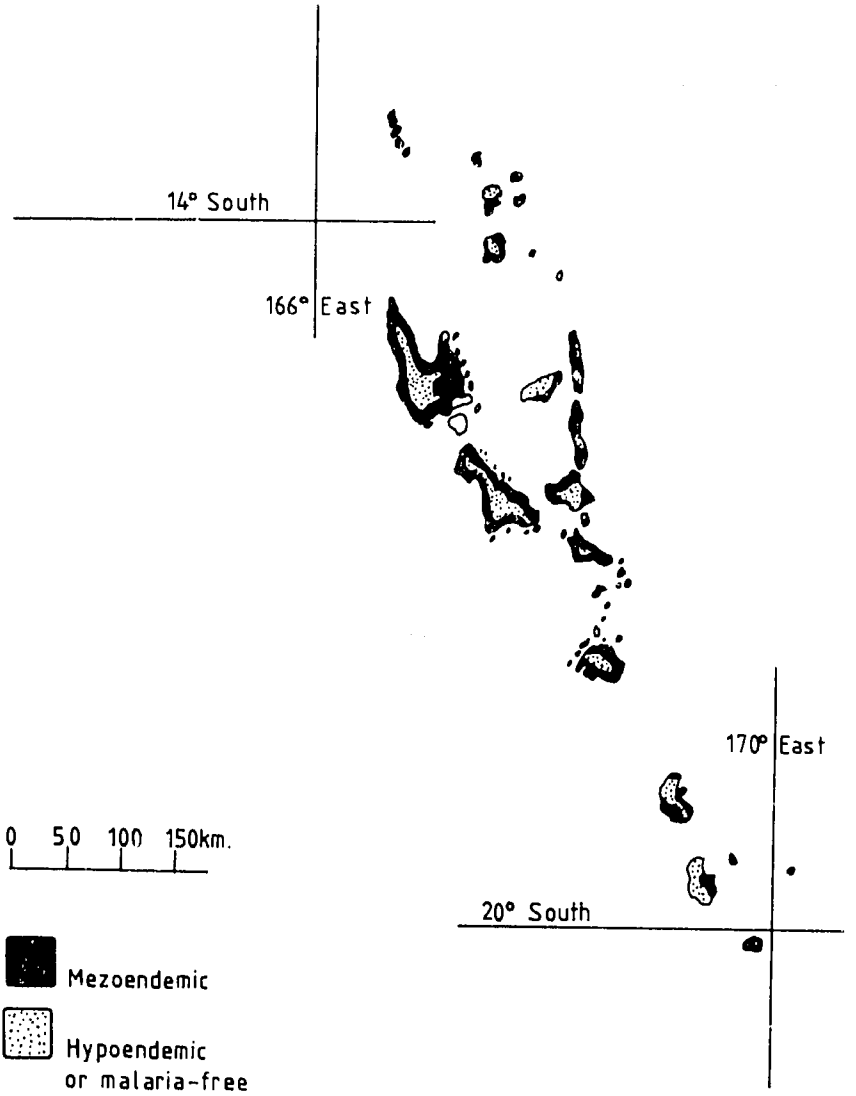

FIGURE 2. Endemicity of Malaria in Vanuatu Prior to Malaria Control Programs.[16]

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Discussion

MEUWISSEN: Dr. Cattani, I have three questions. The first is related to the drug resistance patterns in Papua New Guinea. Is it true that drug pressure could not have been the only factor in the distribution of drug resistance which starts at the upper Fly and goes to Sepik and Madang? Do you know anything about some other factors than drug pressure which may have been responsible for aminoquinoline resistance?

The second question is from, I think, your own observation a number of years ago that you had annual variations in spleen patterns. Has this been confirmed? What might be some of the explanations?

My third question is related to anemia and hemoglobulin and the observation by some that iron treatment actually may increase accessibility of children with anemia thereby causing a paradoxical effect?

CATTANI: May I answer the last question first? We have just begun a trial in schoolchildren using iron comparing iron supplementation and

a placebo to evaluate the question whether or not children are more susceptible with adequate iron stores to parasitemia. We will be looking at several parameters, one being parasitemia followups of morbidity episodes in children. This is to start in fact this very week, with the permission of the education department.

Yes, now that we have more data, the annual variation in spleen rates does appear to hold. But this is true for only one of our areas of endemicity. In fact, this is the lowest level where there does appear to be significant seasonal variation and the variation in spleen rates exists only among adults, not among children. In this area, however, the spleen rates among children is between 50 and 70%. In adults, it tends to rise in the wet season and to decrease by about 15–20% in the dry season.

Concerning the first question about drug pressure, I don't know that anyone can really answer that question. I don't even attempt to try. It is our feeling that the drug resistant situation in Madang has not varied much over the last several years since 1979 and yet, there is enormous drug pressure with chloroquine. Also, all of our resistance, or most of it, remains at the R1 level. That is really about the only comment I can make on it.

BRUCE-CHWATT: I am very much impressed by the thoughtful address of Dr. Cattani to the problem of epidemiology of malaria in Papua New Guinea. I have always been fascinated by the epidemiology of malaria in that part of the world because of its resemblance to the African holoendemic malaria, yet with a number of differences that we still do not quite understand. What impressed me in Dr. Cattani's assessment of the possibility of using this area for a prospective clinical trial is the way she assessed the pros and the cons of the situation. The pros of the situation are that the epidemiology is reasonably well known and that though there are still a number of points that have to be elucidated, it is obvious that there are groups of people who know the area very well indeed and whose attitude to the forthcoming trials, if they are carried out there, is quite unbiased. When it comes to problems, one, in my view, of the most disturbing situations for conducting clinical trials in that area is the mobility of the population. This is true for both Africa and Papua New Guinea. The best proof of the high mobility of the population is the drug pressure of 4-aminoquinolines that are obviously brought to this area from the outside. How to isolate the clinical trial area from these outside pressures and how to make the experiment of clinical trial as foolproof as possible is not for me to tell. It is for local people like Dr. Cattani to assess. This apart, it seems to me that this would be an area potentially susceptible to a clinical trial of antimalarial vaccine. The question I'd like to put forth to Dr. Cattani, assuming that

it is quite obvious that the forthcoming antimalaria vaccine will be related to *P. falciparum* is, "What is the proportion of malaria incidence due to the three species of malaria parasites? I ask this because it will be much easier to assess the results of the trials on *P. falciparum* incidence when done on a mixed incidence. In Africa, the incidence will not be as mixed because 90%–99% will be due to *P. falciparum*. But it seems to me that in Papua New Guinea, the proportion of *P. vivax* is quite high (up to 15% or more) and this might create a disturbing element in the assessment of the results of malaria vaccine.

CATTANI: Thank you, Dr. Bruce-Chwatt, for your kind comments on my talk. The proportion of *P. falciparum* at the moment virtually all over Papua New Guinea is that *P. falciparum* is definitely the predominant species. The parasite ratio, or formula, is approximately 75%–78% *P. falciparum*, 20% *P. vivax*, and from 2%–5% *P. malariae* with the odd case of *P. ovale*. It is interesting, and this is just a preliminary communication, and I would hope not to be quoted, but in looking at sporozoite assays on infected mosquitoes, our entomologists have found that about 50% of the mosquitoes are infected with *P. vivax*. Yet, 75%–78% of our infections in humans are *P. falciparum*. So, yes, I think your point is well taken that there is more *P. vivax* than there appears from the parasite formula and that it also may appear that some of the genetic traits that are present may be evolved in response to *P. vivax* rather than *P. falciparum*. But, there is plenty *P. falciparum* around. There would be no trouble in finding it.

MEUWISSEN: I would like to ask if you have data on the relationship between vector infection rates and gametocyte density, eventually?

CATTANI: We do not have final data on it. Dr. Patricia Graves is looking at feeding mosquitoes in the field on blood samples that we collect routinely for serum samples and then looking at the correlation of her infected mosquitoes with gametocyte positivity in blood slides. No, we don't have data on it yet, but are looking at it.

Status of Malaria in the Philippines, 1984

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I. Introduction

The Philippines lies in the Western Pacific region bounded on the north by Taiwan, on the east by the Pacific Ocean, on the south by Borneo and the Celebes Seas, and on the west by the China Sea. It is composed of 7,100 islands with a total area of 115,707 square miles. The entire country is about 65% mountainous with a tropical climate and divided into twelve (12) health regions (Map 1).

Malaria has long plagued the people. A centralized Malaria Control/Eradication program was established to solve this health problem. In line with the present global strategy, the conventional concept in the campaign to control and eventually eradicate malaria in the country has been modified by utilizing the primary health care approach whose foundations are appropriate technology, sectoral linkages, community participation, and self-reliance. To support this strategy, Executive Order No. 851, dated December 2, 1982, has integrated the Malaria Program to the Field Health Services of the Ministry of Health, wherein the control operation has become a partnership with the community.

II. Epidemiological Features

The general epidemiological picture of malaria is summarized below:

1. Endemicities vary from hypo to hyperendemicity, unstable and prevalent in almost all provinces.
2. Due to breeding preference of the principal mosquito vector, malaria occurs in the rural areas and is absent in the urban areas.
3. Transmission is perennial with two peaks, during the start and during the end of the rainy season.
4. All the three species of plasmodia are found. The predominant

specie at the moment is *P. falciparum* with the ratio: about 65% is *P. falciparum*, 35% *P. vivax*. *P. malariae* is rare and no *P. ovale*.

5. Malaria vectors are zoophilic and prefer to feed on animal blood.

6. There are four (4) anopheline species of malaria vector. The principal malaria vector is *An. flavirostris* and this prefers to breed in slow flowing, clear and partly shaded streams. Other species are *An. mangyanus*, *An. litoralis*, and *An. balabacensis*.

III. Strategy

The strategy is basically control using combinations of insecticide residual spraying of houses, biological control, environmental management, personal protection measures to avoid mosquito bites such as sleeping under mosquito nets, screening houses, use of protective clothing, use of mosquito repellants either chemical or smoke, diverting mosquito bites by tying work animals near houses, and surveillance measures utilizing the primary health care approach.

The priority in implementing the appropriate control measures is on selected communities which are categorized according to endemicity and economic importance. Transfer of technology to the community is done until they develop the capability to implement the appropriate control measures. Health education, information dissemination and training management concerns and biological control is done through community participation under the technical supervision of trained government personnel. Case finding, and drug administration is done by the Barangay health workers (volunteers) in their respective catchment area of twenty (20) families.

Epidemiological investigation of areas and institution of remedial measures is undertaken by a team of government health workers under the supervision of an epidemiologist.

To maintain a regular monitoring and evaluation of the malaria activities in the field, copies of reports are requested from the Health Information Service of the Ministry of Health and quarterly field visits are done from the headquarters level.

IV. Current Status

Malaria, which ranked as number one (1) among the ten (10) leading causes of morbidity and mortality during the 1940s, had decreased to number nine (9) in 1982 in morbidity (Chart 1) and is no longer among the ten (10) in mortality (Chart 2). About 20% of the areas under malaria risks are already cleared of the disease.

The years 1983 and 1984 were transition periods from a centralized program to an integrated program during which time the antimalaria

CHART NO. 1. Leading Causes of Morbidity* (Rate/100,000 Population) Philippines, 1982

Causes	*No.*	*Rate*
1. Upper respiratory tract infections	447,831	882.2
2. Enteritis & other diarrheal diseases	364,780	718.6
3. Bronchitis	353,463	696.3
4. Influenza	300,923	592.8
5. Pneumonia	134,928	265.8
6. Tuberculosis, all forms	127,720	251.6
7. Skin infections	45,890	90.4
8. Intestinal parasitism	45,382	89.4
9. Malaria	41,220	81.2
10. Avitaminosis & other deficiency	40,915	80.6

* Data from Planning Service, MOH, Philippines.

CHART NO. 2. Leading Causes of Mortality* (Rate/100,000 Population) Philippines, 1982

Causes	*No.*	*Rate*
1. Pneumonias	42,349	85.6
2. Tuberculosis, all forms	20,630	41.7
3. Disease of the heart	16,573	33.5
4. Enteritis & other diarrheal diseases	11,676	25.6
5. Malignant neoplasm	8,361	16.9
6. Accidents	8,262	16.7
7. Cerebrovascular accident	6,530	16.2
8. Bronchitis	4,453	9.0
9. Avitaminosis & other nutritional deficiencies	3,661	7.4
10. Cardiovascular diseases	2,968	6.0

* Data from Planning Service, MOH, Philippines.

expertise was transferred to the provincial, municipal, and Barangay health workers causing a temporary interruption in the smooth flow of operations. The disease took advantage of this short period of reorganization and showed an increase in incidence, although not to an epidemic proportion.

Most of the cases were found in Mindanao and Sulu areas followed by northeastern Luzon with the least in the Visayas (Table 1).

P. falciparum resistance to chloroquine was detected as early as 1968. In 1974, a nationwide monitoring of drug resistant cases was started and continued up to the present (1984), using both *in-vivo* and *in-vitro* techniques, which resulted in the delineation of the areas which are sensitive and those which are resistant to chloroquine (Maps 2 and 3). About 70% of the total *P. falciparum* cases were found resistant to chloroquine. This finding is significant since *P. falciparum* is the predominant

TABLE I. Comparative Malaria Data, By Regions, Philippines, 1983 and 1984

	Population		*No. SI Examd*		*No. of Positive*		*P.f*		*P.v.*		*P.m.*		*Mx*		*SPR*		*Case/1000*	
Reg.	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*	*1983*	*1984*
I	1,241,995	1,260,893	13,045	33,423	721	2,857	404	1,767	315	1,070	0	1	2	19	5.5	8.5	.5	2.2
II	798,027	816,920	88,533	66,179	11,970	13,200	6,509	8,320	4,936	4,821	0	0	75	59	12.5	19.5	13.8	16.1
III	1,728,673	1,769,409	24,936	25,107	2,976	5,385	1,530	3,152	1,431	2,211	0	0	15	22	11.9	21.4	1.7	3.0
IV	2,239,194	2,300,421	216,951	177,170	22,099	26,137	12,775	13,576	9,192	12,426	0	0	132	135	10.1	14.7	9.8	11.7
V	1,233,422	1,255,463	23,313	19,975	4,850	3,210	2,278	1,158	2,572	2,052	0	0	6	0	20.8	16.2	2.6	2.5
VI	1,611,305	1,644,201	7,113	2,740	493	350	485	337	6	13	0	0	2	0	6.9	12.7	1.21	.21
VII	1,348,249	1,374,508	13,338	294	198	268		164	26	34	0	0	0	0	2.1	1.4	.2	.14
VIII	991,647	1,010,201	48,623	45,342	1,961	928	1,683	747	274	177	1	0	3	4	4.0	2.0	1.9	.9
IX	904,394	923,394	175,993	185,826	27,257	34,916	19,647	25,197	7,442	9,155	4	3	164	201	15.4	18.9	30.1	37.8
X	1,010,023	1,039,129	69,064	76,111	13,507	8,253	9,028	4,841	4,292	3,336	0	0	197	76	19.5	10.8	13.3	7.9
XI	1,233,577	1,271,705	10,321	35,666	5,087	444	373	5,089	130	2,107	1	248	4	4	4.9	20.8	.4	5.8
XII	803,015	817,086	28,579	22,072	4,583	8,624	2,212	5,994	2,336	2,260	3	120	32	250	16.0	30.1	5.7	10.7
Tot.	15,143,521	15,483,321	719,809	689,905	94,802	104,572	56,924	70,342	32,952	39,662	9	372	626	770	Av 10.8	Av. 14.75	Av. 6.8	Av. 8.25

infection. With the problem of uncontrolled population movements and the temporary nature of the inhabitants' dwellings in the hinterland, the use of antimalaria vaccine as a control method would become an appropriate measure.

V. Operational Research

I. Completed studies as of 1984:
   1. The effectiveness of DDT emulsion for residual spraying of premises did not differ from wettable powder significantly.
   2. "Abate" larvicide is effective in selected low endemic areas.
   3. Mass drug administration could only attain a low level of control.
   4. DDT pressure of the main vector, *An. flavirostris* did not produce any change in their behavior.

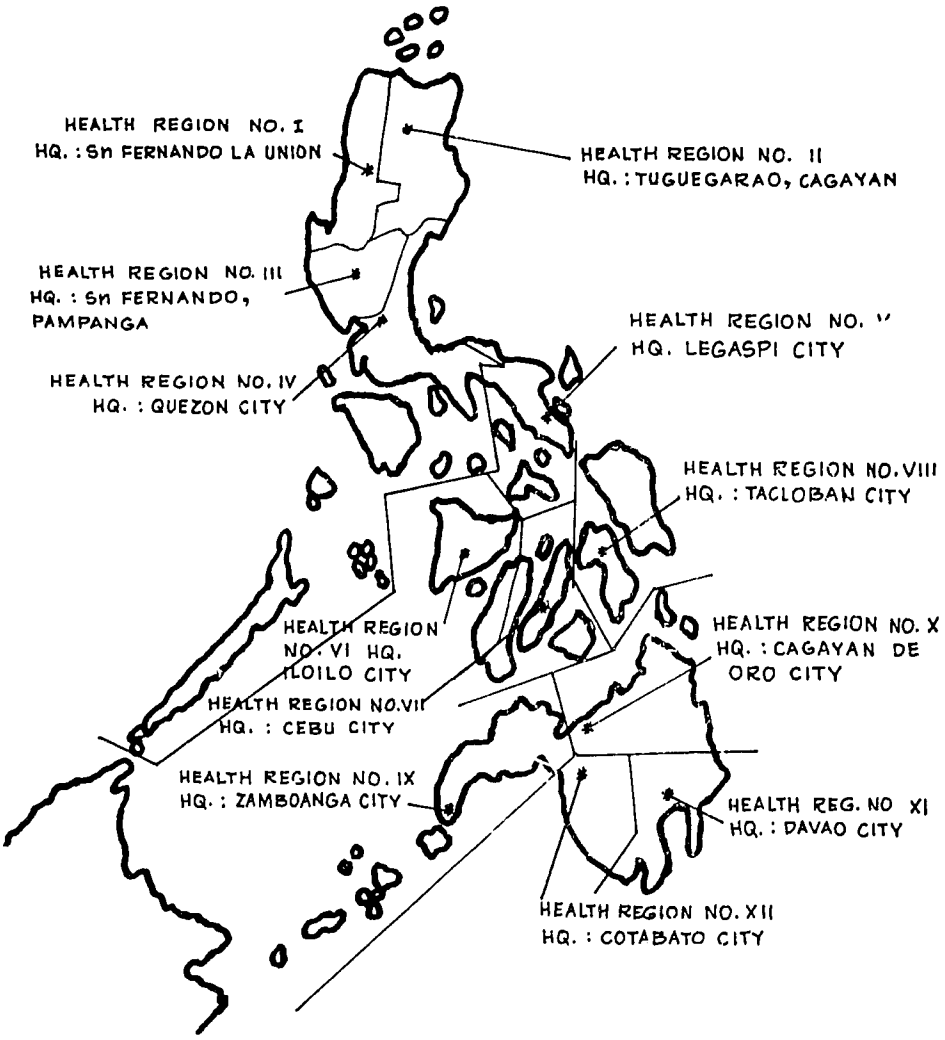
II. Ongoing studies:
   1. Sensitivity of *P. falciparum* to chloroquine by *in-vitro* (micro) technique.
   2. Bionomics of *An. balabacensis*.
   3. Colonization of *An. flavirostris*.
   4. Alternative insecticides and antimalarial drugs.

VI. Problems

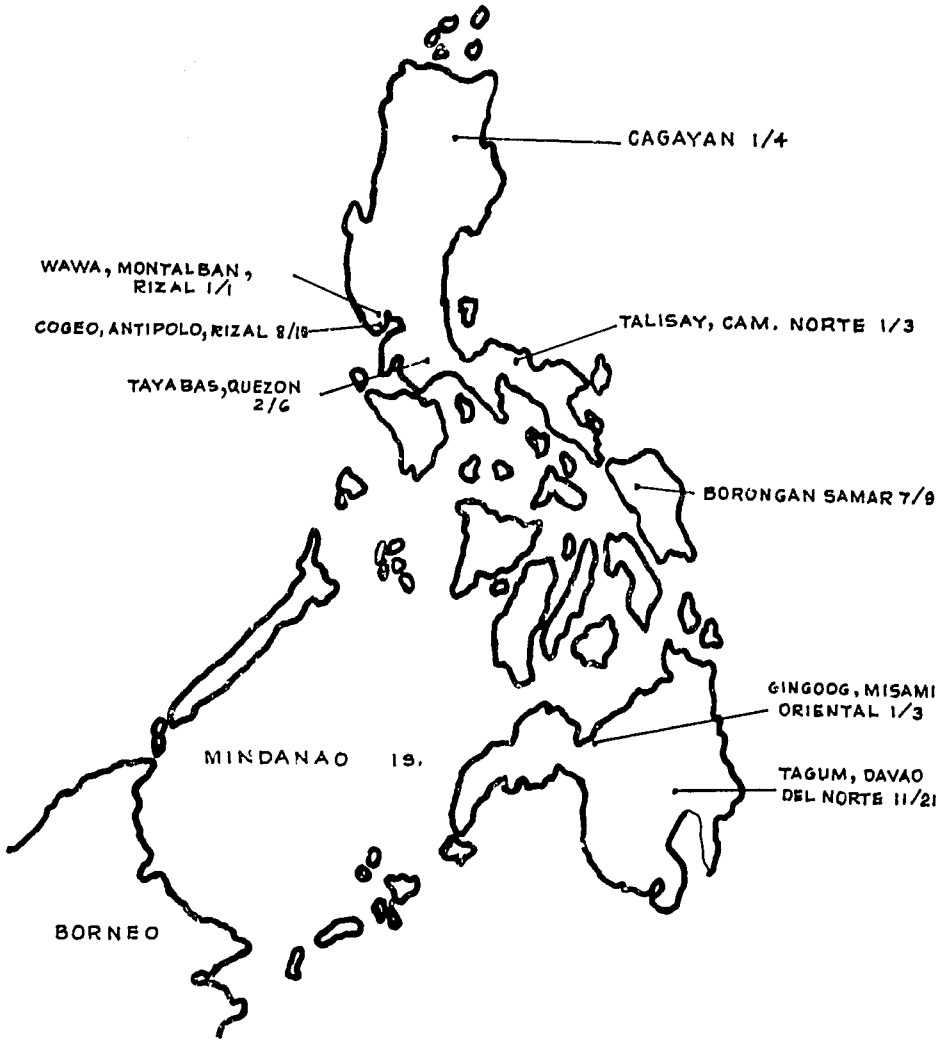
1. Administrative:
   a. Inadequate financial and manpower support, and
   b. Incomplete coverage of areas. Both can be corrected by PHC approach.
2. Operational:
   a. Extensive population movements between malarious and cleared areas make it difficult to maintain the gains already achieved.
   b. Ecological changes, like extensive irrigation projects, have contributed to creation of breeding places for local vectors.
3. Technical:
   a. Increasing areas with *P. falciparum* resistance to chloroquine.

VII. Expectations for a Malaria Vaccine

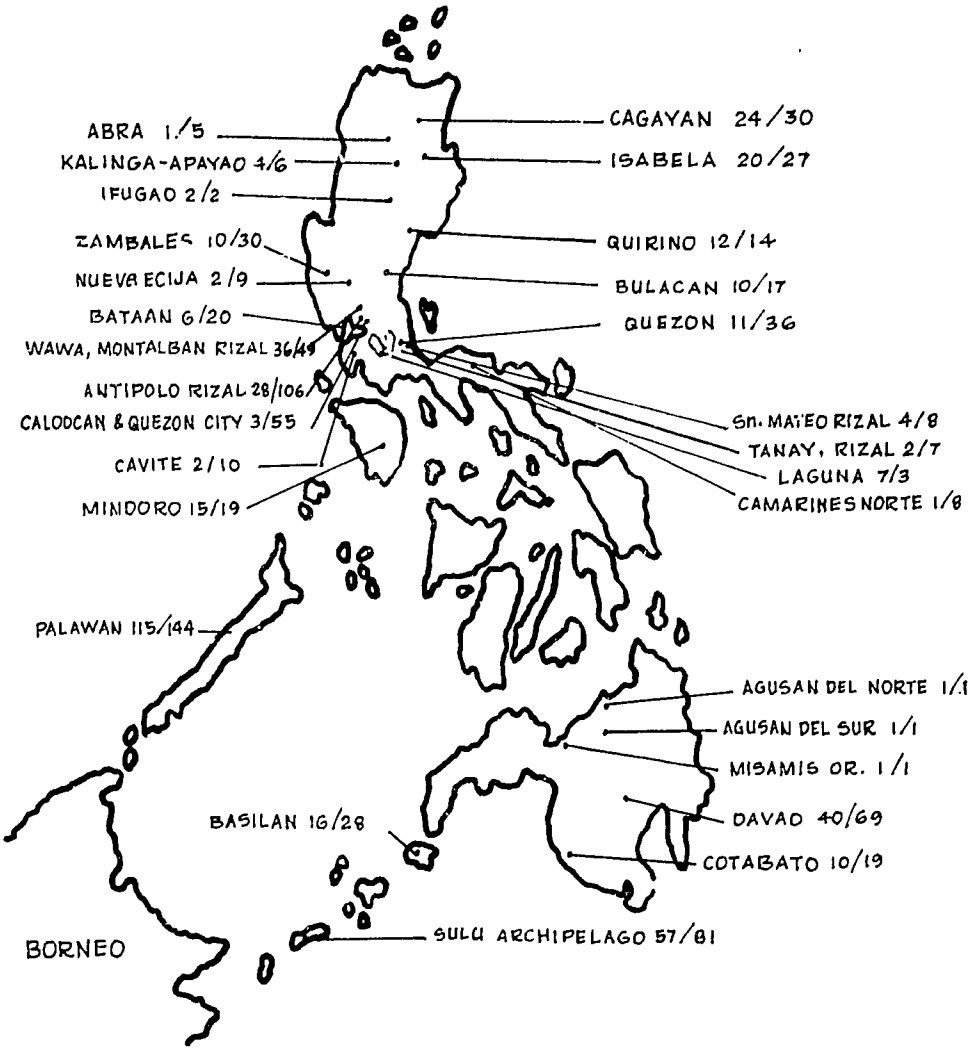
Some factors which caused the failure of an eradication program such as uncontrolled population migration, temporary dwellings, refusal to allow insecticide residual spraying of houses, and incomplete drug treatment are expected to be solved by a nationwide vaccination program, which is expected to be economical, feasible, and acceptable to the people. However, a pilot project is necessary before taking this to a nationwide scale.



MAP No. 1. Map of the Philippines Showing the Twelve Health Regions.



MAP NO. 2. Map of the Philippines Showing the Distribution of *P. falciparum* Tested by the *In-Vitro Micro* Technique (1982–July 1984), No. Resistant/No. Tested.



MAP No. 3. Map of the Philippines Showing the Distribution of Infections of *P. falciparum* Tested by the *In-Vitro Macro* Technique (1974–July 1984), No. Resistant/No. Tested.

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Discussion

TRAGER: I have a question with regard to the incidence of chloroquine resistance, which seems to be very spotty—is that right? Is this related to anything in particular that you know, perhaps to the vector or any other known situation?

RIVERA: The spotty situation shows that evidence of chloroquine resistance of *P. falciparum* depends upon the endemicity. For instance, in Palawan and the southern part of the Philippines, where it is highly endemic, you will note that the percentage of chloroquine resistance is much higher (it goes to around 60%–70%), whereas in the other areas where the endemicity is not as high, the percentage of resistance is also not as high.

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Current Status of Malaria and Anti-Malaria Programme in Malaysia

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1. Introduction

1.1 Malaysia, consisting of Sarawak, Sabah, and Peninsular Malaysia, has an area of 330,426 square kilometers and a population of about 15 million. Owing to different political histories, geographical location, and malaria vectors, the national Anti-Malaria Programme started off as three separate programmes, each with its own developmental history:

a. *Sarawak*

The first malaria survey in 1952 revealed that the annual incidence of malaria in Sarawak was as high as 40,000 cases. A malaria control pilot project was carried out in 1953–1954. The malaria eradication programme was launched in 1961. This was subsequently changed to a malaria control programme in 1970.

b. *Sabah*

A malaria survey in 1956 estimated that the annual incidence of malaria in Sabah was around 250,000 cases. After the malaria control pilot project in 1956–1960, a malaria eradication programme was launched in 1961. It reverted back to a malaria control programme in 1971.

c. *Peninsular Malaysia*

A malaria control pilot project was carried out from 1960 to 1964 in Peninsular Malaysia. A pre-eradication survey in 1965 revealed that the annual incidence of malaria was around 300,000 cases. This was followed by the malaria eradication programme in 1967. Owing to administrative, technical, and operational problems, it was re-defined as a malaria control programme in 1980.

1.2 In 1980, all the malaria control programmes in the three regions

were integrated to form the national Anti-Malaria Programme. This was later incorporated into the national Vector Borne Disease Control Programme (VBDCP).

2. Objectives

The objectives of the Anti-Malaria Programme are as follows:

a. To reduce morbidity and mortality due to malaria so that it will no longer be a public health problem in Malaysia.

b. To prevent the re-establishment of malaria in areas where there are no outbreaks at present.

3. Strategies

The strategies adopted by the VBDCP for the interruption of malaria transmission and depletion of the reservoir of infective cases were through geographical reconnaissance, residual house-spraying with DDT, surveillance, drug chemotherapy, case investigation, case follow-up, and anti-larval measures (especially in Peninsular Malaysia).

4. Malaria Vectors in Malaysia

4.1 Of the 65 anopheline species recorded in Malaysia, only 7 species have been confirmed as malaria vectors, namely: *Anopheles maculatus*, *Anopheles sundaicus*, *Anopheles campestris*, *Anopheles letifer*, *Anopheles balabacensis balabacensis*, *Anopheles leucosphyrus*, and *Anopheles donaldi*. There is no change in the behaviour of the malaria vectors against DDT. All the 7 species are still susceptible to DDT. However, *Anopheles campestris* (in Peninsular Malaysia) and *Anopheles donaldi* (in Sarawak) have shown better tolerance.

4.2 Out of the 7 malaria vectors in Malaysia, 4 species (*A. sundaicus*, *A. b. balabacensis*, *A. leucosphyrus*, and *A. donaldi*) are responsible for malaria transmission in Sarawak; 2 species (*A. sundaicus* and *A. b. balabacensis*) in Sabah; and 5 species (*A. sundaicus*, *A. b. balabacensis*, *A. campestris*, *A. letifer*, and *A. maculatus*) in Peninsular Malaysia. Only 2 species (*A. sundaicus* and *A. b. balabacensis*) are common malaria vectors throughout the whole country.

5. General Review of Malaria Situation in Malaysia

a. Malaria Incidence

Malaria is still a major public health problem in Malaysia especially in the rural areas. There were 32,094 cases reported in the country in 1984. Out of these, 1,012 cases (3.2%) were reported in Sarawak; 21,358 malaria cases (66.5%) in Sabah; and 9,724 malaria cases (30.3%) in Peninsular Malaysia. This is a vast improvement when compared to the annual figures in the fifties and sixties, namely, 40,000 cases in Sarawak; 250,000 cases in Sabah; and 300,000 cases in Peninsular Malaysia.

The malaria cases in Peninsular Malaysia have stabilized to

around 10,000–12,000 cases annually since 1980. Sarawak has achieved its target of less than a thousand cases annually since 1980. The malaria situation in Sabah is volatile. Malaria cases vary from 50,000 cases in 1981 to 11,000 cases in 1983.

b. Breakdown of Malaria by Parasite Species
In Sabah, *P. falciparum* (almost 80%) is the predominant parasite species. In 1984, the number of *P. falciparum* in Peninsular Malaysia was almost the same as *P. vivax*. The falciparum-vivax ratio has changed slowly from 2.6:1 in 1980 to 1:1 in 1984. In contrast, *P. vivax* (about 73%) is the predominant parasite species in Sarawak. *P. malariae* accounts for a very low percentage of the total cases in the country. *P. ovale* is not found in Malaysia.

c. Breakdown of Malaria by Age-Groups
The adult population (15 years and above) constitutes about 47.1% of the malaria cases in Malaysia. There were about 830 infant malaria cases (2.6%). This indicates that active malaria transmission is still going on, especially in the areas under spraying.

d. Malaria Deaths
Malaria caused 54 deaths in 1984, namely: 2 in Sarawak; 40 in Sabah; and 12 in Peninsular Malaysia.

e. Drug Resistant *P. falciparum*
Chloroquine-resistant *P. falciparum* is quite widespread in the northern part of Peninsular Malaysia. In Sabah, it is found throughout the state. No resistance of *P. falciparum* to chloroquine has been reported in Sarawak. There is at present no evidence to support the presence of resistance to Sulphadoxine-Pyrimethamine combination drug (FANSIDAR) and Mefloquine in Malaysia.

f. Imported Malaria from Neighbouring Countries
In 1984, about 488 cases (or 1.5%) were imported from outside Malaysia.

g. Operational Areas/Phases
In 1984, the country was divided into three operational areas/phases for the purpose of controlling malaria.

i. Area under Spraying (Late Attack Phase/Category A)
They are mainly located in the hilly hinterland of the country. In 1984, about 1,258,601 people (8.4%) lived in these areas.

ii. Non-Malarious Areas (Pre-Maintenance Phase/Category C)
They are mainly located along the coastal plains, especially in the highly urbanized areas. In 1984, about 11,609,684 people (77.0%) lived in these areas.

iii. Areas under Surveillance (Consolidation Phase/Category B)

These areas are in-between the areas under spraying (late attack phase/category A) and the non-malarious areas (pre-maintenance phase/category C). In 1984, about 2,199,715 people (14.6%) lived in these areas.

6. Operational Problems and Constraints

   The problems and constraints faced by the programme are as follows:

   a. Lack of community support due to apathy as well as lack of awareness of their role in malaria control.
   b. Lack of staff supervision in the deep hinterland due to curfew, remoteness, poor accessibility, and security problems.
   c. Increasing incidence of drug-resistant *Plasmodium falciparum*
   d. Changing pattern of malaria parasite species.
   e. Refractory nature of *Anopheles balabacensis balabacensis* in Sabah.
   f. Poor housing condition.
   g. *Moving Population Groups*

      There are various types of population that move constantly from one area to another, e.g.

      i. Temporary workers in land development schemes, highway projects, and dam construction projects.
      ii. The Security Forces, e.g., the Police and Army.
      iii. The deep-jungle aborigines population.
      iv. Movement across the border, e.g., with Thailand and with Indonesia.
      v. Others which include illegal traders and so on. It is difficult to contact these groups of population and also to be certain that measures taken among them are effective.

7. Role of Malaria Vaccine

   The malaria vaccine can be a very useful supplementary tool to minimize mortality and reduce morbidity in malaria control. However, it is not a panacea for the control of malaria in Malaysia. It is useful in the following conditions:

   a. High risk groups, e.g., Armed Forces, deep-jungle aborigines, workers in land development schemes, pregnant mothers/infants, and health staff working in malarious areas.
   b. Chloroquine-resistant *P. falciparum* areas.
   c. Prophylaxis for people visiting highly malarious areas.
   d. Remote areas with poor transportation.

TABLE I. Incidence of Malaria and Blood Slides Collection in Malaysia (1980–1984)

Year	*Estimated Population*	*Slides Examined*	*Confirmed Cases*	*API (‰)*	*SPR (%)*	*ABER (%)*
1980	13,434,520	1,799,977	44,488	3.3	2.5	13.4
1981	13,747,295	1,926,058	59,422	4.3	3.1	14.0
1982	14,061,524	2,035,922	43,915	3.1	2.2	14.5
1983	14,373,355	1,873,433	22,218	1.5	1.2	13.0
1984	15,068,000	1,729,322	30,094	2.1	1.8	11.5

Note: API = Annual Parasite Incidence per one thousand population.
SPR = Slide Positivity Rate.
ABER = Annual Blood Examination Rate.

TABLE II. Geographical Distribution of Malaria in Malaysia (1980–1984)

State/ Country	*Year*				
	1980	*1981*	*1982*	*1983*	*1984*
Peninsular Malaysia	9,110 (20.6%)	8,651 (14.5%)	12,311 (28.3%)	10,069 (45.3%)	9,724 (30.3%)
Sabah	34,351 (77.7%)	50,037 (84.2%)	30,540 (69.6%)	11,290 (50.8%)	21,358 (66.5%)
Sarawak	765 (1.7%)	754 (1.3%)	956 (2.1%)	858 (3.9%)	1,012 (3.2%)
Malaysia	44,226	59,422	43,915	22,217	32,094

Note: Figures in parenthesis denote percentages.

TABLE III. Malaria in Malaysia by Parasite Species (1984)

State/ Country	*Total Cases*	*Parasite Species*			
		P. falciparum	*P. vivax*	*P. malariae*	*Mixed Infection*
Peninsular Malaysia	9,724	4,708 (48.4%)	4,704 (48.4%)	9 (0.1%)	303 (3.1%)
Sabah	21,358	17,047 (79.8%)	4,106 (19.2%)	60 (0.3%)	145 (0.7%)
Sarawak	1,012	221 (21.8%)	742 (73.4%)	47 (4.6%)	2 (0.2%)
Malaysia	32,094	21,976 (68.5%)	9,552 (29.7%)	116 (0.4%)	450 (1.4%)

Note: Figures in parenthesis denote percentage. Mixed Infection means *P. falciparum* and *P. vivax*.

TABLE IV. Malaria in Malaysia by Age-Groups (1984)

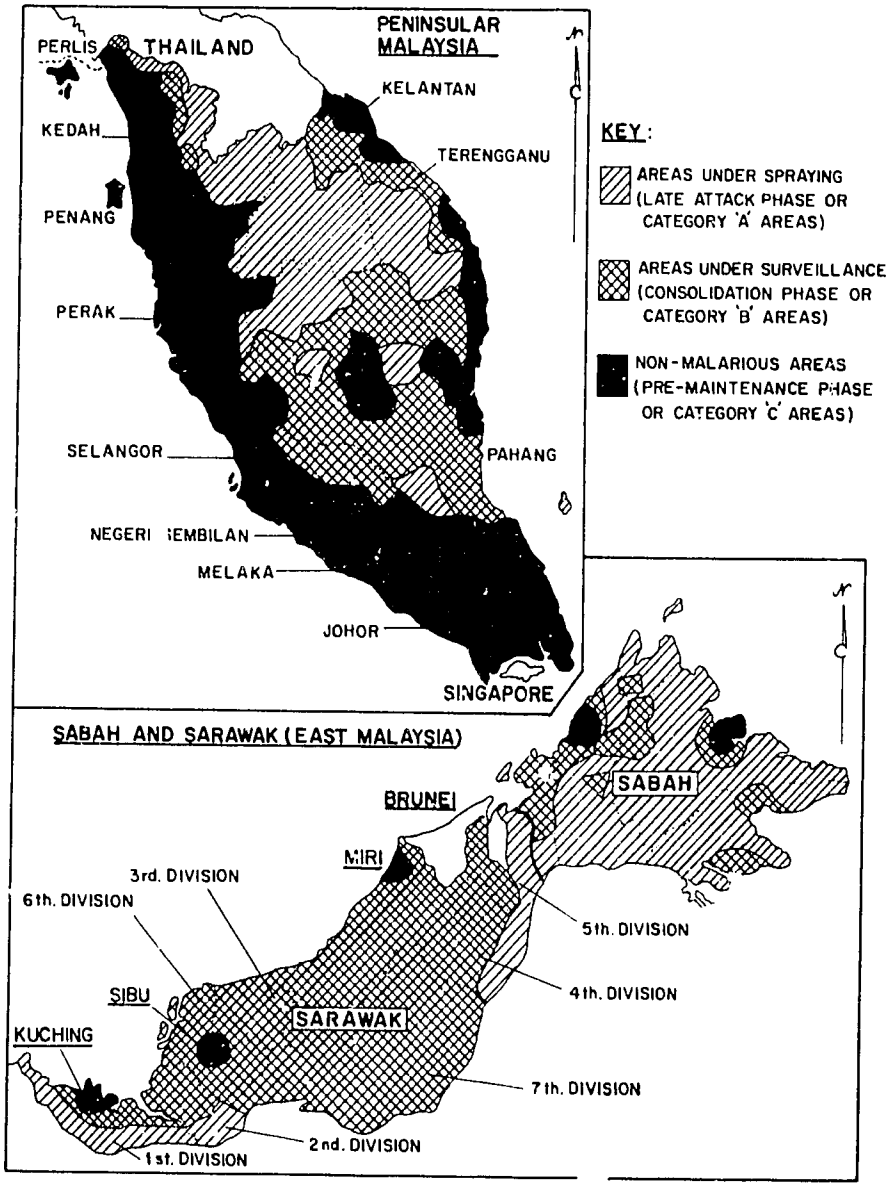
State/ Country	*Total Cases*	*Age Groups*				
		Below 1	*1-4*	*5-9*	*10-14*	*Over 15*
Peninsular Malaysia	9,724	172	1,630	1,721	1,369	4,832
Sabah	21,358	656	1,490	3,492	3,220	9,500
Sarawak	1,012	2	28	73	191	808
Malaysia	32,094	830 (2.6%)	6,148 (19.2%)	5,286 (16.5%)	4,690 (14.6%)	15,140 (47.1%)

Note: Figures in parenthesis denote percentage.

TABLE V. Population (Projected) Living in Various Operational Areas/Phases in Malaysia (1984)

State/ Country	*Total Population*	*Operational Areas/Phases*		
		Areas Under Spraying (Late Attack Phase/ Category A)	*Areas Under Surveillance (Consolidation Phase/ Category B)*	*Non-Malarious Areas (Pre-Maintenance Phase Category C)*
Peninsular Malaysia	12,467,000	536,081 (4.3%)	698,152 (5.6%)	11,232,767 (90.1%)
Sabah	1,140,000	547,200 (48.0%)	467,400 (41.0%)	125,400 (11.0%)
Sarawak	1,461,000	175,320 (12.0%)	1,034,163 (70.8%)	251,517 (17.2%)
Malaysia	15,068,000	1,258,601 (8.4%)	2,199,715 (14.6%)	11,609,684 (77.0%)

Note: Figures in parenthesis denote percentage.


PENINSULAR MALAYSIA
PERLIS
THAILAND
KELANTAN
KEDAH
TERENGGANU
PENANG
PERAK
SELANGOR
PAHANG
NEGERI SEMBILAN
MELAKA
JOHOR
SINGAPORE
KEY:
AREAS UNDER SPRAYING (LATE ATTACK PHASE OR CATEGORY 'A' AREAS)
AREAS UNDER SURVEILLANCE (CONSOLIDATION PHASE OR CATEGORY 'B' AREAS)
NON-MALARIOUS AREAS (PRE-MAINTENANCE PHASE OR CATEGORY 'C' AREAS)
SABAH AND SARAWAK (EAST MALAYSIA)
SABAH
BRUNEI
MIRI
3rd. DIVISION
6th. DIVISION
5th. DIVISION
4th. DIVISION
SIBU
SARAWAK
KUCHING
7th. DIVISION
2nd. DIVISION
1st. DIVISION

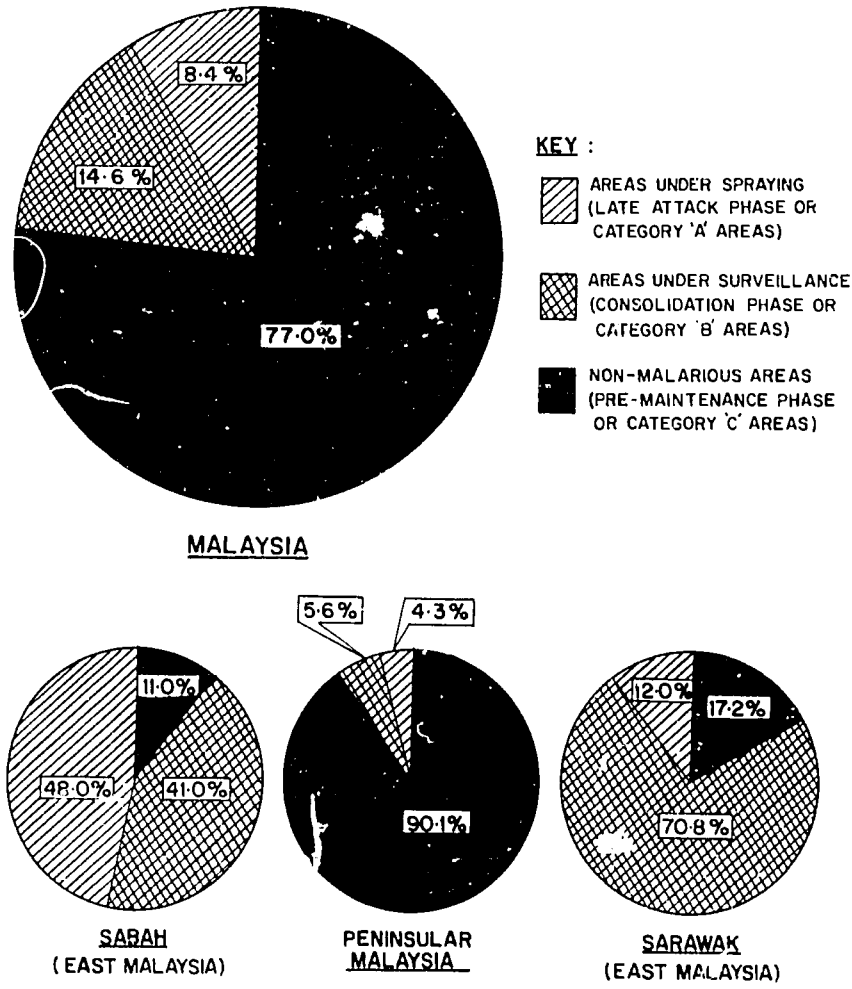


FIGURE 2. Percentage of Population Living in Various Phases/Categories of the Programme in Malaysia, 1984.

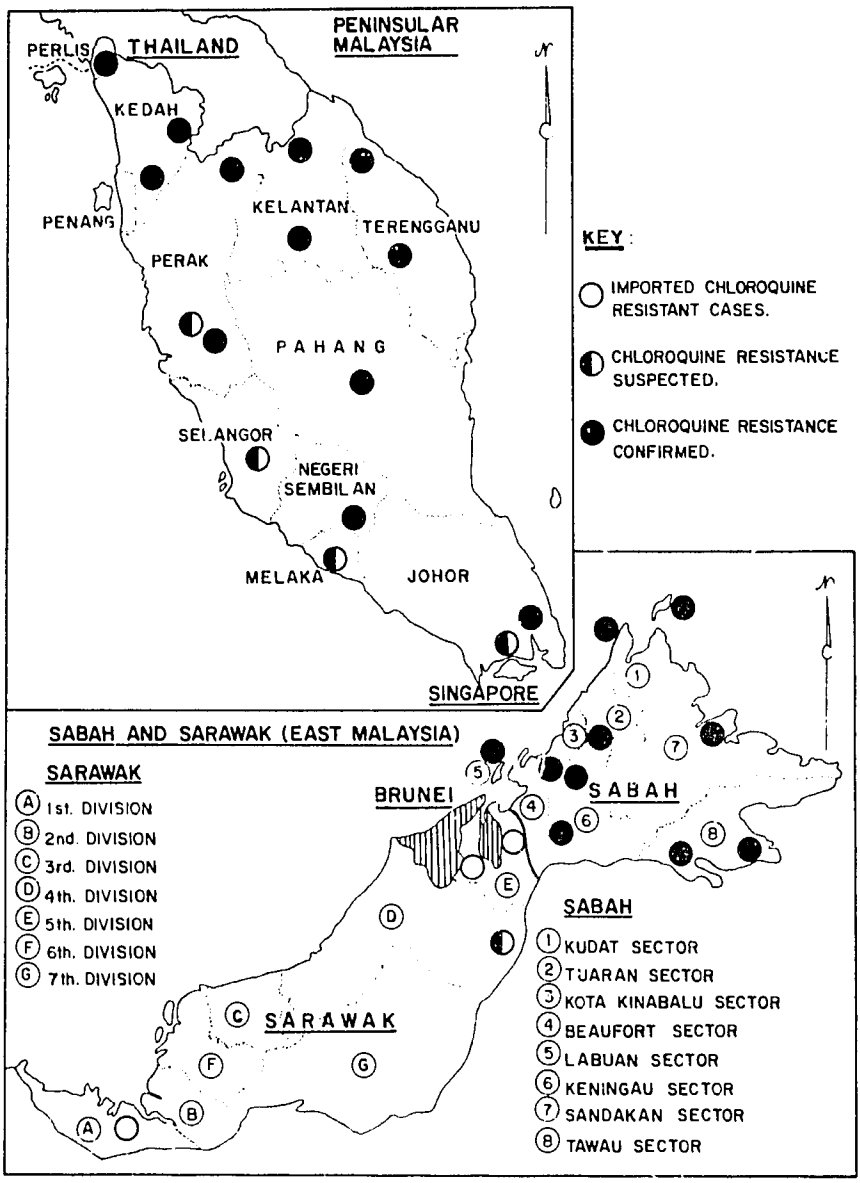
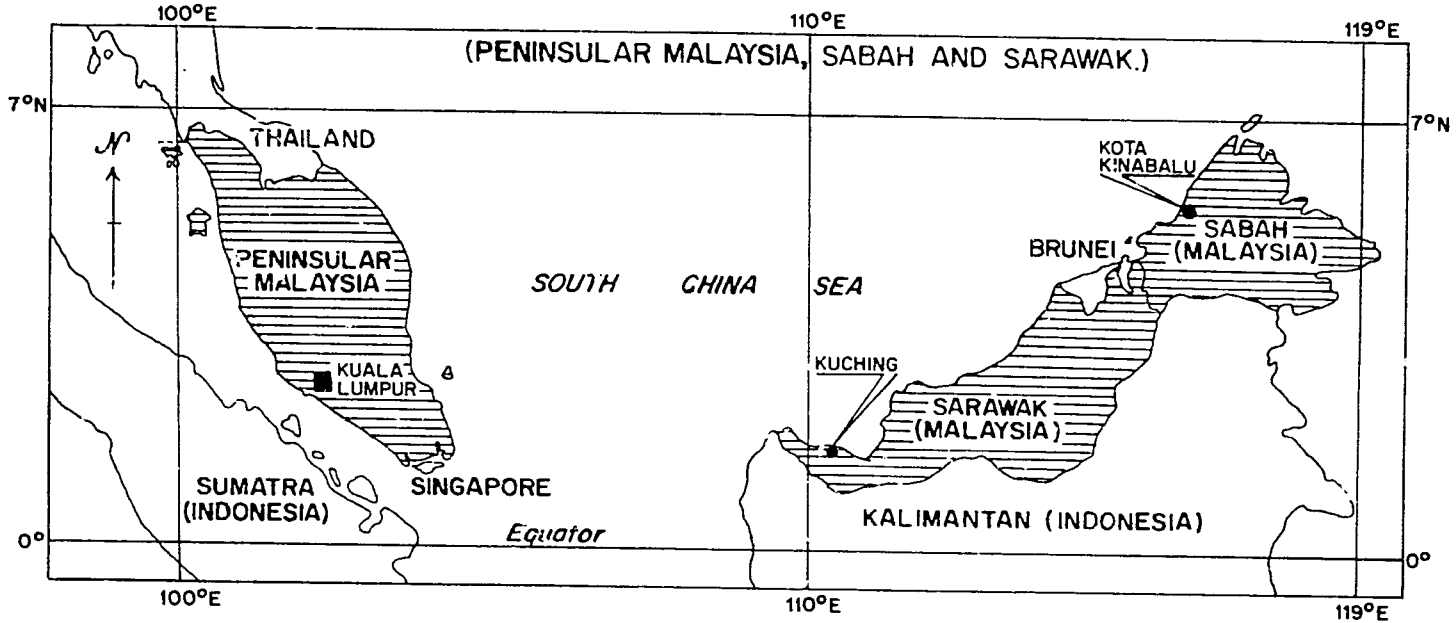


FIGURE 3. Distribution of Chloroquine Resistant Falciparum Infection in Malaysia, 1984.



	AREA (sq. Km.)	POPULATION
SABAH	74,389	1,140,000
SARAWAK	124,450	1,461,000
PENINSULAR MALAYSIA	131,587	12,467,000
TOTAL FOR MALAYSIA	330,426	15,068,000

FIGURE 4. Map of Malaysia.

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2. Plan of Action 1984. Vector Borne Disease Control Programme, Ministry of Health, Kuala Lumpur, Malaysia.

Discussion

BUCK: What is the role of the migration from the Sulu Island and Mindanao at the east coast of Sabah?

HO: I believe these are what they call the "compeet." They come to trade. At the moment, we screen them, and this is what we have seen.

BUCK: The vectors in Sabah, how often do they also serve as vectors for filariasis?

HO: In Peninsular Malaysia, *A. campestris* is a vector. Sabah has only *A. sundaicus*. In the *A. sundaicus* area you do not have filariasis but *A. balabacensis*. Whether it is a vector or not, I do not know.

RIVERA: You mentioned that all Anopheles species in Malaysia are susceptible to insecticides. Could you tell us the method of application of insecticides in the cases of *A. b. balabacensis*, which I believe is exophilic?

HO: We still use the WHO methodology which in indoor house spraying with 2 grams/square meter of technical grade DDT.

BRUCE-CHWATT: In comparison with the remainder of Southeast Asia, Malaysia really does not have a very large problem. To report 30,000 cases altogether for a population of 50 million shows that tremendous progress has been achieved over the past 20 years since the pilot projects in Silah and Silungar were started. How can we explain the very high degree and high incidence of resistance to chloroquine in *P. falciparum* in Sabah, as opposed to Peninsular Malaysia? It really is a puzzle. In northern Peninsular Malaysia one could expect it because of the close contact and migration from Thailand. How do you explain the high degree of resistance in Sabah, which is reasonably isolated both from Indonesia and from the Malaysia Peninsula?

HO: I am as puzzled as you are. Why, instead of using the peninsula as a bridge, does it go straight to the Thai/Kampuchean border? This is a question which I have asked myself many times. Perhaps others here could offer an explanation?

CLYDE: The only comment I can make on that is that when *P. falciparum* resistance initially emerged, it was thought to be *A. balabacensis* trans-

mitted. *A. balabacensis* is predominant in Sabah but not in Peninsular Malaysia. However, this theory has been pretty well disproven since the resistance has spread far beyond the endemic areas of *A. balabacensis* or *A. dirus*. But that was the original thought. That's the only possible explanation I can give.

HO: Yes, I am puzzled also. In Sarawak, we do not have any resistance.

RIVERA: Regarding the high percentage of resistant cases in Sabah, I made mention during my talk of the highest incidence being in the southern Philippines. There is much movement of people from the southern Philippines to Sabah. In fact, we have the highest incidence in that region which supports so much barter and trade between these two areas.

HO: I was postulating from the Thai/Kampuchean border to Mindanao and from Mindanao to Sabah.

Discussant: Malaria Situation in the Western Pacific Region

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Despite nearly three decades of extensive control activities, malaria still remains a major health problem in some endemic countries of the Western Pacific region.

After the initial success in malaria control, primarily through DDT residual house spraying at one time, the malaria situation has worsened in two main areas: one is the area where *A. balabacensis* is the main vector such as countries of the Indochinese Peninsula, some parts of the Philippines, and Sabah State in Malaysia; the other is the area where the main vector is *A. farauti*, such as Solomon Islands and Papua New Guinea.

The disease, however, was eradicated in Australia, Brunei, China (Province of Taiwan), Japan (Ryukyu Islands), and Singapore.

A bright spot in the present gloomy situation is Malaysia. Further progress was achieved in two areas, namely, Peninsular Malaysia and the State of Sarawak. In Peninsular Malaysia, the malaria eradication programme has reached a stage where 95% of the total population are either in the maintenance or consolidation phase of the eradication programme, while intensive surveillance operations in the State of Sarawak have been successful in keeping the incidence of malaria throughout the state well below 1,000 cases per year.

In my opinion, three factors are mainly responsible for the present sorry state of affairs. First, the effectiveness of residual spraying with DDT, a major weapon in malaria control, has decreased.

A. balabacensis and *A. farauti* have not developed physiological resistance to DDT but have adopted different patterns of behaviour to avoid picking up the lethal effect of DDT deposits on the wall inside the house by not going indoors, but not resting on sprayed surfaces, and by biting human beings out of doors. Spraying is sometimes badly done, particularly in remote areas where good supervision is almost impossible. A second factor is drug resistance. A third is the fact that the integration of the former vertically organized malaria services into the

general health services or primary health care system has not gone smoothly. During the past few years, there has been in many places a kind of organizational vacuum.

Changing Approaches in Malaria Control

Efforts to control malaria have moved into a phase of change and reassessment. The time-limited concept of malaria eradication is being replaced with the more realistic but complex goal of long-term control. In recent years, it has also become clear that traditional, semi-military, vertical control programmes could not continue to be supported. The funds for such expensive efforts are not available in most malarious countries. Furthermore, the mandate of "health for all by the year 2000" calls for reliance on multipurpose primary health care workers, especially at the community level, with special emphasis on community involvement in disease control.

Generally, DDT residual indoor spraying, conducted at six-monthly intervals at 2 gms per square metre with wettable powder, is still an effective principal control measure applied in the areas of high transmission potential. However, DDT indoor spraying is only effective when the local vectors are responsive to DDT spraying and human behavioural factors are favourable.

Thus, an inventory should be made to assess the prevailing epidemiological and entomological conditions, physical environment, and even psychosocial factors to decide whether indoor spraying is to be commenced, continued, or discontinued.

In some instances, this can be supplemented with mass drug administration using a combination of chloroquine and primaquine. In areas of low or moderate malaria transmission, potential efforts are generally confined to malaria diagnosis and treatment. Here the general health services often function effectively where the health infrastructure at the grass-roots level has reached a certain standard of development. Where the basic health infrastructure is totally lacking or grossly inadequate, a special effort has been directed recently in several countries to develop a network system of malaria aide posts to provide early diagnosis and treatment for acute infections of malaria. It is the general policy in these countries for the malaria aide posts network to play an essential role in and to function as an integral part of primary health care for the population under malaria risk. The diagnostic and treatment services are important also in areas of high malaria transmission potential where the indoor residual spraying method has little or no impact because of vector behaviour, problems of refusals, or local housing conditions. In the latter areas, special attention is often given to the care of vulnerable population groups, particularly young children.

Source reduction by environmental management and manipulation is often talked about these days but in reality it is still rather of academic interest as in the case of malaria control.

Problems and Constraints

The major problems and constraints confronting the malaria control programmes are technical, administrative, and managerial.

In all malaria control programmes, indoor spraying with residual insecticides (chiefly DDT) has been used (in some programmes for more than two decades) as the principal method of attack. Paradoxically, total dependence on this method alone in the past has been the cause of some major failures. Total and complete spraying coverage of all inhabited premises is a prerequisite if this method is to be successful in interrupting malaria transmission but it has not been achieved in a large part of the endemic countries because of operational factors such as unsatisfactory and inadequate supervision of spraying squads, difficult terrain, lack of transport, primitive housing conditions, etc. Mounting public resistance to spraying over a number of years has made the situation even worse.

The increasing evidence of reduction or loss of sensitivity of certain strains of *P. falciparum* parasites to chloroquine in all endemic countries of the region, with the probable exception of part of China and Sarawak State in Malaysia, has presented another technical challenge. Quite apart from the magnitude of the problem *per se*, it has produced a psychological impact, resulting in confusion in the proper use of antimalarial drugs for treatment of patients and for prevention of infection.

From the administrative point of view, some disorganization in the national malaria control service has been observed recently in several countries due to the transition period in the process of integrating the malaria service into the general health services structure or primary health care system.

Other factors affecting the success of the antimalaria campaigns have been the diminishing financial support and the steadily increasing cost of the programme due to inflation, particularly during the last ten years. The cost of DDT residual indoor spraying for two cycles a year now averages US$4 to US$5 per capita per annum multiplied by the population under coverage, a 1,000 percent increase compared with the cost in the 1960s.

The departure of trained and experienced manpower in all categories of the malaria service, particularly in the professional grade, and the frequent turnover of personnel are other common problems encountered by most countries.

Discussion

PERRIN: You mentioned 221 cases of *P. falciparum* in Sarawak. The summary from China, which is such a large area, reports few cases per year. How is it possible to have continuous and persistent transmission of the disease without foci in these areas when you have so few cases per year?

PAIK: This is a good case with which to compare the situations of Sabah and Sarawak. There is no *A. balabacensis* vector in Sabah. Whereas in Sarawak, the main vector is *A. balabacensis*. That's the main factor in differentiating these two epidemic situations. The total cases now in Sarawak are below 1,000. Not the entire Sarawak is endemic, however, there are some pockets of transmission. That's why we cannot say there is transmission throughout the state. There must be foci. That's why in these foci they still maintain the transmission. But the case total is still very low. This data is quite reliable because specific case detection in Sarawak is well developed. Local epidemiological conditions are very important concerning this data.

LOWRY: Will you comment on the situation of malaria in Vanuatu versus the Solomons? I had heard recently that the increasing prevalence of malaria in Vanuatu was presenting a situation which resembled the Solomon Islands in terms of control and problems of treatment, etc.

PAIK: First of all, the conditions in the Solomons is quite a unique one because there were some epidemics a few years ago. But, now this is on a descending trend. This may be due to the cyclical change noted in health immunity. Once there is an epidemic, everyone acquires the infection and the community immunity drops. Now all notice the decreasing trend. When I was there in 1974, parasitemia was *vivax* 70%, *falciparum* only 30%. Now, the pattern is completely reversed with *falciparum* 70% and *vivax* 30%. The situation we have is quite different from that in the Solomon Islands. In the Solomon Islands, endemicity is almost the same, but still they recognize the highest. In New Hebrides, only the southern part is hypoendemic. In the northern part you have this instance normally high. Also, there is quite a mix of different endemicity by localities. Still, I feel that the situation in Vanuatu is better than that in the Solomon Islands. There is a good chance that malaria can be controlled through other primary health care means. We are now actively thinking about introducing larvivorous fish for which Hawaii has a lot of experience. Our entomologist, Mr. Toi, two months ago, discussed how to transfer the technology of the use of larvivorous fish in Hawaii to the Vanuatu situation.

SURIN PINICHPONGSE: I would like to comment on what Dr. Paik as well as Dr. Ho Keong Bin mentioned earlier about the vectors in the West-

ern Pacific regions, particularly the vector you call *Anopheles balabacensis*. In Thailand, we have reported that *A. balabacensis* is now named *Anopheles dirus*. I understand that some parts of northern Peninsular Malaysia also use the term *Anopheles dirus* instead of *A. balabacensis*. That is my first comment.

The second point that Dr. Paik had mentioned was that because of reduced effectiveness of DDT spraying, he recommends the alternative methods of using drug prophylaxis and treatment in order to reduce mortalities. I am not quite sure about this recommendation. It has been mentioned by Drs. Rivera and Ho that in the Philippines, there exists *P. falciparum* resistant to 4-aminoquinolines. In Thailand, we have used the sulfadoxine/pyrimethamine combination to replace chloroquine. We have found, though, after a few years of using this drug, that *P. falciparum* again develops resistance. Then, the only drugs left to be used are very expensive drugs. Therefore, I think that if we were to follow these recommended methods, we may have to spend more money in the future than if we continued with DDT spraying. It has been our experience in Thailand that while we do have a reduction in the effectiveness in DDT, it still is able to control malaria.

PAIK: With regard to Dr. Surin's question for the scientific name for *Anopheles balabacensis* in Malaysia, I am not an entomologist, so I will leave this question for Dr. Ho to answer. Of course, *A. dirus* is a different name for the *balabacensis* group. But I cannot distinguish how one is called *dirus* and the other *balabacensis*. This is a very specific entomological question, so I will leave this for Dr. Ho.

With regard to the cost of the drugs versus the use of the malaria eight-post system for reduction of mortality/morbidity, we are not recommending the discontinuation of DDT spraying for the whole region. We are only recommending this orienting method in Melanesian countries such as Papua New Guinea and the Solomon Islands. This is because Papua New Guinea, for example, has spent 4 million kinau for DDT spraying for the last 20 years. However, if we assess its usefulness we would find that it is very, very limited. This is because, first of all, *farauti* is highly exophilic. The vectors do not enter the house, they bite outside. The peak time is after sunset. When many villagers come back from the garden, they start to cook or eat outside. They never go inside the house before 9:00 P.M. Therefore, the majority of people are outside just before sunset, which coincides with the peak time of the biting farauti. Also, *farauti* is highly anthropophilic. You find few blood which originates from pig or dogs. This is a very effective vector. You can see, therefore, that there is no use to spray indoors since the people do not enter the house before 9:00 P.M. There is quite a unique area that is surrounded by vast breeding sources where the U.S.

Marine Corps suffered severely from malaria during the Second World War. But, controlling all those rivers, creeks, and lagoons is almost impossible. Here, also, the density of the *farauti* is too high. We used to try three-month cycles of spray operations but we could never bring down to the certain threshold density in which malaria could be interrupted. So, we found that with these two countries, it is almost impossible to gain from DDT spraying. So, why should we recommend that these governments spend money on antimalarial drugs? It is because there is no other alternative than to reduce the morbidity/mortality rates.

Dr. Surin mentioned the chloroquine resistance problem. The intensity of the chloroquine resistance strain is very high in Sabah. Our WHO Inter-country Team found that almost 90% of local strains are resistant to chloroquine at different degrees. However, the chloroquine resistant strains in the Solomon Islands and Papua New Guinea are still very much limited. We found some R1 and R2 but seldom any R3. So, the conventional antimalarial drugs can still be used in these two countries if they can develop a consistent distribution of these drugs for the population.

Malaria Control in Indonesia
Current Status and Trends 1979–1983

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1. Introduction

The malaria control programme in Indonesia (population 167 million) is completely integrated with the Directorate General of Communicable Diseases Control at the central level and with the Sub-Division of Communicable Diseases Control at the provincial level.

In view of the autonomy of the provincial and peripheral health administration, the role of the Sub-Directorate of Malaria Control Programme/CDC has been primarily that of overall countrywide planning, provision of technical guidance and assistance and operational support, monitoring and evaluation, training and manpower development, initiating operational studies and special measures, institution of emergency operation, and intersectoral coordination. It has no directional or controlling authority over the provincial malaria control programme in the periphery. The malaria control activities are to be carried out as part of primary health care approaches with the community participation.

Strategically, malaria control activities in Java and Bali provinces are different than those in the islands other than Java and Bali (Outer Islands).

2. Objectives

In terms of providing "Health For All By The Year 2000," the Government of Indonesia has formulated the following objectives for the control of malaria.

2.1 *Long-Term*

2.1.1 Java-Bali—To reduce the number of high case incidence Kecamatan/Sub-District (API more than 7.5‰) to 20 by the end of Sixth Five Year Development Plan (1994/95–1998/99) and maintenance of

Annual Parasite Incidence/API to less than one case per thousand population.

2.1.2 Outer-Islands—To reduce malaria prevalence to less than 2% in the priority operational areas and to 5–10% in the remaining areas by the end of the sixth Five Year Development Plan. Priority areas cover transmigration projects, socio-economic development areas, areas bordering neighbouring countries, and outbreak areas.

2.2 *Short-Term*

2.2.1 Java-Bali—to reduce the number of high case incidence sub-district from 63 to 37 by the end of fourth Five Year Development Plan (1984/85–1988/89) and maintenance of API less than one case per thousand population.

2.2.2 Outer Islands—to reduce malaria prevalence to less than 5% in the priority operational areas and to 15–20% in the remaining areas by the end of the fourth Five Year Development Plan.

3. Strategy

Type of Activities	*Java Bali*	*Outer Islands*	
		Priority	*Non-Priority*
Surveillance	ACD, PCD	PCD, MMS	PCD
Treatment	Presumptive, Radical	Suppressive	Suppressive
Vector Control	IRS	IRS	—
Anti-Larval Measures	Limited	Limited	—

ACD = Active Case Detection
PCD = Passive Case Detection
MMS = Malariometric Survey
IRS = Indoor Residual Spraying

4. Situation Analysis

4.1 In Java and Bali, evaluation of the epidemiological situation during the past four years of the third Five Year Development Plan (1979/80–1983/84) revealed that there had been a reduction of malaria cases, slide positivity rates, and annual parasite incidences during 1982 than the previous years as follows:

Year	*BLSL Exam.*	*Positive*	*SPR%*	*API‰*	*ABER%*	*Pf.%*
1979	8,042,198	78,854	0.98	0.84	8.6	46.9
1980	9,085,040	176,733	1.93	1.85	9.5	46.6
1981	9,128,356	124,656	1.37	1.30	9.5	45.2
1982	9,196,556	84,266	0.92	0.86	9.4	54.3
1983	9,226,412	133,708	1.44	1.34	9.2	47.9

Number of the houses sprayed and insecticide used during 1979/80–1983/84 is given below:

Year	*Houses Sprayed*	*DDT 75% wdp used (kg)*
1979/80	2,840,037	2,082,798
1980/81	1,942,298	1,226,670
1981/82	1,965,469	1,291,741
1982/83	1,471,936	1,068,012
1983/84*	450,435	339,498

*Incomplete

4.2 *In the Outer Islands* There have not been many changes in the activities as well as in the malaria situation. Malariometric surveys and spraying operations were carried out only in priority areas of transmigration projects, other socio-economical development localities, and bordering areas covering 5.6% of the total population in the Outer Islands. Reports received from the Outer Islands regarding malariometric surveys and spraying coverage are given below:

Malariometric Surveys

Surveys	*1979*	*1980*	*1981*	*1982*	*1983*
Blood Slide exam	155,892	466,355	559,657	600,078	436,243
Positives	6,558	19,283	20,303	31,074	12,458
P.R.	4.2	4.1	3.6	5.2	2.9
P.f.%	34.0	37.1	37.1	35.1	55.7

Spraying Operations

Year	*Houses Sprayed*	*DDT 75% wdp used (Kg)*
1979/80	939,892	466,478
1980/81	1,111,976	525,448
1981/32	1,282,828	596,869
1982/83	1,342,310	606,296
1983/84*	751,966	359,927

*Incomplete

4.3 Health institutions (hospitals, health centres, sub-health centres, and polyclinics) examine clinical malaria cases and give suppressive treatment Reports received from the Outer Islands regarding passive case detection in the health institutions were as follows.

Particulars	*1979*	*1980*	*1981*	*1982*	*1983*
Clinical Malaria	1,075,658	1,241,403	756,771	1,214,496	831,824
Blood Slides Exam.	360,428	488,616	353,788	411,758	421,631
Positive	87,105	130,279	90,780	105,121	84,268
S.P.R.	24.3	26.7	25.6	25.5	20.0
P.f.%	31.9	35.5	40.4	31.8	38.0

4.4 During the last two years, the detection of *Plasmodium falciparum* resistance to chloroquine has revealed that the problem is widespread in

the eastern islands of the archipelago. So far, of 27 provinces, 24 have been tested and 22 have been found having resistant foci.

4.5 Of the 80 anophelines species recorded in Indonesia, 16 species have so far been confirmed as vectors of malaria. *An. aconitus*, the main vector of Java-Bali, has been found resistance to DDT in the inland area of East Java, Central Java, and Yogyakarta, while in the Outer Islands so far all vectors/suspected vectors have been found to be susceptible to DDT.

5. Approaches

For implementation of effective malaria control, the approaches would be based on integrated health services activities, intersectoral collaboration, and coordination and community participation.

5.1 *Integrated Health Services*

At the national level, the malaria control programme is one of five Sub-Directorates of the Directorate of Vector Borne Diseases Control which in turn is under the Directorate General of Communicable Disease Control. The Sub-Directorate is responsible for overall planning, technical guidance, operational support, programme evaluation, special staff training, and some field studies. Sub-Directorate of Entomology works in close cooperation with the malaria control programme along with its other entomological activities.

At the provincial level, the vector control section under the Sub-Division of CDC is responsible for the implementation of the antimalaria activities in the provinces. Active case detection and treatment, malariometric surveys, laboratory examination, entomological activities, spraying operations, and limited larviciding are carried out by the integrated provincial/peripheral staff. Passive case detection and treatment of clinical and confirmed malaria cases are carried out by the network of health institutions (hospitals, health centres, and sub-centres).

5.2. Intersectoral collaboration and coordination have been identified as an essential component in the overall health development including malaria control activities. Examples of areas for such cooperation include the departments of transmigration, village development, agriculture, forestry, fishery, public works, and education.

5.3. Community participation. PKMD (village community health development) is an operational form of primary health care in Indonesia and is progressing satisfactorily. PROKESA (village health volunteers) under the PKMD system are potential workers who could assist in drug distribution, epidemic notification, and motivation of the com-

munity to participate in limited and suitable bioenvironmental methods for malaria control at the village level.

6. Activities Related to Malaria Control Measures

6.1 *Spraying operation in Java and Bali*

Main insecticide of use will be DDT. Primary criteria for spraying operation will be the presence of three or more indigenous cases in a village during previous year. Number of spray cycle and dosage of DDT will depend upon the local epidemiological situation of the area. High case incidence Kecamatan will be covered by two spray cycles a year during January–March and July–September at a dosage of 1 gr/m^2 or 2 gr/m^2 depending upon the length of transmission and the susceptible status of the local vector. Vulnerable and receptive areas will be sprayed once or twice a year depending upon the local epidemiological situation at the dosage of 1 gr/m^2 2 gr/m^2 DDT as stated before. Focal outbreaks of malaria will be sprayed with 2 gr/m^2. Other insecticide for use will be fenitrothion. It will be used only in some selected villages where cases are very high, vector is completely resistant to DDT, and *P. falciparum* are resistant to chloroquine. Dosage will be 2 gr/m^2 twice a year.

6.2 *Spraying operation in the Outer Islands*

Only the priority areas will be sprayed with DDT twice a year during January–March and July–September at a dosage of 2 gr/m^2.

6.3 *Antilarval Operations*

Antilarval operations will be applied to *An. sundaicus* breeding sites of high case incidence areas on weekly basis for 3–5 months during dry season, i.e., June–October. Larviciding agents and dosage will be as follows: Diesel oil + kerosine (2:1) 150 liters/Ha, Diesel oil + spreading agent—Triton x 100 (99.5:0.5) 50 liters/Ha and abate 50% E.C. 0.11 liters/Ha.

6.4 *Active and Passive Case Detection in Java and Bali*

Active case detection (ACD) will be carried out by village malaria workers on monthly or two monthly basis depending upon the availability of staff. Passive case detection (PCD) will be carried out by hospitals, health centres, sub-health centres, and polyclinics. Blood smears will be collected from all current and recent post-fever cases.

6.5 *Malariometric Surveys and Passive Case Detection in the Outer Islands*

Malariometric surveys will be carried out in all the priority operational areas to determine the impact of spraying operation. Blood smears will

be collected from infants and children up to 9 years of age on sample basis once or twice a year. Passive case detection of clinical malaria cases will be carried out in all the hospitals, health centres, sub-centres, and polyclinics. Blood smears will be collected from fever cases depending upon the availability of staff and laboratory facilities.

6.6 *Treatment*

Presumptive and radical treatment of the cases will be carried out all over the country as per guidelines provided. Following is drug regimens used in the MCP:

Presumptive & Suppressive Treatment:

—Sensitive Areas: Chloroquine, single dose, 600 mg base.

—Resistant Areas: Chloroquine, single dose, 600 mg base plus primaquine, single dose, 45 mg base.

Radical Treatment:

—Sensitive Areas: PF—Chloroquine, 1200–1500 mg. base, in 3 days plus primaquine, 15 mg. base, daily, for 3 days.
PV—Chloroquine, 1200–1500 mg. base in 3 days plus primaquine 15 mg. base daily for 5 days.

—Resistant Area: PF—a) Fansidar, single dose, 3 tablets plus primaquine, 45 mg. base.
b) Quinine, 400 mg. 3 times daily for 7 days plus primaquine, single dose 45 mg on day 1.

Prophylaxis:

—Sensitive Areas: Chloroquine, single dose, 300 mg. base, weekly.

—Resistant Areas: Fansidar, single dose, 1 tablet, weekly, plus chloroquine, single dose, 300 mg base, weekly.

6.7 *Laboratory Services*

Blood slides collected by both ACD and PCD in Java and Bali will be examined at health centre level and cross-checked at regency level. Blood slides collected by malariometric surveys in the Outer Islands will be examined at the regency laboratories. Limited blood smears collected by health centres in the Outer Islands will be examined either at health centre or at regency laboratories as per facilities available. Cross-checking of these blood smears will be done at regency laboratories in Java and Bali and provincial laboratories in the Outer Islands. Aim of cross-checking will be to evaluate the reliability of microscopists and provision of refresher training courses if so required. All positive slides and 10% of the negative slides (on random basis) on quarterly basis from each microscopist will be sent for cross-checking. Of these, the cross-checking laboratories will cross-check only 50–100% of positive

and 25–50% of negative slides (selected on a random basis) depending upon the availability of staff and their work load.

6.8 *Monitoring Drug Response*

Though a limited study on monitoring of *P. falciparum* resistant to chloroquine started in 1973, it was intensified during 1981–1983. By now 24 provinces have been tested and 22 have been found resistant to chloroquine as follows (see Map).

a. *Provinces not yet tested* (3):
Jambi, Bengkulu, and West Sumatra.

b. *Provinces tested but found susceptible* (2):
Yogyakarta and Central Kalimantan.

c. *Provinces found resistant with limited foci* (19):

East Java	North Sulawesi	North Sumatra
Central Java	Central Sulawesi	South Sumatra
West Java	South East Sulawesi	Lampung
Jakarta	South Sulawesi	West Kalimantan
Bali	East Nusa Tenggara	South Kalimantan
Riau	West Nusa Tenggara	Maluku
Aceh		

d. *Provinces where resistance is well established and widespread* (3):
East Kalimantan, Irian Jaya, and East Timor.

Monitoring of *P. falciparum* resistant to chloroquine is carried out by using *in-vitro* micro and *in-vivo* technique.

6.9 *Entomological Activities*

Entomological activities and their aims in the malaria control programme are summarized as follows.

—Determination of anopheline fauna and their seasonal distribution
—Identification of principle and secondary vectors
—Monitoring the susceptibility status of anopheline mosquitoes to insecticide
—Evaluation of the impact of DDT residual house spraying
—Investigation of bionomics of the vector/suspected vectors.

In Indonesia, entomological work has been carried out since the early fifties and even before that the Dutch workers had contributed considerably to the knowledge of the anopheline vectors.

Of the 80 anophelines species recorded in Indonesia, 16 species have so far been confirmed as vectors of malaria as follows.

Java and Bali	*An. aconitus, An. sundaicus, An. subpictus, An. maculatus, An. balabacensis.*
Sumatra	*An. sundaicus, An. hyrcanus group, An. maculatus, An. letifer, An. aconitus**

Kalimantan	*An. balabacensis, An. umbrosus, An. hyrcanus group, An. letifer,. An. sundaicus**
Sulawesi	*An. barbirostris, An. sundaicus, An. subpictus, An. letifer, An. hyrcanus group, An. flavirostris*
Nusa Tenggara	*An. subpictus, An. sundaicus, An. barbirostris, An. aconitus**
Irian Jaya	*An. farauti, An. koliensis, An. punctulatus, An. bancrofti, An. karwari.*

*Suspected vector.

6.10 *Field Trials*

6.10.1 Use of larvivorous fish, Panchax-Panchax to control rice water-breeder *An. aconitus* in Central Java and East Java.

6.10.2 Simple drainage scheme to eliminate *An. sundaicus* in the coastal areas of West Java, East Java, and Bali.

6.10.3 Use of bednets in East Nusa Tenggara and Timor Timur.

6.10.4 Use of larvicides such as fenthion, BTI, insect growth regulator (Altosid, Dimilin).

The result of the trials so far are encouraging.

More emphasis will be given to non-chemical measure in controlling malaria.

7. Use of Malaria Vaccine

The introduction of malaria vaccine with its limitation such as species and stage specificity, short period of immunity conferred, and cost will not bring dramatic changes in the control strategies.

In Indonesia, malaria vaccine will be first given to transmigrants from Java-Bali to the Outer Islands. This group of population poses high risk of contracting malaria in their new settlement as they move from very low endemic areas to a hyper or sometimes holo-endemic areas. Gradually, as conditions permit, vaccination can be extended to cover other vulnerable groups such as infants and the under-five in the highly endemic areas throughout the country as an additional effort in reducing infant and under-five mortality rate.

In East Nusa Tenggara, Timor Timur, and Irian Jaya provinces, malaria is the number one killer of infants and the under-five.

8. Future Trends

It is anticipated that with the speed up of development, coupled with higher population movement, more man-made breeding places will be created.

Therefore drastic reduction of malaria prevalence is not expected in the near future. Thus malaria would probably remain a major public health problem. Perhaps the more important short-term objective is to prevent unnecessary death and alleviate suffering due to malaria.

The primary health care approach in malaria control will be extended. Use of appropriate technology and sufficient attention paid to develop income generating activities in the rural areas are very important factors in sustaining community participation.

9. Constraints

9.1 Territorial vastness

9.2 Shortage of skilled manpower, especially in the Outer Islands

9.3 Increased vector resistance *(An. sundaicus)* to DDT in Central and East Java

9.4 *P. falciparum* resistant to chloroquine becomes more widespread

9.5 Inadequate cross-sectoral collaboration.

Discussion

Bruce-Chwatt: I'd like to congratulate Dr. Rai on his splendid presentation on the problems of malaria control and the present epidemiological situation in Indonesia. I wondered if he would expand a little on the problem of malaria, specifically in Irian Jaya, in view of the fact that this has a common frontier with Papua New Guinea and, thus, any sort of malaria control that can be attempted in Papua New Guinea will be influenced to a smaller or larger extent by the malaria situation and control in Irian Jaya.

Rai: With regards to the malaria in Irian Jaya, we do have many things to overcome, especially as the vector is more or less similar to the one in Papua New Guinea. We are now trying to find a better solution. We know that the farauti groups there breed in almost any kind of breeding place, ranging from small ponds to large lagoons. We did larviciding during the last 10 years. Recently, however, we discontinued that because the communities are using the breeding places for "cancoon" growing. "Cancoon" is a very important vegetable for the local communities in Irian Jaya. Therefore, we must find a different control measure. That is why we are now trying the insect growth regulator in Central Java. We have tried "altasit" and like to use them in Irian Jaya. Another measure is the use of bednets. We know now that in Indonesia, bednets seem to be almost forgotten. We are trying this method first in

East Nusa Tenggara with the hope that if it is successful again, we would extend to the other areas. We do realize the problems in Irian Jaya from the vector as well as the parasite point of view. As you can see from the map, *P. falciparum* resistance is most widespread in Irian Jaya. The population there is less than 1 million. They live sparsely, which makes it costly to intervene. I think some help is still needed for Irian Jaya. Although, we do obtain help from the UNDP, we fail to attract USAID and the World Bank to join us in Irian Jaya.

BRUCE-CHWATT: Are these bednets impregnated with pyrimethamine?

RAI: No, these are just ordinary bednets. We will try next with impregnated bednets.

MITCHELL: In the transmigrant populations of Indonesia, do you have people coming from one area of high endemicity to another area of equally high endemicity where you find lots of malaria in adults as related to acquired immunity, etc.? Is there an experiment in Viricomors where you have people coming from one highly endemic area to another highly endemic area of the same country?

RAI: In most instances, the transmigrants come from very low endemic areas such as Java and Bali whose API is only 1–2/1,000. In the Outer Islands, the API average is 2–5/100. So, there is quite a lot of difference. We did have one occasion where the transmigrants brought malaria to the Outer Islands. Unfortunately, we do not have any data on this. Usually, the transmigrants contract malaria from their new settlement areas.

MITCHELL: What sort of numbers are you talking about? Are they all adults? When you say they contract malaria, what do you actually mean?

RAI: I think for all age groups, it depends on the area. If the transmigrants go out of their locality, then all age groups will contract malaria since we do spray the transmigration area. But when adults alone go out of their area, for whatever reason, they alone will contract malaria.

PAIK: With regard to the use of bednets, a study of their effectiveness against malaria has been catalogued recently in Papua New Guinea. The results are rather unsatisfactory. There was a slight reduction of malaria only among children. There was no difference in the mosquito net use groups and the control group among adults. Perhaps this was due to the fact that most young children go to bed earlier, therefore missing the peak biting hour of 7:00 P.M., whereas adults would go to bed much later than 7:00 P.M. We were rather disappointed at this result.

RAI: I think it is very important that we plan to use bednets only in certain areas. We realize that there are some limitations with the use of bednets.

FREEMAN: Do you have an estimate for the present total number of deaths per year from malaria in Indonesia?

RAI: I must admit that the recording and reporting of death in Indonesia, especially from malaria, are not very good at the present. There was an estimate made in Irian Jaya and East Nusa Tenggara that in some areas more than 40% of infant deaths were due to malaria. National figures are not yet available.

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The Current Situation of the Anti-Malaria Programme in Thailand

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Introduction

The Malaria Control Division was established in 1943 with drug distribution as its chief function. At that time malaria was the leading cause of death with a mortality rate of 351 per 100,000 population. Following the encouraging results of a pilot project for malaria control with DDT residual spraying in 1949, the Government developed a countrywide malaria control programme in 1951. In 1964, a countrywide eradication programme was established, with an eight-year plan of action. Although complete coverage by DDT spraying had not yet been achieved, vast areas were cleared of endemic malaria, and the death rate was reduced to 22.8 per 100,000 population. By 1970, this rate had been further reduced to 10.1 per 100,000 population. In 1971 a new policy was developed, as a result of the WHO revised global strategy of malaria eradication, for maintaining the gains already made and for the prevention of the rise of new problem areas.

At present the Anti-Malaria Programme provides services to all the population with operations being divided into two areas as follows:

a. Control Area, consisting of forested hills and mountains, border areas, and insecure areas with a population of approximately 10.5 million.

b. Eradication Area, consisting of the major part of the country with a population of approximately 38.5 million.

The strategy implemented is that of long-term malaria control in the forested and hilly areas of the country (Control Area), and prevention of the re-establishment of malaria transmission in the remaining areas (Eradication Area).

The country has been appropriately stratified according to different levels of receptivity in association with major variations in the terrain. The main control measures are residual insecticide (DDT/Fenitrothion) house spraying once or twice a year, radical treatment centres (malaria clinics), provision of anti-malaria drugs, and health education. Other

measures are supplemented as appropriate including larviciding, space spraying, case detection and treatment, and the use of larvivorous fish. In order to establish appropriate health care delivery to all individuals in all villages, the programme has developed the Village Voluntary Malaria Collaborator (VVC) to assist in taking blood smears from and giving presumptive treatment to any person who is suspected of having malaria, and to assist in spraying operations.

Present Situation

1. *Morbidity.* The incidence of malaria throughout the country has been greatly reduced during the past 35 years. In 1947 the morbidity rate was 286 per 1,000 population. This rate was reduced until the period of 1966–1972 when the incidence appeared to stabilize, varying from 2.2 to 3.6 per 1,000 population. Since that time it has risen annually, to 7.1 in 1979, and 10.6 in 1981 (Table 1). The increase in 1979–1981 in malaria incidence is attributed mainly to population movements and problems of parasite resistance to drugs. However, the increasing trend in malaria morbidity, which reached a peak in 1981, had shown a downward trend in early 1982 which persisted through 1983. The reasons for this improvement are not completely clear, but the most plausible explanation may be the striking increase in the numbers of malaria clinics and productive malaria village volunteers, resulting in earlier diagnosis and appropriate therapy. In 1979, the Malaria Division had established 174 malaria clinics located at various malaria offices and field sites. Based upon results and epidemiological data accumulated, it was determined that the effectiveness of malaria clinics could be increased by extending the services outside the malaria offices to strategic locations in high transmission areas, marketing centres, and on migratory routes. At present there are 454 malaria clinics. The annual parasite incidence for fiscal year 1983 is the lowest in the last 10 years (Figure 1).

2. *Mortality.* In 1947 malaria claimed some 40,000–50,000 lives annually with the death rate of approximately 300 per 100,000 population. Now it is the seventh-ranking cause of death nationwide, preceded by accidents, heart disease, malignant neoplasms, respiratory tuberculosis, pneumonia, and diarrhoeal diseases. The mortality rate from malaria in 1974 was 15.8 per 100,000 population, decreasing to 4.4 in 1984, which is the lowest mortality rate from malaria ever recorded in Thailand (Figure 2). The steady decrease in the mortality rate since 1974 is due to the expansion of peripheral health care delivery and the establishment of malaria clinics providing prompt diagnosis and treatment in areas of high transmission.

3. *Parasitology.* The three species of Plasmodium found in Thailand

TABLE 1. Results of Surveillance Activities (1979–1984)

	1979	*1980*	*1981*	*1982*	*1983*	*1984*
Population covered by surveillance	42,701,040	44,230,292	44,815,133	46,028,197	46,924,325	48,595,751
Blood smears[1] examined	4,287,282	4,852,919	5,583,689	6,094,665	5,365,960	6,666,441
Positives	302,721	395,442	473,210	420,799	243,910	306,569
SPR[2]	7.0	8.1	8.5	7.0	4.5	4.6
ABER[3]	7.2	8.1	8.9	9.1	8.2	8.2
API[4]	7.1	8.9	10.6	9.1	5.2	6.3

1. Blood smears taken by Active and Passive Case Detection and Mass Blood Surveys
2. SPR = Slide Positivity Rate (%)
3. ABER = Annual Blood Examination Rate (%)
4. API = Annual Parasite Incidence per thousand population

are *falciparum*, *vivax*, and *malariae*. The parasite formular in 1984 showed 70.7% *P. falciparum*, 29.0% *P. vivax*, and 0.02% *P. malariae*.

P. falciparum started showing poor response to chloroquine in 1962, and at the present time it is found that more than 95% demonstrate resistance, mainly at R II and R III levels. Furthermore, resistance to sulfadoxine-pyrimethamine occured and spread throughout the country during the last few years.

4. *Entomology*. To date, three species of anopheline mosquitoes have been proven responsible for malaria transmission in Thailand, namely *Anopheles balabacensis (An. dirus)*, *An. minimus*, and *An. maculatus*. *An. minimus*, considered to be the most important vector, is prevalent in forested and cleared forested foothill areas with slow running streams throughout the country. *An. balabacensis (An. dirus)* is the other main malaria vector. Its area of prevalence can be taken to coincide with the distribution of forest land. *An. maculatus* has been incriminated as a vector in southern Thailand. *An. sundaicus* and *An. aconitus* are considered as secondary vectors in some particular areas. The result of DDT susceptibility tests carried on the principal and secondary vectors have shown that they respond favourably to the insecticide. However, in the presence of DDT residual spray, the vectors show a higher density of outdoor biting.

Problems

At present there are three major problems confronting the Anti-Malaria Programme in Thailand. These include the rapid dissemination of *P. falciparum* strains highly resistant to both 4-aminoquinolines and sulfadoxine/pyrimethamine drugs, the exophilic behaviour of malaria vectors, and the occupational migration of the people.

For many years chloroquine was the mainstay of anti-malarial chemotherapy. It is still an effective treatment for *P. vivax* infections. However, in the late 1950s *P. falciparum* clinical treatment failures with chloroquine were noted. Studies in twenty provinces applying the *in vitro* macro technique, in 1977–1981, showed chloroquine resistance in 97% of isolates tested.[1]

The combination of sulfadoxine/pyrimethamine was an effective replacement until the mid-1970s when resistance was first noted along the Kampuchean border. Resistance to this combination is now widespread but is strongest in the Thai-Kampuchean border provinces and in areas involved in the migration to and from these areas.[2]

The regimen which has replaced sulfadoxine/pyrimethamine in areas of high resistance is a combination of quinine and tetracycline. This combined course, while generally effective, is difficult to supervise and, because of the side effects of quinine, compliance in outpatients is poor over the necessary seven days of treatment.

Mefloquine promises to be both an effective and an operationally feasible substitute for quinine-tetracycline. The Anti-Malaria Programme has been involved in a number of field clinical trials of both mefloquine and the triple combination mefloquine-sulfadoxine-pyrimethamine. A large-scale field trial of the triple combination has been carried out in selected border areas. The results show that this drug has a potent blood schizontocidal activity against strains of *P. falciparum* resistant to other anti-malarial drugs. It gives a cure rate of greater than 95% and is very well tolerated. Following this large-scale trial involving some 60,000 falciparum malaria cases, the combination drug has been introduced into the malaria programme from January 1985, to be used as the first line drug for the treatment of falciparum malaria.

Regarding the exophilic behaviour of the vectors, the principal techniques employed to overcome this problem are the use of larvivorous fish, thermal fogging with Fenitrothion in epidemic situations, and encouraging the use of personal protection measures.

The third problem is that of occupational migration of Thai people to the Kampuchean border area for gem mining and farm labour as well as local migration from villages to forests for wood cutting and expanded cultivation in foothill forest fringe areas. This problem has been addressed through increased health education by stressing the importance of personal protection measures such as mosquito nets and repellent.

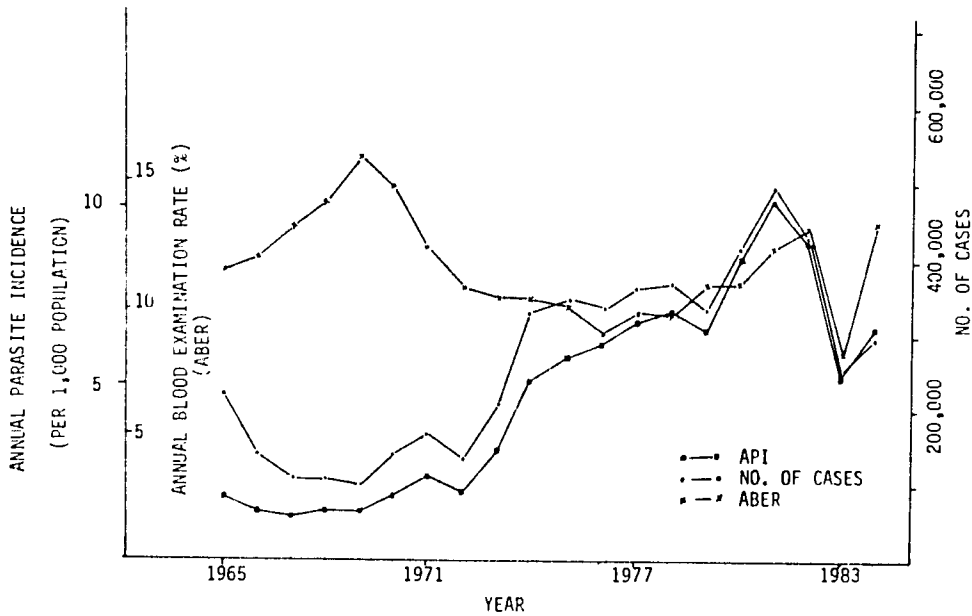

FIGURE 1. Malaria Morbidity in Thailand From 1965-1983.

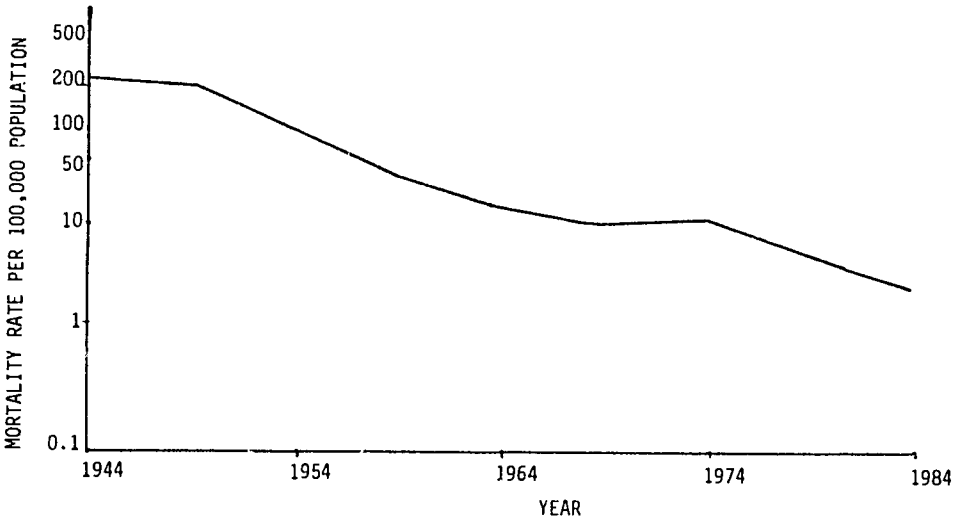

FIGURE 2. Mortality Due to Malaria in Thailand, 1944–1984.

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Discussion

DULAY: Your reduction in mortality appears to be very impressive. You have utilized various combinations of measures to combat the numerous problems. You did spray and you did establish a primary health care worker for nearly every village. This, along with the use of primary health care workers in the village. How would you ascribe the reduction of mortalities?

SURIN: The reduction of mortalities is the result of complex activities, not only from the main activities of the malaria programme, but also from the development of health infrastructures throughout the country. We have what is called a community hospital in every district. One district covers about 50,000 people. There is also a provincial hospital in every province. One province covers about 200,000 people. These are in addition to our malaria control programme. We have not really done studies to show to what effect we can reduce malaria mortality. But, according to our malaria clinics located in most of the rural areas, we

have received very good cooperation from the villagers. They come to the clinics at once if they suspect they have malaria. They then receive microscopy diagnosis and treatment within 20 minutes. This is one of the reasons.

MITCHELL: I am interested in these 500 malaria clinics that you were mentioning just now. Could you expand a little on what the malaria clinic actually is in terms of staffing, facilities, monitoring, etc.?

SURIN: The activity is very simple. We have only one or two microscopists who we train to take blood slides when the suspected people come to visit the clinic. They perform microscopy diagnosis that identifies the malaria species and key treatment. We need only one or two lower level staff to do this plus one microscope. As for the space, we may use a health center or some villager's house. Right now, the people are very supportive in donating or building facilities for this cause.

PERRIN: You have a lot of experience in the treatment of sulfadoxine assisted by pyrimethamine. There have been many questions raised concerning the secondary effects of this combination. What is your experience in Thailand concerning side effects due to the administration of sulfadoxine/pyrimethamine?

SURIN: We have used sulfadoxine/pyrimethamine for the last 10 years covering around 400,000 people per year. That means that during the last 10 years, we have covered at least 4 million people. We have never found serious side effects from that regimen, although there were some minor side effects.

COPPEL: I was interested if you could give us a bit more epidemiological background about areas where multiple drug resistance is common. Have you defined demographically and epidemiologically some areas that may be useful as sites for vaccine trials where drug resistance is extremely common so that you will not have the complicating factor of people self-treating themselves and thus dropping the incidence and confusing your measure of efficacy?

SURIN: Chloroquine is all over the country but sulfadoxine/pyrimethamine has been found first in one area. During the early period, we have found that the resistance to sulfadoxine/pyrimethamine started around the Thai-Kampuchean border. It is a mining area, so a lot of people migrate from there. We had then found other areas which showed high resistance of *P. falciparum* to sulfadoxine/pyrimethamine. Now, however, we find this resistance everywhere. Therefore, we cannot localize which areas would be suitable for vaccine. But as I mentioned, it may be utilized for control of malaria among the migrant population.

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Malaria: Current Situation and Future Control Strategies in India

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Introduction

Malaria was a major public health problem in the entire Indian subcontinent except in the north Himalayan ranges above 5,000 ft. sea level and a narrow coastal belt of western and eastern ghats. The adverse effects of malaria on human life were stated by the public health commissioner of the Government of India in 1931: "The combined effects of cholera, plague and small pox were responsible for less than 5 out of every 100 deaths, whereas 60% of the total mortality in the country was ascribed to fevers of which one third to one fourth are probably deaths due to malaria." Noted malariologist Sinton described the situation: "The problem of existence in very many parts of India is the problem of malaria.[1] There is no aspect of life in this country which is not affected either directly or indirectly by this disease. It constitutes one of the most important causes of economic misfortune, engendering poverty, diminishing the quantity and the quality of the food supply, lowering the physical and intellectual standards of the nation and hampering increased prosperity and economic progress in every way." In 1947 it was estimated that 75 million people suffered from the disease and 800,000 died annually. Mortality due to malaria increased considerably during the epidemic years. Malaria was a major impediment and scourge for the successful implementation of developmental projects and human welfare. This situation took a dramatic turn when DDT was made available for use in public health. From 1946 onwards the Government of India launched several feasibility projects on malaria control. These projects utilized DDT as the residual insecticide and successfully interrupted malaria transmission in U. P. terai, Rayagada, Malnad, and Wynad. This was soon followed by similar demonstrations in Karnataka, Maharashtra, West Bengal, and Assam. By 1952

about 30 million of the population were being protected from malaria. Based on the success of its pilot projects, the Government of India launched the National Malaria Control Programme (NMCP) in 1953. Progress of malaria control under NMCP was so spectacular that in 1958, the Government of India converted NMCP to the National Malaria Eradication Programme (NMEP). As a result of the nationwide spraying of residual insecticides, malaria was nearly eradicated from the country, and at one time it appeared as though the battle against malaria had been won completely. The euphoria of success resulted in the conversion of the one time world famous Malaria Institute of India to the National Institute of Communicable Diseases. Research on malaria was de-emphasized and publication of one time famous *Indian Journal of Malariology* was terminated as there were neither the readers nor the subscribers. Still in 1965 there were at least 100,000 cases of malaria in the country. Slowly and gradually malaria cases started building up, and during these years, there was little preparedness to combat the disease. As a result in 1976, NMEP recorded at least 6.4 million parasite positive cases of malaria. Deaths due to malaria also started to increase (see Table 1).

During the early 1970s, the Government of India realized that eradication of malaria was not a realistic goal, that it was impossible to achieve. Therefore, there was a need for a more realistic approach to malaria control. A revised strategy known as the modified plan of operations (MPO) was launched by the NMEP in 1977 with the following objectives: (i) to eliminate deaths due to malaria, (ii) to reduce morbidity due to malaria, (iii) maintain the green revolution and industrial development, and (iv) retention of the achievements gained. In order to meet these objectives, inter alia, all areas with 2 or more cases per thousand population per year (2 or more API) were to be sprayed with residual insecticides and drugs made available to every fever case throughout the country. There were other technical and administrative changes as well.[2]

Implementation of MPO resulted in the improvement of the malaria situation. Malaria cases gradually declined from 6.4 million in 1976 to 2.0 million in 1983 (see Table 1). The MPO was also augmented with 2 additional schemes, i.e., the urban malaria scheme (UMS) and the *Plasmodium falciparum* Containment Programme (PfCP). Launched in 1971–1972, UMS was implemented in towns with >40,000 population and the API of 2 or more. It was envisaged that 132 towns would be covered under the scheme in a phased manner. As of now, 125 towns covering a population of 43 million are under urban malaria scheme.[3]

PfCP was launched in 1977 with its immediate objective being to improve the system of case detection and laboratory services, drug distribution, treatment, management of serious cases, and insecticidal

TABLE 1. Malaria in India

Year	*Total Positives*	*SPR*	*P. falciparum Cases*	*SFR*	*P. falciparum %*	*Deaths*
1961	49151	0.375	17144	0.131	36.8	—
1962	59575	0.223	16674	0.062	28.4	—
1963	87306	0.225	22750	0.059	26.4	—
1964	112942	0.26	31490	0.073	27.9	—
1965	100185	0.25	26163	0.066	26.3	—
1966	148156	0.37	39618	0.099	26.78	—
1967	278622	0.69	61984	0.154	22.38	—
1968	274881	0.65	92714	0.219	33.74	—
1969	348647	0.83	103217	0.246	29.70	—
1970	694647	1.72	100115	0.245	14.46	—
1971	1312118	2.27	148683	0.255	11.31	—
1972	1430025	3.65	142177	0.363	9.34	—
1973	1934485	4.56	265154	0.626	13.14	—
1974	3172866	7.10	476922	1.069	14.39	3
1975	5166142	9.97	729251	1.395	14.12	99
1976	6467215	11.55	753713	1.346	11.70	59
1977	4740900	8.32	461484	0.807	9.70	55
1978	4144385	6.85	548567	0.907	13.20	74
1979	3064697	4.99	558423	0.909	18.22	198
1980	2896600	4.32	586438	0.875	20.24	207
1981	2566244	3.04	574814	0.871	21.55	170
1982	2182393	3.34	551057	0.84	25.25	187
1983	2018605	3.13	600964	0.93	29.77	239
1984*	1921264	3.23	503100	0.85	26.45	236

* — Provisional
Source: NMEP

spraying. The intermediate objectives were to intensify spraying to intercept transmission and prevent the spread of drug resistant strains. In order to accomplish these objectives, epidemiological teams were established in strategic areas for the monitoring of two-way population movement from areas known to have foci for drug resistant strains and also to take such measures as were necessary to deal with the problem of malaria under each situation.[4]

The scheme (PfCP) was launched in areas with a high rate of *P. falciparum*. During the period of resurgence it was observed that 55 districts had about 70–80% of all *P. falciparum* infections that occur in India. Of these, 18 districts of the northeastern region accounted for 80% of *P. falciparum* infections. PfCP was initially launched in these districts with a view to check the spread of falciparum malaria to other areas. Based on the information elicited by the surveillance, PfCP was extended to several other states. At present PfCP operations are divided into 4 zones covering a total of 79 districts in the country.

Falciparum Malaria in India

Before launching of the NMCP/NMEP, the proportion of *P. falciparum* infections averaged 46% in 1930 and 40% in 1950.[5] Spraying of residual insecticides produced dramatic reductions in malaria incidence. During the heyday of malaria control, the disease was eradicated from more than 50% of the country, in 25% of the country the eradication programme was in an advanced stage, and in the remaining 25% spraying operations were ongoing, which included the hard core areas. It appeared as though malaria was about to be eliminated, as there were only about 100,000 cases in the entire country in 1965. The success in malaria control was spectacular but short lived, and in subsequent years there were focal outbreaks even in areas that were in the maintenance phase. The main reasons for the resurgence were the shortages of DDT, presence of foci in urban areas, persistent malaria transmission in hard core areas which constituted 9% of the population, and general health services which were not mature enough to accept the responsibility of the maintenance phase.[6] As a result, between 1965 to 1976 malaria cases increased 65 times and the proportion of *P. falciparum* varied from 10 to 15% (Table 1). The implementation of the modified plan of operations in 1977 produced a steady reduction in the total number of malaria cases, but the incidence of *P. falciparum* stabilized at about 0.5 million cases annually, i.e., there was an increase in *P. falciparum* proportion in the country from about 10% in 1977 to 30% in 1984. It may be noted that while there was an increase in *P. falciparum* in areas outside the PfCP zones, there was a considerable reduction in *P. falciparum* in areas under PfCP (A. P. Ray, coordinator, PfCP NMEP, Delhi, personal communication). Reasons for this selective drop in vivax malaria could be that (i) *P. vivax* is sensitive to the commonly used drug chloroquine, and the presumptive dose of 600 mg chloroquine (4 tablets) is enough to clear the parasitaemia. Also the drug is available freely in all parts of the country through a network of DDCs, FTDs and other agencies. (ii) In vivax malaria, the relapse rate is low, i.e., 20% of the infections show a first relapse, 8% second relapse, 4% a third relapse and 2% a fourth relapse.[7] (iii) Chloroquine is gametocitocidal in *P. vivax* and not in *P. falciparum*, so that presumptive treatment also stops transmission of vivax malaria. (iv) In *P. falciparum* primaquine is to be administered to kill the gametocytes, and DDCs give only chloroquine as a presumptive treatment, thus all falciparum malaria cases with gametocytes continue to produce secondary cases. (v) There are also fears of primaquine induced haemolysis in G-6-PD deficient cases, which prevent the use of primaquine. Thus the transmission continues, even though the patient is relieved of the fever as a result of the schizontocidal action of the chloroquine. (vi) There is also a clear evidence of the decreasing

sensitivity of *P. falciparum* to chloroquine so that recommended dosages are not enough resulting in recrudescences. (vii) Chloroquine facilitates transmission of resistant Plasmodia through the vectors thus enhancing the spread of falciparum malaria,[3] and (viii) *P. falciparum* has a biological advantage of high multiplication rate, thus increasing the parasite load and enhancing transmission.

In many states NMEP has recorded a considerable increase in *P. falciparum* cases during 1984 over 1983. For example, the increase was: Andhra Pradesh 44%, Himachal Pradesh 675%, Jammu and Kashmir 209%, Maharashtra 17%, Meghalaya 40%, Orissa 11%, Punjab 57%, Tripura 35%, Uttar Pradesh 19%, West Bengal 49%, Chandigarh 90%, Goa 113%, and Mizoram 9%. The situation in Orissa did not improve much and about 40% of all *P. falciparum* infections occurred in one state alone. There are similar examples of the increase in falciparum malaria from several parts in the country.[9,10,11]

The increase in falciparum malaria is also noticeable in areas which were exclusively vivax malaria areas after the near eradication of malaria. A classic example is that of U.P. terai (distt. Nainital). In this area, intensive surveillance on a daily basis in 1981–1982 revealed only 23 cases of falciparum malaria in Kichha PHC (Pf% 1.3) and surveys in other PHCs showed only isolated cases of falciparum malaria. In subsequent 2 years *P. falciparum* increased enormously, outnumbering vivax malaria (Pf% 70–100). Also in certain areas there are epidemics of malaria resulting in deaths like in Shahjahanpur.[12] In this area even a good coverage with 4 rounds of BHC spraying and adequate availability of drugs in the affected villages did not improve the situation. The SPR was 53.8 and Pf% 94 during the epidemic year in 1983 and during 1984 the SPR was 46.2 and Pf% 74.6. Thus the Indo-UK Workshop scientific group which discussed falciparum malaria in India concluded that the independent studies by the Malaria Research Centre (MRC) and by a WHO/PfCP team have indicated that the NMEP figures greatly underestimate the extent of the falciparum problem.[13]

Studies on the epidemiology of malaria showed that vivax malaria cases start increasing from March, reaching their peak in July–August followed by a sudden decline to low numbers. The incidence of falciparum malaria is very low from March to May with cases gradually building up in the following months, peaking in October–November followed by a sudden drop to low numbers during the winter months. During the same period in the same community mass blood surveys showed similar peaks of vivax and falciparum malaria (Fig. 1a & b), and that the proportion of atypical or inapparent malaria cases was equal or more as compared to febrile episodes in the villages.[9] Subsequent studies showed similar peaks of vivax and falciparum malaria from several parts of the northern states.[10,11]

The evolution of insecticide resistance in vector species is resulting in an increasing demand of the replacement insecticides. These insecticides are more toxic and many times more expensive. It is also certain that in the course of time, resistance would evolve against these insecticides as well. The escalating cost of insecticides would alone make the strategy of intradomiciliary spraying unacceptable. Added to this is the fact that several studies in the field areas have shown that ongoing spraying is only helpful in reducing the incidence of malaria to some extent and preventing the onset of epidemics. It is against this background that it appears that the present strategy of malaria control is at best controlling malaria on borrowed time. There is therefore an urgent need to intensify research on the integrated control strategy with minimum reliance on insecticides. Towards this objective a study in Nadiad has shown that it is possible to control malaria by environmentally safe and sound methods without the use of insecticides.[14]

Problems in Malaria Control

Malaria control in India is faced with formidable administrative, operational, and technical problems accentuated with financial constraints. Some of the important technical problems are discussed below.

Development of multiple resistance in vector species is the foremost problem in the interruption of malaria transmission. There are 7 primary vectors of malaria in India. Of these *Anopheles culicifacies* is the vector of rural malaria, others being of local importance. This vector has become resistant to DDT and BHC in most parts of the country. Resistance to malathion has also appeared in Gujarat and Maharashtra. As a result insecticidal spraying as a means to control malaria is not having a cost effective impact on transmission. The second vector *Anopheles stephensi* transmits malaria in urban areas. Although monitoring of resistance has shown that the vector has become resistant to DDT and BHC in many towns of India, under the urban malaria scheme main reliance is on the source reduction and anti-larval methods. Therefore, resistance is not an obstacle in the control of *A. stephensi*. *Anopheles minimus* and *Anopheles fluviatilis* are still susceptible to insecticides and a good spray can interrupt transmission. At one time spraying under NMCP/NMEP almost eradicated *Anopheles minimus*, but in recent years there has been a considerable increase in its populations and its role in malaria transmission has been re-established.[15] *Anopheles sundaicus*, a one time vector in Orissa and West Bengal and particularly notorious in Chilka lake area, is now almost exclusively found to transmit malaria in the Andaman and Nicobar Islands. The vector is susceptible to insecticides but prefers to rest and feed outdoors. *Anopheles dirus* and *Anopheles philippinensis* are also susceptible to insecticides and transmit malaria in northeastern states, and particularly latter species influence transmis-

sion up to West Bengal. Sizable populations of these species are exophilic and exophagic in behaviour, and the intradomiciliary spraying of residual insecticides is not very useful in the control of malaria. The technique of control in these vectors would have to be modified to take advantage of the weak links in the biology of these vectors instead of complete reliance on the spraying. It is therefore obvious that the problem of insecticide resistance essentially relates to the control of *Anopheles culicifacies*.

A serious problem in the control of malaria with the ongoing insecticidal spraying is the poor coverage of houses mainly as a result of refusals. There are many reasons for this, foremost being the poor response of mosquitoes to spray, lack of colateral benefits, and house holders not allowing their cement houses to be sprayed. It may also be pointed out that spraying of chlorinated hydrocarbon insecticides like DDT is not very effective in the control of Culex mosquitoes, whereas DDT spraying may kill the anopheline mosquitoes. The presence of Culex mosquitoes in large numbers make the villagers believe that spraying was not at all effective, thus adding to refusal rates. The spraymen are only half willing to carry out their job. There is also the lack of experienced supervisory staff, so that the quality of spraying is wanting. It has been observed that there are generally no refusals for spraying the cattlesheds, so that in a village more cattlesheds are sprayed than houses. Because of the excito-repellent action of DDT, vectors prefer to enter houses than cattlesheds. As a consequence, there may be some reduction in vector densities, but without any tangible impact on malaria transmission.

A serious problem that is raising its head is the development of chloroquine resistance in *P. falciparum*. The first report of resistance in *P. falciparum* to chloroquine came from Diphu and Nowgong in Assam in 1973.[16] This was followed by several similar reports from Assam, Meghalaya, Arunachal Pradesh, U.P., Mizoram, Orissa, Nagaland, Maharashtra, Andhra Pradesh, Madhya Pradesh, Andaman and Nicobar Islands, Karnataka, Haryana, and Delhi.[17] This delimitation of the problem was possible through a systematic monitoring programme of drug resistance sponsored by the Indian Council of Medical Research under the auspices of the NMEP. It may be noted that under the MPO, chloroquine is being distributed free in the country through the drug distribution centres (DDCs) and the fever treatment depots (FTDs). These have been established at the rate of 3 DDCs and 1 FTD per 10 thousand population, and as of now 2,49,669 DDCs and 1,24,552 FTDs are functioning in the country. DDCs give chloroquine to all fever cases and FTDs while dispensing presumptive treatment also collect blood smears for radical treatment. As a result, chloroquine is the most widely distributed antimalarial. It has now been established from several parts in the world that although resistance may be spreading

geographically as a result of the movement of population and/or vectors, pressure of chloroquine over a long period may bring about poor response of chloroquine in the clearance of parasitaemia.[7-17] In India, although drug resistance is found in many states, the most common type is RI. RII is also occasionally encountered and RIII resistance has been recorded from 8 PHCs of 6 states in the country. There are a number of other problems which are serious obstacles in the successful control of malaria. Some of these are the rising cost of insecticides, antimalarials, and labour, e.g., poor surveillance, lack of intersectoral cooperation, inadequate manpower as the key posts are not filled, and complacency of health staff including the fact that the priority is given to family planning at the cost of malaria work.[18-19]

Among the inputs required to increase agricultural production, irrigation is contributing to the increasing incidence of malaria. Early work in Punjab and Pattukkottai had shown that there was an intimate association of *A. culicifacies* with irrigation water, and that irrigation increases the incidence of malaria[20-21] Rao reported that the opening of Visvesvarya canal in Mandaya district (Karnataka) increased the average enlarged spleen rate from 15.3 to 50-90%.[22] There was a 20-fold increase in deaths due to fever. Within 3 years of the introduction of irrigation the conditions had settled down to a state of hyperendemicity. In India, areas under irrigation have increased from 29m hec. in 1960 to 60m hec. in 1984, and as a consequence of this large epidemic areas in Punjab, Haryana, Delhi, and U.P. have become endemic to malaria. A recent study near Delhi showed that canal-irrigated areas were more prone to malaria than the tube well irrigated areas. Also there was a considerable increase in falciparum malaria in the canal-irrigated areas during the latter part of the year.[23] Russell stated that it was not the irrigation *per se* but defective and untidy irrigation which, by misplacing to the advantage of certain anopheline mosquitoes, generates malaria.[24] It is commonplace that canals are not lined and are poorly maintained in the country. Sharma and Mehrotra stated that the unplanned growth in urban and rural housing and lack of adequate water disposal facilities create mosquitogenic surroundings and increase the receptivity to malaria.[24] Irrigation also increased the average humidity of the atmosphere and made the regions more conducive for the mosquitoes' survival. This had the most profound affect on the basic reproduction rate. As a result there was resurgence of malaria in large parts of the country at one time freed from the disease.

Tackling the Problems

One method to tackle the problem of insecticide resistance is the use of replacement insecticides. However, mosquitoes have the ability to evolve multiple resistance. The newer insecticides are more toxic and

expensive. As an example, the cost of spraying 1 million population in 1985 would be Rs. 2.2 million for DDT, Rs. 3.2 million for BHC, and Rs. 20.0 million for malathion, and the cost of some newer insecticides may be many times more. During 1984–1985, India's budget for malaria control was Rs. 1,650 million, of which Rs. 1,000 million was for the purchase of insecticides, i.e., Rs. 340 million for DDT to cover 210 million population, Rs. 300 million for BHC to cover 110 million population, and Rs. 360 million to cover 21 million population. Any significant increase in the purchase of insecticides, particularly malathion, or change over to newer insecticides like fenitrothion is not possible because of the financial limitations. It is therefore absolutely essential to extend the useful life of the cheaper insecticides like DDT and BHC. Towards this objective, a study was launched in Faridabad villages (Haryana) to demonstrate the usefulness of DDT in DDT resistant *A. culicifacies* areas. In this area, WHO tested the susceptibility to insecticides and showed that 88% mosquitoes survived one hour exposure on 4% DDT impregnated papers. DDT was sprayed under the supervision of the MRC staff and a 1 gm/m^2 DDT deposit was ensured in 70 - 80% structures. Spraying reduced vector densities, and the slide positivity rate was reduced to half.

The study was repeated in 1984 in Loni PHC (Ghaziabad, U.P.). In this area due to DDT resistance in *A. culicifacies* BHC was being sprayed for several years. But this spraying has not produced a tangible impact on malaria transmission. Spraying of DDT @ 1gm/m^2 and BHC @ 200mg/m^2 in these areas reduced vector densities and malaria to low levels as compared to BHC sprayed @ 200mg/m^2 by the health department. The study revealed that correct dosage application and increased coverage of houses were important to produce epidemiological impact of spraying and that DDT spraying was better than BHC spraying.

The problem of drug resistance is being tackled by the NMEP/PfCP by implementing alternate drug schedules in areas with resistant foci.[18] The spread of drug resistance to other regions of the country is being prevented by a systematic action-oriented programme such as (i) the area is demarcated for monitoring, treatment, and spraying; (ii) insecticidal spraying is intensified to curb transmission; (iii) monitoring is extended to delimit the problem; and (iv) the treatment schedule is changed to (a) presumptive treatment (ACD, DDC and FTD): Amodiaquine 600mg and PCD sulphalene 1000mg + pyrimethamine 50mg as a single dose; (b) radical treatment: sulphalene 1000mg + pyrimethamine 50mg + 45mg primaquine 45mg in a single dose. The migratory labour population leaving the PfCP zones or high *P. falciparum* areas are given mass drug administration of chloroquine 600mg + primaquine 45mg in a single dose. For radical cure chloroquine 1500mg (600 day 0, 300 day1 & 2 each) + primaquine 45mg on day 0 is given. If there is no response within 72 hours, radical cure of long-acting sul-

phonamide in a single dose is given as radical treatment. Primaquine is not given to pregnant women and children. The implementation of the above measures were highly successful in reducing/eliminating the drug resistance foci from many parts in the country. Major focus of drug resistance in Karbi Anglong (Assam), Chandrapur (Maharashtra), and Mirzapur (U.P.) was curbed.

A long-term strategy to control malaria by integrating methodology without the use of insecticides is being developed in Nadiad Taluka, distt. Kheda, Gujarat. The main components of this strategy are (i) source reduction, (ii) biological control using larvivorous fish, (iii) chemotherapy, (iv) health education, (v) community participation, and (iv) environmental improvement by introducing social forestry, alternate energy sources, etc.

Alternate strategy is being integrated with other developmental projects to make it a holistic approach for the evolution of villages towards the Gandhian way of life. The study aims to generate village economy to help sustain vector control at the village level by social forestry, fish culture, and other schemes. It was initially launched in a population of 25,000. In one year (1984) malaria incidence was reduced by 64% from the previous year or from the comparison area, and if one compares the reduction from 1981 or 1982, when malaria was controlled using BHC and malathion, the incidence was reduced dramatically to low levels. The study has now been extended to 100 villages (0.35 million population) and research is being directed to make it cost effective. Cost projections to control malaria in 100 villages of Nadiad Taluka revealed that a 3-year expenditure of malaria control would be Rs. 2.4 million with DDT, Rs. 3.6 million with BHC, Rs. 21.0 million with malathion, and Rs. 5.5 million with integrated control methodology. The cost is therefore affordable, and if benefits from other schemes that are integral to the control strategy like fish culture, social forestry, environmental improvement schemes, etc., are taken into account, the cost of malaria control may be absorbed almost entirely. The adoption of this method in large parts of the country would, therefore, on its own eliminate the problems of insecticide resistance, drug resistance, and those related to the behaviour of mosquitoes.

Role of Malaria Vaccines

It is against this background that the importance of malaria vaccines comes out. An ideal vaccine should provide long-lasting immunity from the clinical attacks of all Plasmodia, perferably in a single dose and without side effects. Malaria vaccines should first be tried in regions of stable malaria. During the heyday of malaria control, the technique of intradomiciliary spraying of insecticides failed in 9% of the population

which remained in attack phase for more than 11 years. In such areas, malaria vaccines could be tried to elicit information on the true value of immunization against malaria. Vaccines would also find easy acceptability for clinical trials in areas prone to epidemics such as Shahjahanpur, Mathura, and similar areas where deaths due to malaria occur periodically.

Vaccines would be of immense value in protecting the tourists who come from non-immune areas and become victims of serious episodes of malaria. The estimated tourist flow in India is about 1 million. The industry earns foreign exchange of about Rs. 11,000 million annually. Tourists may have a genuine fear of malaria, and this may adversely affect the tourist flow. Even a short protection period of a few weeks would be a great help and save life. Similarly vaccination of migratory labour from endemic to non-endemic areas could prevent the spread of drug resistant and other variant strains. The urban population and defense personnel would make greater use of malaria vaccines. In rural India, even if mass vaccination is not possible, immunization of children would be highly beneficial, as this age group is most vulnerable to malaria. Integration of malaria vaccines with the ecologically safe methods of malaria control under development in Nadiad would be very useful in protecting areas from introduced malaria. Vaccines could be used in immunizing the incoming and outgoing populations and also other vulnerable groups to prevent the onset of secondary cases. A notable feature of the malaria situation in India is the selective decline in the vivax and not the falciparum malaria, and therefore vaccine against *P. falciparum* can play a vital role in lowering falciparum malaria by mass immunization of people living in *P. falciparum* dominant areas.

In India, areas above 5,000 ft. sea level and a narrow belt along the western and eastern ghats are naturally free from malaria, and other regions have variable endemicity. These areas could be stratified according to the vector influence or graded according to the endemicity. At present, the National Malaria Eradication Programme of the Government of India is protecting 350 million population living in rural areas. This does not include areas/sections under 2API, and in the same geographical area there may be sections (section=10,000 population) with <2API which are not sprayed. Therefore these regions with malariogenic potential continue to be the vulnerable groups. A sudden spurt in vector populations due to favourable conditions may bring an epidemic. Macdonald had shown that with an initial parasite rate of 0.1% (i.e., 1 introduced case in a village of about 1,000 population), how epidemics affecting 50% of the population can occur in 7 months even if the reproduction rate is as low as 5, and only 3 to 4 months if the reproduction rate is 20.[26] Therefore even in areas where either the malaria control measures have been successful or in areas with <2API, a con-

stant surveillance is important. Malaria vaccines can play an important role in preventing the fulminating epidemics to occur.

Success in immunization against malaria would depend on the protection provided by the vaccine, side effects, and the application procedures. At present immunization against malaria in large parts of rural India is difficult unless special preparations are made. This may take time in training and integration of the immunization programme with the primary health system.

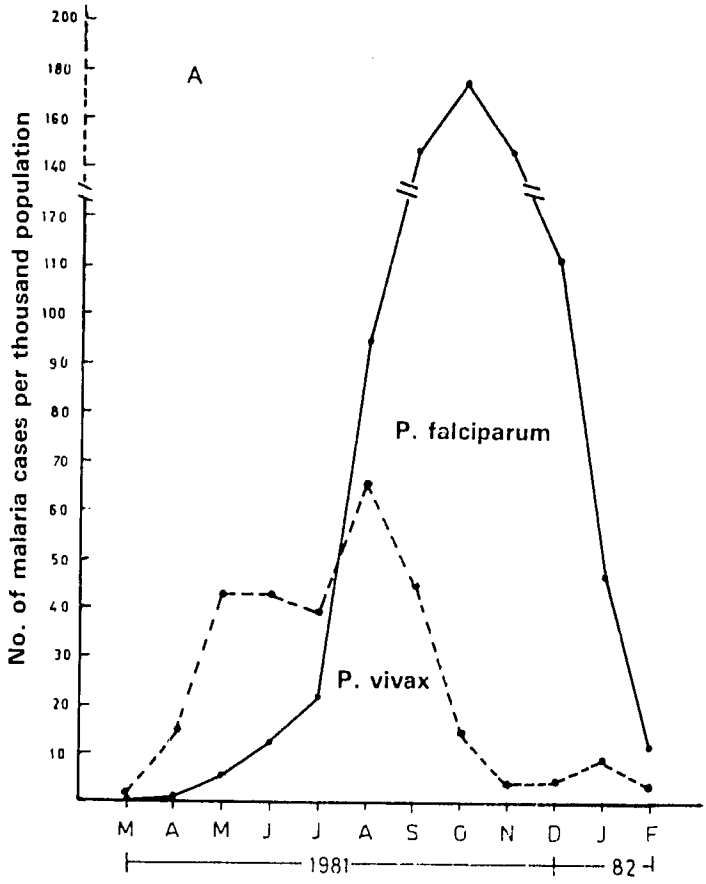
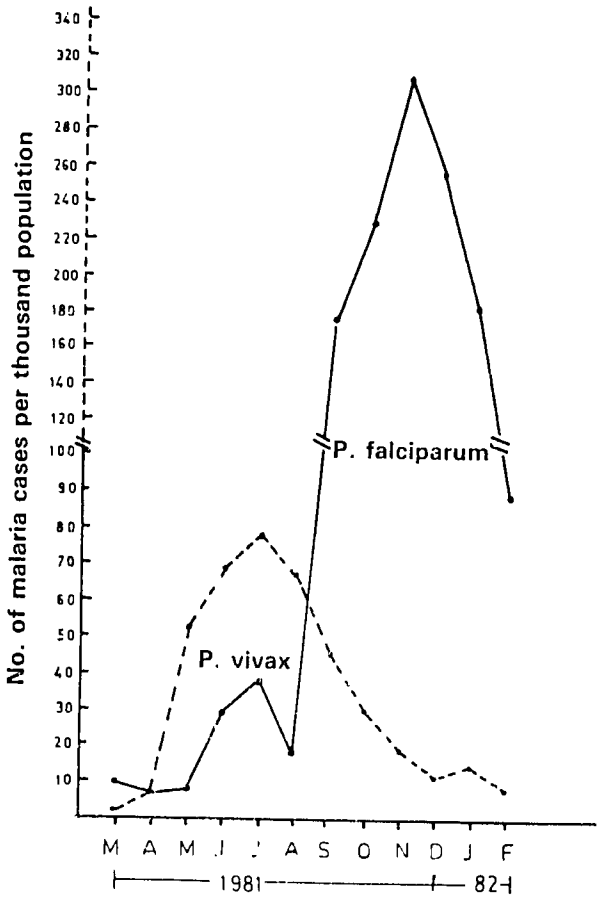


FIGURE 1. Showing peaks of vivax and falciparum malaria in Kharkhoda PHC (Haryana) as detected simultaneously by (A) active



surveillance in fever cases, and (B) mass blood survey in afebrile or inapparent cases of malaria."

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Discussion

MEUWISSEN: What agents would you use for the preliminary control?

V. P. SHARMA: What was happening was that villagers were auctioning ponds for the collection of fish. These villagers were removing all the fish for periods of 3 to 4 months, thus supporting the breeding of mosquitos. Our health sanitation program advised the villagers to use instead mesh nets of at least one inch so that the larvae fish would remain in the ponds. So far the villagers have done a fantastic job. Wherever we have introduced fish, there is no larvae in the ponds.

MEUWISSEN: Can you tell me something about the role of *A. stephensi* in India?

V. P. SHARMA: This is a known vector for urban malaria in India. Any town that has more than 40,000 population is under urban malaria control scheme. Control measures are directed against *A. stephensi*. *A. culicifacies* is the vector in peripheral areas only. The main control methods are anti-larval ones. There is no problem of resistance. It is only because of operational problems that malaria transmission continues.

MEUWISSEN: Is there any role for *A. stephensi* in the rural areas of India as there is in Pakistan rural areas where this vector transmits falciparum malaria?

V. P. SHARMA: In some parts of Kutch area there is some high density of *A. stephensi*. It is not a problem, though, in that it is only a serious vector of malaria in urban and not rural areas. What we have found, as I showed you in my talk, is that where peaks of falciparum and vivax malaria were present, malathion had been used. Therefore, the vectors were resistant to DDT and BSG. The population of *A. culicifacies* was then replaced by *A. stephensi*. Now, while *A. stephensi* is not a vector of malaria in these rural areas, it is a known vector which may take over the transmission of malaria if the infection is around. Luckily, there is no present infection as the parasite load from the community has been completely eliminated. However, even under the pressure of malathion, there is no effect on *A. stephensi*.

SURIN: I am very impressed with your attempt at integrated control without spraying. If I remember correctly, before you started a modified plan of operation in 1977, you had a malaria eradication program. During the eradication program, the DDT spraying was withdrawn from many areas with millions of people. Without DDT spraying, there were very active survival activities for 4–5 years. Then the malaria situation in India increased tremendously to perhaps one million to 6 million? You then had to come back to spray again to control malaria. So, I am not quite sure if you have an integrated control program without spraying. Also if you do implement these activities in some areas, won't malaria reappear?

V. P. SHARMA: Well, this is true. Even if near eradication were achieved, as was done in the past, as soon as spraying was stopped, malaria would return. So, even in the integrated control of malaria, if people don't work with the strategies of control and containment, the end result will not be eradication. To achieve eradication requires an effective insecticide. In a control strategy, you have to keep the pressure on all the time.

Discussant: Current Status of Malaria and Malaria Control Programs in Asia and Pacific Countries

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Knowledge of the current status of malaria and malaria control programs in the littoral countries of Asia and the Pacific is essential for the planning of further interventions, including incorporation of vaccination procedures. This review is based on the most recent report of the World Health Organization, supplemented with information supplied by the countries concerned.[1]

Western Pacific Region

Despite nearly three decades of extensive control activities, malaria still remains a major health problem in some endemic countries of the Western Pacific Region. After the initial success in malaria control, primarily through DDT residual house spraying, the situation has worsened in two main areas: one is the area where *Anopheles balabacensis* is the main vector such as countries of the Indochinese Peninsula, some parts of the Philippines, and Sabah State in Malaysia; the other is the area where the main vector is *A. farauti*, such as Solomon Islands and Papua New Guinea.

The disease, however, has been eradicated in Australia, Brunei, China (Province of Taiwan), Japan (Ryukyu Islands), and Singapore. In Malaysia, further progress has been achieved in Peninsular Malaysia and the State of Sarawak. In the former, the program has reached a stage where 95 percent of the population are in the maintenance or consolidation phase, while in Sarawak intensive surveillance operations have succeeded in keeping the incidence below 1,000 cases a year.

Three factors are mainly responsible for the poor state of affairs now existing in most of the countries. First, the effectiveness of residual spraying using DDT has decreased. While *A. balabacensis* and *A. farauti* have not developed physiological resistance to DDT, they have adopted different patterns of behavior to avoid picking up the lethal effect of DDT deposits, by not going indoors, by not resting on sprayed sur-

faces, and by biting people out-of-doors. Spraying is sometimes badly done, particularly in remote areas where supervision is difficult to accomplish. A second factor is drug resistance. A third is organisational. Integration of the former vertically structured malaria services into the general health services or primary health care system has not been smooth, and the command structure has been weakened.

Other factors affecting the success of the antimalaria campaigns have been the diminishing financial support and steadily increasing cost of the program due to inflation. The cost of DDT residual indoor spraying for two cycles a year now averages US$5 per head per year, a 1,000 percent increase in cost since the 1960s. Departure of trained and experienced manpower in all categories of the malaria service, particularly in the professional grade, and the frequent turnover of personnel are problems encountered by most countries.

In *China*, the number of malaria cases has been decreasing to a low level, but the position is unstable as local outbreaks occur. In 1983 there were reported to be 1,377,647 cases, morbidity being 0.14 per thousand, and in 1984 the number was below 1 million.

Of the total cases reported, 78 percent (mainly attributable to *P. vivax*) occurred in Huanghuai and Jianghan plains, which are now the major malaria endemic areas in the country. In the south, formerly the high endemic areas, prevalence has been declining steadily although foci of high endemicity remain especially on the southeast border and in inaccessible mountainous areas of poor socioeconomic conditions. Annual prevalence in the south has declined to less than 5 per ten thousand, and in the north and northeast to less than 1 per ten thousand.

Falciparum malaria was distributed in 8 provinces and autonomous regions in 1983, accounting for 15 percent of the total cases and in some counties reaching 30–50 percent. The vectors of *P. falciparum* in China are *A. minimus* in the south, *A. dirus* in Hainan Island, and *A. lesteri anthropophagus* in the middle latitudes.

In 1983, out of 29 provinces, autonomous regions, and municipalities in China mainland, 22 with 62.3 percent of the total population experienced a malaria morbidity below 5 per ten thousand. Three factors hinder the progress of antimalaria activities. The first occurs in Huanghuai and Jianghan plains, where *P. vivax* predominates and *A. sinensis* is the only vector. This anopheline is exophilic, thus making indoor residual spraying ineffectual, while contact between man and mosquito is frequent because most people sleep out-of-doors in the summer and autumn. A second factor is the prevalence of chloroquine-resistant *P. falciparum* in the south, and a third is the tendency of this strain to spread northward.

In the *Philippines*, about one-third of the population of 54 million are living in endemic areas. Despite intervention measures, malaria incidence remains high, on the average 7.2 per thousand population. The

predominant species is *P. falciparum* in the ratio of 65 percent to 35 percent *P. vivax*. Of the former, about 79 percent of infections are resistant to chloroquine, the incidence being higher in the southern islands.

Malaria endemicity varies widely in almost all provinces, transmission being generally perennial with peaks at the beginning and the end of the rainy season. The vectors tend to be zoophilic, the principal, *A. flavirostris*, breeding in slowly flowing, clear, and partly shaded streams, and the others being *A. mangyanus*, *A. litoralis*, and *A. balabacensis*.

Control strategy includes use of combinations of residual spraying of houses, biological control, environmental management, personal protection measures against biting, and surveillance activities involving the primary health care organization. The years 1983 and 1984 were transition periods from a centralized to an integrated program, causing a temporary interruption in continuity of operations and a corresponding increase in disease incidence, most cases occurring in Mindanao and Sulu areas.

In *Malaysia*, each of the three constituent regions, namely, Peninsular Malaysia, Sabah, and Sarawak (the two latter forming Eastern Malaysia), has individual sed programs related to local epidemiological situations. In 1984, in the country as a whole, 32,095 malaria cases were reported, the API (annual parasite incidence per thousand population) being 2.1, and the SPR (slide positivity rate per hundred fever cases) being 1.8.

Peninsular Malaysia in 1984 contributed 30.3 percent of the cases, the vivax-falciparum ratio being 1:1. Resistance of *P. falciparum* to chloroquine is widespread especially in the north, but more than 90 percent of the population live in malaria-free areas. All the five vectors, *A. maculatus* (the main vector), *A. campestris*, *A. sundaicus*, *A. letifer*, and *A. b. balabacensis* remain susceptible to DDT.

Sabah contributed 66.5 percent of the cases, of these *P. falciparum* being responsible for nearly 80 percent. Three-fourths of malaria deaths in Malaysia occur in Sabah. Resistance of *P. falciparum* to chloroquine is widespread, and only 11 percent of the population live in malaria-free areas. Both the two vectors, *A. b. balabacensis* (the main vector) and *A. sundaicus*, remain susceptible to DDT.

Sarawak contributed 3 percent of the cases, more than 60 percent of these occurring due to population movement across the border with Indonesia. The predominant parasite is *P. vivax*, producing 73 percent of the cases within Sarawak. Resistance of the local *P. falciparum* to chloroquine does not occur. Despite the small number of malaria cases, however, only 17 percent of the population live in malaria-free areas. All the four vectors, *A. leucosphyrus*, *A. donaldi*, *A. sundaicus*, and *A. b. balabacensis*, continue to be susceptible to DDT.

The problems facing the program in Malaysia include lack of community support due to apathy and ignorance, active population move-

ment (construction workers, security forces, migrants), increasing incidence of drug-resistant *P. falciparum*, changing pattern of malaria species predominance, lack of supervision of control measures in the remote areas, refractory nature of *A. b. balabacensis* in Sabah, and poor house construction with incomplete walls.

In *Papua New Guinea*, the *Solomon Islands*, and *Vanuatu* the current status of malaria may be outlined as follows.

Throughout Papua New Guinea malaria endemicity varies. The country may be divided into four regions in this respect, the highlands, the New Guinea mainland, the New Guinea islands, and the Papua region. The principal vectors are *A. farauti*, *A. punctulatus*, and *A. koliensis*, transmission being perennial with some seasonal variation. A change in parasite species predominance from *P. vivax* to *P. falciparum* has been observed since 1972, the proportions now being *P. falciparum* 75%, *P. vivax* 20%, and *P. malariae* 5%. Chloroquine-resistant strains of *P. falciparum* have become widespread since they were first confirmed in 1976, and resistance to sulfadoxine-pyrimethamine is also being reported.

In the Solomon Islands, since 1977 there has been an extremely vigorous resurgence of malaria characterised by the same predominance of *P. falciparum* occurring in Papua New Guinea. Chloroquine-resistant strains, first seen in 1980, are now widespread. The malaria situation in Vanuatu is, to a lesser extent, following that of the Solomon Islands.

South-East Asia Region

Countries of the WHO South-East Asia Region that are affected in varying degree by malaria are Bangladesh, Bhutan, Burma, India, Indonesia, Maldives, Nepal, Sri Lanka, and Thailand. The disease is widely distributed, particularly in the foothill districts of Bhutan, Burma, India, Nepal, and Thailand. In Bangladesh it is mainly confined to the eastern hilly districts; in Indonesia it is more prevalent in the outer islands than in Java and Bali where vector control measures are undertaken; in Maldives it has virtually been eradicated by 1985 (the transmission of *P. falciparum* having been eliminated by 1976); in Sri Lanka perennial transmission occurs in belts in the north-center and the southeast, and seasonal transmission elsewhere.

The incidence profile in these countries combined may be highlighted as follows:

Year	*API*	*SPR*	*SFR*	*Pf%*
1970	1.96	2.37	0.47	19.8
1976	8.86	9.74	1.37	14.0
1983	2.59	3.05	0.99	32.5

These data parallel those of India, whose involved population greatly exceeds those of the other countries. Following failure to maintain the

impetus of the eradication campaign during the late 1960s, incidence reached a peak in 1976; since then, it has been reduced by twice yearly house spraying using DDT (or malathion or HCH two to four times a year in parts of western India, Nepal, Java, and throughout Sri Lanka where vectors have reduced susceptibility to DDT), and by surveillance with treatment adapted to local patterns of drug resistance in *P. falciparum*. Despite intensification of antimalarial measures, however, or possibly because of the massive increase in distribution of vivax-inhibiting chloroquine, the proportion of *P. falciparum* malaria has been steadily increasing in most countries of the region.

The trend towards malaria reduction is now leveling off due to a number of constraints additional to reduced financial allocations in several countries: (1) decreased spray coverage due to householder resistance and rising costs of insecticides and personnel; (2) deficiencies in surveillance and underreporting of incidence; (3) increased vector breeding due to deforestation, flooding, unregulated irrigation, gemming excavations, shifting cultivation; (4) migration of gametocyte carriers to receptive areas, and susceptibles to endemic areas; (5) vector insusceptibility to insecticides in Burma, India, Indonesia, Nepal, Sri Lanka, and avoidance in Thailand and Burma; (6) *P. falciparum* resistance to chloroquine widely in Bangladesh, India, Indonesia, Thailand, and focally in Bhutan, Nepal, Sri Lanka (and resistance similarly developing to alternative drugs).

The malariometric data now available suggest strongly that unless new methods are introduced and increased funding provided for improvement of existing programs, there will be little if any further reduction of malaria by the year 2000, and indeed there may be a great deal more, particularly falciparum malaria.

Individual countries of this region that may be further considered here are Bangladesh, Burma, India, Indonesia, Sri Lanka, and Thailand.

In *Bangladesh*, malaria occurs in the north-east (Mymensingh areas), east (Sylhet), and southeast (Chittagong Hill Tracts): in the hilly districts both forested (where *A. balabacensis* is the principal vector) and cleared (*A. minimus*). In the central, western, and southern plain/estuarine districts (*A. philippinensis*, *A. sundaicus*) there is much less malaria. The incidence in the country may be highlighted as follows:

Year	*API*	*SPR*	*SFR*	*Pf%*
1970	0.13	0.14	0.07	49.6
1979	0.61	3.62	0.76	20.9
1983	0.44	1.74	6.71	40.8

The peak of post-eradication incidence occurring around 1979 has been brought down by intensified control measures. Increase in the SFR since that peak reflects continuing control of transmission in the

plains where *P. vivax* predominates, in contrast with the hilly areas where *P. falciparum* is rampant and is increasingly resistant to chloroquine, and to which areas transmigration is in progress involving the introduction of relatively non-immune settlers from the plains.

Control measures in Bangladesh are adapted to three defined epidemiological strata: (1) all forested areas where the principal vector is *A. balabacensis*, cleared foothill areas having *A. minimus*, and areas dominated by *P. falciparum*—in this stratum the objectives are to prevent mortality and reduce morbidity in high-risk groups by means of treatment and residual spraying with DDT twice a year; (2) mainly plains areas having *A. philippinensis* and *A. sundaicus* as the principal vectors—in this stratum the objectives are to prevent mortality and reduce greatly the incidence of *P. falciparum* infections indigenous and imported, by means of one round of pre-monsoon DDT spraying and surveillance, (3) plains areas where the vector is *A. philippinensis* and the parasite *P. vivax*—in this stratum objectives are to maintain gains through surveillance and contain focal outbreaks of vivax malaria through radical treatment of cases and (where there are clusters of cases) pre- and post-monsoon DDT spraying. In this major part of the country the primary health care system (thana health complex) is largely responsible for the program.

In *Burma*, two-thirds of the population in the malarious areas are covered by malaria control activities, and the rest have access to treatment facilities. The following data show progress of the work:

Year	*API*	*SPR*	*SFR*	*Pf%*
1975	0.50	4.24	2.73	64.5
1980	0.99	7.71	6.35	82.5
1983	1.10	4.21	3.60	85.4

While the significance of the API is vitiated by an extremely inadequate sampling (ABER), it is apparent that the incidence has not changed dramatically in recent years.

The main areas of malaria distribution in Burma are the forested foothills where *A. balabacensis/dirus* predominates. In the plains *A. annularis* and *A. culicifacies* occur, while along the coast *A. sundaicus* is found. Control measures are diversified and increasingly undertaken at community level under supervision of expert personnel. The measures include DDT spraying, anti-larval operations, and distribution of chloroquine, with vigilance and surveillance in areas freed of the disease. Measures have not yet been significantly impeded by reduced vector susceptibility to DDT, although this does occur focally in some *A. culicifacies* and *A. annularis* populations. Avoidance is exhibited by *A. balabacensis*, and in some places this mosquito may have become physiologically resistant to DDT.

A major problem throughout the country is resistance by *P. falciparum* to chloroquine and now in the southeast at the border with Thailand to sulfadoxine-pyrimethamine. To this the national experts attribute the increase in incidence of falciparum malaria—prior to 1979 the species ratio was F2:V1, but since that year it has become F5:V1.

In *India*, the control program is faced with the formidable problems of insecticide resistance in the rural vector *A. culicifacies* and the urban vector *A. stephensi*, drug resistance by *P. falciparum* spreading westwards across the country, and escalating costs of all components of the program, particularly insecticides. The problem is aggravated by a 100 percent increase in irrigation in the last two decades, which together with extensive deforestation and flooding has resulted in proliferation of *A. culicifacies* and the hillside stream-breeding *A. fluviatilis*, rendering vast areas endemic to malaria. Although the incidence was reduced by 1984 to less than a third of the cases seen in 1976 this reduction has been in respect of *P. vivax*, falciparum malaria cases remaining constant at around half a million annually.

Year	*API*	*SPR*	*SFR*	*Pf%*
1976	10.97	11.55	1.35	11.7
1980	4.47	4.32	0.88	20.3
1983	2.78	3.11	0.88	28.1

By 1984, however, the downward trend had leveled out, and now a 12 percent increase in cases, due to *P. falciparum*, is officially reported (spot studies reveal considerable underreporting). The present nationwide strategy of house spraying pre- and post-monsoon, using DDT or other appropriate insecticide, with surveillance and chloroquine distribution through more than 250,000 peripheral workers is decentralized to primary health care at the district level. But ground is no longer being gained even though the nationally funded campaign is being intensified in the northeast, east, and center by a special donor-supported Plasmodium Falciparum Containment Programme.

In *Indonesia*, a comprehensive program of malaria control is being undertaken in Java and Bali, with less complete operations in the outer islands. The malariometric data obtained in Java and Bali may be highlighted as follows:

Year	*API*	*SPR*	*SFR*	*Pf%*
1975	1.53	1.51	0.54	35.4
1980	1.85	1.93	0.91	46.6
1983	1.34	1.44	0.69	47.9

These data indicate continuing success of the program in Java-Bali, where the main insecticide used for spraying operations is DDT; in areas where the principal vector, *A. aconitus*, is resistant to DDT, feni-

trothion is used. Anti-larval operations are employed in *A. sundaicus* breeding sites during the dry season.

The outer islands for malaria control purposes are divided into non-priority and priority areas. The latter includes transmigration projects, socioeconomic development areas, areas bordering with neighboring countries, and outbreak areas, and activities consist of case finding and treatment, spraying with DDT, and anti-larval measures. In the non-priority areas, the DDT spraying is omitted.

Falciparum malaria accounts for 30 to 56 percent of the malaria infections. Resistance to chloroquine is widespread, foci being detected in 25 of 27 provinces. Other constraints to the program include territorial vastness, shortage of skilled manpower, especially in the outer islands, vector insusceptibility to DDT in Java, and inadequate inter-sectoral collaboration.

In *Sri Lanka*, malaria occurs at lower altitudes in most of the country excepting the greater Colombo area. It is endemic in years of normal rainfall, but epidemic in dry years when pooling occurs in the river beds promoting breeding of the only vector *A. culicifacies*, which is resistant to DDT. Following an epidemic in 1968, the incidence may be highlighted by the following data:

Year	*API*	*SPR*	*SFR*	*Pf%*
1970	50.96	31.21	0.09	0.3
1975	39.12	26.86	4.28	15.9
1981	4.52	5.31	0.14	2.6
1983	11.50	12.06	0.43	3.5

In 1977, an intensive control program was introduced using quarterly residual house spraying with malathion, chemotherapy, source reduction, particularly in river beds by flushing from dams, and larviciding, surveillance, and space spraying in pilgrimage cents on festive occasions. In 1981 stratification differentiated seasonal from perennial transmission areas, the former to receive only selective not regular quarterly house spraying. However, an upsurge of *P. vivax* transmission commencing at the end of 1982 and peaking in 1984 resulted in re-establishment of quarterly malathion spraying in all malarious areas, together with larviciding, river-pool management by flushing from dams, source reduction including filling of abandoned gem mining pits, and space spraying in pilgrimage areas. Chemotherapy using chloroquine was intensified, but late in 1984 the first cases of falciparum malaria resistant to this drug appeared, while indications are being found of reduced susceptibility of *A. culicifacies* to malathion. Because of the epidemic potential, integration of the anti-malaria program and primary health care is being developed cautiously.

In *Thailand*, in the 40 years since establishment of the anti-malaria

program, malaria incidence has been reduced from 300 cases per thousand population to 5.2 cases. In recent years emphasis on expansion of case detection and treatment facilities, notably establishment of malaria clinics throughout the country and of a network of village malaria voluntary collaborators, has brought about a 42 percent reduction to a current 10-year low level. Malariometric data may be selected as follows:

Year	*API*	*SPR*	*SFR*	*Pf%*
1978	7.7	8.3	4.59	57.2
1981	10.6	8.5	5.87	69.3
1983	5.2	4.5	2.67	65.3

The parasite species formula in 1983 was *P. falciparum* 65.3 percent, *P. vivax* 34.4 percent, and *P. malariae* 0.02 percent. At the present time, more than 95 percent of the *P. falciparum* in the country show some degree of resistance to chloroquine, while an increasing number of strains are also showing resistance to sulfadoxine-pyrimethamine, especially in the areas near Kampuchea. Some resistance to quinine and, most recently in one or two cases, to mefloquine is also appearing. This major constraint to control of the disease is supplemented by other problems: widespread population mobility across the borders, changing patterns of vector bionomics, particularly involving exophily of the principal vector *A. dirus*, and financial constraints.

The strategy being implemented is that of long-term malaria control in the forested and hilly areas where the disease is endemic (population 10.5 million) and prevention of re-establishment of transmission in the remaining areas in which eradication strategy is being applied (38.5 million population). Actual control measures depend upon the local epidemiological situation, for operational purposes the country having been stratified according to differing levels of receptivity. Particular attention is paid to the development of primary health care components within the program, in relation to radical treatment centers (malaria clinics providing fast slide diagnosis and immediate treatment) and a network of village voluntary malaria collaborators.

REFERENCE

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General Discussion

BUCK: Dr. Clyde, could we have figures on the corresponding changes in mortality rates for these countries? Some crude estimates for the same years?

CLYDE: As was mentioned by a speaker a few moments ago, mortality figures are of very little use in many of these countries, particularly when they are ascribed to a particular disease. In Burma, however, the figures are more accurate. There was a considerable reduction in mortality from about five thousand deaths attributed to malaria (it was the number one cause) down to two or three thousand a few years ago. But the latest figures indicate that malaria deaths have risen again. Malaria is still not first on the mortality list, but it has risen again. This is largely because of the presence of *Plasmodium falciparum* in the areas that are beyond the effective control of the authorities. There are many areas where immediate health care is not accessible. As we all know, the whole battle against falciparum cerebral malaria is having immediate treatment facilities or even temporary treatment facilities which are currently lacking.

In Bangladesh, the figures are not really worth mentioning.

In Sri Lanka, as I have said, there is virtually no falciparum except in one area where we now have up to 25%. It is usually 4% with the mortality negligible because the clinical treatment centers are so immediately available. They have excellent roads, transport systems, etc.

BRUCE-CHWATT: I would like to commend the Government of Sri Lanka in maintaining the vertical structure of their malaria control program. I've been in Sri Lanka, and I've seen how it works. I would like to compare their attitude to the attitude of the Government of Pakistan which has introduced a premature integration of malaria control with so-called primary health care, which meant a complete destruction of a good vertical program of malaria control that existed before the integration.

This should be a warning to any government in Asia not to hasten with so-called fashionable integrations of malaria control with primary health care before the primary health care structure is sufficiently built up.

BUCK: Concerning Quinghasou, a new indigenous medicine derived from Chinese practices, which I think is chemically completely different from drugs that are now used as anti-malarials, what is the state of affairs? Where are we now in the development of the Artemisine and its salts and derivatives?

CLYDE: Well, let me refer this question to Dr. Wernsdorfer, who is here with us. But first, let me say that the Ayurvedic compounds that I am talking about are made of four herbals including roots that have a tremendous tradition in India, going back for 2,000 years. They are drugs that have been used for many different conditions with some success. But they are not specifically anti-plasmodial at all. Quinghasou and the Artemisine derivatives from that are a totally different situation. This is a very highly active chemical constituent that has been isolated and is highly effective. As you know, it is similar to quinine. Again, before I ask the moderator if Dr. Wernsdorfer could possibly comment, I might say that as Quinghasou is being developed in China, our Chinese colleagues here could speak to this point. Other countries are searching industriously to try to find this drug because the plant Artemisine is commonly found in many countries. The Burmese particularly have been isolating and analyzing many plants and doing mouse and simian malaria tests to try to get this particular drug. Other countries are also doing the same.

COLLINS: Dr. Clyde, you said that *P. vivax* from India is a poor relapser? Will you define what you mean by a "poor relapser"?

CLYDE: I think it was Dr. V. P. Sharma who said that the *P. vivax* in India is a poor relapser. But, let me not pass the buck like that. There have been tremendous numbers of studies in India on the short courses of primaquine. Five-day courses of primaquine were the standard. They have now been reduced to three days in many areas because of the impossibility of getting people to come back for a proper primaquine course. Now, concerning work done by the Indian Council of Medical Research with Dr. Sharma's participation, and by the National Malaria Service personnel, they have studied more than 7,000, possibly 8,000, *P. vivax* cases who received 5 days of primaquine and were followed for up to a year. These cases, which appear to be scientifically authentic, showed a 97% radical cure with the 5-day course of primaquine. This suggests either that they are not being followed up properly and are taking chloroquine when they get a bit of a headache, (which doesn't seem

to be the case, because these are authentic workers), or that there is something rather strange about the parasite, the absorption, or the action of primaquine. We can knock out the absorption, action, and primaquine, which leaves us with a parasite which must be a little peculiar.

COLLINS: I would think a definition of a relapser is when you don't give primaquine. What happens when you do give just chloroquine? We know that Chesson strain is a frequent relapser. St. Elizabeth doesn't come down for 6 months and multinucleatum for a year. You are describing here that the parasite is a poor relapser—not that it has a susceptibility to a drug. That is what I want to know. What is the pattern of this particular parasite without primaquine?

CLYDE: I am coming to this. Primaquine was the initial index that showed that this parasite seemed not to relapse after almost homeopathic doses of primaquine. Studies with chloroquine and follow-ups of these cases showed that there have been far fewer numbers of relapses with Indian *P. vivax*. In southern India now, it is interesting that when you cross the border into Nepal, you appear to have a parasite that relapses much more vigorously but only on chloroquine. There have been a number of papers (two or three) published which show this difference. I can follow this up by something that I think you, Dr. Collins, were probably personally involved in. Some years ago, this strain was taken from South India and brought either to Glaxo or one of the testing centers where it was found that when primaquine was given that it required the full 14-day course of primaquine to prevent relapses.

COLLINS: This strain is from West Pakistan and Dr. Contacos handled it.

CLYDE: Anyway, that would be my answer. Again, one would possibly ask Dr. V. P. Sharma to elaborate a little on this since he is the one who has done the work.

V. P. SHARMA: On a 5-day course with a population of nearly 30,000 people, we found that the clearance rate was about 98%. Very few cases relapsed. There are no authentic studies of follow-up cases without primaquine, but we estimate that there is no more than a 30–35% relapse rate.

BRUCE-CHWATT: I remember a discussion I had about three years ago with Dr. Rai concerning the pattern of relapses with primaquine for 5 days only. To the best of my recollection, he said that they were giving primaquine for 5 days only because, operationally, they were unable to maintain it for 14 days and that a great deal of the drug would be wasted if they did. I asked him what was the frequency of relapses at this different from other methods' radical cure of 5 days and he replied that they

get about 10% relapses on a dosage for 5 days, and that with 10% relapses with this type of semi-radical cure they were able to follow up with their surveillance system and eventually give the drug for the second time. This is what Dr. Rai told me. I don't know to what extent it has been proven by actual epidemiological data.

PAIK: Dr. Clyde, do you have any case studies of good malaria control with so-called primary health care in your regions? I am curious about the primary health care approach.

CLYDE: Yes and no. It has been mentioned by several speakers that premature integration has been a disaster. This has happened in Bangladesh and Nepal as well as a number of countries in our region. They have reverted to a vertical system and are now experimenting with primary health care more carefully. Studies are being carried out on integration through primary health care, particularly in India. These studies have also been done in Thailand on a semi-scientific basis since it is very difficult to establish what parameters of success are at the village level. I would possibly ask you to refer to Dr. V. P. Sharma and Dr. Surin Pinichpongse on that basis.

WERNSDORFER: I noticed some common denominators in the problems of vector control in the countries of the Western Pacific and Southeast Asia, this being efficacy of vector control, be it caused by resistance or exophilic habits of the vectors. I also heard mentioned the problem of drug resistance in *P. falciparum*. As I recall, the common denominator when this drug resistance first occurred was that chloroquine was used in areas where transmission was actively going on unchecked. The real propagation of resistance occured when chemo-prophylaxis and haphazard use of chloroquine occurred in place of vector control. I think that this is one thing which should be considered as we think of future strategies of malaria control.

I think that one very impressive item mentioned in Dr. Clyde's summary was the leveling out effect. I think that this effect is caused by undermaintained malaria control. I wonder if further slackening of the efficacy of control measures or slackening in the effort to continue these measures will cause this leveling out effect to be only temporary? If so, then I would expect a swing up. This may have justification in the fact that the falciparum ratio is rising. In this part of the world, *P. vivax* is also a major part of the problem which should be addressed.

The situations in China and Thailand have given me some hope for the future. China described a consistent reduction of malaria incidence and prevalence. Thailand, in spite of major difficulties with vectors and multi-resistant falciparum, has also been able to reduce malaria incidence. I think that if chemo-therapeutic means are used, they should be

aimed at fully curative action based on diagnosis. The role of the community in these two countries also seemed to contribute greatly to the successful control of malaria. I think that these factors should certainly be considered when planning for the future. Also to be considered are the problems that exist now with vector control and the use of antimalarial drugs, which has become a great motivation for vaccine development and deployment. It was said by one of the speakers this morning that those points which have been responsible for shortcomings in the application of malaria control are also going to militate against the correct deployment of vaccines. I think this is where corrective action has to come in and create the best possible conditions for the use of antimalarial tools, be they to induce immunity, to control vectors, or to deploy drugs.

COPPEL: I have a question to be directed to all of the speakers who have spoken today. I think that one of the cited aims of this conference is to discuss the soon-to-occur clinical trials for a vaccine, initially based on sporozoites. I think that a prerequisite for any clinical trials occuring on indigenous populations is a large amount of careful prior research to define study populations entomologically, demographically, and parasitologically. I was wondering from the speakers who have spoken today if they have such study groups already defined? We heard from Dr. Cattani in Papua New Guinea that there was such a group available. Are such groups available in other countries?

SURIN: Regarding these studies, there are quite a few topics to be studied in Thailand related to social and economic research. Some groups have studied the cost effectiveness of malaria activities and some groups are now studying the behavior of migration. So I would like to say that there are some groups studying in Thailand. In addition to these, we can get information from our Malaria Surveillance Agency. From our Surveillance Agency, we can conduct the required investigations to identify available groups in the area.

MODERATOR: There were questions raised earlier about Quinghasou. I wonder if Dr. Mao would care to tell us a little about that?

MAO: First of all, I should say that my institute is directly involved in the chemical experimentor for clinical trial of Quinghasou. As far as I know, Quinghasou is effective against *P. falciparum* and is especially useful for the treatment of strains resistant to chloroquine. But as my colleague mentioned this morning, 78% of malaria cases in China are due to *P. vivax*. So, Quinghasou is not widely used in my country. Even in the case of *P. falciparum*, I believe that 10% relapsed after one month. That is what I know about Quinghasou.

I am personally more interested in schistosomiasis. I would like to make a relation of plant molluscides to plant anti-malarials. I think studies on plant molluscides are important just to give proof of effectiveness. A new molluscide should be synthesized and not to be used in its plant state. To extract and purify the active ingredient from a plant is very expensive. So, I think that for Quinghasou to be used more widely, it should be synthesized first.

SAVANAT: I would like to respond to the previous question concerning whether any place had been identified as a suitable candidate for a vaccine trial. One of the activities of the Faculty of Tropical Medicine in Bangkok is on the epidemiology of malaria. We have identified a number of suitable sites. Even though my map showed many red areas as parasite infested, the infection rate in those areas is quite low. In fact, since we have gone through these studies identifying the population and various parameters which are involved in the spread of malaria in that region, we have been able to identify what people are good candidates for vaccination. It turns out that the migrants who moved to the northeastern area are not the main problem. The main problem itself is due to the local people who go from the village to work elsewhere, contract the infection, and then spread it locally in their region. You can see that in this particular area, we have identified what is the most important factor and how to approach it. We have people who approach this problem by examining those who come from working outside and administering chloroquine to them. In this way, we can control incidence to zero in that particular region. Since we have done zero epidemiology in that area, we know their background immune status, even though it is not related at all to protection.

SULEMAN: I have a comment which the experts here may consider. It concerns those places where transmission of malaria is seasonal, such as India and Pakistan. The current method of studying the proportion of falciparum and vivax in those areas is that monthly data is accumulated from January through December for a particular year. These data are then compared from prior data on cases of falciparum versus vivax incidence. I think this is not an accurate measure, because falciparum transmission is mainly limited to a couple of months. There is also no relapse in falciparum. Vivax has a relapsing nature which causes the accumulative data to ultimately show that there is not a high proportion of vivax and falciparum. I feel that this results is bias in the data. Does anybody have a comment on this?

V. P. SHARMA: I think that the comparison is accurate. For example, when we were involved with U.P. Terai, we found that in one year in cases done on a daily basis there were only 19 cases of falciparum. Two

years later, we found 90% falciparum. This would definitely show that falciparum is increasing. There are peak seasons which can be compared season-wise. Of course, if you want to compare the incidence of falciparum last year to this year, you would naturally have to take the entire year into consideration.

SULEMAN: Exactly. That is what I am pointing out. The comparison would be made on a monthly basis, not on accumulative annual data which could create a bias because one is relapsing and one is non-relapsing.

SIDDIQUI: I would just like to take a moment first to thank the moderator, Dr. Trager, for the excellent job he has done. One of the objectives of this conference was to provide a means for effective interchange between so-called classical parasitologists, immunoparasitologists, and molecular biologists. I am particularly very gratified that since the beginning of this conference, this goal has been achieved. I have observed scientists in the fields of immunoparasitology, molecular biology, and "classical" parasitology actively listening, learning, and participating together. Thank you very much.

SESSION II

CURRENT STATUS ON THE DEVELOPMENT OF MALARIA VACCINES

A. CANDIDATE ANTIGENS

Studies on the Development of a *P. Falciparum* Malaria Vaccine for the Induction of Transmission Blocking Immunity

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Introduction

The induction of transmission blocking immunity in avian malaria models as a potential tool in malaria control was reported by Gwadz and by Carter and Chen following earlier experiments by Huff.[1-3] The efficiency of immunization with extra cellular gametes was also demonstrated in a *P. yoelii* rodent model and in a *P. knowlesi* monkey model.[4,5]

The introduction of methods for *in vitro* culture of *P. falciparum* was followed by procedures for the production of gametocytes and gametes as well as the establishment of routine *in vitro* infection of mosquitoes.[6-9]

The availability of gametocytes, gametes/zygotes, and ookinetes was basic for biochemical and molecular biological studies of these developmental stages of *P. falciparum*. In the NIH research group Rener and co-workers were the first to demostrate that monoclonal antibodies (MoAbs) against male and female *P. gallinaceum* gametes could interfere with the sporogony of the parasite in mosquitoes.[10] In most experiments effective transmission blocking activity was achieved only in the presence of complement or by the synergistic action of two different MoAbs. In later studies in this avian model it was shown that the MoAbs prevented the fertilization of macrogametes by microgametes.[11] Similar results were obtained with *P. falciparum*.[12] A combination of two MoAbs against gametes of a Brasilian isolate 7G8 suppressed the infectivity of gametocytes in the absence of complement, whilst in the absence of complement (with one exception) individual MoAbs were not able to block transmission.

The MoAbs were used for the identification of target antigens of anti-

gamete immunity. Three gamete proteins of *P. gallinaceum* of Mr 240, 56, and 54 k, were identified by specific MoAbs by the application of surface labelling techniques, SDS polyacrylamide gel electrophoresis of parasite extracts under reducing conditions, and followed by autoradiography. In the corresponding experiments with *P. falciparum* sexual stages a similar set of gametes surface proteins of Mr 260, 59, and 53 k, were precipitated by the available MoAbs.[12] As demonstrated in biosynthetic labelling experiments it was found that these gamete surface proteins were synthesized in gametocytes before gametogenesis. In the *P. gallinaceum* model it was shown that these surface proteins were synthesized into the surrounding medium and that a new set of proteins, Mr 26 and 28 k, were synthesized and expressed on the surface of ookinetes.[11,13] Meanwhile similar studies on the sexual stages, zygotes, and ookinetes of *P. falciparum* have been carried out independently at our institute. The results are reviewed in greater detail in order to indicate to what extent they confirm the above results and at what points new information can be added.

1. Surface labelling of macrogametes of a *P. falciparum* isolate NF 54 resulted in a number of ^{125}I labelled bands after separation by SDS PAGE. The electrophoretic mobility of some proteins was different in the presence or absence of reducing agents like DDT. Usually four major polypeptides of 250, 58, 47, and 40 k could be detected under reducing conditions; under non-reducing conditions the above proteins demostrated a Mr of 230, 48/45, 45, and 40 k. The 48/45 kD doublet did not separate under reducing conditions. Minor bands were seen at 33, 25, and 16 kD. The Mr under non-reducing conditions will be used for the rest of this review when reference is made to the various proteins.

Several MoAbs (32F1, 32F3, 29F12, 32F5) reacted with the 48, 45 kD doublet, some of these, however, coprecipitated also the 230 protein. Although a protein was labelled in the 25 kD region this could not be precipitated by any of these MoAbs.

2. Immunoblotting experiments using polyclonal as well as monoclonal antibodies showed a result somewhat different from the above. Polyclonal rabbit serum did not bind to a 230 kD protein whereas it precipitated this protein after surface labelling. On the other hand, the serum did react prominently with a 25 kD protein which was not labelled in the surface iodine ion. Some of the MoAbs were obtained by selecting hybridoma lines that positively reacted to the blotted 25 kD protein. Similarly MoAbs reacting with the 40 kD protein were selected and these recognized also a 45 kD protein.

3. Seven transmission blocking and eight non-blocking anti-macrogamete antibodies were identified. The molecular weights of proteins of macrogametes/zygotes recognized by the MoAbs and polyclonal rabbit

serum are given in Table 1. Table 2 gives information with regard to the transmission blocking potency of the different blocking antibodies.

4. The strength of transmission blockade depends on two factors: the concentration of the specific antibodies and the quality of gametocytes that are involved. The intensity of the blocking effect appeared inverse-

TABLE 1. Action of a selected number of monoclonal and polyclonal antibodies directed against *P. falciparum* macrogametes/zygotes

		Molecular weight of target proteins ×10⁻³			
		230	48/45	45/40	25
Transmission blocking antibodies	7F11				+
	29F432	+	+		
	32F3		+		
	32F5		+		
	32F61				+
	32F81				+
	1771*	+	+	+	+
Non-blocking antibodies	7F3	+	+		
	28F1	+			
	32F1	+	+		
	32F62			+	
	32F63			+	
	32F71				+
	32F72				+
	32F84			+	

* rabbit polyclonal antibody

TABLE 2. Degree of transmission blockade by monoclonal and polyvalent antibodies directed against *P. falciparum* macrogametes/zygotes

Antibody	*Mean percent reduction in oocysts compared to control*	*Total no. of test mosquitoes dissected*	*Mean oocysts in control mosquitoes*
7F11	97 (3)	79	17
29F432	97 (2)	25	21
32F3	100 (11)	173	24
32F5	95 (5)	103	14
32F61	74 (4)	53	16
32F81	95 (6)	74	18
1771*	99 (4)	76	19

The number in parenthesis indicates the number of experiments carried out with each antibody.

* rabbit polyvalent antibody.

ly related to the transmission potential of the gametocytes cultures. It should be considered that in the laboratory experiments the number of infectious gametocytes in the mosquito blood meal—about 20,000 per mm^3—is much higher than in normal field conditions. Although complete transmission inhibition could be achieved by single MoAbs, we confirm that combination of MoAbs does potentiate transmission blockade. Combination of a complete blocker used at a dilution which on its own could not totally inhibit transmission with a non-blocking MoAb was able to potentiate the transmission blocking action of the former.

Combinations of non-blocker MoAbs recognizing different proteins had no effect on transmission.

5. In biosynthetic labelling experiments (using freshly purified macrogametes incubated in methionine-free RPMI 1640 medium supplemented with 35S methionine, for a period of three hours (at 25 °C), it could be shown that predominantly 25 kD protein was produced. This protein could be solubilized almost completely (in 25 mM Tris 0.5% Triton X100 pH 8), and precipitated by blocking MoAbs 32F61, 32F81, 7F11 as well as by non-blocking MoAbs 32F71 and 32F72. The 25 kD protein was not present in asexual parasites but it could be demonstrated in gametocytes and in much larger quantities on gamete/zygote surface. Using surface immune fluorescence with different MoAbs it was demonstrated that the surface of the zygote transforming into the ookinete in the mosquito midgut is gradually replaced by the 25 kD protein.

6. By application of a two-site immunoradiometric assay (IRMA) using microtiter plates coated with MoAbs against 45 kD or 25 kD protein and using protein extracts of gametes/zygotes as the antigen it could be shown that transmission blocking and non-blocking MoAbs recognize two different epitopes on each protein. Both epitopes appeared to be non-repetitive in the IRMA.

7. The SDS PAGE pattern of surface labelled proteins of *P. falciparum* NF54 macrogametes showed great similarity with that described by Rener.[12] We confirm that some labelled proteins showed a difference in migration in SDS PAGE under reducing and non-reducing conditions. Since all three proteins of 230-48-45 kD showed a higher Mr value in the reduced stage, they consist of no more than one polypeptide chain. The presence of intra-chain disulfide bonds probably influences the binding of SDS to the polypeptide backbone resulting in shift in their relative molecular weights.

8. The three proteins of 230, 48, and 45 kD might possess a common antigenic site on account of their reactivity with a set of MoAbs (11C5-B10, 32F1, 29F132, 7F3; only MoAb 28F1 was monospecific for the 230 kD protein). However, the quantity of 230 kD precipitated relative to that of the lower Mr proteins differed from one MoAb to another. This suggests that the 230 kD protein probably coprecipitated in a non-

specific way. This is supported by the finding that in the immunoblotting experiments the 230 kD protein could not be detected. The 230 kD, 48/45 proteins are no longer detectable on the surface of developing ookinetes. A precursor-product relationship of the 230 kD protein with any of the lower Mr antigens has not yet been demonstrated. The importance of the 230 kD protein in transmission blocking action is not clear. Only one MoAb (29F432), which recognizes the 230, 48, and 45 kD proteins, blocked effectively, but it is not clear whether this was due to its reactivity with the 48, 45kD proteins as well.

9. The 48/45 protein doublet is present in the gametocytes and later expressed on the surface of the newly emerged macrogametes. Since transmission blocking MoAbs that are interacting with this protein doublet completely block formation of retort cells/ookinetes, it appears that these proteins play a significant role in the process of fertilization of the macrogametes. There was no indication in biosynthetic labelling experiments that one acted as a precursor for the other. This doublet, however, is no longer present on the intermediate stage (retort cell) between zygotes and ookinete. On account of similar findings in *P. gallinaceum* it seems likely that also the 49/45 kD proteins are shed from the surface during ookinete development.[11]

The relevant epitopes of the 48/45 kD proteins are not sensitive to the action of SDS as was demonstrated by immune reaction on Western blots. These epitopes, however, are sensitive to reduction by DTT. The reduced proteins not only showed a difference in Mr but reactivity of these blotted DTT-treated proteins with blocking MoAbs as well as non-blockers was totally abolished. This suggests that these epitopes have a conformational nature.

10. The 45/40 kD set of proteins could also be detected in the gametocytes stage by MoAbs that were selected on Western blots. These MoAbs (32F62, 32F63, and 32F84) also showed a positive immunofluorescence on live gametes. Following surface labelling of gametes, however, none of these MoAbs precipitated any of these labelled bands. Apparently the radio-iodination interferes with the binding of the antibody.

The significance of these proteins remains unclear since none of these MoAbs blocks the formation of oocysts. The 40 kD protein is predominantly present with respect to the 45 kD one.

In two-dimensional PAGE the 45 kD protein appeared identical to the 45 kD protein that reacts with the blocking MoAb 32F3.

Reduction of this protein by the DTT resulted also in a shift in Mr to 58 k. The epitope present on both the 45 and 40 kD proteins, however, was not sensitive to reduction.

11. The 25 kD protein is already present in mature gametocytes.

Biosynthetic labelling experiments in synchronized gametocytes cultures, containing gametocytes only (Ponnudurai et al., 1985, in prepa-

ration) indicate that the 25 kD protein is already being synthesized in small quantities in early stages of gametocyte development. MoAbs against this protein were selected on Western blots of macrogamete proteins. Some of these block transmission (7F11, 32F61, 32F81).

Non-blockers probably bind to another determinant in the molecule. Using surface immunofluorescence on live cells it was demonstrated that this protein is hardly detectable on the surface of newly emerged macrogametes. It becomes, however, progressively expressed on the surface of the macrogamete/zygote over a period of two hours. The suggestion that this protein plays an important role in the development of the parasite in the mosquito was further confirmed by the fact that although the presence of the transmission blocking MoAb 32F81 in the mosquito blood meal did not significantly influence the number of retort cells/ookinetes formed, yet almost no oocyst resulted. The presence of the antibody on the surface of the transforming zygote apparently did not hamper its further development into a mature ookinete. Does the 25 kD protein act as a ligand for a receptor on the mosquito midgut cell wall?

12. The NF54 isolate has been obtained from an airport infection in the Netherlands and is, therefore, of unknown geographic origin. The presence of the 48/45 and 25 kD proteins and their relevant epitopes in isolates from different geographic areas was tested using immunoblotting and transmission blocking assays.

Isolates from Vietnam (NF 117), Ghana (NF 7), Central Africa (NF 115), and Brasil (7G8) were probed with the anti-NF 54 MoAbs 32F3 and 32F81. All isolates demonstrated positive reactions with the MoAbs and no differences were found with regard to the Mr of the relevant proteins.

The transmission blocking capacity of several (NF54) MoAbs was tested in three different isolates (NF54, NF7, and 7G8) in parallel experiments. All MoAbs tested, either blockers or non-blockers, produced similar results.

13. Blots of NF54 macrogamete proteins were incubated with sera of patients with a *P. falciparum* infection. From a set of serum samples collected from Dutch patients with an infection, one serum gave a strong reaction with the 48/45 kD protein. That the reaction of MoAb 32F3 and of this patient's serum was identical was demonstrated in a two-dimensional blotting experiment. Moreover, the patient's serum was shown to block transmission of the isolate NF 54. The serum was collected from an elderly missionary who had lived in East Africa for the greater part of his life. He was accustomed to treat the repeated attacks of malaria with chloroquine.

Positive reactions with 48/45, 25, and 10 kD protein were seen also with sera obtained from indigenous people from Burkina Faso and Liberia. These preliminary results indicate that the development of

transmission blocking antibodies might occur also in natural conditions. Further studies are needed to investigate under what circumstances this takes place and especially what the epidemiological significance is of these antibodies.

14. Genomic DNA libraries as well as cDNA libraries prepared from NF54 gametocytes and macrogamete RNA, respectively, have been cloned in *E. coli* expression vectors. Fusion proteins were obtained which did not react with any of the MoAbs. However, by using the "antibody select" method, fractions of a polyvalent rabbit antigamete serum (1771) monospecific for certain proteins, we were able to identify several bacterial clones relating to the 48/45 as well as the 25 kD proteins.

Summary

In summary we conclude that proteins of the 48/45 doublet and 25 kD are involved in transmission blockade. They are produced already in gametocytes—although expressed later on the surface of gametes/zygotes and ookinetes. This might have relevance for the boosting of induced immunity by natural infections. It might be expected that DNA recombinant technology would lead to the production and application of transmission blocking vaccines, preferably as components of a polyvalent malaria vaccine. Such vaccines should be applied in combination with existing and new methods for the control of malaria. There is need now for thorough epidemiological studies in the field in order to identify the sites where these vaccines can be applied in well-controlled field trials.

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Discussion

MCCUTCHAN: Are you using the same clone strain as Dr. Carter?

MEUWISSEN: No, we are using an NF54 isolate that was obtained from a patient infected in the neighborhood of Amsterdam Airport. That's the NF54 line and we use this routinely because tests prove it to be a very prolific and reliable producer of gametocytes.

QUESTION: Is there any possibility that some of the differences in the results between you and Dr. Carter are due to strain differences?

MEUWISSEN: I think our results are almost similar. If we talk about various minor proteins that could be detected, then there are differences with Dr. Carter's observation. With regard to the 230K that he thinks to be more or less involved in protection, we cannot find that. All our monoclonals react with 45K, but the one that reacts with 230K doesn't block at all. We have not obtained, although we tried to find a blocking monoclonal against 230K. In addition, there are differences with regard to the transmission blockade. Some of their monoclonals needed complement. All our monoclonals react without the addition of complement in the transmission blockade.

MITCHELL: In your two-site immunoradiometric assay, the details of that I missed to some extent. Do you always get a signal when you have a non-blocking and a blocking monoclonal antibody in combinaion as either the capture or the target, and do you never get a signal when you have two blocking antibodies or, conversely, two non-blocking monoclonals in that particular assay?

MEUWISSEN: So far, all the anti-NF45s recognize the same epitope and the anti-NF25s do the same, whether they are blocking or not. I can only say that we have so far obtained evidence for a single epitope led to a non-blocking and another epitope to a blocking monoclonal.

V. NUSSENZWEIG: Did you say that the 220K may be coprecipitated, but is it possible, then, by using this two-site assay to demonstrate that if

you capture with one and you come with anti-220K, you get a reaction?

MEUWISSEN: We have not done that yet.

PEREIRA DA SILVA: Do you know if the 45K protein, which is recognized by the protective monoclonal called the blocking monoclonal, is the same as the one recognized by the non-blocking one?

MEUWISSEN: It's the same protein but different epitope. And the epitopes are different because the blocking one is sensitive to reduction and the non-blocking one is not.

ZAVALA: With respect to the epitope also, the fact that one epitope may apparently disappear with the treatment you do, may explain the different epitope but may also be related with the problem of affinity of your monoclonal epitope. So, my question is have you done an inhibition study with the monoclonal epitope to actually identify the epitopes?

MEUWISSEN: I cannot answer your question. We say that the 45K epitope recognized by the monoclonal actively contains a disulfide bridge that's been reduced and thereby disturbs the epitope. That's our opinion at the moment. But, please, understand, I am not a biochemist and there should be a biochemist here to give you more details and information on that.

V. NUSSENZWEIG: Do you know anything about the mechanism of inhibition of zygote formation? Is it simply by agglutination of your gametocytes or gametes?

MEUWISSEN: We are saying that the 45K is related in one way or another to blockage of fertilization of the macrogamete. We have not observed so far any agglutination of macrogametes by the monoclonals, but it might be that microgametes do, but we never see any inhibiton of exflagellation. Anti-25K is present on the ookinetes where they are in the midgut without doing any harm to the ookinetes. And so we presume that the internalization of the ookinete into the midgut cell wall has something to do with the point where the blockage is occurring.

ZAVALA: I am curious about the stage specificity of your monoclonal antibodies. Are they specific for the gametocytes or are they expressed in asexual forms?

MEUWISSEN: I showed that none of our monoclonals reacted whatsoever with asexuals and they are specific for gametes and for gametocyte internal protein.

PERRIN: Can you comment on the fact that you tell us that you need 2 mg/ml of monoclonal antibody to get inhibition. Why so much?

MEUWISSEN: Yes, it's 1–3 mg/ml to get complete blockage. With dilution up to 4x or 5x, you still get inhibition, but not up to 100% and that relates probably to the number of gametocytes that are present in the blood meal that we offer them. Because it is about 10–20 times as high as you would see under natural conditions. So, it is a very direct relationship between the number of gametocytes and the quantity of monoclonal. We are carrying out dilution experiments to do the transmission blocking of our experiments with lower number of gametocytes and with reduced amounts of immunoglobulin.

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Development of a Malaria Vaccine Based on the Structure of the Circumsporozoite Protein

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Introduction

In this presentation we will discuss the rationale for the development of a malaria vaccine containing sporozoite antigens. Such a vaccine, if successful, would be truly prophylactic: it would prevent disease by inhibiting the penetration or development of sporozoites into the host's hepatocytes.

In mammalian hosts, sporozoites develop exclusively in hepatocytes. The penetration of parasites into these non-phagocytic cells is remarkably fast and efficient. Between 10^1 and 10^3 sporozoites can infect susceptible hosts and liver uptake occurs in a few minutes.[1] It seems very likely therefore not only that specific surface recognition processes take place, but also that the membrane molecules of the parasite involved in this interaction would be good candidate antigens for the development of vaccines.

Vaccination with Irradiated Sporozoites

Immunization with sporozoites was first undertaken by Mulligan and co-workers, using an avian malaria system.[2] They found that ultraviolet irradiated sporozoites are highly immunogenic, since they induce sporozoite-specific antibodies and protect immunized birds against sporozoite challenge. More recently it was shown that irradiated sporozoites of rodent, simian, and human malaria also induce sterile immunity (reviewed in reference 3). Immunized animals and volunteers developed high titers of anti-sporozoite antibodies measured by the circumsporozoite (CSP) reaction, by indirect immunofluorescence, and by a more recently developed radioimmunoassay. These antibodies are directed against the sporozoite surface and have biological activity, since they neutralize sporozoite infectivity *in vivo* and *in vitro*.[4,5]

Protection against sporozoite challenge and the corresponding anti-

body response are both stage and species specific. A small number of human volunteers have been successfully vaccinated by repeated exposure to the bite of *P. falciparum* or *P. vivax*-infected irradiated mosquitoes. Of interest and relevance for vaccine development was the finding that immunization with one geographic isolate of the parasite induces protection against challenge with sporozoites of a series of different isolates of the same plasmodial species.[6]

IMMUNOGENICITY OF VIABLE SPOROZOITES

Animals can also be immunized with live non-attenuated sporozoites, followed by suppressive drug treatment to prevent progression of the infection. A large proportion of individuals who live in areas of high malaria endemicity, where they are frequently exposed to the bite of *P. falciparum* and/or *P. vivax*-infected mosquitoes, display anti-sporozoite antibodies. A high percentage of positive reactions occurs even in areas where the transmission season lasts only several weeks or months. Adults have a much higher level of anti-sporozoite antibodies than young children. In the Gambia, West Africa, more than 90% of the sera of individuals over twenty years of age produce surface fluorescence upon incubation with *P. falciparum* sporozoites. Only 40% of the sera of five- to eight-year-old children are positive, under the same conditions.[7]

IMMUNOCHEMICAL AND BIOLOGICAL ACTIVITIES OF CS PROTEINS

The outer membrane of mature infective salivary gland sporozoites is uniformly covered with acidic 30,000 to 60,000 Mr polypeptides, which have been designed as circumsporozoite or CS proteins.[8] It is probable that these polypeptides are involved in the interaction between sporozoites and the hepatocyte membrane. Monoclonal antibodies to CS proteins and their Fab fragments neutralize sporozoite infectivity *in vivo* and *in vitro*, and prevent sporozoite attachment and penetration into target cells.[9,7]

Immature sporozoites found within oocysts, in the midguts of Anopheles mosquitoes, bear little or no CS protein on their outer membrane and are not infective.[10] There is a close temporal relationship between the development of sporozoite infectivity and the appearance of CS protein on the membrane of the parasite. More importantly, the appearance of CS protein upon maturation of the sporozoites also correlates well with their capacity to induce protection in otherwise susceptible hosts.[11]

The CS protiens of rodent, simian, and human malaria parasites have similar structural, biosynthetic, and immunological properties.[1] One interesting feature is that these parasites shed their CS proteins when cross-linked by antibodies, forming a long tail-like precipitate on

the posterior end of the parasite, the CSP reaction.[13] The significance of the CSP reaction is not clear, but it is certain that most or all parasites which have undergone this reaction lose their motility and their infectivity.[14]

The CS proteins constitute a major biosynthetic product of sporozoites. About 5–15% of the ^{35}S-methionine incorporated into proteins by salivary gland sporozoites occurs in the CS proteins. These polypeptides fail to occur in the blood stages of malaria parasites, i.e., they are strictly stage specific. Pulse-chase experiments have revealed the presence of two intracellular precursors of the membrane protein, which have higher molecular weights and more basic isoelectric points. The membrane form of the CS proteins appears to be generated from the precursors by sequential removal of two small basic peptides.[8]

All CS proteins display the same unusual immunological properties. They have a single immunodominant epitope, which is present multiple times in each CS molecule. All monoclonal antibodies obtained by immunization with sporozoites are directed against this epitope. Preincubation of sporozoite extracts with these monoclonal antibodies inhibits by 70 to 100% the binding of antibodies present in the sera of sporozoite-immunized animals and human volunteers.[15] It is therefore certain that most or all anti-sprozoite antibodies of polyclonal antisera are directed against the repetitive epitope of the CS protein.

Structural Characteristics of the CS Proteins

The structure of the CS proteins was clarified by cloning and sequencing of various CS genes. Only one copy of the CS gene is present per haploid genome and its DNA sequence is uninterrupted. The central area of all CS proteins is formed by tandemly repeated sequences of amino acids, which vary in number and in composition according to the plasmodial species. The first plasmodial gene which has been cloned, that of the CS protein of the H strain of *Plasmodium knowlesi*, contains 12 repeats of 12 amino acids each.[16]

The repeat of the CS protein of *P. falciparum*, a human malaria parasite, consists of only four amino acids.[17,18] This sequence is reiterated 36 times in a Brazilian clone of this parasite.[17] The immunodominant epitope of the CS protein of *P. flaciparum* is represented by three consecutive repeats of NANP, i.e., $(NANP)_3$. This was determined by comparing the reactivity with monoclonal antibodies of synthetic peptides containing different numbers of repeats.[19] This epitope $(NANP)_3$, occurs in sporozoites of a large number of isolates of *P. falciparum* from widely different areas of the world. All these isolates react with the same set of monoclonal antibodies.[20] Furthermore, the DNA extracted from blood stages of 23 isolates of *P. falciparum* was found to hybridize with a DNA probe corresponding to the repeat region.[21]

IMMUNOGENICITY OF A SYNTHETIC PEPTIDE CONTAINING THE IMMUNODOMINANT EPITOPE AND OTHER CONSTRUCTS OF THE CS PROTEIN OF *P. FALCIPARUM*

Immunization of experimental animals, rabbits and mice, with the synthetic peptide $(NANP)_3$, coupled to a carrier protein, induces antibodies which produce a CSP reaction and, most importantly, neutralize sporozoite infectivity *in vitro*.[19-22] It has also been found that an *E. coli* fusion protein, containing a series of NANP repeats, is very immunogenic for rodents and induces neutralizing antibodies.[23]

Taken together these findings indicate that the repeats of the CS molecule provide a promising candidate for vaccine development. This view is further strengthened by the finding that the repeats of the CS proteins are highly immunogenic and are conserved among different isolates of human malaria parasites. It remains to be determined whether protection might best be accomplished through the use of a synthetic peptide or recombinant products produced by bacteria, yeast, or a genetically engineered attenuated virus vaccine.[19,23,24]

ACKNOWLEDGMENTS

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Discussion

WERNSDORFER: That the structure and the amino acid sequence in the *P. falciparum* sporozoite surface antigens, from different geographical origin, are the same, is encouraging. Of course, there was also immune pressure, apparently, on at least one of these geographical origins because one, I believe, was from Gambia. The observations in *P. cynomolgi* indicate that there are structural amino acid differences in the

sequence between different strains. Could they possibly express differences between different species?

R. NUSSENZWEIG: I think your comments refer to two points. Let me take the later one related to *P. cynomolgi*. Yes, indeed, the *P. cynomolgi* have evolved in very different regions. They are apparently very separate isolates. The question is if these are different species or are they simply different isolates? The pattern which I showed on my slide was on the data on the falciparum antibody response from the Gambia, but the two CS proteins which were clones so far one was from Brazil and the other one was from Thailand. We have compared a relatively large number of isolates, extracts of sporozoites from very different regions—some from practically all continents where malaria does occur, and the upshot of this is that all are recognized by the same set of monoclonal antibodies. There are some small differences, but it looks as if these are differences which are not related to the composition of the repeats.

BRUCE-CHWATT: Your set of slides showed us this morning that the immunization with sporozoites produces an immunity. Normally, this is a stage-specific immunity. What would happen if those mice were infected with blood merozoites?

R. NUSSENZWEIG: They behave like naive animals. They would not have any protection against blood stages and this is the basic issue in remission to all the protective antigens as far as I understand malaria parasites. Each one of them is stage-specific.

BRUCE-CHWATT: Is the spleen involved in this protection against sporozoites?

R. NUSSENZWEIG: The spleen is probably, because of the amount of immunization and the fact that these are organisms which circulate in the blood, a very important locus of antibody formation. Splenectomized animals produce a much lower type of antibody and a lower percentage is protected, but there are still some animals in which we barely detect antibodies which are protected, indicating that there are certainly other cell-mediated mechanisms which may be important.

DIGGS: Two questions: Did I understand correctly that there are data, neutralization data, on falciparum in humans?

R. NUSSENZWEIG: Data on neutralization in relation to falciparum and vivax malaria come from one of the modes that I showed to detect neutralizing antibody; namely, to incubate the sporozoites with the monoclonal antibody directed against the CS antigen and then look at the adherence attachment to these and penetration and development into

the hepatoma cell. I think that's the only easily feasible way that one can look at it. And, under these conditions the monoclonal antibodies, regardless of the origin of the sporozoites, abolish their infectivity for hepatoma cells.

DIGGS: The second question is a general one. Is there any data yet on co-existence of antibodies and parasitemia in nature in actual populations with CSP specificity?

R. NUSSENZWEIG: No, the data which exist have been generated in a rodent malaria system because of the difficulty in the past to look for anti-sporozoite antibodies due to the limitation of the availability of parasites. What these data say is that the parasitemia at a certain level not only abolishes or reduces the immune response to unrelated, non-malarial antigens—which has been known for some time—but that it also reduces the immune response to malarial antigens. If you induce the parasitemia in a sporozoite immunized animal, it loses the antibody response, it loses protection. If you manipulate that and treat the animal so that you abolish the blood infection, you again get the anti-sporozoite response. The conclusion which one is tempted to draw is that in malaria endemic areas, if the individuals are not treated and have patent malaria, this interferes and delays the anti-sporozoite response. In the Gambia, where people normally are not treated unless they are very ill and go to the hospital, under these conditions this delays and is responsible for this age profile which I showed.

REESE: Ruth, have any of you gone back to the original human sera that you accumulated from the irradiation studies and tried to look at the serum levels of anti-sporozoite antibody to try to determine what level is necessary to provide protection so that once you start immunizing, you could then say, "I have to have this level to have protection, and when it gets below a certain level, we start seeing breakthroughs?"

R. NUSSENZWEIG: Yes, it certainly has been done. These sera have a relatively high anti-sporozoite antibody titers, but one can, by immunization, reproduce this titer in immunized animals.

PERRIN: How strong are the observations that there is an inverse relationship between the antibody titer against sporozoite and against asexual blood stage?

R. NUSSENZWEIG: No, I did not want to imply that there is an inverse correlation. Besides, I am the first to say that these data are messy, because what we were comparing were data against a very well-defined sporozoite antigen versus immunofluorescence with blood stages which has all kinds of antibodies. But, what I meant to say was that there was

no direct correlation, so that you can have very high levels of anti-sporozoite antibodies in the absence of antibodies in the blood stages and vice versa. They are not related. That's all I meant to say.

MITCHELL: Are there any restraints imposed by route of injection? You emphasized the intravenous route in certain experiments with irradiated sporozoites.

R. NUSSENZWEIG: The intravenous route, I would say, is as good as the bite of infected mosquitoes, which might, in a way, end up being intravenous route. Now, I think the story is quite different. One has to keep in mind, when one inoculates the intact parasites as compared to when one uses a purified antigen or a peptide. And then, the story is quite different. But yes, with intact parasites, the intravenous route is better than, let's say, subcutaneous administration of sporozoites.

DIGGS: Concerning my question about parasitemia and antibody, what I was trying to get at is that there is a lot of data being collected on anti-CSP antibody levels in human populations and I wonder if anyone has yet been able to correlate those data with antibody in nature that have malaria?

R. NUSSENZWEIG: Well, this is related at the same time with the question of Dr. Reese. Basically, the volunteer which has been immunized and protected has higher titers of antibodies against the synthetic peptides representing the main immunodominant epitope than any other adult in the Gambia. This is the highest titer—about 3 or 4 times higher than the highest titer in the Gambia. This volunteer was immunized with irradiated sporozoites. Few numbers of irradiated sporozoites mimic the bite of mosquitoes. That would be one level to be achieved if, perhaps, we want complete protection.

RISTIC: Ruth, just a question. Has your group or anybody else that you know of ever shown active protection by immunization with killed sporozoites in any type of animal to any species of malaria?

R. NUSSENZWEIG: Very little work has been done with killed parasites, but the work with the synthetic peptide shows that you obtain neutralization with these protective antibodies.

POWELL: I believe it was true that back around 1940, Paul Russell worked with formalinized sporozoites in chickens and obtained some partial evidence of protection. I'm still having some trouble visualizing why, in nature, if sporozoites have these types of effects, there should not be more pronounced, more complete immunity established in younger children and less of a problem with older children, adolescents, and adults given the types of exposure that occur and the phenomena that I think occur in nature.

R. NUSSENZWEIG: One, at this point, can only speculate about it, but the idea is that in nature what you see is obviously not only the effect of anti-sporozoite immunity, but also the combined result of the protection to blood stages and sporozoites. Second, you have to consider that although the exposure in number of bites in highly endemic areas is very high, the number of parasites which are inoculated during the bite is really a very small number. If you then consider the amount of antigen introduced, this is certainly less than what you would do by immunization. In the third place, an important consideration is the antigenic competition of an individual which is being exposed to all the antigens in the blood stages, which, I mentioned a moment ago, have been sure to cause immunosuppression. I think this is why you see the result in nature. It takes relatively a long time to develop a protective immunity and, therefore, the target population which one would like to reach by vaccine is really young children or individuals who come from a non-endemic area.

NICHOLS: Have you ever done IFA assays on infected liver cells to see if they are showing different surface proteins than normal liver cells?

R. NUSSENZWEIG: They certainly do not expose their sporozoite antigen on the surface. We do not find the CS protein. The CS antigen is conserved; that is, when the sporozoites enter liver cells, you can still detect the presence of the antigen within the exoerythrocytic stages—primarily earlier exoerythrocytic stages, but it's not on the surface of the liver. I have no information about other alterations on the liver cell surface.

SIDDIQUI: Ruth, I would like to ask do you have enough CS protein to have any kind of direct proof for protection or any kind of experiments for the induction of protective antibodies?

R. NUSSENZWEIG: Victor is going to speak about this tomorrow, so I don't want to preempt his talk, but the answer is yes, it's easy to make quite a bit of synthetic peptide.

TRAGER: Ruth, I seem to remember some nice micrographs of Hollingdale's that show a reaction of the monoclonal antibody with the parasitophorus vacuole as well as with the sporozoite surface. Is that correct?

R. NUSSENZWEIG: No, but I think the interpretation of this EM data is that the sporozoite surface antigen is in contact at first with the liver cell in the process of penetration, and that later on, one does find this material within the liver cells. It's carried into the liver cell. I don't know if that answers your question. The precise localization, I really don't think we have data in the liver cell to say where this antigen is.

MARTIN: Could you elaborate on measuring antibodies in vaccinated individuals and correlating antibody levels. . . .

R. NUSSENZWEIG: What I meant to say is that in this plasmodium analysis system, not all of the antibodies which reacted with the peptide also seem to react with the sporozoites. I think that it's a common finding that you have not precisely the same specificity but you have a microheterogeneity of these antibodies. The situation is different in falciparum and there you have very good coincidence or very similar levels of reactivity with the peptide and with surface of the sporozoite.

COPPEL: Is it possible in the past speculation of yours, about the repeat being the binding ligand, did you look at all the peptides to see if these were binding?

R. NUSSENZWEIG: No, these experiments are not being done. It's more than speculation, but I think that it's a plausible one.

MEUWISSEN: I would like to ask you, Ruth, you said in your hybridization experiments that you could detect 50 parasites. What were you referring to? Were these schizonts or were you referring to 50 sporozoites?

R. NUSSENZWEIG: No, the standardization is made on the basis of using the DNA of blood stages. It's based on parasite nuclei, so it detects 50 parasite nuclei in the case of falciparum.

MOSS: What is your appraisal of the immune response to the CS protein presented to the host in vaccinia compared with the responses to sporozoites given in FCA? It was noteworthy that the responses in two rabbits were variable and low compared to the control rabbit repeatedly injected with sporozoites in FCA. This may be antibody to contaminants of the sporozoite extract coated to plastic detecting Ab to the same contaminants after immunization of the rabbit with the same sporozoite extract. That is, I'm sure the sporozoite extract contains say salivary gland proteins which are more immunogenic in the rabbit than the CS protein.

R. NUSSENZWEIG: A quote from a Workshop Report on the Molecular Biology of Host-Parasite Interactions indicated (and I quote), "immunization of animals with irradiated sporozoites provides total protection with no patency in the immunized host." Could you clarify which malaria parasites and which hosts are being referred to?

Discussant: The Circumsporozoite Protein of *Plasmodium Falciparum*

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INTRODUCTION

The circumsporozoite (CS) protein of malarial parasites is the major surface antigen of the sporozoite stage of the *Plasmodium* life cycle.[1] It has been shown to cover the entire surface of the sporozoite.[2] Protective immunity against malaria has been achieved by inoculation of irradiated sporozoites[3] (reviewed in 3). The dominant epitope recognized by antibodies to the CS protein and the one involved in the protective response is included in a single immunodominant region of the protein.[1] In the case of *P. falciparum* this immunodominant region consists of a four amino acid sequence repeated forty-one times in a row.[4] Here we will discuss the identification of the immunodominant repeat of *P. falciparum*, the study of other potentially important regions of the gene, and some important questions that remain with regard to a sporozoite vaccine.

Identification and Isolation of the Circumsporozoite Protein Gene

The CS protein has been the subject of investigation in the laboratory of Ruth Nussenzweig for many years. From these studies it is clear that this protein is an important candidate for a malarial vaccine. Therefore the cloning of this gene and the production of this protein *in vitro* seemed to be one direct step towards a vaccine. Cloning of the CS protein gene, however, presented a particular problem to molecular biologists because of the lack of availability of the sporozoite stage of the parasite. The first CS protein gene was cloned from the simian malaria *P. knowlesi*.[5,6,7] To do this, Drs. Nussenzweig, Godson, and their collaborators collected a large number of sporozoites from the salivary gland of infected mos-

quitoes. *P. knowlesi* was chosen because in the laboratory it produces approximately ten times more sporozoites in an infected mosquito than any of the human malaria species. From these sporozoites, mRNA was isolated and used to make a cDNA library in *E. coli* which contained copies of sporozoite specific messages. The cDNA containing a fragment of the CS protein gene was identified using a monoclonal antibody to the CS protein. This gene segment was then used to identify the entire CS protein gene. Unfortunately the *P. knowlesi* gene was of little use in identifying CS genes of other species of malaria because there were no apparent sequence similarities among genes carried by different species of parasites. Hence, it appeared that the most direct approach to isolating genes from different species was to start from the beginning and collect sporozoites from each of the parasites of interest. The NYU group undertook this work, while we designed a new approach to circumvent much of the difficulty involved in cloning stage-specific genes.

We collected DNA from asexual parasites grown in culture and used this to prepare a library containing intact genes from all stages of the parasite. We were able to do this because of an earlier discovery indicating that the mung bean nuclease cuts *Plasmodium* DNA in areas flanking genes, thereby reducing the genome to fragments containing intact genes.[8] We detected bacterial clones containing the CS protein genes with a monoclonal antibody to the CS protein which was supplied by researchers at Walter Reed. Most DNA fragments that were detected using this approach contained the complete CS protein gene of *P. falciparum*. Two other cloned DNA fragments were identified that encoded other peptides that were weakly reactive with the monoclonal antibodies but had little sequence relationship with the CS protein.[4]

Circumsporozoite Gene Structure

The cloning and DNA sequencing of the *P. falciparum* CS protein gene allowed comparison of this sequence with that of the *P. knowlesi* gene. Although there were only two small regions of DNA sequence similarities (Fig. 1) between the two, there did appear to be a certain structural relationship. Evolutionary conservation of structure at some level was of course expected between two analogous proteins that are presumably responsible for the same functions. For example, a concentration of hydrophobic amino acids at the amino- and carboxyl-terminals of the protein probably serve as signal and anchor regions for the CS proteins. Regions of the two CS proteins that contain a concentration of charged amino acids can also be aligned and probably result from structural restraints on the molecule. The two regions of sequence homology between the *P. knowlesi* and *P. falciparum* genes flank the repeated or immunodominant portion of the molecule. It seemed possible that the

conservation of sequence in these two areas reflected their involvement in some essential parasite function such as reception for liver invasion.[4] The repeated area in the center of the molecule (Fig. 1) is the species specific immunodominant area of the protein and the subject of the most intensive investigation regarding vaccines.

Gene Segments

Knowledge of the gene structure has enabled researchers to produce particular segments of the CS gene for study. Investigation of the repeated peptide that is the immunodominant area of the protein has been of importance because it appears to be a target for vaccine development. Initial experiments by our group with chemically synthesized peptides indicated that as few as two repeats containing seven amino acids mimicked the antibody binding site of the CS protein.[4] This suggested that the overall native configuration of the protein was not necessary to display the appropriate antigenic site and that short chemically synthesized molecules might be useful as a vaccine. Longer segments of the repeated peptide, which have been produced in genetically engineered bacteria, were immunogenic for mice and rabbits.[9] Antibody to the repeating peptide recognized native CS protein and blocked sporozoite invasion of human hepatoma cells *in vitro*. Immunization using areas of the CS protein other than the repeats has also been investigated. Victor Nussenzweig discusses these experiments in this volume. Experiments of this type have also been reported by a group at Walter Reed.[10] Two regions of the protein that appear to be accessible to the host immune system are at or near the sequences that are conserved in *P. knowlesi* and *P. falciparum*. The conserved areas are labeled Region 1 and 2 in Figure 1. The Walter Reed group finds that antibodies to these regions do not block sporozoite invasion. Victor Nussenzweig and collaborators have studied peptides from overlapping areas, labeled N-2 and C-2 in Figure 1. They find that antibodies to region N-2 neutralize sporozoites.

DNA Sequence Drift

The short region of homology between the CS genes of *P. knowlesi* and *P. falciparum* (Region 2 in Figure 1) also appeared to have some potential as an oligonucleotide probe that would be useful in identifying the CS genes of other important malarias.[5] Southern blot analysis of DNA from a number of different malarias using a Region 2 oligonucleotide probe revealed one or two strong bands of hybridization in each (Fig. 2). The bands in the *P. knowlesi* and *P. falciparum* lanes clearly resulted from homology to the CS gene. We therefore screened a mung bean library of *P. berghei* DNA and isolated cloned segments of DNA that had

homology to the Region 2 probe. We have sequenced the different clones and find that, although the Region 2 homology is good, neither contains the *P. berghei* CS gene (Fig. 3). This indicates that the sequence of so-called conserved regions is drifting rapidly and may not be very useful in finding other CS genes. Further, this work indicates that CS protein sequences in general are very plastic and that many alternative sequences may function similarly.

Antigenic Variabilty

Other questions of importance are currently being investigated. The extent and rate of variability in the repeat region seems to be an area of importance in vaccine research. Perhaps much can be learned from the work done on *P. falciparum* S-antigen that is discussed in this volume by Coppel et al. Clearly the repeat area of this molecule varies rapidly enough that there are antigenically distinct populations of *P. falciparum*. The work of Cochrane et al. on *P. cynomolgi* shows antigenic variability in the CS protein of several different isolates of these parasites.[11] This work certainly raises at least two questions: 1) Are there reasons to believe that the *P. falciparum* CS protein gene will be more stable than the S antigen gene or the CS protein gene of *P. cynomolgi*? 2) What effect would small subpopulations containing variant CS protein epitopes have on the effectiveness of a vaccine? In this volume Victor Nussenzweig discusses the potential use of peptide portions of the CS protein other than the repeated region being used for immunization. These regions are probably more stable than the repeats and are certainly accessible to the immune system. The work that we have described above, however, suggests that even these portions of the CS protein gene are subject to sequence alteration or drift. If no portion of these genes are tightly selected in evolution, one must wonder about the long-term effectiveness of a vaccine that is CSP derived.

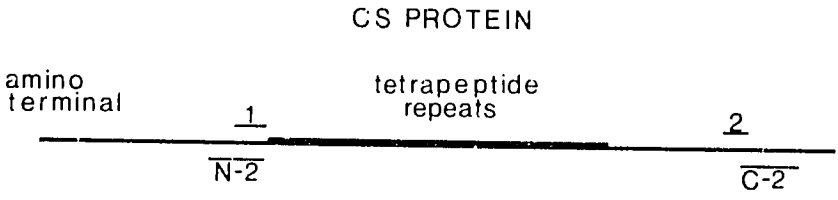

FIGURE 1. *A Diagram of the Plasmodium falciparum Circumsporozoite Protein.* The numbers 1 and 2 indicate regions of DNA sequence homology between the *P. knowlesi* and *P. falciparum* CS protein genes (4). N-2 and C-2 indicate regions of the *P. falciparum* protein used for immunization studies by Victor Nussenzweig and collaborators which are described in this volume.

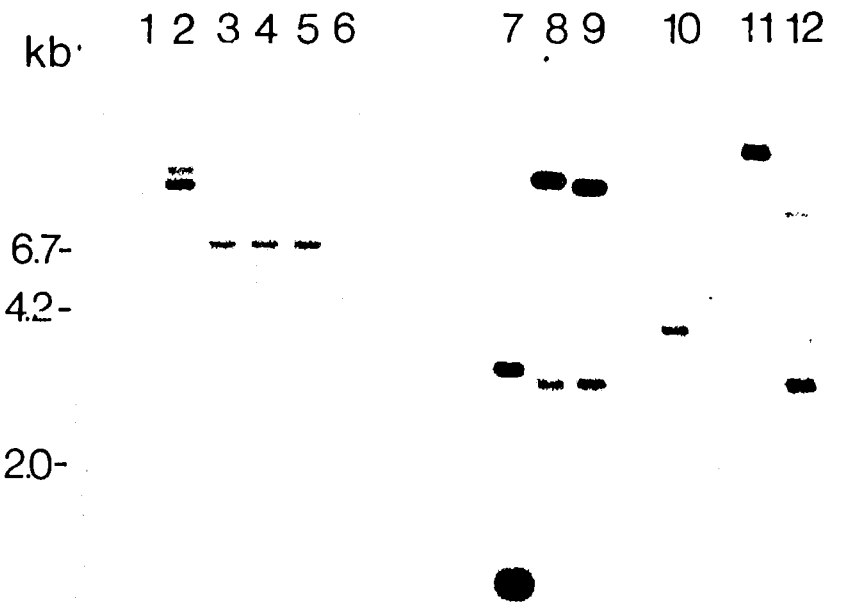

FIGURE 2. *A Comparison of Region 2 DNA Sequence Homology with a Plasmodium berghei Sequence*

	Region 2
P. falciparum	CCA TGT AGT GTA ACT TGT GGA AAT G
P. knowlesi	CCA TGC AGT GTA ACC TGT GGA AAT G
P. berghei	CCA TGG ACT GAA ACC TGT GGA AAT G

FIGURE 3. *Southern Blot Analysis of Several Restricted DNA's Using a Region 2 Probe.* 1. *P. vinckei*, 2. *P. yoelii*, 3., 4. and 5. Three strains of *P. berghei*, 6. Mouse, 7. *P. knowlesi*, 8. and 9. Two strains of *P. cynomolgi*, 10. *P. brasilianum*, 11. *P. falciparum*, and 12. *P. fragile*

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Schizont/Merozoite Antigens of *P. Falciparum*

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The three major surface antigens of *P. falciparum* merozoites appear to be derived by proteolytic processing from a single precursor polypeptide synthesized in schizonts.[1] In the Wellcome (Lagos) strain of *P. falciparum* the precursor has an apparent molecular weight (MW) of 195,000. A polyvalent antiserum raised against this polypeptide immunoprecipitated labelled bands of 83,000 MW, 42,000 MW, and 195,000 MW from extracts of ^{125}I surface-labelled merozoites. Peptide mapping indicated that the 83,000 MW and the 42,000 MW bands are non-overlapping fragments of the 195,000 MW precursor.

Satisfactory peptide maps were not obtained for the 19,000 MW polypeptide, and our conclusion that it is also a fragment of the 195,000 MW precursor is tentative. We have made three monoclonal antibodies that immunoprecipitate the 42,000 MW and the 19,000 MW fragments from extracts of merozoites (these also precipitate the 195,000 MW precursor from schizont extracts). However, these antibodies always precipitate both fragments together. The two are not disulphide-linked, and our working hypothesis is that our antibodies bind to only one fragment, the other being coprecipitated due to non-covalent association.

The 83,000 MW fragment seems to be shed from the merozoite surface. It can be recovered in soluble form from culture supernatant and cannot be detected in newly invaded ring-forms.

On the other hand, the monoclonal antibodies specific for the 42,000/19,000 MW complex react with ring-stage parasites in the immunofluorescence test using acetone-fixed cells, and these fragments are not present in culture supernatant. The functions of these merozoite surface antigens are not known, but their abundance suggests that they may be constituents of the merozoite surface coat. As such they may be involved in the initial interaction of the merozoite and the red cell, or they may serve the function of protecting the merozoite from immune attack.

The 195,000 MW precursor of these three merozoite surface components shows considerable antigenic diversity between different isolates of *P. falciparum*, although some epitopes are conserved in all serotypes.[2] Where the variable epitopes are situated with respect to the 83,000 MW, 42,000 MW, and 19,000 MW fragments has yet to be elucidated.

Whether the 195,000 MW protein is a protective antigen is not yet known, but is interesting to note that young children with acute malaria may have a high titer of antibody against it, whilst it is not uncommon to find sera from immune adults that is low in antibody titer against the 195,000 MW antigen (Fig. 1). However, it is encouraging that in a recent study squirrel monkeys immunized with purified 195,000 MW protein (referred to as 200K polypeptide) in Freund's adjuvant were partially protected against challenge with a heterologous *P. falciparum* strain.[3] It is possible that this antigen can elicit a protective response via a cell mediated immune effector pathway, in which case the titer of antibody specific for the 195,000 MW antigen in the serum of individuals living in endemic areas need not correlate with their immune status.

Two groups have recently described a 155,000 MW protein that is deposited on the red cell surface as the merozoite invades.[4,5] The Swedish group have reported that there is an "excellent correlation" between invasion inhibitory activity and antibody titer against the 155,000 MW protein in sera from Liberian donors.[6] The Australian group have called the protein RESA (ring-infected erythrocyte surface antigen) and they have shown by cDNA sequencing that the protein contains extensive stretches of peptide repeats, which appear to be conserved between strains of *P. falciparum*.[4] In free merozoites RESA is located within the rhoptries (R. L. Coppel, Walter and Eliza Hall Institute, personal communication).

We have recently identified an antigenic protein that is located within the rhoptries of *P. falciparum* merozoites.[7] Monoclonal antibody 61.3 stained free merozoites in the IIF test, apparently binding to internal paired organelles. The antibody immunoprecipitated a 140,000 MW protein and a 155,000 MW protein from schizont extracts. We purified the antigen from a detergent extract of schizonts by affinity chromatography using antibody 61.3 coupled to Sepharose. After washing away unbound material, the 155,000 MW band was eluted using 2M KSCN. Then the 140,000 MW antigen was eluted using 50 mM diethylamine pH 11.5.

We raised a polyvalent antiserum in rabbits against the 140,000 MW protein, and this antiserum reacted only with the 140,000 MW band on Western blots of schizont and merozoite proteins. The antiserum bound to the rhoptries of cryosectioned merozoites, demonstrated by immunoelectron microscopy, indicating that the 140,000 MW protein is located within the rhoptries. It has been suggested (L. Q. Tam, University of

Hawaii, personal communication) that the 155,000 MW protein that is noncovalently associated with the 140,000 MW rhoptry protein might actually be RESA.

On the basis of immunoprecipitations using immune and nonimmune sera, as well as monoclonal antibodies that inhibit the growth of *P. falciparum in vitro*, Perrin and co-workers have defined a subset of schizont/merozoite proteins that are likely to be protective antigens.[8] Their sizes are 200,000 MW, 140,000 MW, 82,000 MW, and 41,000 MW. The 200,000 MW protein is identical to the 195,000 MW schizont protein described above. The 41,000 MW protein is localized in merozoite rhoptries and in purified, non-denatured form has been used to immunize squirrel monkeys against *P. falciparum* challenge with notable success (L. H. Perrin, University of Geneva, these proceedings).

Perkins has identified two schizont/merozoite polypeptides, of 155,000 MW and 130,000 MW, that seem to have a high affinity for erythrocyte glycophorin.[9] It is possible that these proteins may be involved in the initial attachment of the merozoite to the red cell. Again, it will be noticed that one of these proteins is of the same size as RESA.

Finally, Dubois et al. found that squirrel monkeys protected against *P. falciparum* challenge by immunization with a gel-eluted schizont protein fraction made a good antibody response against a 90,000 MW parasite protein.[10] Recently these workers have made a monoclonal antibody against this protein.[11] Fluorescent antibody staining studies indicate that the 90,000 MW protein is located within the parasitophorous vacuole of the schizont and that it is not associated with free merozoites.

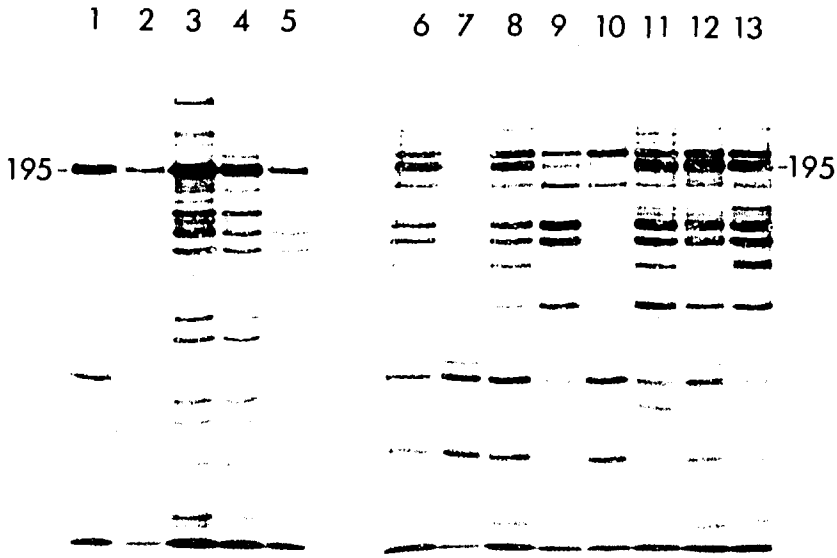

FIGURE 1. *P. falciparum* Polypeptides Immunoprecipitated by Individual Gambian Sera. *P. falciparum* polypeptides were biosynthetically labelled with ^{35}S-methionine and solubilized in a buffer containing 1% NP40. Individual sera from Gambian donors were used to immunoprecipitate labelled parasite polypeptides, which were separated by SDS-polyacrylamide gel electrophoresis and detected by fluorography. The position of the 195,000 MW protein is indicated.

Tracks 1–5: Immunoprecipitations using sera from non-immune children

Tracks 6–13: Immunoprecipitations using sera from immune adults.

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Discussion

COPPEL: From the rabbit serum you raised against the 195K—did you ever use that for any growth inhibition studies *in vitro?*

FREEMAN: Yes. Negative results—if it would have been positive, I would have mentioned it.

GREEN: I have a comment and question for Dr. Freeman. First I'd mention that we also have been working with a 43Kd antigen which stains by immunofloresecence identically to the one he's looking at and we've also worked with a 25Kd antigen. We have managed to raise a polyclonal serum against the 25Kd antigen and tried that in parasite inhibition assay, and we've found that it is not protective. We've not yet done that with the 43Kd. I wonder if Dr. Freeman could comment whether he has used either of these in protective assays. I'm asking if you have looked at the monoclonal or polyclonal serum against the 43Kd antigen in protective assays?

FREEMAN: We have a monoclonal that recognizes the 42K processing fragment and it doesn't do anything in terms of *in vitro* inhibition of growth. And as I mentioned in response to Ross Coppel's question, a rabbit polyvalent antiserum against the whole protein also doesn't inhibit *in vitro*.

SIDDIQUI: Just a small comment with reference to Ted Green's comment. I think we should be very careful when we say "*in vitro* inhibition"; we should not use this synonomously with protection.

REESE: We have monoclonal antibodies against the 83K which see epitopes which are found on virtually every isolate we have looked at. I think if you look at the right place on the molecule, you are going to find that it is conserved. We've got about 150 against it though, and it's taken a while. It probably is a reasonable candidate, and what you have to make sure is that the immune response is against the right portion of the molecule.

FREEMAN: Fair enough. I am not trying to argue against the possibility that there might be extensive genetic diversity or polymorphism along the whole sequence. I think that the strain specific determinants are going to be quite restricted. But if the critical antigens do vary, or if they

are diverse because they are trying to escape an immune response, then they could be the very ones we want to go for.

TAM: Dr. Freeman has been asked several times this morning whether the monoclonal antibody to the high molecular weight merozoite surface coat precursor inhibited *in vitro* and the answer was no. He has also reported an experiment in which the homologous *P. yoelii* antigen was isolated with monoclonal antibody and used to immunize mice and the mice were protected. But when he did passive transfer with serum from those mice, the mice were not protected. These are two pieces of evidence which indicate that antibody to this antigen is not protective. Should we exclude this as candidate antigen? I think not. Perhaps because none of these data measure CMI.

FREEMAN: Thanks very much for bringing that point up because it's very pertinent to this discussion. Just to recap on that, the high molecular weight glycoprotein from *Plasmodium yoelii* when in purified form and presented with an adjuvant such as saponin, provides really impressive protection in mice against challenge. I have avoided talking about that because when we started out in this work, that was our first success and we wanted to cross over to falciparum. But that's where we ran into trouble. I'm not quite sure whether you can work in one system (the mouse) and expect to get the same sort of responses in monkey. You really run into problems. Still we used the same adjuvant, saponin, and immunized some monkeys with this purified falciparum, 195K molecular weight protein and those monkeys made good antibody but were not protected. So I think perhaps if the high molelcular weight glycoprotein is going to be a protective antigen, it'll probably be operating via a cell-mediated immune pathway. This is somewhat disillusioning to me because the whole basis of the way I look at the problem starts from the early McGregor passive transfer experiments where taking gammaglobulin from adult Africans and injecting it into young children with acute malaria produced very rapid cure and clearance of parasitemia. So it seems to me that there is an antigen associated with the blood form, either on the merozoite or the surface of the red cell, that is highly susceptible to being blocked in its function by the action of antibody binding, but I suspect from all this that it's probably not this high molecular weight glycoprotein but another one.

PEREIRA DA SILVA: Dr. Freeman did you use in your immunization experiment the 195K protein? Was it native or denatured protein? Was it native protein or did you use detergents and denaturing agents, reducing agents?

FREEMAN: Yes, it was the native protein largely in undegraded form.

PEREIRA DA SILVA: Nondegraded, just purified by immunoabsorbents, and not using SDS in general?

FREEMAN: No, it was just purified by affinity chromatography.

SIDDIQUI: I'm very pleased that Dr. Freeman has discussed this experiment and made this public. This really points out the important point that induction of antibody does not necessarily mean that we're talking about protection. CMI is going to play a very important role. And I think that it's not only just limited to the erythrocytic stages, but it may have some relevance to the sporozoite also. If we want to call a sporozoite antigen as a protective antigen, induction of antibody may not be enough. We have to have some evidence that antibody is enough, and the evidence is the direct challenge.

RISTIC: I'd just like to go back to Dr. Freeman. I for one share his opinion that there is a big difference in the immune response and protective immunity between a mouse model and a monkey model. I know that at one time people have induced protective immunity in *P. berghei* with nonspecific means such as BCG or as we have done with *Babesia rhodesi* in mice with BCG injection. You cannot induce protection of this highly nonspecific type with either the monkey host or with *Babesia* in cattle. It doesn't work. So protective immunity in mouse versus monkey with malaria or mouse versus cow with *Babesia*, it's completely different.

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Changes Induced in the Surface of Erythrocytes by the Human Malarial Parasite *Plasmodium Falciparum*

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I. Introduction

Invasion of erythrocytes by species of *Plasmodia* initiates the phase of parasite growth which gives rise to the symptoms of malaria. During the blood stages, there i tremendous amplification of parasites which increase in number every 48 hours. In order to accomplish this growth, each parasite must convert the highly specialized, metabolically inactive red blood cell host into a rapidly metabolizing factory for new parasites. This change implies modification of the erythrocyte membrane to, first, admit the parasite itself and, later, to allow entrance of metabolites and expulsion of wastes. The nature of these stage-specific changes is of considerable interest in understanding and potentially controlling the disease. This review addresses biochemical and morphological changes in the parasitized erythrocyte membrane starting with invasion by merozoites and progressing through the 48 hours of the intraerythrocytic cycle.

II. Alterations Due to External Parasites

A. Merozoite Surface Proteins Which Bind to the Red Blood Cell Surface

Electron microscopic data suggest that at least one component on the merozoite surface binds with components of the erythrocyte membrane.[1-6] Based on indirect evidence, a number of merozoite surface proteins have been implicated in this interaction (Table I). These parasite proteins have been purported to bind to glycophorin, to the erythroglycan of red blood cell band 3, or to N-acetyglucosamine which is found on both of these erythrocyte components.[7-12,13,9] A variety of

TABLE I. Erythrocyte Structures Which Bind Parasite Proteins*

Possible Erythrocyte Receptor	*$M_r \times 10^{-3}$ of Parasite Protein*	*References*
Glycophorin	(155?) 130	7–11
Erythroglycan of Band 3	?	13
N-Acetylglucosamine	140, 70, 35	9

* For a detailed discussion of the properties of each of these molecules, please see "Use of biotechnology to study *Plasmodium falciparum* asexual blood stage antigens," these volumes.

techniques used in different laboratories have implicated a series of distinct parasite proteins suggesting the existence of many possible binding sites of similar energy or that the energy of interaction of individual parasite proteins with components of the erythrocyte surface is generally low. As yet, it is not known if any of these merozoite surface molecules remain attached to the erythrocyte surface after penetration is completed. In addition, there is no evidence that any merozoite surface molecules, which are often found free in the culture supernatant, bind to the surface of erythrocytes on their own. Whether these soluble merozoite surface components can be used as a means of identifying and attacking the parasitized erythrocytes is still unresolved.

B. MEROZOITE SECRETORY MOLECULES INSERTED INTO THE RED BLOOD CELL MEMBRANE AS PART OF ENDOCYTOSIS

At present, there is no rigorous evidence that during endocytosis any proteins of parasite origin are inserted into the membrane of the erythrocyte. Nevertheless, electron microscopic studies beginning with those of Ladda et al. suggest that an electron-dense material normally contained in organelles (rhoptries and micronemes) found at the apical end of the merozoite may be secreted during the invasion process.[1-6] These investigators suggested that endocytosis was driven by "membrane-active" materials released from the polar organelles which caused the erythrocyte membrane to invaginate.[1,2] This stimulated formation of a cavity within the erythrocyte into which the parasite moved. Consistent with such a hypothesis is the observation that, whereas these organelles are easily identifiable at the segmenter through merozoite stages, they are no longer distinguishable once entry has been completed and "ring" morphology is assumed.[2,6] Examination of time-lapse photographs suggests that entry can be completed in under a minute; thus, invasion probably does not require the synthesis of new proteins.[3] The electron microscopic studies of Ladda and Bannister suggest that the "membrane-active" substances are first released from the micronemes and then from the rhoptries.[1,2] This is understandable if the

smaller micronemes are truly connected with the rhoptries as some studies suggest.[1,2]

During endocytosis, the portion of the erythrocyte membrane with which the parasite interacts undergoes a series of rapid changes. This was established by Langreth in a cytochemical study in which she found that within a three-minute period during parasite entrance (the shortest time measured), certain enzymes normally found in the erythrocyte membrane had reversed their orientation.[3] Specifically, one enzyme, which had been located on the outside of the erythrocyte membrane, was oriented into the erythrocyte from the parasitophorous vacuole membrane, and a second enzyme, which had been found on the inside of the red blood cell membrane, faced into the parasitophorous vacuole.

Evidence that the rhoptry material plays a role in generating the parasitophorous vacuole membrane comes mainly from electron microscopic data.[1-3,9] These studies suggest that although most of the rhoptry's content is secreted during invasion, the remainder is expressed into the parasitophorous vacuole, perhaps to enlarge it, and then the rhoptries disappear. The rhoptry components have been observed outside of the merozoite by Bannister et al., who suggested that they condense into membraneous lamellae.[3] However, we must await biochemical characterization of the proteins involved before we can determine whether these results are likely to be real or artifactual.

Group I Rhoptry Polypeptides. Monoclonal antibodies have been isolated which react with antigens concentrated in two discrete regions within the merozoite. Because of the bipunctate immunofluorescence pattern and the observation that these antigens are located in an area distinguishable from the nucleus, it is believed that the antibodies recognize antigens contained within the rhoptries.[11-19] These antibodies bind various members of what we will refer to as either Group I or Group II rhoptry proteins (Table II). Polypeptides in the first group, characterized by relative molecular weights (M_rs) of 82K, 70K, 67K, 39K, and 37K, are found in merozoites and schizonts.[17] If we normalize to the concentration of protein 82 (p82) found in schizonts or in rings shortly after merozoite penetration, then the p70 and p67 polypeptides are either lost or sufficiently altered during erythrocyte invasion so that the monoclonal antibodies (McAbs) no longer detect their presence in the newly invaded erythrocyte. Polypeptides p39 and p37 appear to be quantitatively reduced at the ring stage relative to their concentrations in the schizont or merozoite (Fig. 1). As yet, we do not know if these polypeptides are organized into one or more protein complexes.

Group II Rhoptry Polypeptides. A second group of proteins, which appear to be found in the rhoptry during the merozoite stage, have molecular weights of approximately 155K, 135K, 100K, 60K, and 35K.[14,16,18,19] At present, there is no evidence upon which to propose a

TABLE II. Alterations in the Erythrocyte Surface by Proteins Secreted by the Merozoite During Penetration

Group	$M_r \times 10^{-3}$ *of Rhoptry Proteins at Merozoite Stage*	$M_r \times 10^{-3}$ *of Rhoptry Proteins at Young Ring Stage*
I		
Low Molecular Weight	82, 70, 67, 39, 37	82, 39, 37
II		
High Molecular Weight	155, 135, 100, 60, 35	

specific role for these molecules, but data should be available shortly since several laboratories have monospecific reagents which detect them. As yet, it is unclear whether the proteins referred to as 155 by different laboratories are the same or not.[14-16,18-22] This is confused even more by the realization that the molecular weights may in part be artifacts of the gel system or the molecular weight markers used in their determination. These problems, together with the real variance in molecular size which may come from isolate diversity, are not likely to be resolved until protein and DNA sequence data are available or reagents to all of the proteins can be tested in a single laboratory. Nevertheless, based on the sequences of the DNAs encoding two of these proteins and the assumption that they both represent the carboxyl terminus of each molecule, it is likely that the 155K protein which is purported to be part of the merozoite surface is different from a 155K protein which may be associated with the rhoptry.[21,14,16,18-22] This second 155K protein has been referred to as RESA. Based on information available now, it is our opinion that it may well be the 155K rhoptry protein.[23] The protein encoded by this parasite gene first appears in trophozoites and then increases in concentration in schizonts and merozoites. It was conserved among the four isolates from Papua New Guinea, as well as the one from Thailand and another from Ghana which were tested.[20]

III. Alterations Due to Internal Parasites

Once entrance into the erythrocyte has been completed, there is no available evidence for further modification of the erythrocyte membrane until approximately 24 hours, or halfway through the growth cycle. At this point, a dramatic change generally occurs (Fig. 2). The parasitized cell develops a form somewhere between an echinocyte, having evenly distributed projections ranging from stage I (biconcave) to stage III (spherical), to an acanthocyte, where the almost spherical cell has surface protrusions whose shape and spacing are irregular. These morphological changes of erythrocytes are known to be induced

by low intracellular potassium and pyruvate kinase deficiency, as well as uremia, and are also found in a series of disease states where protein and lipid metabolism are altered.[25] Uremia can also induce them.[25] Since the disease malaria often leads to uremia and metabolic disturbances, it is not surprising to find red blood cells undergoing such changes in infected individuals. Parasite infection of an erythrocyte may also alter the potassium levels in the compartment outside of the parasite. This would become most pronounced beginning at the trophozoite stage as the parasite completes synthesis of new ribosomes and is able to greatly expand protein synthesis.[24-26] Any lipids it might steal from the erythrocyte membrane might also contribute to induction of irregular erythrocyte morphology.[27] With these factors in mind, it is impossible to unequivocally link alterations induced in the surface of parasitized erythrocytes with the insertion of any specific parasite protein or proteins. However, it has been undeniably established with a variety of independent techniques used in different laboratories that the membrane is altered, possibly by the insertion or exposure of new antigens.

A. KNOB-RELATED CHANGES

Electron micrographs (Fig. 3) of parasitized erythrocytes during the last 24 hours of the intraerythrocyte cycle clearly show the uninterrupted lipid bilayer covering electron-dense material localized just under the parasitized erythrocyte plasma membrane.[28-31,34] This material was initially believed by some workers to be in the membrane, in regions of the cell where protrusions occur.[32,33] These electron-dense deposits with the protrusions above were first described by Trager et al. and called knobs.[28-30] Cloned cell lines have been derived which no longer produce knobs (K^-).[34,35] The existence of such cell lines is the first evidence that some of the changes in the erythrocyte morphology are not related to indirect metabolic or ionic changes such as potassium levels but are more probably due to insertion of new parasite-derived components or parasite-induced modification of the structures already existing in the erythrocyte membrane.

When we compared a K^- clone with the K^+ clone from which it derived, a major change was observed. The K^+ clones had a histidine-containing protein of approximately 88K which was not detectable in the K^- progeny. With selection, it was possible to observe K^+ progeny arising from the K^- clones. These K^+ revertants contained a similar protein of M_r 82K. The rates at which K^+ changed to K^- and K^- reverted to K^+ were of the same general magnitude, i.e., 5×10^{-6} per cell per generation. This is consistent with the rate at which mutational events often occur. That in going from K^+ to K^- and then back to K^+, there was a significant change in the apparent M_r of this molecule suggests loss of sev-

eral amino acids which might best be explained by a recombinational event rather than a point mutation. Data such as these have been used to link this 85–95K protein with the occurrence of knobs.[32,33,36–39]

When it was first suggested that there might be a correlation between the presence of a 90K protein and the presence of knobs, the protein was believed to be located in the erythrocyte membrane.[33] The important piece of information which was used to localize this protein in the membrane was the immunoelectron microscopic reaction of a rabbit antiserum immunized with a putative avian analogue of this protein.[33] The avian protein has been referred to as histidine-rich protein (HRP) because of its high histidine content.[44,45] This was not necessarily consistent with the electron microscopic data which showed that K^+ cells contained an electron-dense area in the membrane skeletal region. Biochemical data which should also be considered demonstrated that the 90K protein was not easily extractable in nonionic detergents but was highly soluble in SDS, which is known to dissolve the membrane skeleton and other poorly soluble membrane proteins.[33,36] These biochemical and electron microscopic data have now been reproduced in at least three different laboratories.[33,36,39] It is possible, therefore, that an 85–90K protein which is produced by the parasite is inserted into the erythrocyte membrane skeleton causing or contributing to dramatic changes in cell structure. It is also possible that this protein extends across the erythrocyte plasma membrane. There is precendent for this in such proteins as glycophorin and Band 3. There is, however, no rigorous proof that the 85–90K protein does extend through the membrane into the aqueous environment outside of the cell, nor is there any proof that it is responsible for knob formation.

More than one parasite-derived protein which is associated with the erythrocyte skeleton or membrane may be present in K^+ organisms and absent in K^- (Table III). At least three or four proteins made by the parasite become associated with the erythrocyte membrane at approximately the trophozoite stage of development (Table III).[36–38,40] Of these, more than one may be lost in K^- cells, making it inappropriate, based on currently available data, to suggest that a single parasite protein is responsible for knob formation.[37–39]

That K^+ organisms can directly give rise to K^- at a significant rate and vice versa suggests that during the course of most human infections an individual will be exposed to both.[34] Nevertheless, organisms isolated from the blood are typically K^+, suggesting a strong selective advantage for these organisms *in vivo*. This selective advantage does not exit *in vitro* and may even shift in favor of the K^-.[35] What happens to K^- organisms *in vivo* is at present unknown. It is likely that forms of the parasite which are K^- and perhaps have other changes as well are less lethal to their host. Recent work by Langreth and Petersen has demonstrated

TABLE III. Alterations of the Erythrocyte Surface Caused by Internal Parasites

IFA Pattern	*Distribution*	K^+	K^-	*Cytoskeletal Linkage*	$M_r \times 10^{-3}$	*Isolate Specificity*	*References*
Confluent RBC Surface or Spotty*	Trophozoites, Schizonts	+	+	—	300–400	Broad	37, 58
Confluent RBC Surface or Spotty	Trophozoites, Schizonts	+	—(?)	?	260–300	Limited?	36
Confluent RBC Surface or Spotty	Trophozoites, Schizonts	+	—	+	84–94 (Band Typically Indistinct)	Broad	31, 34, 37

* One McAb gives spotty fluorescence with some isolates; spots appear membrane-associated but may be intracellular also.[37]

that the presence of knobs may be a virulence factor.[41] A K^- clone was found to cause a very limited infection in intact monkeys while splenectomized animals developed infections equivalent to those seen in animals infected with noncloned K^+ organisms. Trager has data suggesting that K^- organisms may have the capacity to live for extended periods in intact animals.[42,43] With this potential as well as the ability to revert to its K^+ form as the *in vitro* studies demonstrate, the organism clearly has the capability to exist in a subacute fashion, which has not been widely appreciated. Thus, if we hope to effectively rid the body of malarial parasites, we must bear in mind that there may be the potential of clearing the body of one form of the organism only to create a less visible carrier state from which acute disease may arise later.

B. Antibody Binding to Infected Red Blood Cells

A substantial amount of evidence exists that parasitism actually alters the surface of the erythrocyte membrane. Although most of this evidence is immunologic, it derives from a series of different techniques; thus, the data from each reinforces the other (Table IV).

In the first study, it was demonstrated that immune serum from animals that are susceptible to human malaria can recognize parasite-induced alterations on the erythrocyte surface.[46] Several important points were made by these experiments. It was shown that sera from owl monkeys immune to *P. falciparum* always contained antibodies which could inhibit *in vitro* growth of the parasite and bind to the surface of erythrocytes containing live parasites.[46,47,48] Such sera have also been found to provide passive protection to other owl monkeys.[49] Some of the antibodies in these sera were localized in the regions of the knobs on K^+ cells from cultured parasites. The antibodies were so low in concentration that they could not easily be visualized by immunofluorescence but generally required immunoelectron microscopy. K^- cultured cells did

Table IV. Evidence for New Antigens on the Surface of Parasitized Erythrocytes

1. Antibodies from immune monkey serum have been demonstrated by immunoelectron microscopy to bind to K^+ parasitized erythrocytes; this binding did not appear isolate-specific, although quantitative differences among isolates would not have been detected [Langreth and Reese].[44]
2. Antibodies from immune monkey serum can blok the *in vitro* binding of parasitized erythrocytes to certain culture cell lines [Leech *et al.*].[46]
3. Antibodies in immune human or monkey serum can block the uptake of ^{35}S-methionine into trophozoites and stop the intracellular development of the parasite [Stanley and Reese].[50]
4. Parasitized erythrocytes activate the complement system in the absence of immune serum; in the presence of antibody, the complement system is activated more efficiently [Stanley and Reese].[47]

not appear to bind antibody. To rule out possible artifacts arising from the use of cultured cells, K^+ parasitized cells were obtained directly from monkeys. Again, antibodies from immune monkey serum appeared to localize in the regions associated with the knobs. Antibody binding was observed regardless of whether the cell examined was infected with homologous parasites or an organism from a different area of the world than that used to immunize the monkey.[16] Because electron microscopy is not easy to quantitate, no conclusions were drawn about degrees of cross reactivity, but it was clear that there was substantial conservation of certain epitopes on the surface of parasitized cells. Conservation of the epitopes was reasoned to be highly likely if these antibodies were recognizing a receptor-like structure which might play a role in allowing parasitized cells to bind to endothelial cells.[44,50,51] Immobilization of schizonts in capillaries due to specific interactions between the knobs and the endothelium had previously been postulated.

At this point it became clear that attention should be focused on those epitopes which were conserved among various isolates of *P. falciparum* since they might provide useful targets for a protective immune response. Structures which the parasite could alter to the extent that many forms are permitted or so that substantial regions of three-dimensional structures are not retained are obviously poor candidates for immunologic protection. The parasite has already developed a method of modulating these and, thus, can readily protect itself against responses to them.

More recently, it has been demostrated that passage of a K^- clone through an owl monkey can cause the expression of parasite-induced antigens in the erythrocyte surface. Since the parasitized cells still lacked knobs, the expressed antigens are clearly *non*-knob associated.[52] In addition, immune human sera, which contain much more specific antibody to a high molecular weight erythrocyte membrane-associated antigen than immune monkey sera, detect antigens on the parasitized red cell surface of both K^+ and K^- cells.[53,54]

Inhibition of Intracellular Growth of the Parasite. The premise that antigens on the surface of the parasitized erythrocyte should be considered a target for any form of immunologic protection is supported by several groups of experiments. Firstly, these antigens appear to be available as recognition sites for 24 hours in contrast with the relatively short-lived merozoite surface antigens; thus, several arms of the immune response can expected to be effective agains. them.[10,55-58] Secondly, evidence exists that an effective immune response to malaria is composed to varying degrees of many different factors. Specifically, preliminary data suggest that in addition to cellular immunity, antibodies from immune human serum can react with the parasitized erythrocyte surface and alter the uptake and perhaps the disposal of essential metabolites such

as the amino acid methionine and in so doing inhibit the intracellular development of the parasite.[55–57,58] The antibodies involved are not isolate-specific, which is consistent with the idea that they may bind to the orifice of some channel involved in membrane transport. They are often found in individuals who have not yet produced high levels of antibody to internal schizont or merozoite antigens, suggesting that production of antibodies to these structures may be one of the earliest responses which is triggered. Thus, these antigens may be among the first targets of the host's immune response.[58]

Complement and the Red Blood Cell. Binding of antibodies to the surface of the parasitized red cell not only has the direct effect mentioned above but they also trigger a series of indirect effects. Among the most important is the substantial activation of the body of plasma proteins, many of which are enzymes, which are collectively referred to as complement.[59] Complement activation leads to deposition of biologically reactive peptides on the surface of infected cells and causes the continued release of biologic activators which are called "chemotactic factors." This stream of chemotactic factors attracts various groups of white blood cells to the infected erythrocytes even though they are sequestered in the deep vasculature.[46,50,55–57] That phagocytic white blood cells can effectively recognize, engulf, and destroy parasitized erythrocytes is clearly documented.[46,55–57] How effective this aspect of the immune response is in providing protection in primates or man has never been rigorously established. There are, however, suggestions from studies on rodent malarias that the importance of this aspect of immunity should not be underestimated.

In studying the interaction of parasitized erythrocytes with the complement system, two other facts were learned. First of all, it was found that the parasite altered the erythrocyte surface so that even before significant antibody levels were stimulated, the complement system was activated by the "alternative" (nonantibody) pathway.[59] This would suggest that trophozoites and schizonts (rings have not been examined) contain some complement components on their surfaces regardless of the level of immunity of the host at that point in time. Does the parasite subvert this basic protective mechanism in order to create channels through the erythrocyte membrane by which it can more easily obtain extracellular metabolites and discharge toxic by-products? This theory is consistent with information suggesting that beginning with the trophozoite stage, the outer red blood cell membrane becomes far more porous. Both complement fixation and permeability changes occur at a point in the parasite cycle when it escalates its metabolic level, but, as yet, there is no formal evidence to link them. This whole area clearly needs a great deal more study if we are to achieve any fundamental understanding of what occurs after parasitism of an erythrocyte.

Morphologic Changes of Parasitized Erythrocytes Appear to be Independent of Other Surface Changes. A second important point learned from studying the effects of complement on parasitized cells was that the dramatic physical changes in the erythrocyte structure, which we referred to as knobs, occurred independent of the expression of changes in the plasma membrane of the parasitized erythrocytes. This meant that these changes were likely to be under the control of different molecular events. The data demonstrating this are the following:

Rouleaux-inducing agents such as gelatin compounds have for some time been used to concentrate K^+ parasitized cells because the knobs physically preclude such cells from stacking, and they do not easily deform. Thus, these agents cause non-knob-containing cells (normal RBCs, rings, or K^- cells) to stack in rouleaux.[31] This leaves the K^+ cells in suspension. When such a procedure was used so that concentrated K^+ cells could be studied, it was observed that with continued culture, some antigens on the surface of the parasitized RBC membrane were lost.[58] This was demonstrated using an assay which showed that Ab-induced inhibition of intracellular development decreased with increased time in culture, even though the cells being assayed were always selected on the basis of having knobs (Table V). Another assay using the fluorescent-activated cell sorter suggested that there was a complete spectrum of levels of antibody binding to parasitized K^+ cells.[59]

These data all suggest that the parasite is contributing at least one molecule to the erythrocyte membrane, is altering existing erythrocyte membrane molecules, or both. That the lipid composition of the RBC membrane is changed by the parasite is well documented.[27]

That this could alter the relationship of normal erythrocyte proteins to one another is quite possible.

Binding of Trophozoites to Endothelial Cells. An assay which may have considerable biological significance has also suggested the independence of knobs and particular membrane changes. These experiments demonstrated that although trophozoites from infected monkeys could adhere in culture to endothelial and specific tumor cells, this capacity was rapidly lost during culture of the parasite.[51] Significantly, the *in vitro* adher-

TABLE V. % Inhibition as Function of Length of Time Parasites Maintained in Culture

Nigerian Serum	*1 Month*	*2 Months*	*3 Months*
1	27%	9%	
2	35%	20%	
4	33%	11%	
36	47%	26%	12%

Reference (56).

ence could be blocked by immune owl monkey serum.[38] This assay thus appears to provide an independent system by which the endothelial cell receptor on the trophozoite can be distinguished.

A very large protein of approximately 250K–300K has been implicated as the possible parasite receptor for endothelial cells.[38]

This molecule is associated with the erythrocyte membrane as suggested by attempts to surface label with ^{125}I. Interpretation of such experiments is complicated since virtually all of the ^{125}I labeling techniques have allowed labeling of globin. If globin is labeled, then it is difficult to be sure whether other proteins within the erythrocyte are not also being labeled. Thus, rigorous biochemical localization of a protein to the parasitized red blood cell membrane is difficult.

A 930 base pair cDNA clone which encodes part of a molecule which is likely to be associated with the erythrocyte membrane has been examined.[60] Antibodies to the protein, which were produced against the product of a cDNA clone expressed in *E. coli,* produced a speckled intracellular immunofluorescent pattern in both trophozoites and schizonts.[39,60] On Western blots, these antibodies bound to a very large molecule of perhaps 300K and in a lesser degree to smaller peptides.[39,60] This antigen reacted with 93% of the 65 human immune sera tested. Nevertheless, an antiserum to the expressed protein detected parasite proteins whose sizes varied depending on the isolate from which they were obtained.

IV. Summary

As a result of infection with the human malarial parasite, *P. falciparum*, molecules on the merozoite surface or those contained within the rhoptries and micronemes may bind to or become inserted into the erythrocyte membrane. Two families of molecules (Groups I and II) have been identified as possible candidates. After approximately 24 hours within the host cell, a series of changes are detectable. One or more parasite proteins are inserted into the erythrocyte membrane skeleton, and at least one high molecular weight protein which may be linked with the erythrocyte membrane skeleton appears to be inserted through the erythrocyte membrane into the aqueous environment outside of the cell. The membrane skeletal alterations are likely to be responsible for the dramatic surface changes referred to as knobs. Membrane changes take place which are independent of the membrane skeletal changes. They may play a role in producing the receptor by which the trophozoite can bind to endothelial cells and sequester. There are, therefore, a series of parasite-induced alterations which appear in the host erythrocyte membrane. Further study is likely to identify specific membrane structures which may be useful in providing immunologic protection for humans.

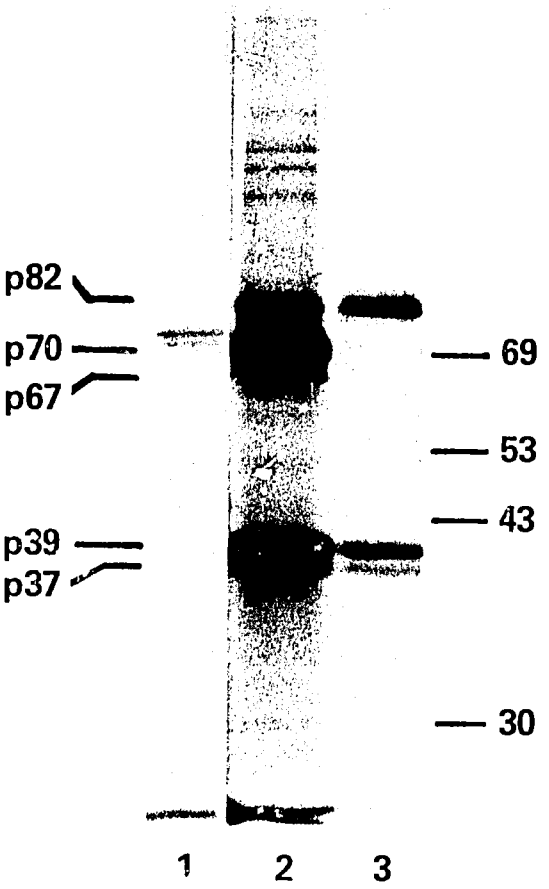

FIGURE 1. Immunoprecipitation of Radiolabeled Rhoptry Polypeptides from Schizonts and Schizont-Labeled Ring Stage Parasites. Lane 1, ^{35}S-methionine-labeled schizont proteins bound by ascites fluid with nonmalarial antibody; lanes 2 and 3, ^{35}S-methionine-labeled schizont and schizont-labeled ring stage proteins, respectively, precipitated by anti-group I punctuate monoclonal antibodies (from reference 17 with permission of the editor).

FIGURE 2. Scanning Electron Micrograph of Normal Trophozoite-Infected and Schizont-Infected Human Erythrocytes.

FIGURE 3. Transmission Electron Micrograph of Trophozoite Infected Erythrocyte Showing Knobs on Erythrocyte and Electron Dense Deposits Under the Lipid Bilayer.

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Discussion

DIGGS: I'd like to go back to an experiment by Dr. Stanley that you described in which he looked at a synchronized system and intracellular incorporation of the precursor. I'm interested in the specificity, the antibody specificity of that result. Could you tell us more about how you exclude nonantibody mediators?

REESE: It's a good question Carter. We have not affinity purified the antibody and shown that it was necessarily due to antibody. We have done the easy experiment, if you will. We've taken and done an ammonium sulfate cut, put that on DEAE, and used that globulin fraction. You get that reaction. You also find that in fact if you use a K^-, you don't see the affect. So if you're going to argue that it's some low molecular weight thing, then it should still permeabilize the K^- organism. We also find the correlation that it only occurs at that point. You can titrate it back to the point at which we know knobs are expressed. By EM, those same antisera contain antibodies which can bind to the surface of the erythrocyte. Thirdly, we know that if we take the same antiserum and culture the organisms for increasingly longer lengths of time, expression of that antigen is lost. This fits too closely with what Lou Miller and some other people have found. There are changes which occur on the erythrocyte surface, on what I'm going to assume is the binding site for these antigens. So I think, if you're asking me if I've got rigorous proof that it is antibody mediated, the answer is no, but we've got a lot of circumstantial evidence that certainly points in that direction.

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Soluble Antigens of the Asexual Blood Stages of *Plasmodium Falciparum*

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Introduction

During schizogony, rupture of the infected erythrocyte releases many parasitic proteins into the blood stream. These include: proteins secreted into the parasitophorous vacuole, proteins released from the secretory organelles of merozoites, and soluble cleavage fragments (secondary to processing or degradation) of proteins that are not otherwise soluble. Many of these proteins will elicit immune responses (Fig. 1) and the term "soluble antigen" is therefore an imprecise one covering a wide spectrum of antigens. For example, the ring infected erythrocyte surface antigen (RESA) is transferred to the surface membrane of a newly infected erythrocyte during the process of merozoite invasion.[1] A proportion of merozoites fails to invade and die, releasing RESA which can subsequently be detected as a soluble antigen. Antibodies against soluble antigens have been demonstrated to inhibit parasite growth *in vitro* but the target antigen has not been clearly identified.[2]

The S Antigen System

An early attempt to classify soluble antigens of malaria identified antigens differing in their stability to heat.[3] Those proteins that retained their antigenicity after boiling were designated S antigens. S antigens which are found in the plasma of infected individuals and in culture supernatants exhibit extreme serological diversity. Although many different S antigens may be present in a localized region, individual S antigens are found in widely separate geographic areas.[3,4] The S antigen serotype of a particular parasite strain appears to be stable, persisting

unchanged after numerous *in vitro* and *in vivo* passages.[5] Thus this antigen system differs fundamentally from the variant surface glycoprotein of *Trypanosoma brucei*, as a cloned population of trypanosomes switches readily from one VSG to another. As it has proved difficult to raise antisera against purified native S antigens and the acquisition of anti-S-antigen antibody in man is age dependent, S antigens appear to be poorly immunogenic.[6,7] Immunoblotting experiments using culture supernatants from *P. falciparum* lines maintained *in vitro* have enabled us to identify the S antigens of several isolates.[8] S antigens show remarkable size polymorphisms with apparent molecular weights ranging from less than 100kd to about 250kd. Even antigenically related S antigens from different clones of a single isolate may vary in size.[8] Biosynthetic labelling experiments have demonstrated that the S antigen is a gene product encoded by the parasite[6] and ^{3}H-glycine has been a particularly useful biosynthetic label for identifying S antigens of different isolates.[9] The S antigen is synthesized in the mature parasite and released at the time of schizont rupture. Immunoelectron microscopy suggests that S antigen is located in the parasitophorous vacuole (Fig. 2).

S Antigen Gene Structure

cDNA libraries that express the antigens of *P. falciparum* have recently been constructed. An expressing clone from such a library was found to produce a portion of the S antigen from FCQ27/PNG (FC27), a Papua New Guinean isolate of *P. falciparum*.[10] This fragment was entirely composed of a repeating 33 base sequence coding for tandem repeats of 11 amino acids. This 11 amino acid sequence was found to be highly strain specific—the sequences were absent from other *P. falciparum* isolates that did not express that S antigen specificity.[10] This cDNA was subsequently used to identify a genomic clone from the FC27 strain that encoded the S antigen sequence. Although the repeats were absent from other parasite isolates, flanking regions of the gene could be used to find the corresponding S antigen in other isolates. Thus, the S antigen gene from NF7, a Ghanian isolate, was also isolated. Both genes were completely sequenced and demonstrated a basic structure for S antigens.[11] The structure of the S antigen gene is summarized in Figure 3. The central portion of the protein is composed of tandemly repeating oligopeptides, 11 amino acids in the case of FC27 and 8 amino acids in the case of NF7. The number of these repeats is estimated to be 100 for FC27 and 43 for NF7. There are two distinct versions of the NF7 octapeptide due to a single base change in the 24 nucleotide repeating units resulting in an Arg to Leu change. The repeat sequences are summarized in Figure 3. The vast bulk of the natural antibody response to S antigens appears to be directed towards epitopes encoded by these repeat

sequences. Variation in the composition and number of amino acids in the repeats accounts for the serological diversity of the S antigens. Size polymorphisms are accounted for by variations in the number of repeats and the number of constituent amino acids. Surrounding the repeat regions are stretches of highly charged amino acids, a structural feature also found in the circumsporozoite proteins of *P. falciparum*.[12] The amino and carboxy terminal portions of the sequences are up to 70% homologous in these two isolates. There is a signal sequence present at the amino terminus. A mechanism for generation of the diverse S antigens appears to be a process of spreading point mutations, deletion and insertion throughout the repeat units, probably by unequal crossing over. This has resulted in a series of genes that are maintained by selection as a polymorphism in natural populations of the parasite. The selection pressure that maintains this polymorphism would appear to be the human immune system.

S Antigen Families

The isolation of genes encoding the S antigen has enabled a subclassification of the S antigen family. The repeat portion of the gene can be used as a probe to identify cross-hybridizing S antigens, e.g., the repeats of the NF7 antigen will hybridize to the S antigen of K1, a Thai isolate. However, antibodies to the corresponding S antigens from these isolates do not cross-react immunologically. These findings are summarized in Table 1. Thus, the S antigens can be classified into subgroups in which the repeat portions demonstrate nucleotide homology but not immunological cross-reactivity. This may reflect the way in which the proteins have evolved. For example, a single second base change exemplified by that resulting in an Arg to Leu substitution in the octapeptide repeats of the NF7 S antigen would drastically change the immunological properties of the antigen if it spread to all the repeats, even though the sequence remained 96% homologous. This grouping of antigens deriving from a presumed common ancestor is also suggested by the observation that S antigens that share cross-hybridizing repeat regions show very great homology in the flanking regions (Tables 1 and 2).

Function of the S Antigen

The function of the S antigen system is unknown. There are several striking and even paradoxical features about this system that are yet to be explained. These center on the observations of extreme diversity coupled with low immunogenicity as native molecules (cf., fused polypeptides). As has been mentioned there are very many S antigen types

TABLE 1. Relationship between S antigen repeats

	Nucleic acid probe		*Antibody probe*	
Strain	*FC27*	*NF7*	*FC27*	*NF7*
FC27	+	—	+	—
NF7	—	+	—	+
K1	—	+	—	—
V1	—	—	—	—

Four *P. falciparum* isolates, FC27 from Papua New Guinea, NF7 from Ghana, K1 from Thailand, and V1 from Vietnam were cultured *in vitro* to provide DNA and culture supernatants. The DNA was digested with a restriction enzyme, size fractionated by agarose gel electrophoresis, and tranferred to nitrocellulose. The filters were probed with labelled DNA sequences corresponding to the S antigen repeats of the indicated strain, followed by washing at high stringency. Positive hybridization is denoted by a +. In a parallel experiment boiled culture supernatants were size fractionated on polyacrylamide gels and immunoblotted to nitrocellulose followed by reaction with antibodies against the repeat units of S antigens of the indicated strains. Detectable antibody binding is denoted by a +. The table demonstrates that the four isolates produce non-cross-reactive S antigens but the K1 and NF7 repeats share considerable homology at the nucleotide level.

TABLE 2. Conservation of the 68 amino acid 5' region of the S antigen

Strain	*Exact Matches*	*Conservative Changes*
Compared to the 68 amino acid 5' region of NF7 (Ghana)		
K1 Thailand	68 (100%)	0
FC27 (Papua New Guinea)	48 (71%)	13 (90%)

Note: Naturally occurring antibodies of these three S antigens are non cross-reactive.

Genomic clones encoding three non-cross-reacting S antigens (Table 1) from three *P. falciparum* isolates have been sequenced. 5' to the central core of repeats is a region of 68 amino acids. Analysis of this region reveals high homology between the three strains. The 68 amino acid regions are highly charged with >35% of charged amino acids. Therefore these regions are unlikely to be buried within the S antigen molecule, but rather exposed to the external environment. We have not been able to detect naturally occurring antibodies against this 68 amino acid region and it is possible that the repeats camouflage this flanking region from the immune system.

which have been maintained in the naturally occurring parasite population. This bespeaks some strong selective pressure. However, one of the main findings in the seroepidemiology of S antigens has been the observation that antibody responses to these antigens are rare. This is precisely what one might predict for a soluble antigen made up of repeating epitopes if it behaves as a T independent antigen giving rise to short lived IgM responses together with suppression. Immunization with vaccinia virus expressing the S antigen in its native form as a soluble antigen has resulted in very low antibody responses in experimental animals (C. J. Langford, The Walter and Eliza Hall Institute of Medical Research, unpublished observation). It is difficult therefore to understand the need for such extreme serological diversity in a molecule that is so poorly seen by the immune system.

One seemingly obvious conclusion about S antigens is that because they are so variable they cannot be the target of protective immunity as there exist many parasite strains that do not express a particular specificity. This conclusion seems less obvious after the discovery of conserved regions amoag S antigen molecules. The K1 and NF7 strain specific S antigens do not show any demonstrable cross-reactivity when reacted with naturally occurring antibodies. The 68 amino acid 5' conserved region (Fig. 3), however, is identical in both S antigens. Thus conserved regions have apparently been rendered invisible to the immune system as evidenced by seroepidemiological studies with cloned antigen fragments. That immune responses directed against S antigens can have some host protective value has been suggested by the report that a monoclonal antibody directed against the repeats of the FC27 S antigen inhibits the growth of the homologous strain *in vitro*.[14]

ACKNOWLEDGEMENTS

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190'

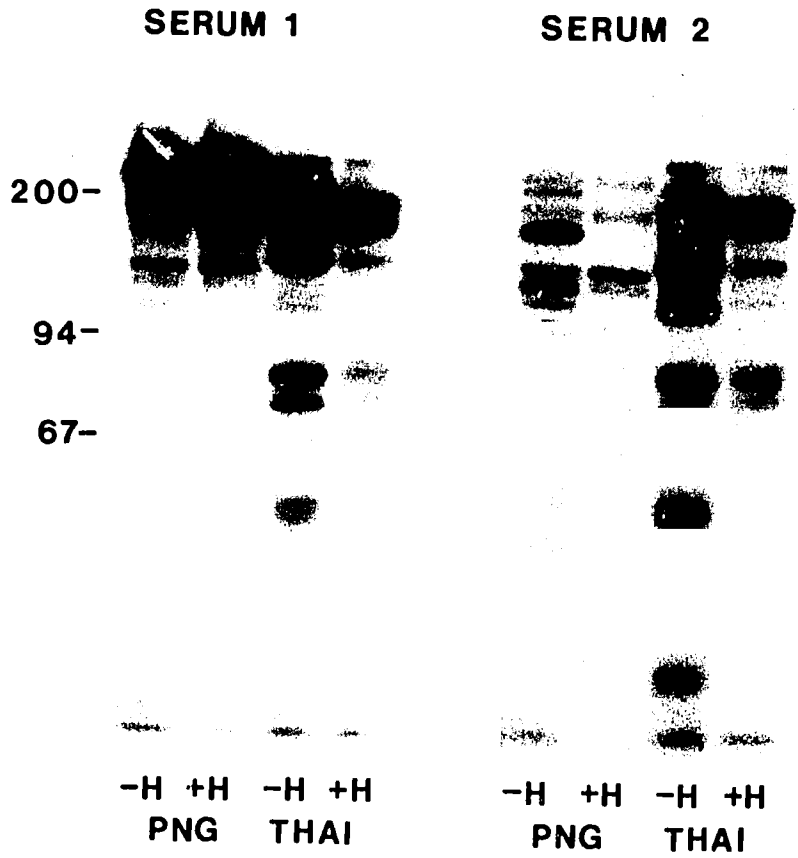

FIGURE 1. Immunoblot of SDS-PAGE of Culture Supernatant Antigens from the FC27 Isolate (PNG) and the K1 Isolate (Thai) of *P. falciparum*.

The antigens were concentrated from 4mls of heated (+H) or unheated (-H) culture supernatant applied to 100μl of Papua New Guinea (PNG) IgG-Sepharose prior to electrophoresis. The immunoblots were probed with 1:500 dilutions of two PNG sera, serum 1 containing a high titre of antibodies to the FC27 S antigen and serum 2 which has no reactivity to the FC27 S antigen. Although the S antigen is the dominant soluble antigen there are many other soluble antigens recognized.

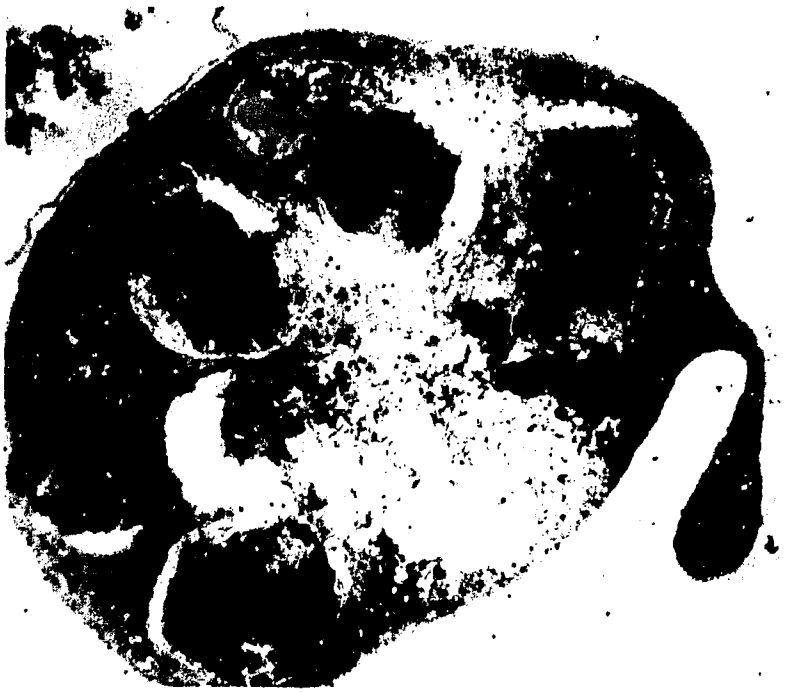

FIGURE 2. Immunoelectronmifrograph Showing the Location of S Antigen Throughout the Parasitophorous Vacuole of a *Plasmodium falciparum* Schizont. S antigen has been labelled using a rabbit antiserum raised against the repeat region of the FC27 S antigen followed by Protein A gold. Magnification by 44,300.

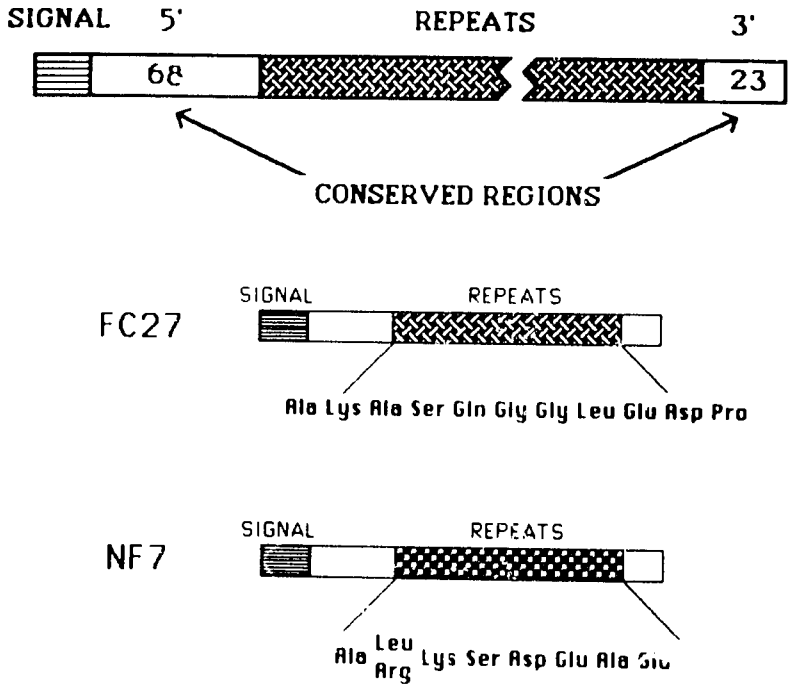

FIGURE 3. Generalized Structure of the *P. falciparum* S Antigen.

The upper panel illustrates the large central block of repeats surrounded by conserved regions both 5′ and 3′. The numbers of amino acids in the conserved regions are listed. The lower two panels show the sequences of the repeats found in the S antigens of two isolates of *P. falciparum*; FC27 from Papua New Guinea and NF7 from Ghana.

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Discussion

ZAVALA: What is actually, why are you thinking that this protein can help the parasite to evade the immune system? You would agree that you have no evidence for this? And even more, if this antigen is poorly immunogenic, does it make any sense to believe that it helps to evade the immune system? My question is on the theory.

COPPEL: Let me turn around the question and ask it the other way. Can you make any sense of the observation that if an antigen is poorly immunogenic, why should it be so variable? I mean, what is the point of 50 different isolates in nature—in these repeats? To me, the whole observation is extremely puzzling. I think that, in my personal prejudice, there are in fact two mechanisms to protect the constant region from recognition. It is not particularly immunogenic to start with, and when it does induce antibodies, it focuses them on internal repeats, which can vary between isolates. But I think it is an extremely puzzling combination of features.

PEREIRA DA SILVA: I think it is a very exciting question. I hope that it will be discussed in the general discussion. Many people would like probably to ask and discuss the problem.

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Culture-Derived *Plasmodium Falciparum* Exoantigens: Isolation and Characterization of Protective Polypeptides

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I. Introduction

Exoantigens are soluble proteinaceous moieties naturally released into the blood plasma of animals infected with *Plasmodium* and *Babesia* or into the supernatant medium of *in vitro* cultures of these organisms.

Our knowledge of these antigens commenced some three decades ago when we first observed their presence in the plasma and serum of several nonbovine animal species infected with *Babesia spp.*[1] During these studies an interrelationship between parasitemia levels, concentration of plasma antigens, and the level of antibodies to these antigens was demonstrated. A direct relationship between the levels of parasitemia and plasma antigens was found[1] while an inverse relationship existed between these two elements and serum antibody levels. Recognizing the immunogenic potential of these antigens, we initiated vaccination studies in dogs against infections caused by *B. canis* utilizing soluble exoantigens as immunogens. It is in the course of these studies that we, for the first time, recognized the protective nature of these antigens. However, while such potential was clearly evident, it was also recognized that the immunogenicity of plasma-derived soluble antigens was low, probably because of immune-complex formation and enzymatic degradation.

The development of methods for *in vitro* propagation of *B. bovis* renewed our interest in soluble exoantigens as immunogens against bovine babesiosis.[2,3] During the last six years, well-controlled babesiosis vaccination experiments using culture-derived soluble exoantigens of *B. bovis* were conducted in the United States, Mexico, Venezuela, and Australia.[4-9] The evidence of protection against virulent *B. bovis* challenge using infected blood or ticks was demonstrated in all of these studies.

Exoantigens of parasitic origin have been detected in the plasma or serum of rodents, chickens, monkeys, and man with acute malaria.[10] Several groups of plasmodial exoantigens from human plasma have been characterized according to heat stability molecular weight, enzyme sensitivity, and antigenicity.[11,12] In addition, exoantigens have been isolated from the culture supernatant medium of *P. falciparum* parasites propagated *in vitro*.[13,14] Partial protection against challenge infection has been demonstrated in chickens and monkeys with blood plasma-derived exoantigens of *P. gallinaceum* and *P. knowlesi*, respectively.[15,16]

Development of a method for continuous *in vitro* propagation of *P. falciparum* provided a means for the production of soluble exoantigens unaffected by the host's metabolic and immune systems.[17] It appears that the antigen to which the host must respond if it is to develop protective immunity against the asexual blood phase of malaria is a component of the surface coat covering the outer membrane of the merozoite. This surface coat appears necessary for the attachment of the merozoite to erythrocytes and mediates the first essential step for entry of this organism into the erythrocyte.[18] There is sufficient evidence that most of the exoantigens originate from the merozoite surface.[19] Accordingly, the humoral immune response to these exoantigens should be capable of blocking an invasion of erythrocytes by the merozoite as recently demonstrated with *B. bovis* and *P. falciparum*.[10]

II. Vaccination Experiments with Exoantigen-Containing Crude Culture Supernatant: Homologous Challenge

Squirrel monkeys *(Saimiri sciureus)* can be protected from clinical reactions and death caused by *P. falciparum* by vaccination with a cell culture-derived immunogen.[10] The immunogen consisted of filtered, concentrated (10x) supernatant fluid collected from *in vitro* cultures of *P. falciparum*. Challenge infection was administered 2 to 9 months after vaccination. Challenge material consisted of 2.0×10^7 *P. falciparum*-parasitized (Indochina I strain) erythrocytes contained in whole blood collected from an acutely infected monkey. The immunogenicity of vaccinal antigens was fortified by the use of saponin or aluminum hydroxide adjuvants. Strong humoral immune responses measured by the indirect fluorescent antibody (IFA) test were in evidence post-vaccination and challenge. No mortality occurred among 11 vaccinated animals which included 4 preimmunized and 7 malaria-susceptible monkeys, while 2 susceptible control monkeys developed typical signs of malaria following challenge and died.

Pathological and clinical observations demonstrated substantial protective immunity in vaccinated animals. These monkeys maintained

stable hematocrit levels and consistently lower parasitemias, accompanied by the presence of parasite crisis forms. Further corroborative evidence of the vaccine efficacy was the specific inhibitory capacity of sera collected from vaccinated animals prior to challenge. This was established by *in vitro* growth inhibition studies.[10]

III. Partial Purification and Characterization of Culture-Derived *P. falciparum* Exoantigens

Previous work has led to the isolation and partial purification of an antigenic fraction from supernatant fluids of asynchronous cultures (average parasitemia=2–3%) of the Geneve/SGE-1 strain (Senegalese isolate) of *P. falciparum*.[20] This fraction, referred to as the SP fraction, was purified by cation-exchange chromatography on an SP-trisacryl column (0.01 M acetate buffer, pH 3.7, with a step-wise NaCl gradient). Antigenic fractions in this and subsequent purification procedures were monitored by the indirect ELISA utilizing human anti-*P. falciparum* IgG and normal human IgG, both of placental origin. Antigenic activity was found in fractions which eluted between 0.2 M and 0.3 M NaCl concentrations. Malarial antigens contained in the SP fraction were then identified by the Western immunoblot technique using rabbit anti-SP antiserum. This antiserum was highly schizont-specific, showed an IFA titer of 1:1280 and inhibited merozoite invasion of human erythrocytes *in vitro* (C. Fajfar-Whetstone, unpublished data). Analysis was also conducted with an appropriate control antigen and antiserum to an SP fraction from supernatant fluids of noninfected cultures (rabbit anti-negative SP fraction). Western blot analysis revealed a major malarial antigen of MW 83K in the SP fraction. Subsequently, additional *P. falciparum* antigens of 100K and 70K were identified as being present in this fraction.

An examination of the SP fraction by crossed immunoelectrophoresis demonstrated that it was free of erythrocytic components and IgG immunoglobulin. We estimated that 80% of contaminating serum proteins was removed with the SP-trisacryl fractionation procedure.

Fraction "SP" was further purified by preparative size exclusion high performance liquid chromatography (HPLC).[20] Based on the ELISA monitoring of proteinaceous fractions eluted from the column, apparent molecular weights of antigenic moieties were 350 kd, 170 kd, 130 kd, and 40 kd, respectively. However, subsequent characterization by SDS-PAGE and Western immunoblotting demonstrated that these proteins were actually being eluted in the dimeric form. Immunoblots of HPLC fractions showed unique bands representing major malarial proteins at 100 kd, 83 kd, and 70 kd with minor antigens observed at molecular weights 185 kd, 43 kd, and 20 kd.

Antigenic fractions from size exclusion HPLC which contained the

83K exoantigen were pooled and additionally purified by DEAE anion-exchange HPLC. ELISA reactivity corresponded to one major protein peak, demonstrated on Western blots to be the 83K antigen. This antigen was found as a single homogeneous protein by SDS-PAGE with Coomassie Blue staining. One liter of the original pooled supernatant fluid yielded approximately 50 micrograms of the 83K protein.

Automated Edman Degradation was performed on an Applied Biosystems, Inc. (Foster City, CA, USA), Model 470A Protein Sequencer using 500 picomoles of purified 83K exoantigen. Phenylthiohydantoin (PTH) amino acids were detected by reverse phase HPLC on an Altex ultrasphere ODS column (Beckman Instruments, Inc., Palo Alto, CA, USA). The N-terminal amino acid sequence of the *P. falciparum* 83K exoantigen was determined for the first 35 residues. There were no repeating units in the N-terminus, in contrast to that seen for some other *P. falciparum* blood stage antigens.[21-24] Residues 16 through 22, Trp-Asp-Gln-Asn-Glu-Lys-Phe, contain the least codon ambiguity and represent a sequence appropriate for constructing a synthetic oligonucleotide probe. Tryptophan has a single codon and the other 6 amino acids—aspartic acid, glutamine, asparagine, glutamic acid, lysine, and phenylalanine—are only 2-fold degenerate.

The strong hydrophobic nature of the N-terminal sequence suggests that it is associated within an interior portion of the protein or alternatively it could serve as a membrane binding site. The possible existence of a membrane binding site on the 83K protein coupled with the schizont-specificity of the rabbit SP antiserum is suggestive that the 83K antigen may be derived from the 190–200K protein identified by other investigators.[25-27]

A mixed 20mer derived from amino acid codons for residues 16 through 22 was selected as a hybridization probe on the basis of the relative stability of G . T versus A . C mismatches and the fact that *Plasmodium* DNA is very rich in A . T base pairs. Thus, a mixed probe with 4-fold degeneracy was synthesized on an Applied Biosystems Model 380A DNA synthesizer having the composition: 3'ACC-CT$^{A}_{G}$-GTT-TT$^{A}_{G}$-CTT-TTT-AA 5'. The oligonucleotide probe was purified by reverse phase HPLC.

More recently, the 100K exoantigen has been isolated in pure form.[20] Preliminary sequence data appear almost identical with those of the 83K polypeptide. Monospecific rabbit antiserum raised against the individual pure polypeptides confirm their cross-reactivity. We postulate that the 83K antigen is a specific processing product of the larger 100K antigen. Additionally, these exoantigens have been shown to be glycoproteins. Subsequent purification and characterization of a 70K exoantigen has shown it to be proteinaceous in nature with an N-terminal amino acid sequence distinct from those of the 83K and 100K glycoproteins.

IV. Recombinant DNA Studies

Sequencing of the 83K peptide N-terminus had led to the synthesis of a 20-nucleotide probe with only 4-fold degeneracy. This oligomer is being employed to search for sequences encoding the 83K protein by Southern blot hybridization to *P. falciparum* restriction digests (J. Nichols, unpublished data).

The DNA was isolated from erythrocytes of *in vitro* cultures infected with the Geneve strain of *P. falciparum*. Infected cells with a 3–5% parasitemia were harvested and centrifuged to remove serum, buffy coat, and the upper erythrocyte layer. Remaining erythrocytes were washed several times in PBS to remove any remaining serum contaminants. Cell-free parasites were obtained by lysing infected erythrocytes in saponin and washing parasites 3x in PBS. The DNA was recovered by solubilizing the parasitic fraction in sarcosyl detergent buffer and repeatedly extracted with phenol, chloroform, and mixtures of the two. The DNA was then dialyzed, ethanol-precipitated, pooled, and checked for purity from enzyme inhibitors by restriction digestion and agarose gel electrophoresis.

Preliminary hybridization results show the oligomer probe to be specific to the *P. falciparum* genome. Since the probe does not significantly cross-react with λgt 11, λ1059, pUC, or *E. coli* DNA, a small genomic library has been constructed in the pUC 19 (plasmid). Prospective clones are being screened by hybridization to the oligomer, and sequence analysis will be performed to confirm isolation of those fragments coding the 83K peptide. Isolated fragments will be expressed by recombination to λgt 11. Application of our ELISA detection system to colony screening has been tested, and no cross-reaction to λgt 11 host strains using anti-exoantigen antibodies has been noted. Optimization of antigen detection by antibodies is currently being investigated in preparation for isolated gene expression. Prospects for mRNA isolation are also being considered but are awaiting further results from clone sequencing.

V. Vaccination with Partially Purified Culture-Derived Exoantigens: Homologous and Heterologous Strain Immunity

Recently, we investigated the induction of protective immunity with an SP-trisacryl cation-exchange purified antigenic fraction derived from supernatant fluids of asynchronous cultures of the Indochina I and Geneve/SGE-1 strains of *P. falciparum* in susceptible Bolivian adult male squirrel monkeys.[28] This SP fraction is approximately 80% pure and contains *P. falciparum* polypeptides of 100K, 83K, and 70K (see section

III above). An SP-purified fraction from noninfected cultures was utilized as a negative control immunogen. This study involved duplicate experiments of identical design. In total, 4 monkeys were vaccinated with the SP fraction derived from the Indochina I strain, 8 animals were immunized with the SP immunogen derived from the Geneve/SGE-1 strain, and 6 animals received the control preparation. Each dose consisted of 2 mg total protein in a 1 ml volume. These preparations were gently mixed with 127.5 μl of 1.86% aluminum hydroxide adjuvant for 1 h at 25° C. Two subcutaneous inoculations were administered at a 4-week interval. Challenge exposure consisted of an intravenous injection of fresh infected blood containing 1×10^7 virulent monkey-adapted Indochina I organisms approximately 2½ months following the second vaccination. Hereafter, SP fractions derived from Indochina I and Geneve culture supernatants will be referred to as homologous and heterologous immunogens, respectively.

A complete hematological profile was considered essential for assessing potential indicators of protection post-challenge. In addition to hematology, blood chemistry, and serology, parasitemia levels were monitored. Special emphasis was also placed on the development of parasite morphological alterations, termed crisis forms.[29]

The critical period in the course of infection occurred 14 days post-challenge as decreases in hematocrits paralleled rises in parasitemia. Recovery to normal hematological levels was notably accelerated in the animals which received the homologous SP fraction, was moderate in monkeys immunized with the heterologous SP fraction, and was considerably delayed in the negative control animals. Mortality was observed with two monkeys of the control group on days 13 and 15 post-challenge. At this time, we felt that to provide an unbiased evaluation of protective immunity, treatment of experimental animals should be avoided. Significant differences in clinicopathological responses were seen, particularly with regard to animals immunized with antigenic fractions prepared from the homologous strain. Hematocrit decreases were limited, as well as the degree of parasitemia. Antibody levels in vaccinated monkeys increased rapidly and remained elevated 4 weeks post-challenge at the time titers in control animals began to decline. Crisis forms were first observed between 11 and 13 days post-challenge with mean parasitemias on those days notably lower in vaccinated monkeys. Two types of intracellular crisis forms were observed: (1) point or pyknotic forms and (2) abnormal, vacuolated organisms.

Potential criteria for protection against disease included parasitemia levels, parasite killing as evidenced by the appearance of crisis forms, hematocrit and hemoglobin values, and importantly, SGPT and BUN levels as indicators of liver and kidney function, respectively. Fourteen days post-challenge, nonvaccinated animals showed dramatic increases

in these specific parameters (SGPT=+119%, Bun=+58%). This is consistent with the hepatomegaly and proliferative glomerulonephritis seen upon necropsy of control animals which succumbed to clinical malaria (see section VI below). In conclusion, this study demonstrated that purified *P. falciparum* exoantigens induce significant protective homologous and moderate heterologous strain immunity to clinical malaria caused by a virulent challenge exposure.

VI. Pathology of Fatal *Plasmodium falciparum* Infection in the Squirrel Monkey *(Saimiri sciureus)*

Our laboratory has studied the pathological alterations associated with experimentally induced fatal acute falciparum malaria in mature Bolivian squirrel monkeys (J. Everitt, manuscript in preparation). Pathologic changes in the pulmonary, hepatic, renal, and hematopoietic systems suggest that the disease in this simian model closely resembles the human infection.

Marked splenomegaly with lymphoreticular hyperplasia was a constant feature of fatal infections in squirrel monkeys. Lymphoreticular changes were prominent in the liver, bone marrow, and lymph nodes and were identical to those described in human infections and other simian falciparum models.

Prominent microvascular sequestration of parasitized erythrocytes was present in adipose tissue and cardiac musculature. Occasionally, parasitized red blood cells were adherent to microvascular endothelial surfaces in the lung, liver, and adrenal gland. The capillaries of the brain were notably free of erythrocyte sequestration.

The animals which died had severe interstitial pneumonia believed to be associated with a pulmonary microvascular disturbance. This phenomenon was not associated with sequestration of parasitized erythrocytes. Similar pulmonary pathologic changes have been reported in a variety of hemotropic parasitic diseases. The pulmonary disease in the squirrel monkey appears analogous to human "malaria lung" and may serve as a useful model in understanding the pathophysiology of this unusual and fatal complication of falciparum malaria.

Renal changes in fatal squirrel monkey infections included tubular nephrosis and mesangioproliferative glomerulonephritis. Initial ultrastructural studies have shown that the glomerular changes are similar to those described in human and *Aotus* monkey falciparum malaria.

Retinal hemorrhages were present in a single animal. This is an interesting finding which has been described in human cerebral malaria and claimed to be of prognostic significance. Additional ocular studies are warranted in the simian model as the pathogenesis of the retinal lesions is unknown.

The similarity noted in the pathology of *P. falciparum* in the human patient and the squirrel monkey suggests that this simian model will prove useful in characterizing and understanding the sequential changes in uncomplicated cases of acute malaria.

VII. Summary and Conclusions

In summary, we have now isolated and characterized *P. falciparum* exoantigens of 100K, 83K, and 70K. These polypeptides were purified from an "SP" fraction that induces protective immunity in squirrel monkeys against a virulent challenge *P. falciparum*. These exoantigens react with antibodies which are present in human placental immunoglobulin IgG from individuals who have recovered from *P. falciparum* infections. These results suggest that the SP antigenic fraction of culture supernatant fluids, or pure malarial polypeptide(s) contained therein, may be good vaccine candidates for immunoprophylactic regimens against falciparum malaria. Studies are currently in progress in which the individual pure exoantigens will be tested for their ability to induce protective immunity in experimental monkeys.

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Discussion

MEUWISSEN: What do you call a crisis form?

RISTIC: We call the crisis form the biologically abnormally appearing parasites present either outside or inside the erythrocytes, which we never see in nonvaccinated control monkeys, and I've shown some of the pictures here. The crisis form is a very interesting question. You may know of the work of Dr. Jensen from Michigan State, in which he claims that besides IgG, there is some other substance present in the serum of patients from, I think it was, Southeast Asia. And we are looking into these monkey serums now which we are working with, if they contain similar type of substance as he spoke of.

COPPEL: Dr. Ristic, despite the differences, do you feel that you are working on a similar protein to both Dr. Perrin and Dr. Freeman? Would you say that you are all looking at the same protein?

RISTIC: If I remember right, Dr. Perrin has focused on 42K and 43K protein and I do identify that specific protein in the supernatant culture medium. I would say that probably we are looking at the same protein.

BRUCE-CHWATT: I was interested to hear today that the first commercial vaccine against *Babesia* is already produced and is available. That is one thing that interested me. But another question, perhaps naive, is there any community of any antigens between *P. falciparum* and *Babesia?* They are so similar morphologically, of course, they are completely different when it comes to host and transmission. Thank you, sir.

RISTIC: As you know, Dr. Bruce-Chwatt, there have been reports in the past of serologic cross-reactivity between *Plasmodium* and *Babesia*. Dr. Nichols would you like to comment on that?

NICHOLS: Using that radioactive probe, we noted that using a four-cutter restrictive enzyme, we see very similar hybridization patterns between a *Babesia* digest and *P. falciparum* digest, but when you use more specific enzymes six-cutters you don't see that level. So the gene level may be similar but at the nucleotide level it doesn't appear that they are the same identical sequences.

MITCHELL: Could I ask Dr. Ristic and also Robert Freeman: Dr. Ristic has emphasized the 83K and now a 100K or 96K, whichever you'd like. Robby, do you find the 100K as a major cleavage product of 195 or not?

FREEMAN: No. We've got one processing intermediate at about 110K but it's certainly not a terminal processing fragment.

MITCHELL: According to Dr. Ristic's work, the 100K is the same molecule as the 83K, just a little bit more of it.

FREEMAN: I'm aware that's an inconsistency between us. It seems to me Dr. Ristic described about six processing products on the surface, whereas, we only have found the three.

MITCHELL: He's describing six in the supernatant. Is that right?

REESE: I think we're confusing whether they are on the surface or simply react whether the epitopes are shared among a series of different molecular weight proteins. I'm going to try to provide some evidence tomorrow which will address that this high molecular weight protein can be processed probably by two independent pathways, one giving rise to a glycosylated group of end products of which 40K and 46K are the terminating ends and end up on the merozoite surface and of which the 83K and perhaps 90K are in the second pathway. They share epitopes with the large molecule.

PEREIRA DA SILVA: The questions we are raising are on the identity of those molecules as described by Dr. Ristic as a processing product of the 195K antigen. Dr. Ristic, want to comment on this?

RISTIC: Let me first say that I explained that we used cation exchange chromatography to obtain the fraction from which we identified these antigens. The isoelectric points of these antigens are about pH 5, that's why we are using cation exchange. Now I made it very clear that two of these antigens, 100K and 83K, first based on the amino acid sequence and then Western blotting, have identical immunochemical specificity. So we think that the same antigen may be processed to these two antigens. Now 70K appears to be a quite different antigen. The 100 and 83K are both glycoproteins by Schiff staining. The 70K is pure protein. There is no sugar portion in the 70K, and it appears that the amino acid sequence based on 55 amino acid residues from the N-terminus is completely different polypeptide.

Another question I'd like to mention, because it was raised in a previous discussion. Somebody asked about the immunogenicity of these exoantigens or soluble antigens. I should state generally that based on long-term experience not only on malaria but with *Babesia*, these are poor antigens. They're poor antigens. Without an adjuvant, they are not very immunogenic. However, with the appropriate combination of adjuvants they become immunogenic. I would like also to stress that going back to whoever created the whole system, the biological system, it was not created to protect the host, but rather to protect the parasite. So these antigens basically protect the parasite by complexing with the antibody in the blood plasma and preventing the antibody from being beneficial for the host. And as the final result you have immunoprecipitation glomerular nephritis of these exoantigens.

PERRIN: In relation to this question, the purification process used by Dr. Ristic is not specific. It's based on molecular weight and at this level there are two antigens present that I can see. We have shown for example that 82KD, which is coprecipitated with the 41KD using monoclonals, is present in the supernatant. On the other hand, Bob Freeman tells me that the 83KD is one of the main components present in the supernatant. So there are at least two antigens in the same area. So the question is: Is it the 82KD, which is coprecipitated by monoclonal recognizing both the 41 and the 82? Or is it the processed product from the 200KD? I think now that you've given us sequence information eventually somebody can answer later if in fact they derive from one or the other. But I don't see any way otherwise to answer the question.

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Characterization of Erythrocytic-Stage Antigens of *Plasmodium Falciparum* Recognized by Monkey Immune Sera

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Based on the evidence that antibody response plays an important role in the resistance to malaria,[1] extensive work has been carried out in various laboratories with polyspecific sera to identify and characterize *Plasmodium falciparum* antigens ever since an *in vitro* culture method of the parasite became available.[2] Some workers used human sera obtained from malaria patients and from people living in endemic areas.[3-9] Others used either sera from *Aotus* monkeys[10-13] or from *Saimiri* monkeys.[14-16]

The *Aotus* sera used in our laboratory were from experimentally vaccinated monkeys.[17] Serum samples from protected and unprotected monkeys were analyzed by immunoassays and immunoprecipitations of metabolically labeled parasite proteins. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) of immunoprecipitates showed that sera from protected monkeys had a higher reactivity to all the identified protein antigens than those from unprotected monkeys.[11] However, when differences in antibody titer between protected and unprotected monkeys were negated by applying equal radioactivity per sample onto the gel, there was no evidence of a unique antibody response to a particular parasite protein antigen in protected monkeys (Fig. 1). Twenty-one SDS-PAGE bands of polypeptide antigens, relative molecular weight (M_r) range from 185 to 30 Kilodaltons (K), were identified. These bands were designated with alphabets *a* through *u*.

Synchronized cultures obtained by treatment with colchicine[18] were metabolically labeled with ^{3}H-isoleucine and labeled parasite proteins from various blood stages were immunoprecipitated with *Aotus* sera from protected monkeys. Figure 2 shows that bands *a* through *g* (M_r

185–102K) occur mainly at the schizont stage.[13] Since the vaccine we used in vaccination experiments[17] was schizont-stage enriched, our attention was mainly focused on such high M_r protein antigens, viz., *a* (M_r=185K), *b* (M_r=152K), *c* (M_r=143K), and *d* (M_r=132K).

^{3}H-isoleucine labeled *P. falciparum* was released from erythrocytes by saponin lysis, sonicated and separated into a particulate and a soluble fraction by centrifugation at 100,000 x g for 1 hour. Figure 3 shows the SDS-PAGE patterns of the two fractions solubilized by Nonidet-P 40 (NP-40) and immunoprecipitated by an *Aotus* immune serum. High M_r bands *a* (185K), *b* (152K), and *d* (132K) are mainly found in the particulate fraction (Fig. 3A) whereas bands *g* (102K), *l* (75K), and *o* (53K) are relatively stronger in the soluble fraction (Fig. 3B).

SDS-PAGE of immunoprecipitates of ^{14}C-glucosamine labeled and solubilized *P. falciparum* extract shows that band *a* (185K) is heavily glycosylated (Fig. 4). In addition, one of the two proteins in band *d* (132K) and other proteins in bands *j* (83K), *k* (77K), *n* (57K), and *p* (47K) are lightly glycosylated. Similar findings using ^{3}H-glucosamine were reported by other workers.[7,19,19]

Since antigens in SDS-PAGE bands *a* through *d* are of rather high M_r, we would like to find out if they are composed of subunits linked by disulfide bonds. Diagonal SDS-PAGE was carried out with immunoprecipitates from ^{35}S-methionine labeled lysates by *Aotus* immune serum as well as two monoclonal antibodies (Mab's), 5.2 (which reacts with bands *a*, *b*, *c*, and *j*.) and 219.5 (which reacts with bands *c* and *d*) raised in our laboratory. It can be seen from both *Aotus* serum and Mab autoradiograms (Fig. 5) that no distinct spots fall below bands *a* through *d*. That such high M_r polypeptides are not formed by disulfide-bond-linked subunits is quite a surprise.

Since antigen bands *a*, *b*, *c*, and *d* occur in schizont stage, and since most of them are presumably membrane-bound and some are glycosylated, they are most likely to be the candidate antigens for protection. Thus we have been directing our efforts on the purification of these bands for further studies. One approach is to use preparative SDS-PAGE. IgG from pooled *Aotus* hyperimmune sera was isolated and immobilized onto CNBr-activated Sepharose 4B beads. NP-40 extract of ^{35}S-methionine-trace-labeled, schizont-enriched *P. falciparum* cultures was made to react with the affinity beads. Bound antigens eluted from the beads were separated by slab SDS-PAGE (Fig. 6). Strips of visualized antigen bands in acrylamide gel were excised, electrophoretically eluted, and concentrated. This method is time-consuming and the yield is often low. Also, antigens are denatured by SDS. Nevertheless in both Pereira da Silva's laboratory[14] and Perrin's laboratory,[20,21] such a method was used to isolate antigens for their vaccination experiments using *Saimiri* monkeys.

Another approach is to raise monoclonal antibodies to these high M_r bands which may then be isolated by affinity chromatography, using appropriate Mab's immobilized on Sepharose beads. The Mab's raised in our laboratory reactive with antigen bands *a* through *d* include two groups. One group, represented by Mab 5.2, recognizes bands *a* (185K), *b* (152K), and minor bands *e* and *f*. Another group, represented by Mab 219.5, recognizes bands *c* (143K) and *d* (132K). Figure 7 shows that bands *a* and *b* are depleted after passage of ^{3}H-isoleucine labeled *P. falciparum* lysate through Mab 5.2 affinity column; and *c* and *d*, likewise, are depleted after passage through Mab 219.5 affinity column. This clear result was obtained only when electrophoresis was in 6.25% polyacrylamide gels rather than our standard 7.5% gels. With the lower gel concentration, the high molecular weight proteins in band *d* resolved into a doublet, only one of which was membrane bound and glycosylated. These indicate the coidentity of bands *a*, *b*, *c*, and *d* recognized by *Aotus* immune serum and monoclonal antibodies. Mab 5.2 was found to immunoprecipitate a heavily glycosylated 180K band whereas Mab 219.5 failed to immunoprecipitate a glycosylated antigen (Fig. 8). Immunogenicity and protection studies with antigens isolated by these two Mab immunosorbants have been conducted, and the results appear elsewhere in this symposium (cf., Siddiqui, W. A. et al.)

As has been mentioned, the yield of preparative SDS-PAGE is not satisfactory and the antigen preparations from each Mab immunosorbant consist of multiple SDS-PAGE antigen bands. So further methods of antigen purification to obtain individual SDS-PAGE antigen bands in reasonable yields should be pursued. Recent rapid advances in protein and peptide separation by high performance liquid chromatography indicate that it maybe the method of choice. Once this is achieved, characterization of the individual bands by peptide mapping, amino acid composition, and sequencing can be carried out to access the relationship of multiple bands recognized by each Mab. Individual antigens can also be used for immunological and vaccination studies. Polyspecific antisera raised with individual antigens will be invaluable reagents for the screening of clones in recombinant DNA work.

ACKNOWLEDGEMENTS

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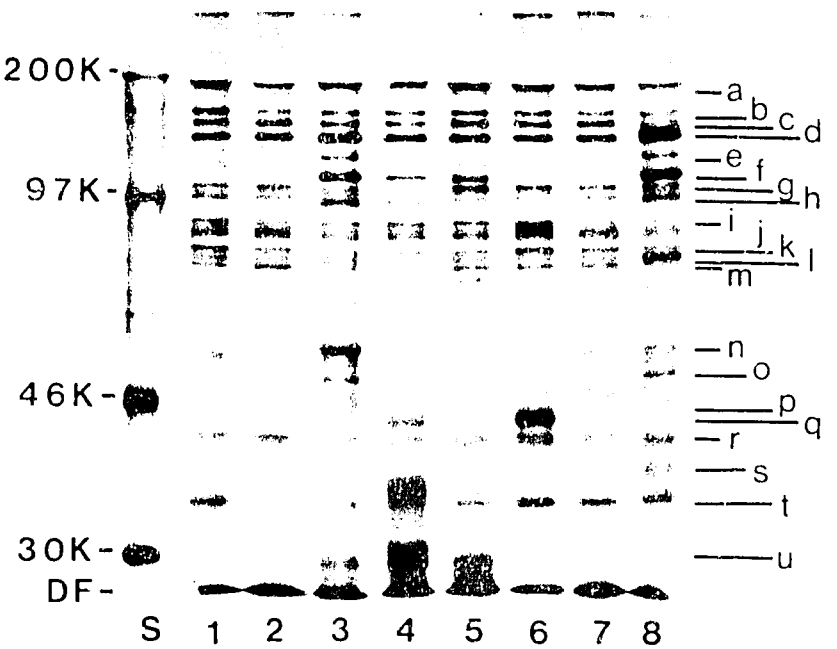

FIGURE 1. SDS-PAGE of Immunoprecipitants of ^{3}H-isoleucine Labeled *P. falciparum* Antigens Reacted with Secondary Sera From Vaccinated *Aotus* Monkeys. Lanes 1 & 2, immunoprecipitants by sera from unprotected monkeys; lanes 3 through 8, immunoprecipitants from protected monkeys. 54 nCi per lane. S, protein standards.

210'

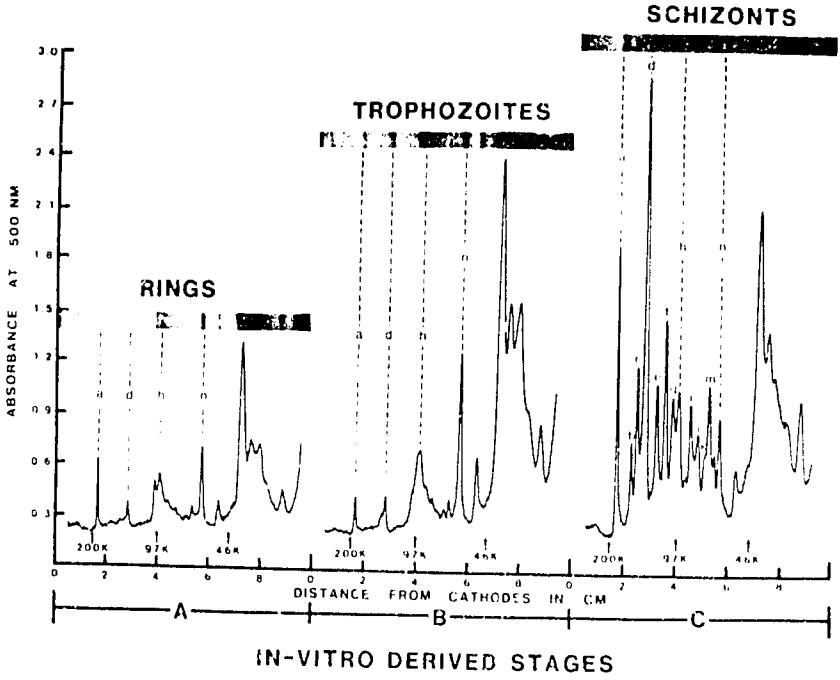

FIGURE 2. Densitometric Tracings of Autoradiograms of ^{3}H-isoleucine Labeled Antigens from Synchronous Cultures of *P. falciparum* at Ring, Trophozoite and Schizont Stages Reacted with Hyperimmune *Aotus* Serum and Separated on SDS-PAGE.

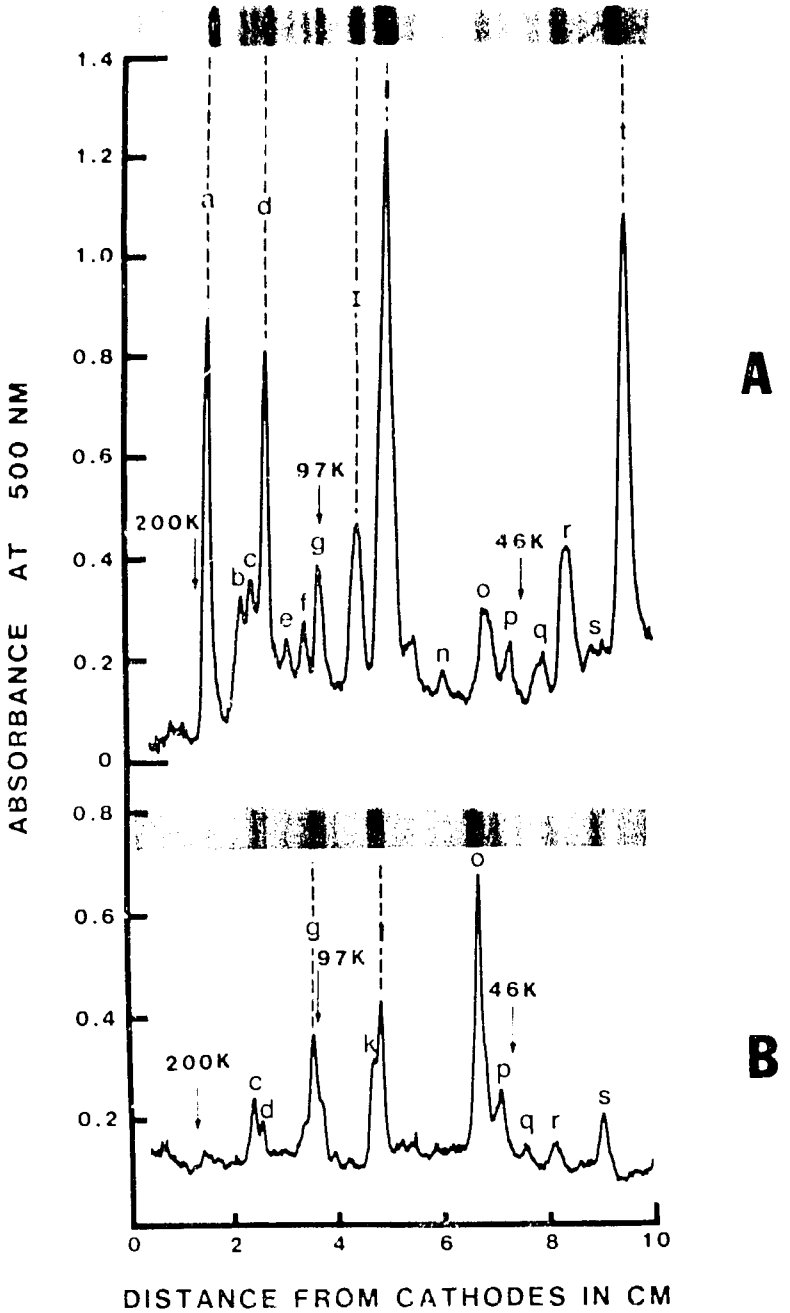

FIGURE 3. Densitometric Tracings of Autoradiograms of ^{3}H-isoleucine Labeled Antigens in a Particulate Fraction (A) and a Soluble Fraction (B) of a *P. falciparum* Culture Reacted with Hyperimmune *Aotus* Serum and Separated on SDS-PAGE.

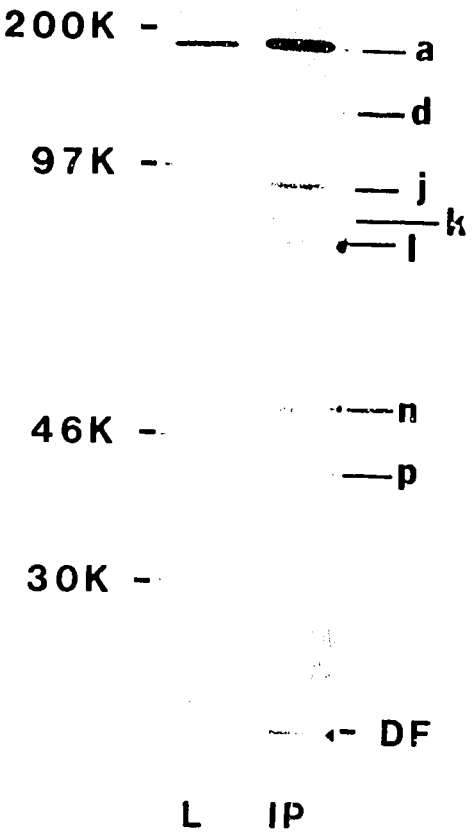

FIGURE 4. SDS-PAGE of ^{3}C-glocosamine Labeled Extract of *P. falciparum*. Lane L, lysate; lane IP, immunoprecipitants with *Aotus* hyperimmune serum.

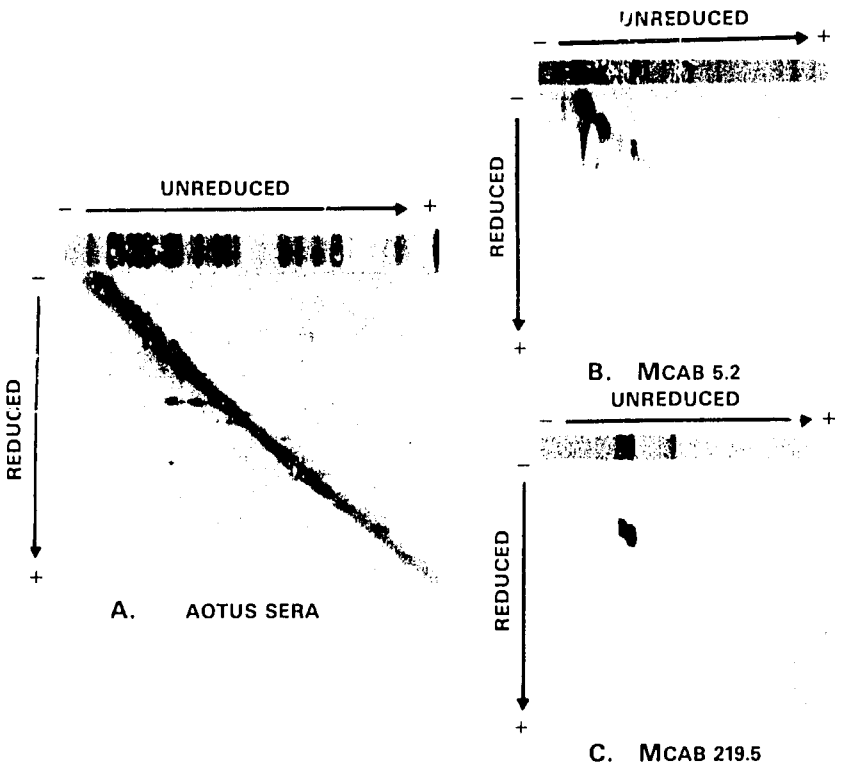

FIGURE 5. Autoradiograms Showing Results of Diagonal Electrophoresis of Immunoprecipitants from ^{35}S-methionine Labeled *P. falciparum* Antigens Reacted with *Aotus* Hyperimmune Serum and with Mab 5.2 and 219.5. Horizontal arrows indicate direction of 1st electrophoresis under unreduced conditions. Vertical arrows indicate direction of 2nd electrophoresis under reduced conditions.

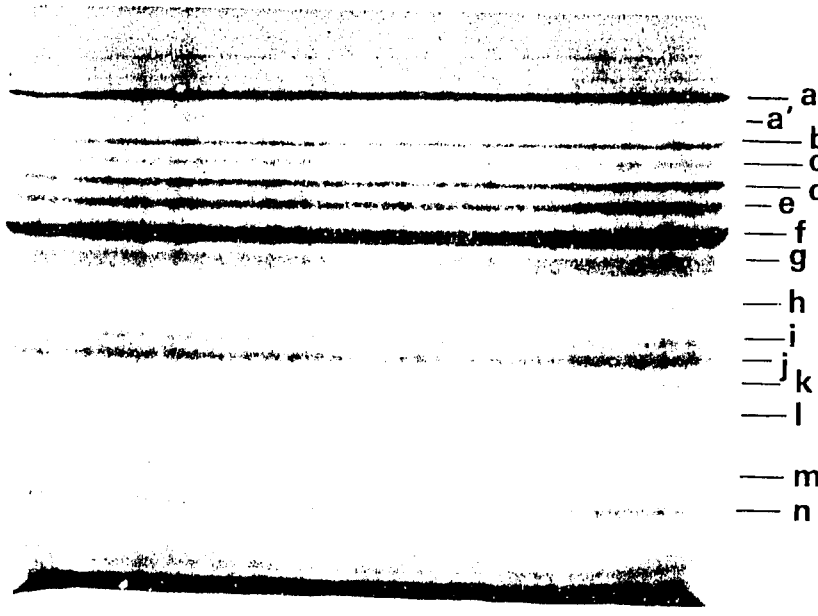

FIGURE 6. Separation of ^{35}S-methionine Labeled *P. falciparum* Antigens Reacted with *Aotus* Hyperimmune Serum by 3 mm Slab SDS-PAGE.

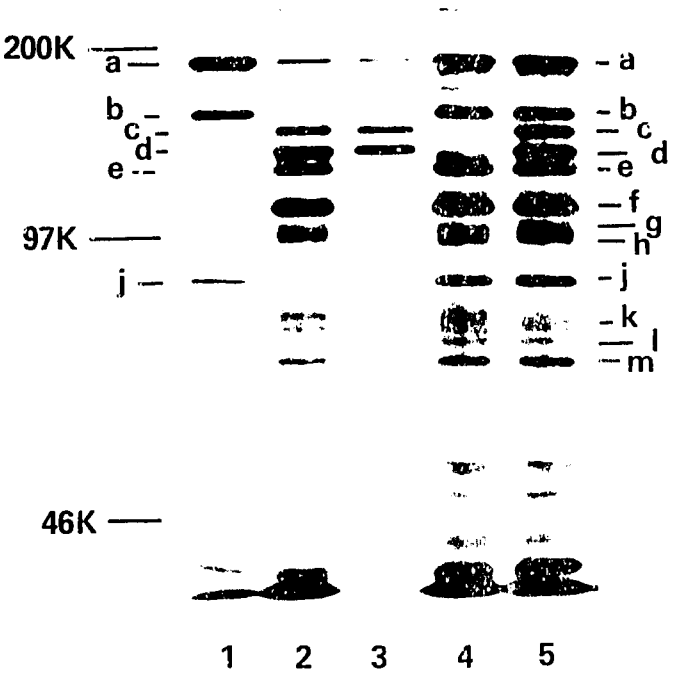

FIGURE 7. ^{3}H-isoleucine Labeled Antigens of *P. falciparum* Isolated by Affinity Beads with Immobilized IgG from Hyperimmune *Aotus* Serum or with Mab's 5.2 and 219.5. Lane 1, antigens eluted from 5.2 beads after passage of ^{3}H-isoleucine labeled lysate; lane 3, antigens eluted from 219.5 beads after ^{3}H-lysate passage; lane 5, antigens eluted from hyperimmune IgG beads after ^{3}H-lysate passage. Lane 2, antigens eluted from hyperimmune IgG beads using ^{3}H-lysate pre-absorbed with 5.2 beads, showing depletion of 5.2 antigens. Lane 4, antigens eluted from hyperimmune IgG beads using ^{3}H-lysate pre-absorbed with 219.5 beads, showing depletion of 219.5 antigens.

FIGURE 8. SDS-PAGE of Immunoprecipitants of ^{14}C-glucosamine Labeled *P. falciparum* Lysate Reacted with Mab 5.2 (lane 1) and Mab 219.5 (lane 2).

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Discussant: Asexual Blood-Stage Candidate Antigens

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Introduction

The prospect of a modern, molecular vaccine to the asexual blood stages of the malaria parasite is based on three important developments. In 1976, Trager and Jensen at the Rockefeller University reported the continuous cultivation of the human malaria parasite *in vitro*.[1] This enabled the large-scale production of parasite material, from which native antigens could be identified, characterized, and tested as vaccines in limited tests with susceptible monkey models. Second, in 1977 reports from the University of Hawaii and University of London revealed that schizont- or merozoite-enriched preparations of the human malaria parasite *P. falciparum* could induce protective immunity in owl (*Aotus lemurinus*) monkeys against a lethal challenge infection, when given with a strong adjuvant.[2,3] This indicated that susceptible animal models existed, although in limited numbers, in which purified candidate vaccine antigens could be evaluated experimentally. Third, the advent of the new biotechnology and the refinement of gene cloning techniques led to a new breed of malariologists–molecular biologists who have provided new and exciting information about the malaria antigens (reviewed in 4,5) and promised a method for the large-scale production of protective antigens in a magnitude compatible with worldwide distribution.

A large number of candidate antigens have been described for an asexual blood stage vaccine. As the characteristics of these antigens are clarified by different laboratories, it is probable that the actual number of strong candidate antigens will be small, possibly as few as four or five. The candidacy of a potential vaccine antigen may be evaluated by asking four important questions: a) Is there indirect evidence that immune mechanisms may retard parasite growth *in vitro*? b) Are analogous antigens in non-human malaria species protective in their respec-

tive hosts? c) Is there direct evidence the candidate antigen, given with appropriate adjuvant, can induce protective immunity in susceptible monkey models? d) Have the genes for these antigen been cloned? In this discussion, the major candidate antigens will be outlined, and the indirect and direct evidence for protection emphasized.

Pf185–200

The initial recognition step of the merozoite with the erythrocyte surface is a weak interaction between surface molecules. The most prominent of the merozoite surface molecules and probably the most well-characterized of the *P. falciparum* blood stage antigens has been characterized by numerous laboratories worldwide. Work from the Wellcome Research Laboratories has revealed that the major antigen on the merozoite surface is derived from a precursor molecule (185k–200k), which is synthesized throughout schizogony and is proteolytically processed into numerous polypeptides. The three major derived polypeptides on the merozoite surface have molecular weight 83K, 42K, and 19K. The 83K precursor is shed from the merozoite surface, while the 42K/19K complex is carried through into new ring forms.[6] The high molecular weight precursor in the schizont is antigenically polymorphic and this, in part, may be responsible for the variability in molecular weight reported by different workers.[7] It is not known where the variable epitopes are situated in relation to the smaller processing fragments on the merozoite surface. Some epitopes are conserved in all serotypes, and if these are exposed on the merozoite surface, these regions may be reasonable epitopes for a molecular vaccine. The abundance of this antigen on the merozoite surface and the fact that it represents almost a third of the metabolically labeled *P. falciparum* polypeptides indicates that it may be a constituent of a surface coat, similar to the sporozoite surface coat and may have some function of the immune system.[12]

The evidence that this molecule might be a good protective antigen is considerable. It has been reported (these proceedings) by the Wellcome and University of Geneva research teams that monoclonal as well as polyclonal antibodies do not inhibit parasite growth *in vitro*. This was very much surprising since it has been largely accepted that antigens of the merozoite coat would be good targets for neutralizing antibodies. However, there is some evidence that protection by a cell-mediated immune pathway is possible, based on work in a rodent malaria.[8,21] Therefore, the evidence showing a lack of inhibition by antibody *in vitro* need not detract from the candidacy of this antigen. But more importantly, there is considerable direct evidence that this antigen is an important protective antigen. In *P. yoelii* rodent malaria, the processed

polypeptides are protective upon immunization.[9] Last year, researchers at the University of Geneva reported that a 200K polypeptide, isolated by conventional means, protected squirrel monkeys against heterologous infection.[10] Subsequent studies confirmed that the major antigen isolated was the merozoite surface coat precursor protein.[22] In addition, work at the University of Edinburgh in the same year showed that the monoclonal antibody-isolated 195K precursor molecule partially protected squirrel monkeys.[11]

It is unknown if this antigen can protect karyotype II and III *Aotus lemurinus* monkeys, generally regarded as a more rigorous monkey model for infection by the human malaria parasite. At the University of Hawaii, we have found that the molecule in the Uganda Palo Alto isolate has a relative molecular weight of 185K. A monoclonal antibody against this antigen absorbed the precursor and two other major as well as numerous minor metabolically labeled antigens co-identified by sera from *Aotus* which had been vaccinated and protected against challenge infection in previous vaccination experiments. In addition, the monoclonal antibody isolated almost one-third of the polypeptides co-isolated by hyperimmune *Aotus* sera.[12] When the polypeptides were given in combination with a high molecular weight rhoptry antigen complex an apparent sterile immunity was induced in a single *Aotus lemurinus azure* (karyotype VI).[12] Induction of the protective immunity was extremely adjuvant dependent, and the apparent sterile immunity was achieved with the polypeptides given three times in Freund's complete adjuvant, but not when given with synthetic adjuvant MDP-Lys(L18).

At least four groups have published gene sequences for this prominent protein.[11,13,14,4] Comparison of sequences between different isolates will provide a molecular basis for diversity and the selection of possible conserved, constant regions.

Glycophorin-Binding Protein

Two other relatively minor metabolically labeled proteins, having molecular weight 155K and 130K, are apparently also on the merozoite surface and are believed to bind to glycophorin on the erythrocyte surface with high avidity. This interaction may be involved in the initial attachment of the merozoite to the erythrocyte surface. Work from the Rockefeller University has indicated that a monospecific polyvalent antisera to these molecules inhibits parasite growth *in vitro*;[15] however, there is no direct evidence that these molecules can induce protective immunity in monkey models to date. The gene for this protein has been cloned by the Sloan-Kettering and Walter and Eliza Hall Institute research groups.[16,4] A conventionally isolated 140K antigen has been isolated by the University of Geneva group and shown to be protective

in squirrel monkeys, but its relationship to the glycophorin binding protein is unknown.[10]

Related to the glycophorin-binding proteins may be proteins, having molecular weight 140K, 70K, and 30K, which bind to n-acetylglucosamine on glycophorin.[17] The relative candidacy of these molecules awaits direct evidence of protection in monkey models.

Pf75

At the Pasteur Institute, workers reported last year that a 75K protein efficiently protected splenectomized squirrel monkeys against a heavy challenge of the Uganda-Palo Alto isolate.[34] The Scripps Institute group has cloned about half of a gene coding for a merozoite surface protein of 75K, but its relationship to the Pasteur 75K protein remains to be clarified.[18] It is reported to be highly conserved among isolates from widely different regions of the world.[7] It is unknown whether antibodies are inhibitory *in vitro*, but this information is eagerly awaited for comparison with the information on the other merozoite surface antigen, pf185-200.

High Molecular Weight Rhoptry Complex

These polypeptides are localized in a seemingly unlikely site of immune attack, the internal parasite rhoptry organelles. After the initial recognition step involving weak interaction of the merozoite and erythrocyte surfaces, a specific re-orientation ensues such that a pair of apical internal organelles become localized at the point of erythrocyte invasion. It is believed that the contents of the rhoptry play an important role in the attachment and invasion process, but the mechanism is unknown.[19]

Work in a *P. yoelii* rodent malaria system from the Wellcome Research Laboratories identified rhoptry antigens as possible targets as early as 1980. Monoclonal antibodies against rhoptry antigens protected a *P. yoelii* rodent malaria upon passive transfer.[20] Moreover, mice immunized with the monoclonal antibody-isolated antigen were protected.[8] The Wellcome group has produced a monoclonal antibody which gives a punctate or spot immunofluorescence staining pattern, in contrast to the even linear staining of the merozoite surface by the previous group of antibodies. The monoclonal antibody isolates two major polypeptides (155K and 140K) as a weakly associated complex.[21] These have been localized by immune electron microscopy to the rhoptry. We have produced a monoclonal antibody which gives a similar immunofluorescence pattern, although it immunoprecipitates 143K, 132K, and 102K polypeptides in the Uganda-Palo Alto isolate. We have exchanged reagents with the Wellcome group and found the antibodies to be identi-

cal. The later polypeptides represent two of the major metabolically labeled parasite proteins and up to 13% of the total polypeptides isolated by antisera from immune *Aotus* monkeys.[12]

We have used these polypeptides in combination with the merozoite surface coat protein and its precursor (described above) to induce an apparent sterile immunity in a single karyotype VI *Aotus* monkey. The University of Geneva group reported that serum from squirrel monkeys which were immunized and protected with proteins from the 140K region of preparative SDS-gels gave a spot immunofluorescence pattern.[22] The third polypeptide having molecular weight 102K is poorly metabolically labeled and has been isolated by other monoclonal antibodies to the high molecular weight rhoptry complex.[24] The gene for the 102K rhoptry polypeptide and two other high molecular weight rhoptry polypeptides probably have been cloned by the Walter and Eliza Hall Institute group.[4]

In an unexpected development, the Australian group also reported that an antigen previously described on the infected-erythrocyte surface and having molecular weight 155K has been localized in the micronemes adjacent to the merozoite rhoptries (see RESA below). In discussions with R. R. Freeman (Wellcome Institute), it is possible that RESA is the larger of the high molecular weight rhoptry complex isolated by monoclonal antibodies 61.3 and 219.5.

Low Molecular Weight Rhoptry Complex

Monoclonal antibodies have been produced to a second set of rhoptry polypeptides.[25-27-24] Some of the antibodies have been shown to be inhibitory *in vitro* by the University of Geneva group.[24-25] A 41K rhoptry polypeptide isolated by the same group gave very strong protection in squirrel monkeys recently.[26] On the basis of the protection experiment in the squirrel monkeys, these antigens appear to be very promising candidates.

Ring-Infected Erythrocyte Surface Antigen

As a result of the merozoite invasion process, parasite antigens may adhere to and remain temporarily on the infected erythrocyte surface. One such antigen identified by the Walter and Eliza Hall group as a ring-infected erythrocyte surface antigen (RESA) has recently been localized in the micronemes proximal to the merozoite rhoptries.[28-29] Independently, a group at the University of Stockholm has developed an antisera to this 155K protein and shown that it inhibits parasite growth *in vitro* very efficiently.[30] There is no direct evidence that this antigen is protective as yet, but its origin in a rhoptry protein and the

indirect evidence of protection *in vitro* makes it a strong candidate. Part of the RESA gene has been cloned.[31]

S-Antigens

During schizogony, rupture of the infected erythrocyte results in the release of merozoites. This is accompanied by the release of numerous proteins from the parasitophorous vacuole which can then be detected in the culture supernatants. These have been termed soluble antigens. Many of these proteins are immunogenic and will elicit immune responses. A well-characterized family of antigens found in culture supernatants or in the plasma of infected individuals are the soluble proteins which retain their antigenicity after boiling and are termed heat-stable or S-antigens (reviewed in 32). Recent immune electron micrographs from the Walter and Eliza Hall Institute in Australia have shown that S-antigen is in the parasitophorous vacuole.[32] There is no evidence that these molecules are protective, as yet.

Supernatant Antigens

Workers at the University of Illinois have isolated an 83K antigen from culture supernatants.[33] The antigen appears to be the same 83K antigen on the merozoite surface coat that has been efficiently isolated directly from schizont-enriched preparations. The possibility that it may in fact be an 82K dimer of the 41K rhoptry complex awaits comparison of amino acid sequences. Crude preparations induced partial protective immunity in squirrel monkeys with alum adjuvant.[35]

Erythrocyte Surface Antigens

There is some evidence that aside from those merozoite molecules which remain transiently following the endocytosis process, new parasite antigens appear on the surface of erythrocytes infected with trophozoites and schizonts (reviewed in 36). Among those recently reported by the NIH group is a strain-specific antigen having molecular weight ranging from 250K to 300K.[37] This molecule may be responsible for the ability of schizont-infected erythrocytes to adhere to and sequester in the endothelium of capillaries and venules. This may be related to a 300K molecule recently reported by the Australian group and having interspersed blocks of repeating units.[38] Despite the lack of direct protection experiments, these molecules are likely to be very important antigens to be included with merozoite surface coat and rhoptry polypeptides in a polyvalent vaccine against the asexual blood stages.

Premunition and Antigenic Diversity

A key host-parasite relationship in malaria is premunition, the co-existence of blood parasites, sporozoites, and gametocytes in the presence of circulating, parasite specific antibodies. As pointed out by Professor Clyde, immunity in nature is maintained by repeated infections in holoendemic areas. The extent of protection and clinical symptomatology is dependent on the frequency and persistence of homologous species infections and diminishes if these infections cease. These observations warn that a successful malaria vaccine may be dependent on the identification of protective epitopes on protective protein antigens. These epitopes might differ between strains (antigenic diversity) and might be weakly immunogenic.

Studies from the Walter and Eliza Hall Institute on the structure of the S-antigen from a Papua New Guinea isolate, based on data from gene cloning, has revealed much about the structure of the malaria parasite antigens. These molecules exhibit extreme molecular diversity.[33] Although the molecules are weakly immunogenic, naturally occuring antibodies are directed at variable, tandemly repeated sequences which differ among isolates from different parts of the world. The variable repeat sequences are flanked by conserved sequences found in many different isolates throughout the world. The conserved regions of Papua New Guinea and Thai isolates are identical at one end of the molecule. Very little naturally occuring antibodies are directed at the conserved regions of the molecule. It would appear, therefore, that a vaccine which is to be effective in different areas of the world must consist of conserved epitopes, rather than variable, repeat sequences. Whether a molecular vaccine consisting of these conserved regions will be protective is unknown.

Conclusion

Direct challenge experiments with isolated proteins from the human malaria parasite have shown that varying degrees of protection can be achieved in susceptible monkey models. Researchers working on an asexual blood stage vaccine appear to be going through a period of convergence. The list of candidate antigens, once believed to be alarmingly extensive, is being reduced to a small number. To date, the most promising candidates, based on direct evidence in monkey vaccination experiments, lie on the merozoite surface and in merozoite rhoptries. Antigens on the surface of the infected erythrocyte will also likely be very important antigens. To date, there are probably five strong candidates on which to base a polyvalent asexual blood stage vaccine.

TABLE 1. Indirect and direct evidence for protection by candidate *P. falciparum* vaccine antigens

Candidate antigen	*Ab inhibits in vitro*	*Homolog protective*	*Protective in monkeys*	*Gene cloned*
pf185–200	—[a]	+[9]	+[10,11]	+[11,13–15]
GBP[b]	+[16]	?	?	+[17]
pf75	?	?	+[18]	+[19]
rhoptry[c]	+[25,26]	+[1]	?	+[4]
rhoptry[d]	+[25,26]	?	+[27]	—
RESA[e]	+[31]	?	?	+[29]
S-antigens	?	?	?	+[31]
cytoadherence	?	?	?	—

[a] Monoclonal and polyclonal antibodies do not inhibit growth, but a cell-mediated immune mechanism may be involved.
[b] Glycophorin-binding protein.
[c] High molecular weight rhoptry complex (155k, 140k, and 110k in the Welcome Lagos isolate vs. 143k, 132k, and 102k in the Uganda Palo Alto isolate).
[d] Low molecular weight rhoptry complex includes a 41k polypeptide.
[e] Ring-infected erythrocyte surface antigen which may be the 155k protein of rhoptry.
[f] A high molecular weight rhoptry polypeptide has been shown to protect in a rodent malaria[28].

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Rapporteurs' Summary

GRAHAM MITCHELL AND LESLIE TAM

The candidacy of blood stage antigens stands in sharp contrast to that of gamete and sporozoite antigens. Whereas in the sporozoite/sexual stages, a restricted number of surface protein antigens have been identified and are obvious candidates to be tested as vaccines preventing sporozoite invasion of hepatocytes or blocking transmission, the nature or priority rating of numerous blood stage antigens is less clear. A major task confronting participants in the workshop session was to clarify the relationships between antigens identified through conventional, monoclonal antibody or gene cloning approaches in numerous laboratories. This was felt necessary in order to facilitate discussion on the vaccination efficacy of different antigen preparations. The following table represents a consensus opinion on the major blood stage antigens reported to date:

Asexual Blood Stage Antigens of P. falciparum

Designation	*Identifying Characteristic*	*Cloned*	*Reference*
I *Merozoite/schizont membrane-associated antigens:*			
Pf 185/200 83	Merozoite surface	+	1–9
40/46			
(42)	Glycosylated, product carried into rings		
N-acetylglucosamine binding protein	140/70/35	—	10
Glycophorin binding protein	low methionine, 130K	+	11, 12
Pf 96/100 doublet	—	—	13
Pf 75	major surface protein of merozoite	+	14
Pf 76			15
II *High M, rhoptry proteins:*			
RESA	rhoptry + ring stage	+	16,17,18,19
Pf 155/140	punctate fluorescence of merozoite	—	20, 21, 6

Designation	*Identifying Characteristic*	*Cloned*	*Reference*
III *Low M_r rhoptry proteins:*			
Pf82 (76,75) 41	punctate fluorescence of merozoite	—	22,23,24,25
IV *Membrane-associated antigens of infected erythrocytes:*			
A knob associated protein	80–95K	—	26,27
SICA-like protein	sequestration protein? variable (± knob associated)		28
V *Antigens in the parasitophorous vacuole:*			
S-antigens	variable, heat stable	+	29–31
90K protein	—	—	32
CRA	23K	+	33,34
FIRA	300K, highly immunogenic	+	35

*As yet it is unclear whether the 83/100K purified exoantigen of Ristic et al. (this proceedings) is identical to the 83K major merozoite protein or, in fact, is the PF 83 (76,65) 41K antigen (low M_r rhoptry proteins). N-terminal sequence data will resolve this shortly.

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General Discussion

MITCHELL: I think just falling on one of the comments from Ross Coppel—this long list that Karen (Yamaga) gave us—which I can imagine is terrifying for someone not in the field; one would be intimidated by it and say, "What are these merozoite people on about. They've got just 50 antigens there. We're not going to bother thinking about them until they get their act together and get that list down to something workable." I should say at least there's a bit of consensus amongst the groups. Now Ross already hinted that that long list of Karen's can get down to around about 4 or 5 antigens when the chips are down, perhaps. So I would not be intimidated by this long list and go away with the conclusion that there are going to be hundreds of proteins of *Plasmodium falciparum*, which at the moment are in the top bracket of the sort of candidate vaccine molecule. That's not the case at all. I think that number is in the 3 to 5 region, rather than the 30 to 40 region, and we will see a consolidation of this listing as we understand what is a cleavage product of a larger protein. As time goes on, of course, the only way to is to get away from molecular weights and get down to sequence data. A year from now, I think that list will look very different from what it does now.

TAM: I think that will be shown in the afternoon session when Dr. Siddiqui will show evidence where two monoclonal antibodies can absorb approximately 46% or nearly half of the total polypeptides which are recognized by immune *Aotus* sera. So that indicates that there's not the great amount of antigens that we had expected.

COPPEL: Probably one point that is worth clarifying is to make the statement now that in fact Robbie Freeman, Luke Perrin, Roger Hall, and John Scaife are all working on the same processed, 185K to 185K surface glycoprotein. The discrepancy has been in molecular weight, which varies among different isolates. They are all accumulating data about

the same gene product, and I think that is conclusive from sequence information now. I just think that perhaps to this list you might add the work of Roger Hall. In their paneled monoclonals there are several that give a characteristic rhoptry appearance and you might add in fact the ring-infected erythrocyte surface antigen RESA, as we now have EM data that puts it in the rhoptry, rather than on the surface of the erythrocyte.

DIGGS: Just a point of information. Is it true that this spotty pattern of immunofluorescence means rhoptry and vice versa now, or are there exceptions?

YAMAGA: Anyone in the audience want to comment on this point?

COPPEL: I think that what you'd prefer as evidence is EM data. I think that this sort of punctate fluorescence is really only second best. It is probably a good working definition to start with. Punctate fluorescence indicates rhoptry until proved otherwise. We don't see the punctate pattern with RESA, but we can see it very clearly on EM. Several people have monoclonal antibodies that in fact recognize the same antigen and this antigen has already been described in 1982. The protein inhibits *P. falciparum* and has been shown now to be in the rhoptry by Dr. Aikawa.

REESE: I don't think there is any evidence at this point in time that the 80K protein is at all on the surface. Likewise, in the work of Perlmann, I don't think that there is any evidence that shows the 155K protein on the surface, because he had to glutaraldehyde fix those cells. When he in fact tried to use live erythrocytes and looked for the binding, he got no binding. So I think one should be skeptical about both of those. I don't think there is rigorous data that suggests either are on the surface.

The only thing that I would suggest, if there is any data at all that is on the surface—maybe Ross, you could comment on this—is the 150K rhoptry protein, which I thought was transiently on the surface. When I looked through your paper, I interpreted your data differently than you did at the time. I thought it was a rhoptry protein, and I said it was transiently on the erythrocyte surface as I could recall.

COPPEL: I think you are probably more astute than we were, because we didn't pick it as a rhoptry protein to start with. Just to keep going on the process of simplification here, Perlmann's group and we are describing the same antigen when we talk about the ring-infected erythrocyte surface antigen (RESA). Now there is, I think, a real difficulty in knowing precisely at the moment what its relationship to the surface is. In our hands we got fluorescence against intact unfixed erythrocytes, with a rabbit serum we originally raised, against the cloned molecule. Now when we tried to repeat that with subsequent immunizations, we did

not get lighting up on unfixed cells, and we don't understand why that was. The EM data we had suggest that it is certainly, if not exactly, on the outside of the surface. It is so close on the EM that you can't pick it as being on, in, or under. But certainly from Peter Perlmann's work, they would tend to put it somewhere surface available, I believe is how they described it. It is not actually on the surface to start with, but perhaps somewhere just inside or under. We think it must be external at one stage because it starts in the rhoptry of the merozoite and ends up somehow at the surface. And I can't see any mechanism for getting to its final location which doesn't put it at some time external to the merozoite and at the surface, but we don't understand exactly what is going on.

Reese: Dr. Coppel, did you exchange reagents with Perlmann's group or with the Perkin's group?

Coppel: We've done some exchange of reagents, but not to address the topic of location. We were trying to clarify their findings of growth inhibition and our own findings in that particular respect, which were not compatible.

V. Nussenzweig: We have obtained a monoclonal antibody, originally by immunization with the gametocyte rich fraction, which is against the polypeptide of similar molecular weight. Our data indicate that this monoclonal reacts with repetitive epitope in the Perlmann protein, and it also reacts with the material which was provided by the Australian group. So, I think the evidence is pretty good that they are the same materials which are being seen and, which I mentioned, are expressed in different stages, not only in all of the asexual blood stages, but it is also present in gametocytes.

Pereira da Silva: So you had a symmetrical phenomenon as Dr. Coppel, who showed that you have a cross-reactivities at the antibody level, but not cross-reactivity at the DNA level?

V. Nussenzweig: No, we believe that it is against the very same protein, and in fact that it is against the same epitope which is on most proteins.

Reese: Ross, could you answer a question as to how long this antigen is available on the erythrocyte surface? It wasn't clear to me from the manuscript whether it was a transient thing or that it stayed there throughout. With the ones that we have looked at we know they are there in the ring stage and it is hard because of the intensity of the staining to tell. We are just to the point where we are trying to boot up and use a biotin system to get up a greater signal to noise ratio.

COPPEL: I can't really address the question of how long it lasts on the outside, but as far as how long it lasts around about the membrane, whether that is inside, under, or on top of, is that in our hands it essentially lasts for at least 24 hours. We think we don't find it by the time the parasite enters the trophozoite stage, by immunofluorescence. By Western blotting, however, you can find RESA reactive proteins present in all stages. In fact in almost any stage specific proteins so described by fluorescence, if you go to the increased sensitivity of Western blotting, it is in all stages. So it becomes a bit fuzzy exactly how long—it depends how sensitively you look.

REESE: Yes, but the Western blot isn't going to tell you where it is. That could be the new denovo synthesized, and, in fact, it is made fairly early. What I wanted to know was—I know when we did the original labeling, looking for antigens on the surface, we were never able to find any evidence, using immune human or monkey serum, for anything at the ring stage.

COPPEL: Right.

REESE: You're saying that you now have one. That was what I was trying to address.

COPPEL: You are saying you're surface labeling and you can't see it. That is presumably to the outside. We are just not sure how long it is on the outside, rather than in, that membrane. It is somewhere around the surface of the cell for quite a long period of time, but how available that is to labeling procedures, being right out and exposed, we are not sure. We have evidence that it's trypsin sensitive, so that seems to suggest that some of it is available for digestion. But I don't think the situation is at all clear.

V. NUSSENZWEIG: If you can stand some additional information about these two antigens, I have some additional data which Ruth (Nussenzweig) perhaps did not mention, but it is being presented now. This is the work of Dr. Masuda and Dr. Zavala on the same antigen which points out how difficult it is to classify these antigens into groups, because according to that piece of work, the same antigen described, the RESA antigen, is found in large amounts in culture supernants, can be clearly classified as an S-antigen because it is heat stable, secreted in large amounts when the schizont breaks up. I don't know what the difference between this and an S-antigen is really.

COPPEL: Just let me clarify that point of Victor's. That is, we agree completely that it is found in the supernatant, but our interpretation is that merozoites have a high degree of failure of penetration. Only a small fraction actually get in to complete the next round, and we feel

what is going on is that the merozoite attaches, the rhoptry disgorges RESA. If it is a defective invasion process that RESA finds its way out into the supernatant, so I don't believe it is secreted in the same way as the S-antigen.

RISTIC: I'm like Dr. Bruce-Chwatt and tried to learn more about molecular biologists. I'm not one. I'm directing a group and trying to make a compromise between clinical immunology and molecular biology. It's my sincere feeling that one side without the other side will not work well in developing a vaccine against malaria. I've seen some molecular biologists being carried away for several years identifying all kinds of antigens. But when it comes to, in the States they say, "Where's the beef?" that antigen doesn't do any good. So what I'm trying to say, while the monoclonal antibody cross-absorption, cross-neutralization is fine, and immunochemically acceptable too, I would not agree that just because an antigen reacts with an antibody it's a proper immunogen, as a protective vaccine. There's no substitute in putting the right antigen in an animal and showing the protection.

MARTIN: One of the missing components of the immune analysis, at least from my point of view, is what is a cell-mediated immune system looking at in these various antigens? I think that kind of issue may have to perhaps add a degree of complexity to this analysis. Yet from the point of view of vaccine development, this may in fact be far more important than what a monoclonal antibody may recognize. I could follow with question: "What in fact is the status of cell-mediated assays for the detection of the various antigens that we've heard mentioned this morning?"

REESE: I think the reason that people use antibodies is because they're specific. They can be used as very good analytical tools. They can tell you specifically the molecule with which they're interacting and they're not so cumbersome and in fact, as I'll provide evidence tomorrow, you can passively transfer it and show protection. I'm not saying that you can't have a role for cellular immunity, but in fact there is good evidence that antibody plays a large role and when you're trying to look for appropriate antigens, cellular assays are terribly cumbersome.

MITCHELL: I think the other point about cell-mediated immunity is that it's very easy to find blast transformation. We could do this tomorrow with repeat structures of antigens or nonrepeat, but what do you do next? That's the problem with cell-mediated immunity. I couldn't agree more with Bob that we can't ignore it, and, of course, you can't stop it from happening, but when it comes to analyzing the actual antigens involved, I think we all would like to stay with antibody for as long as possible. Especially in view of the evidence that antibody can protect.

Why invoke another mechanism which is almost unassayable at the moment? This, of course, will provoke a lot of comment because there are people who, based on negative evidence, if you can't ascribe it to antibody why not invoke cell-mediated immunity? That's sort of what we've been doing for a long, long time and I don't think it's got us very far, I might say.

PEREIRA DA SILVA: This is a very provocative question to the chairman.

YAMAGA: Yes, I think the role of CMI is very important and I think this is why I echo Dr. Ristic's feeling that you have to isolate the antigen and inject it into the animal. Dr. Siddiqui for a long time has advocated the necessity for a potent adjuvant, especially Freund's complete adjuvant which is required to elicit very strong CMI. So the fact that you're using an antibody to isolate antigens does not necessarily mean you're not going to evoke a potent immune response if you administer it into the animal in an appropriate manner. So the role of CMI is obviously very important. That's why the best evidence for protection is direct experimentation within a susceptible animal.

SAVANAT: I'm thinking of a compromise between CMI, classical CMI, and the role of antibody. If one examined the combination of antibody and cell in the so called ADCC, one can have an assay system which one can measure and see whether the combination of these two factors have any effect in the control of malaria.

MITCHELL: I think your comment raises the problem we have when different people talk about cell-mediated immunity. Now, one person, of course, can be talking about a K-cell or ADCC type of mechanism. Another might be talking about a non T-cell, antibody-dependent killing event which again will be present in malaria and impossible to stop, I should say. Another group of people will be thinking of delayed skin reactions, a T-cell response. Another group might be thinking of suppressor T-cell effects, which, of course, will be cell-mediated immunity. So that is exactly the problem with cell-mediated immunity. That when someone invokes it, you have to be much more specific. So are you then saying the repeat antigens that Ross Coppel is talking about or the Nussenzweigs? Are they determinants which are particularly good at addressing themselves to suppressor T-cells or to helper T-cells or whatever?

B. EVIDENCE OF PROTECTION

Immunization of Experimental Monkeys with Defined Schizont and Merozoite-Specific Polypeptides of *P. Falciparum*

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Introduction

The development of acquired immunity to malaria in man depends on the frequency and duration of exposure to the parasite, but sterile immunity is probably never achieved.[1] To develop a malaria vaccine against asexual blood stages therefore seems to be a difficult task and, in the past, few people considered vaccination against malaria to be a reasonable objective.

Pioneer experiments by several groups in the later 1970s demonstrated, however, that immunization of experimental monkeys with *P. falciparum* schizonts and merozoites may lead to a better degree of protection than that induced by natural infection.[2–5] Despite the development of *in vitro* culture of *P. falciparum* asexual blood stages, this approach is inapplicable for large-scale vaccination in man.[6]

Recent investigations have focused on the identification of *P. falciparum* antigens exposed at the surface of red blood cells containing schizonts and at the surface of merozoites, the invasive form of the asexual

cycle. Such antigens are obvious candidates for the development of malaria vaccines based on asexual blood stages. One essential step in malaria vaccine development is the purification of defined antigens of the asexual blood stages followed by evaluation of the ability of these antigens to raise a protective immune response. The evaluation of the potential value of purified antigens can be carried out *in vitro*, either indirectly by checking the biological activity of antisera directed against the malaria antigens of interest, or directly using the antigens themselves in a variety of *in vitro* assays, e.g., the assay for inhibition of reinvasion and assays for measurement of cell-mediated responses. In a more direct approach, selected, defined antigens may also be tested for their capacity to elicit a protective immune response *in vivo* by immunizing and challenging with asexual blood stages monkeys susceptible to *P. falciparum* infection. In this short review, we will concentrate on this latter approach.

Criteria for the Selection of "Protective" Antigens

The selection of *P. falciparum* antigens for experimental immunization can be based on the following criteria:

a. synthesis of antigens late in the asexual cycle at the time of schizogony;
b. exposure of the polypeptides at the surface of erythrocytes containing schizonts and/or at the merozoite surface;
c. preferential recognition of these antigens by monkey sera which transfer protective immunity in naive recipients, or by sera of human individuals with a high degree of resistance to malaria;
d. monoclonal antibodies directed against these antigens inhibit the growth of *P. falciparum* asexual blood stages *in vitro*, inhibit binding of merozoites to erythrocytes, inhibit the binding of erthrocytes containing schizonts to endothelial cells;
e. involvement of these antigens in merozoite-erythrocyte interaction.

Several antigens of polypeptides meet some of these criteria (as mentioned in previous presentations on antigens of the asexual blood stages), but only the following have been used for experimental immunization:

1. 185–200 kd polypeptide which fulfills the following criteria: a, b (for a processed product of 83 kd), (c+), (d+), e?[7-10]
2. 140kd polypeptide: a, (b+), c, d, e.[7,8,11]
3. Two other polypeptide fractions containing more than one polypeptide have been used: the 96–100 kd and the 75 kd, c.[12,13]
4. 41 kd polypeptide: a, b, (c+), d, e?[8,10]

Purification of these Polypeptides

These four polypeptides have been purified by affinity chromatography and/or electroelution from SDS PAGE strips identified by autoradiography.[13,14] In the experiments concerning the 41 kd polypeptide, two preparations have been used, one purified by affinity chromatography (native 41 kd) and a second one which was purified by affinity chromatography followed by electroelution from SDS PAGE (SDS 41 kd).

Immunization and Experimental Protocol

Groups of four *Saimiri* monkeys, intact for the 200, 140, and 41 kd and splenectomised for the 96–100 kd and 75 kd fractions, were immunized at 2–3 week intervals with low amounts of purified polypeptides (less than 100 μg/injection) emulsified in Freund's complete adjuvant for the first immunization and Freund's incomplete adjuvant for the others. Intact or splenectomised control animals were injected with NaCl or SDS mixed with adjuvants according to the same schedule. All monkeys were challenged by an intravenous injection of 5×10^7 parasitised monkey erythrocytes (Palo Alto, FUP strain of *P. falciparum*). The course of parasitemia in individual monkeys was scored on Giemsa-stained blood smears. Immunofluorescence assay was carried out on acetone-fixed smears of schizont-rich preparations of various isolates of *P. falciparum*.[13] Immunoprecipitation analysis of (^{35}S)-methionine-labelled *P. falciparum* culture by antisera of immunized monkeys was performed as indicated in reference 14.

Course of Parasitemia

Nine out of ten splenectomised monkeys and six out of eight intact control animals showed the typical acute course of parasitemia which required antimalarial therapy to avoid death (treatment when parasitemia is higher than 20%). In all the immunized monkeys a delay in the appearance of patent parasitemia was observed; all but one of these monkeys had at least a low level of parasitemia, and they all recovered spontaneously without antimalarial therapy. Peak parasitemia observed in the immunized monkeys is reported in Table 1.

Reactivity of the Monkey Sera

The humoral response of the control and immunized sera was tested by indirect immunofluorescence and immunoprecipitation. The antibody titers and specificity of the sera collected before the challenge infection

TABLE 1. Peak parasitemia observed in various groups of immunized monkeys[1]

Immunogens		*Peak parasitemia (%)*		*Day*
	1	13	(intercurrent infection)	15
	2	6		13
***200kd	3	2		16
	4	2		16
	1	4		8
	2	2		15
***140kd	3	2		16
	4	1		11
	1	19		10
	2	8		9
96-140kd	3	6		13
	4	5		7
	5	5		14
	1	25	(treated day 7th)	
	2	6		5
75kd	3	0.5		12
	4	0.5		12
	5	5		13
	1	1[illegible]		12
native	2	0.2		34
	3	0.08		35
	4	0.05		28
***41kd				
	1	13		14
	2	11		16
SDS	3	3		16
	4	2		14

[1] 9 over 10 splenectomized control monkeys had parasitemia higher than 25 % by day 8.
6 over 8 intact control monkeys had parasitemia higher than 20% by day 14, the two last monkeys had peak parasitemia of 11 and 12% before recovery.
[*] Excluded following intercurrent infection unrelated to malaria infection.
[***] Purified from SGE2 isolate (Zaire), challenge with FCP Palo-Alto.

are reported in Table 2. One example of the reactivity of the monkey's sera immunized with native 41 kd is presented in Figure 1A. The same sera were analysed for their capacity to precipitate (^{35}S)-labelled *P. falciparum* polypeptides (Table 3).

Synthetic Peptides as Immunogens

We have recently cloned a fragment of the gene coding for the 185–200 kd polypeptide and determined the DNA and the corresponding amino

TABLE 2. Prechallenge antibody titres measured by indirect immunofluorescence

	Ab Titres		
Immunogens	*Mean*	*Range*	*Specificity*
200 kd	1/256	1/64 — 1/1024	Surface of schizont Schizont diffusely Merozoite surface
140 kd	1/900	1/512 — 1/1024	Schizont diffusely Merozoite diffusely and spots
96–100 kd	1/160	1/20 — 1/320	Schizont
75 kd	1/160	1/20 — 1/320	—
[1]41 kd ↗native	1/320	1/160 — 1/640	Schizont diffusely[1]
[1]41 kd ↘SDS	1/320	1/160 — 1/640	Merozoite: 2 spots / merozoite

[1] By using a solid phase ELISA assay with plastic wells coated with either native or SDS 41 kd, it was found that both groups of monkeys have reciprocal Ab titres in the order of 1/5000 using wells coated with native 41 kd. On wells coated with 41 kd SDS the Ab titres increased up to 1/200,000 for monkeys immunized with the 41 kd SDS (Perrin *et al.* in press 1985, *J. Clin. Invest.*).

TABLE 3. (35S)-Labeled *Plasmodium falciparum* polypeptides immunoprecipitated by prechallenge sera

Immunogens	*MW of polypeptides precipitated (KD)*
200	200, 155[1], 83[1], 31
140	140
96–100	110, 96–100, 90, 72
75	90, 72
41	41 (105)

[1] Detected on western blott using a pool of sera of the four monkeys.

acid sequences of the inserted gene (Table 4).[15] Rabbits immunized with lysate of *E. coli* containing the plasmid coding for and expressing a portion of the 200 kd polypeptide, immunoprecipitated (^{35}S)-labelled *P. falciparum* polypeptides of 200, 155, and 83 kd. The same sera reacted with an 83 kd band of merozoites as shown by Western blot analysis and with the surface of merozoites using indirect immunofluorescence (Fig. 1B).[15] Three synthetic polypeptides were derived from the sequence presented in Table 4, coupled with tetanus toxoid by the glutaraldehyde method (ratio 1/10) and rabbits were immunized with these various

TABLE 4. Amino acid sequence of the peptide expressed by the cDNA clone coding for part of the 200 kd.

1
Ser — Tyr — Gln — Glu — Leu — Val — Lys — Lys — Leu — Glu — Ala — Leu — Glu — Asp — 15 Ala —
Val — Leu — Thr — Gly — 20 Tyr — Ser — Leu — Phe — Gln — Lys — Glu — Lys — Met — Val — 30 Leu —
Asn — Glu — Gly — Thr — Ser — Gly — Thr — Ala — Val — Thr — Thr — Ser — 43 Thr — Pro — 45 Gly —
46
Ser — Gly — Gly — Ser — Val — Thr — Ser — Gly — Gly — Ser — Gly — Gly — Ser — Val — 60 Ala —
Ser — Val — Ala — Ser — Gly — Gly — Ser — Gly — Gly — Ser — Val — Ala — Ser — Gly — 75 Gly —
Ser — Gly — Asn — 79 Ser — Arg — Arg — Thr — Asn — Pro — Ser — Asp — Asn — Ser — Ser — 90 Asp —
Ser — Asp — Als — Lys — Ser —

The amino acid sequence of the 3 synthetic peptides is as follows:

Peptide 1 : 43 aa no 1 — 43
Peptide 2 : 24 aa no 20 — 43
Peptide 3 : 34 aa no 46 — 79

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reagents. All rabbits had the same type of reactivity as the rabbits immunized with the *E. coli* lysate (H. Leban et al., submitted for publication), suggesting that the three peptides are present in the 83 kd polypeptide which is one of the main components exposed at the merozoite surface.[16] The 43 aa peptide coupled to tetanus toxoid was used to immunize four Saimiri monkeys (3 × 1.5 mg/monkey in Freund's complete adjuvant the first time and subsequently with Freund's incomplete adjuvant). Four control monkeys were immunized according to the same schedule with glutaraldehyde-treated tetanus toxoid. All the control monkeys and one immunized monkey required antimalarial treatment when their parasitemia exceeded 20%. All the immunized monkeys presented a delay in the appearance of patent parasitemia and the peak parasitemia for the three monkeys who recovered spontaneously was 19%, 9.2%, and 3.4%, respectively. The prechallenge sera of the immunized monkeys had a low antibody titres as measured by indirect fluorescence (between 1/50 and 1/200) and relatively good antibody titres against the purified peptide produced by the plasmid-containing part of the gene for the 200 kd polypeptide, as measured by solid-phase ELISA test (1/5.000–1/10.000).

Discussion

The results reported here present several interesting features: (a) Immunization with defined polypeptides of asexual blood stages induced a degree of protection similar to that induced by immunization with intact merozoites and/or schizonts. (b) Protective immunity in the experiments reported here is not isolate-specific. It is possible, however, that better results may have been achieved by using polypeptides purified from the isolate used for challenge. (c) The question of relationship between antibody titres and protective immunity is still open. What is the role of cell-mediated mechanisms? (d) Experiments conducted with the 41 kd polypeptide suggest that the conformation of the polypeptides used for immunization is an important factor. This may be of special relevance to the use as immunogens of peptides produced by DNA recombinant technology. (e) Additional experiments mainly *in vitro* or in cheap laboratory animals are necessary to check the potential of synthetic polypeptides.

These experiments pose more questions than they resolve: (a) Did we select the right candidates? (b) Is it possible to obtain sterile immunity by immunization? In the experiments reported here, we use a low amount of antigens diluted in a relatively large volume. In addition, antigenic diversity among isolates of *P. falciparum* may seriously limit the efficiency of immunization. This may be of importance if the variant epitopes are those exposed at the surface of the parasites. (c) Can we

improve the efficacy of immunization by using a mixture of several putative "protective" antigens as immunogen? (d) Are Saimiri and Aotus monkeys good animal models for the testing of malaria vaccines? (e) Freund's adjuvant is not acceptable for human use. What is the best alternative? Other adjuvants? Insertion and expression of malaria genes in virus vectors?

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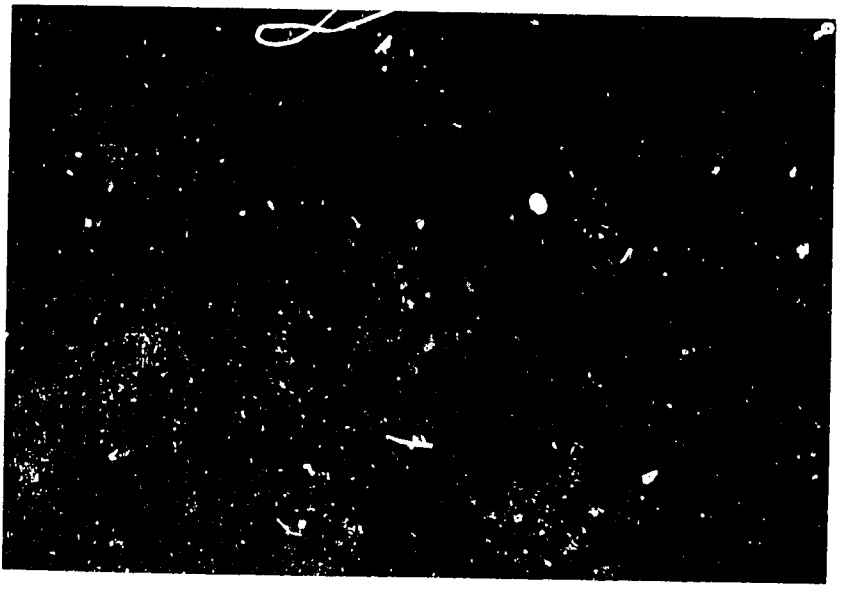

FIGURE 1A. Specificity of Monkey Antibodies Directed Against the Native 41 kd Polypeptide. Acetone fixed *P. falciparum* SGE2 schizonts and merozoites were incubated with a 1/100 dilution of the prechallange serum of a monkey immunized with the native 41 kd polypeptide followed by a 1/50 dilution of fluoresceinated anti-monkey antibodies. These antibodies react with two spots on each merozoite.

FIGURE 10. Specificity of Rabbit Antibodies Directed Against Lysate of *E. coli* Containing a Plasmid Expressing Part of the 200 kd Polypeptide. Acetone fixed *P. falciparum* SGE2 schizonts and merozoites were incubated with 1/500 dilution of the rabbit serum followed by a 1/50 dilution of fluorescinated affinity purified anti-rabbit antibodies. These antibodies are evenly distributed over the entire surface of merozoites (centre of the figure) and also react with the membrane and internal structures of schizonts shown at various developmental stages. The reactivity of sera of monkeys immunized with the 200 kd polypeptide and with the 43 aa coupled to tetanus toxoid present the same pattern. The magnification is 2,000x.

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Discussion

KENNEDY: Two quick questions. First in your peptides, acquisitions 44 and 45 had proline and glycine, two very difficult amino acids to synthesize synthetically when put together in that configuration. It was obvious from the peptides that were made that you avoided that area. Yet, those two peptides may impart a tremendous amount of conformation on the overall structure of your peptide. In other words, it's a difficult amino acid sequence to synthesize, but there is a lot of conformation in that area.

PERRIN: I agree with you. We may have selected a better area to do the experiment.

KENNEDY: Second, when you coupled your peptides to tetanus toxoid with the 1–43, you had aspartic acids, glutamic acids, and lysines there. If your method of coupling involved going through amino terminus or carboxy terminus residues, you may have blocked the epsilon amino group or the carboxyl groups on those peptides.

PERRIN: We did not choose a very elegant method. We chose the butyl aldehyde method, with coupling at 100, in fact.

KENNEDY: So, it'll potentially block all epsilon amino groups?

PERRIN: I can just tell you that in rabbits the titers were fantastic. More than 1/2000 by immunofluorescence and with a precipitate of protein 200 kd and 83 kd. In monkeys, the antibody response was very poor when we used immunofluorescence tests, around 1/200 dilution. The earlier tests ran 1/5000, which is not so high, in fact. I don't know why we had so much difference between rabbit and monkey, besides this technical problem.

COPPEL: Luc, the immunization with the native 41 kd, gave really the most fantastic results I've ever seen in a monkey experiment. How far along are you with bringing that molecule to the same sort of stage you've taken the 200 kd? How well characterized do you have it and have you cloned it?

PERRIN: We tried very hard to clone it but we've had not succeeded up until now.

COPPEL: Do you know much more about where it's made, other than it being a rhoptry protein?

PERRIN: It's in the rhoptry. I sent a molecule out to Dr. Aikawa and he sent me back a picture showing that it's present in the rhoptries without any discussion.

MITCHELL: It came up this morning of a lack of correlation between antibody titers and protection in certain situations. Therefore, some mechanism other than antibody was invoked as being related to protection. Luc gave us a very interesting example of a 41K molecular weight protein, which as a denatured protein, was much less effective than the native molecule. He emphasized the comparable titers in those two groups of monkeys. My question to Luc is, how was that antibody measured? Of course, everything would depend on the means of measuring the antibody titers. If that antigen is denatured by drying it

onto a plate, then, of course, one may not expect to detect that additional complement of antibodies to the native molecule, which are being induced by the native antigen vaccine. The specific question, Luc is, how are those antibody titers measured?

PERRIN: The titer was measured by indirect immunofluorescence and we have done ELISA as well. In fact, the titer in monkeys immunized with SDS antigen against SDS antigen were at least one log higher than the native antigen. Against native antigen, a comparable titer was shown.

The Induction of Protective Immune Responses to *Plasmodium Falciparum* in *Aotus* Monkeys

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In 1977, it was shown independently in two laboratories that immunization of *Aotus lemurinus griseimembra* (karyotype II and III) monkeys with schizont- or merozoite-enriched preparations of the human malaria parasite *P. falciparum* in Freund's complete adjuvant conferred protection against lethal challenge.[1,2] Recently, Mab-defined,[3,4] preparative SDS-PAGE-isolated,[5,6] or ion-exchange chromatography enriched[7] polypeptides have been shown to protect *Saimiri* monkeys to varying degrees. To date, no defined polypeptides have been shown to be protective in *Aotus* monkeys.

Work from the Wellcome group in *P. yoelii* malaria indicated as early as 1981 that a Mab-isolated 230K merozoite surface coat precursor and its processing fragments as well as a 230K rhoptry protein protected mice upon challenge.[8] An analogous 195K *P. falciparum* merozoite surface coat precursor and its processing fragments isolated by monoclonal antibody[4] or the 200K SDS-PAGE isolated precursor[5] has been shown to protect *Saimiri* monkeys. In addition, a Mab-isolated 41K rhoptry polypeptide[4] has also recently been shown to confer protection in *Saimiri* monkeys.

We have produced Mab's to the same processed, high molecular weight merozoite surface coat precursor glycoprotein (Mab 5.2) and to a non-covalently associated high molecular weight rhoptry protein complex (Mab 219.5). We have pursued these polypeptides based on the initial reports of the Wellcome group with *P. yoelii*.[8]

Antibody 5.2 isolated 185K, 152K, 121K, 83K, and other smaller polypeptides associated with the major merozoite surface coat protein (cf. Kan et al., these proceedings). The same polypeptides were immunoprecipitated by antibody 89.1 from the Wellcome group (not shown). The 83K polypeptide is on the merozoite surface along with 42K and

19K processing fragments (cf. Freeman and Holder, these proceedings). Antibody 219.5 immunoprecipitated the 143K, 132K, and 102K polypeptides (cf. Kan et al., these proceedings), as did antibody 61.3 from the Wellcome group (not shown). The latter antibody has been shown to immunoprecipitate 155K, 140K, and 110K non-covalently associated polypeptides located in the merozoite rhoptries by immune electronmicroscopy.[9] The rhoptry polypeptides do not appear to be related by processing but are the complexed products of unrelated genes. The differences in reported relative molecular weight (M_r) may be due to real differences between the Uganda–Palo Alto and the Wellcome Lagos isolates. The relationships of the Mab-identified polypeptides to the total polypeptides identified by hyperimmune IgG obtained from previously vaccinated and protected *Aotus* were reported earlier (cf. Kan et al., these proceedings). The antibody to the merozoite surface coat pre-absorbed prominent 185K, 152K, and 83K polypeptides, while the antibody to the merozoite rhoptries pre-absorbed prominent 143K and 132K polypeptides identified by hyperimmune *Aotus* IgG. In total, five of the seven prominent [^{3}H]-isoleucine labeled polypeptides were pre-absorbed. The merozoite surface coat precursor and rhoptry polypeptides are among the major polypeptides of the parasite. This was supported by direct isolation experiments. Insolubilized hyperimmune *Aotus* IgG isolated 920 μg polypeptides from 125 mg crude parasite antigen cleaned of contaminating hemoglobin. Antibody 5.2 isolated 312 μg and antibody 219.5 isolated 113 μg polypeptides, respectively, from a separate 125 mg aliquot of crude antigen. These figures represent 33% and 13%, respectively, of the total polypeptides identified by hyperimmune *Aotus* IgG. The merozoite surface coat and rhoptry polypeptides represented 46% or nearly half the polypeptides immunoprecipitated by immune *Aotus* IgG.

Immunogenicity of the Mab-isolated polypeptides were determined in *Aotus lemurinus azure* (karyotype VI) monkeys. *Aotus* 381 received 80 μg merozoite surface coat polypeptides combined with 30 μg merozoite rhoptry polypeptides emulsified in Freund's complete adjuvant (FCA) on days 0, 21, and 42. *Aotus* 391 and *Aotus* 390, respectively, received 240 μg immune *Aotus* IgG affinity-purified polypeptides and 1400 μg unpurified FUP antigen similarly in FCA. To minimize the adverse granuloma formation, the amount of mycobacterium in secondary and tertiary immunizations was successively halved. By solid phase RIA, the reciprocal 14d tertiary antibody titers were >31250, >31250, and <156250, respectively, for *A*381, *A*391, and *A*390 (Table 1). By SDS-PAGE, the tertiary sera A381 immunoprecipitated polypeptides having M_r 185K, 152K, 143K, 132K, 121K, 102K, and 83K (Fig. 1). By IFA the punctate IFA pattern expected for the rhoptry polypeptides was obscured by heavy staining of the merozoite surface coat.

Since the titered antibody responses to the respective polypeptides

had been evoked, the monkeys, along with an unimmunized control, *Aotus* 385, were challenged with 1×10^6 FUP freshly passaged K^+, and the course of infection was monitored. As expected from previous studies, the unimmunized control *Aotus* 385 developed a 2–4% peak parasitemia, which was followed by spontaneous cure. This transient infection has been consistently observed with other *Aotus* of this karyotype infected with FUP K^+ over the years in previous karyotype adaptation experiments. Most strikingly, however, all immunized monkeys failed to reveal even a single parasite, scanning thick blood films (Table 1) over a 60-day period. (A fourth *Aotus* given the polypeptides with synthetic adjuvant MDP-Lys [L18] was not protected.)

This pilot experiment further establishes the candidacy of merozoite surface and rhoptry polypeptides for a human malaria vaccine. It appears to be the first successful vaccination experiment with defined polypeptides in *Aotus* monkeys. It need not be emphasized that the positive result was obtained with a single *Aotus*, and that a larger experiment in the karyotype II and III monkeys must be performed to confirm these findings. Nevertheless, it appeared that a sterile immunity had been achieved. It is doubtful that blood from any of the three protected *Aotus* could have initiated an infection had serial passage to naive *Aotus* been performed. Absolutely no parasites could be detected in thick blood films, and a challenge inoculum of 1×10^6 parasites is required to produce an infection in this karotype.

Whether the complete protection was achieved as a result of having a polyvalent antigen preparation (merozoite surface coat plus rhoptries) is conjecture. Antibody against the rhoptries might retard merozoite reinvasion and phagocytosis of the merozoites might then be accelerated by opsonizing antibody to the surface coat. In addition, the use of FCA

TABLE 1. Immune response and parasitemia in karyotype VI *Aotus* immunized with *Plasmodium falciparum* merozoite surface and rhoptry polypeptides and challenged with FUP K^+

			Ab Titers[a]		
Aotus	*Antigens*	*Adjuvant*	*IIFA*	*RIA*	*Parasites*[b]
385	None	None	16	250	220[c]
381	surface + rhoptry	FCA	4096	31250	0[d]
391	total IgG isolated	FCA	8192	31250	0[d]
390	whole cell	FCA	8192	>31250	0[d]

[a] Reciprocal dilution of the highest 14 d tertiary serum giving a positive result.
[b] Parasites per 10,000 erythrocytes.
[c] Monkey A385 became patent on day 4, developed a peak parasitemia of 2.2% on day 13, and cleared the infection on day 17. This transient infection has been consistently observed in numerous other karyotype VI adaptation experiments over the past several years.
[d] No parasites were detected up to 60 days after challenge.

three times would have resulted in strong immune responses, even to weakly immunogenic epitopes. It is known that semi-immunity in nature requires repeated infections in holoendemic areas. The coexistence of parasites in the face of circulating antibody (premunition) may indicate that antibody to weakly immunogenic or antigenically diverse epitopes is required. These might be induced only after repeated infections in the wild or with strong adjuvants under experimental conditions. These and other questions must be resolved in future vaccination experiments.

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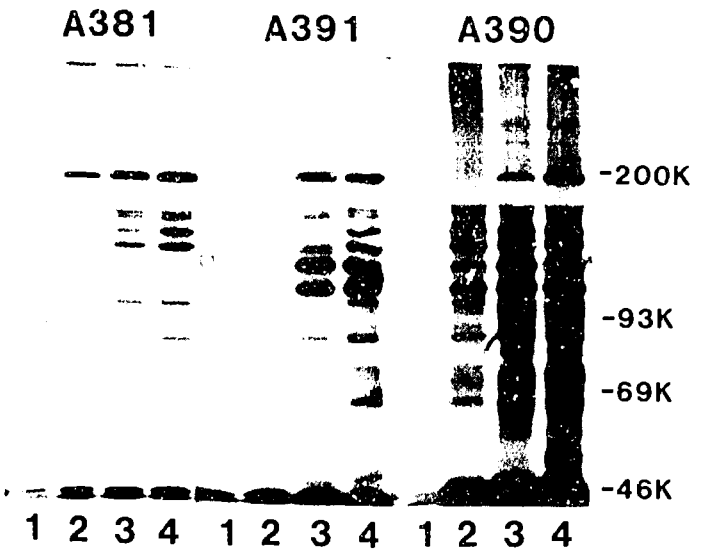

FIGURE 1. SDS-PAGE Analysis of [^{35}S]-methionine-labeled *P. falciparum* Polypeptides Immunoprecipitated by Tertiary Sera from *Aotus* Vaccinated with *P. falciparum* Polypeptides. The polypeptides were complexed with 7.5 μl pre-immune (1), primary (2), secondary (3), or tertiary (4) sera from *Aotus* immunized with a combination of surface and rhoptry polypeptides in Freund's complete adjuvant (A381), the set of polypeptides affinity purified by immune parasite material in FCA (A391), or unpurified schizont-enriched parasite material in FCA (390). Complexes were collected with formalin treated *Staphylococcus* A and polypeptides electrophoresed in 6.25% gel. The molecular weight standards are indicated on the right.

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Non-Human Primate Models for Testing Malarial Vaccines

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With the adaptation of the human malarias to development in New World monkeys almost 20 years ago,[1-4] it was recognized that a new system was available for the testing of anti-malarial drugs and possibly, for the development and testing of anti-malarial vaccines. There has been considerable use of monkeys in the development and testing of drugs[5-7] and a number of limited tests of vaccines in New World monkeys.[8-19] With the rapid identification of the proteins which may be reponsible for protection, these animal model systems may well play an important role in the development of vaccines for the control of malaria.

Two basic approaches to the use of monkeys as models for the development of vaccines exist. The first is the use of plasmodia which are naturally infective to monkeys. These are biologically similar to the plasmodia that infect humans and provide a natural host-parasite relationship. The second approach is to use human malaria parasites growing in certain species of susceptible monkeys. Here, immunogens specific to the human malarias can be examined. The investigator must realize, however, that monkeys are not natural hosts of human malaria parasites and biologically, infections with human plasmodia in these animals are very dissimilar from that which occurs in humans. However, the use of such an animal model can provide important information regarding the protective effect of a vaccine and, depending upon the adjuvant used, the immunogenicity/toxicity of the antigen involved.

It is also important to consider the role of non-human primates in the development of vaccines. One must recognize that not only is the availability of animals somewhat limited, but that laboratories should not use primates in studies for which other small animals might suffice. The initial studies with rodent malarias (*P. berghei, P. chabaudi,* etc.) and avian malarias (*P. gallinaceum, P. lophurae,* etc.) provide the basic principles for studies in non-human primates and eventually in humans.

Only after the basic principles of a study have been investigated in rodents, rabbits, or birds should tests be considered in primates. Some types of vaccine development will require the extensive use of primates, whereas others should require only limited studies before human trials. The development of sporozoite vaccines will probably require only limited testing in non-human primates to establish efficacy. However, studies on compounding variables (previous malarial exposure, concurrent infection, multiple infections, boosting effect of sporozoite inoculations, etc.) could well be done in a selected primate malaria, non-human primate model system.

The selection of effective blood-stage antigens may be extremely important in primates. The most promising peptides must be selected early before any extensive phase I trials in humans. With a myriad of antigens or combinations to be tested, extensive trials in non-human are anticipated. It is our opinion that these antigens should not be examined in a haphazard manner and that the available supply of animals be used wisely. Again, these hosts will be used extensively to determine the effect of the compounding variables (see above) on the vaccines.

It is anticipated that the use of non-human primates for the testing of transmission-blocking vaccines will be very limited. Initial studies will select those antigens which can induce the production of antibodies in lower animals (i.e., chickens or rabbits). These can then be tested for efficacy by combination with culture-produced gametocytes and then membrane feeding to mosquitoes. Once an effective immunogen has been selected, movement to phase I human trials would appear necessary for safety and vaccine potency; the potential effectiveness of the immunization can be determined using the same membrane-feeding techniques and culture-produced gametocytes.

In this report, we present our proposals for the testing of both sporozoite and blood-stage vaccines in the different model systems as they are presently available.

Sporozoite Vaccines

Immunization against the sporozoite stages has the potential for preventing normally infective sporozoites from penetrating the liver.[20] Therefore, with an effective anti-sporozoite vaccine, complete protection of the host from any malarial infection either in the form of exoerythrocytic development in the liver or from subsequent infection of the red blood cells (RBCs) would be expected. Once the barrier has been breeched, however, protection against blood-stage development would not be expected. Thus, trials which test the efficacy of these vaccines, should be straight-forward. An animal which has been immunized with

an effective anti-sporozoite vaccine will fail to develop any infection. Those animals not protected will develop the infection.

MONKEY MALARIAS

Two species that cause monkey malaria, *Plasmodium knowlesi* and *P. cynomolgi*, are the most likely candidates for the testing of sporozoite vaccines. Rhesus monkeys, *Macaca mulatta*, inoculated with adequate numbers of sporozoites can be expected to develop detectable parasitemias within a predictable time frame.[21] With *P. knowlesi*, the prepatent period is between 5 and 9 days; with *P. cynomolgi*, it is between 7 and 15 days. Standardization in these studies has been based on the number of bites from a fixed number of heavily infected mosquitoes. In our studies, we have used the bites of 4 or 5 mosquitoes; for the testing of anti-malarial drugs in humans, the standard has been the bites of 10 heavily infected mosquitoes.[22-23] An alternative method is the intravenous injection of known numbers of sporozoites from dissected salivary glands. From 10,000 to 20,000 sporozoites are usually inoculated.

The experimental animals *(M. mulatta)* needed for such trials with the monkey malarias are readily available. However, the housing requirements for these relatively large animals coupled with the current initial cost of from $600 to $1,000 per animal make these studies very expensive.

One major requirement in sporozoite vaccine trials is a ready supply of sporozoites to challenge the animals. Fortunately, both *P. knowlesi* and *P. cynomolgi* readily infect certain species of laboratory-reared mosquitoes. In addition, the salivary glands normally contain massive numbers of sporozoites; it is therefore not difficult to obtain millions of sporozoites for challenge of large numbers of animals.

Since the peptide sequence of the *P. knowlesi* circumsporozoite protein (CSP) has been identified,[24] this parasite would appear to be a prime candidate for vaccine studies. *Plasmodium cynomolgi* would also appear to be of major importance since it causes a relapsing malaria (as do the human malaria parasites *P. vivax* and *P. ovale*) and could be used in studies on the effect of immunization on the resting stages and the effect on relapse potential.

The proposed testing procedure for the sporozoite vaccines of the monkey malarias would be: 1) to immunize intact *M. mulatta* monkeys twice using an effective adjuvant; 2) to challenge the animals either by mosquito bite or intravenous inoculation of sporozoites from dissected salivary glands at various periods after immunization; and 3) to determine the antibody responses by various techniques to relate vaccine efficacy with one or more serologic results. It is very important to examine and select the most effective serologic technique(s) from among those available.

Human Malarias

The testing of sporozoite vaccines against the human malarias using monkeys is more difficult. A relatively few animals have been successfully infected via sporozoite inoculation. In fact, only the Colombian *Aotus* (karyotypes II, III, and IV) have been tested in sufficient numbers to establish a statistically valid base for the conduct of these studies.[25-32] However, only the Santa Lucia strain of *P. falciparum* has been transmitted with sufficient frequency to establish transmission rates. In this model system, 25% of intact and 85% of splenectomized monkeys inoculated with relatively large numbers of sporozoites have subsequently developed patent parasitemias. Transmission rates in other parasite-monkey model systems have either been considerably lower or too few experiments nave been attempted to draw sound conclusions.

With the Colombian *Aotus*, the results of transmission studies with *P. vivax* are similar to those of *P. falciparum*. Preliminary studies with a small number of Bolivian *Saimiri* monkeys indicate that sporozoite transmissions to splenectomized animals may be relatively high, and this host may have considerable potential for testing sporozoite vaccines against *P. vivax*.[32] Although studies are presently being made with other types of South American monkeys to determine their potential for sporozoite vaccine trials, the results of these studies to date have been discouraging.

Consideration of the differences between the New World monkey model systems and human infections will influence the design and conduct of the trials. In addition to a relatively low transmission rate, the prepatent periods are greatly extended over those seen in humans or with studies of the monkey malarias. Thus, vaccine trials may be lengthy, extending over several months. In addition, the infection of mosquitoes with the Santa Lucia strain of *P. falciparum* from culture has been very low; it has also been very low from *Aotus* monkeys other than those from Colombia. Thus, additional already scarce Columbian animals are also required to serve as the source of infection of mosquitoes to provide the sporozoites necessary for challenge. Another compounding difficulty is that the number of sporozoites in the salivary glands of mosquitoes infected with *P. falciparum* and *P. vivax* are usually much lower than are seen with the monkey malarias; thus, many more mosquitoes must be fed to obtain adequate numbers of sporozoites for the challenge studies.

The proposed testing procedure for sporozoite vaccines to the human malarias calls for the immunization of animals twice using adjuvants. For the challenge, it is anticipated that a minimum of 20,000 sporozoites obtained at dissection will be injected into each animal. Since intact animals have such a low rate of susceptibility to infection, ani-

mals will be splenectomized, but only after the sporozoites have been injected and the antibodies have been allowed to exert their effect. Splenectomy will be performed 6 or 7 days after inoculation. Thus, in a vaccine failure, merozoites released from schizonts maturing in the liver will be in a splenectomized host and more likely to produce a detectable blood-stage infection.

In our view, the development or absence of detectable parasitemia is the major criterion when assessing the protection afforded by a sporozoite vaccine. Other methods for the assessment of vaccine effectiveness may be delayed prepatent periods and a decrease in the number of exoerythrocytic stages. The latter may be measured by examining stained tissue sections or using DNA probes or homogenized, extracted liver tissue. Again, antibody tests would be required to correlate, if at all possible, protection with a measurable immune response.

Since not all animals injected with sporozoites, even after splenectomy, would be expected to develop a patent parasitemia, the number of animals in each test group must be sufficiently large to detect differences between a vaccinated and control group at an acceptable level of statistical signficance. Using the best host-parasite model systems available (i.e., the Santa Lucia strain of *P. falciparum* and Colombian *Aotus* monkeys), a minimum of 10 animals is required per test group to detect a true difference of 0.65 between the proportion of non-immunized ($p=0.85$) and immunized ($p=0.20$) animals developing parasitemia at a level of significance of 0.05.

Blood-Stage Vaccines

MONKEY MALARIAS

Immunization against the blood stages of the monkey malarias has been attempted on numerous occasions for more than 40 years.[33-54] An advantage for investigators is that these parasites produce infections that are biologically similar to human malarial infections.[21] Although 10 species of monkey malaria exist that are useful as models for vaccine studies, (*P. cynomolgi, P. gonderi, P. fieldi, P. simiovale, P. coatneyi, P. fragile, P. knowlesi, P. inui, P. brasilianum,* and *P. simium*), most of the effort has been with *P. knowlesi, P. fragile,* and *P. cynomolgi* in rhesus monkeys. *Plasmodium knowlesi* is a quotidian parasite which almost always produces a fulminating infection; death occurs in approximately 97% of the infections. With this parasite-monkey model system, it is very easy to establish a criterion for vaccine effectiveness—the survival of the host. *Plasmodium fragile* is a malignant tertian parasite with many biological similarities to *P. falciparum*. The infections are fulminating and death occurs in about 30% of the animals. If an animal survives, the infection

is characterized by long-term persistence with frequent recrudescences. *Plasmodium cynomolgi* is a benign tertian parasite with maximim parasitemias of between 5 and 10% which seldom produces infections that are fatal.

In these model systems, susceptible animals (rhesus monkeys) are readily available, and the course of the parasitemia is predictable within certain limits of time and parasite count. Each of these parasites has previously been used in previous vaccine trials with crude antigens using a number of different adjuvants. Characteristically, all animals developed parasitemia which was modified to a varying degree by the type of immunization and the challenge. Adjuvants have been required for successful immunization with these crude antigen preparations. The adjuvant providing the best immunologic response has been Freund's complete adjuvant.

The proposed testing procedure for blood-stage antigens to the monkey malarias is to immunize at least twice with a vaccine containing Freund's complete adjuvant. Since challenge with large numbers of parasites often results in an abnormally rapid rise in parasitemia (especially with *P. knowlesi*), challenge is usually done with less than 10,000 parasites. In addition, since death is an unacceptable outcome, animals which have parasite counts above certain limits must be treated. The criteria for vaccine effectiveness are: 1) extended prepatent period; 2) reduced maximum parasitemia; 3) shortened length of patent parasitemia; 4) presence or absence of recrudescences; 5) reduced infectivity to mosquitoes; and 6) protection against rechallenge with homologous or heterologous strains of the parasite. Serologic and *in vitro* tests are correlated with protection if at all possible. A minimum of 6 test animals per group is considered adequate for such trials. Once an antigen has been shown to be effective, alternative, less pathogenic adjuvants, can be assessed for comparative efficacy.

Human Malarias

The testing of blood-stage vaccines against the human malarias in monkeys is considered to be a major part of the selection process for promising human malaria vaccines. Of the thousands of infections with the human malarias which have been studied in South American monkeys, most have been done with the Colombian *Aotus*. These animals, however, are now very difficult to obtain. Since they are the only monkeys which are suitable for testing sporozoite vaccines and the only ones which can be used for mosquito infection with some strains of *P. falciparum*, the demand far exceeds the supply. Recent studies have indicated, however, that certain strains of *P. falciparum* can be adapted to development in other monkeys, many of which are available, at least in limited quantities. Among these are *Aotus* monkeys from Peru and

Bolivia of karyotypes I, V, and VI, and *Saimiri* monkeys from Guyana, Peru, and Bolivia.[19,55] Several immunization trials have been reported using *Saimiri* monkeys and the Uganda-Palo Alto and Indochina I strains of *P. falciparum*. We have taken the approach of adapting at least one strain of *P. falciparum* for development in all types of animals to obtain some uniformity of parasite challenge. In our studies to date, only the Indochina I strain has been adapted for growth in all of the monkeys examined.[56] Other strains of *P. falciparum* will no doubt be adapted for growth once adequate numbers of linear passages in other monkey types have been made. Some strains of *P. falciparum* have failed to adapt to some of the animal systems. All of the parasites appear to lose certain characteristics upon passage; however, since they appear to retain their high level of virulence once adapted to a particular type of monkey, they can be used successfully for challenge.

At present, based on studies in rhesus monkeys and limited studies in *Aotus* monkeys, adjuvants are required to produce a protective immune response in monkeys against the blood stages. It must be recognized, however, that these previously used vaccines were very crude mixtures, and only limited studies have been made with purified, defined preparations. All animals that are immunized with an effective vaccine can be expected to develop a detectable parastemia since the effect will not be exerted against either the sporozoite of the exoerythrocytic stage. Thus, only after the parasites have been released from the liver and into the bloodstream will the defense mechanisms be activated. The mean maximum parasitemia should be less than that of the control animals and parasitemias should be of much shorter duration than those in the controls. This depends, of course, on the rapidity with which the host reacts to the infection and the number of parasites inoculated. A massive parasite inoculum could result in a very high parasitemia or even death if the host's defense mechanisms are slow to react.

The proposed testing procedure for the blood-stage vaccines to the human malarias would use only those strains of *P. falciparum* which have been adapted to produce parasitemias of 10% of higher within a period of 14 days following inoculation of approximately 500,000 parasites in homologous *Aotus* or *Saimiri* cells. This requirement should be met for any strain adapted for development in a particular model system. In our case, we anticipate that the Indochina I strain will be adapted to meet these criteria in all the animal systems in which the vaccines will be tested. Other strains from other geographic areas are being studied and can be used for heterologous challenge studies in certain animal systems. Since these animals are too valuable to purposefully be allowed to die, an infection which meets or exceeds the 10% level is treated.

Since *P. vivax* rarely produces high-level parasitemias, a maximum

parasitemia of 1% or higher within the same time frame would be considered sufficient. Since animals infected with *P. vivax* would not be expected to die, all of the control and immunized animals would remain in the study.

In the initial trials, animals will be immunized twice, using an appropriate adjuvant (preferably Freund's complete adjuvant). Challenge will be with adapted blood-stage parasites growing in homologous RBCs. To correlate protection with serologic results, a number of different parameters will be used as indicated in the testing procedure for the monkey malarias. To determine if protection is strain specific, investigators can perform secondary studies and rechallenges with heterologous strains of the parasites. At present, it appears that for statistical significance, 6 animals would be required per test group. This number might be reduced as the results of additional immunization trials using standard inocula with adapted parasites are accumulated.

In summary, presented here are various model systems for the testing of sporozoite and blood-stage vaccines against monkey malarias and human malarias. It is believed that such trials will help to determine the efficacy of different vaccine formulations and regimens which may also be effective in humans. The accumulation of these results using standard protocols and selected adapted strains of parasites should help to provide a logical basis for selecting of formulations and regimens for an effective vaccine against malaria.

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Discussion

BRUCE-CHWATT: What is the reason for using Freund's complete adjuvant first and then Freund's incomplete adjuvant?

COLLINS: I'm not an immunologist, but that was the procedure we thought would give us the least adverse response in our monkeys—Freund's complete adjuvant followed by Freund's incomplete, given intradermally. The Freund's incomplete was used with the sporozoite vaccine, because we could get an adequate humoral response with Freund's incomplete adjuvant. But these adjuvants are transitory. I am sure once we find effective immunogens, then we'll move on to other adjuvants.

SIDDIQUI: I might just make a comment. If you recall experiments by Cohen and our experiments, protection has been very unsatisfactory when you have only one injection of Freund's complete adjuvant followed by incomplete adjuvant. I think, if you want to evaluate the candidate antigen, that could serve as a vaccine, and in the absence of a synthetic vaccine adjuvant which could be applicable or could be used for humans, I think you'll want to give that candidate antigen the best chance that you can. I think, by using one injection of Freund's complete adjuvant followed by incomplete adjuvant, you are diminishing the chance for the candidate antigen to serve as a good candidate antigen.

COLLINS: I probably agree with you there. We have a problem. Our monkey-use committees are more interested in the welfare of the animals right now than they are in developing an effective vaccine for malaria. The use of just one Freund's complete adjuvant immunization is probably causing quite a bit on their part to say whether they will let us do it. I don't think they're going to allow us to use Freund's complete adjuvant more than once.

SIDDIQUI: But, just to pursue that, if you reduce the mycobacterium titer to half or less, I think you are giving less insult and the response at the local level is less dramatic.

COLLINS: You've worked a lot with the *Aotus* monkeys. Have you had adverse reactions? We have had two, which haven't done our public relations much good. Have you had adverse reactions, do you think, to the adjuvant?

SIDDIQUI: Well, at the local level, yes. But that's why we are much more interested in looking for synthetic adjuvants.

COLLINS: We've had two that weren't at the local level. And Dr. Pappaioanou, if she could comment on that. We're kind of leery about Freund's adjuvant, yet we want to use the best adjuvant.

PAPPAIOANOU: We've put Freund's complete adjuvant into several monkeys. One of six had severe adverse reactions, an ulcerative, necrolytic lesion over the entire abdomen. It was chronic; it progressed over several months. It came and went and came and went. We had sterile abcesses at one of the sites of injection. We finally euthanised the animal. The liver and kidneys were completely shot. The sinusoids of the liver were completely filled with mitotic cells. There was a question in that particular animal in that prior to inoculation with the adjuvant, the animal had a gingival ulcer which came and went also, and there was the thought that this animal had noma. So, it may have been compromised immunologically before we injected it. That's a question that remains. More recently, we had a monkey that we put Freund's incomplete adjuvant into, and twelve weeks after injection, again, there was epidermic hemorrhage, and it looked like it was going to do the same thing that happened with the first monkey, except that this was over the entire abdomen, up into the thorax area. However, the monkey recovered. The lesions regressed and we did a skin biopsy and, essentially, the second monkey had a non-inflammatory response. There were many erythrocytes and edema in the skin layer, but, essentially, the animal recovered. And what was interesting, I think, is that in the first monkey's lesions from the skin, it was clear that there were lipid granules all throughout the histopathology. And it seemed like the monkey was fighting a losing battle in trying to get rid of the lipid component. The second monkey, which recovered, histologically anyway, appeared to have cells that were competent in taking in this lipid and then removing it from its system. So, I think that the question that came to our mind—even very, very recently, too—is that with the severe pathology that we saw with this first monkey, it would raise real question as to what might be learned from a study where, as far as the pathology goes, what the effect of a vaccine would be if, in fact, an adjuvant is used that

can cause that severe a reaction in that many organs. But these are only two animals. And we're interested in learning whatever experiences other people have had with these adjuvants.

SIDDIQUI: I think we are going to have tomorrow a full session on adjuvants by two leading immunologists who are with us. We know that there is not much use of Freund's complete adjuvants, but the only problem is how to test the candidate antigen in animal model system and to give the candidate adjuvants the best possible chance.

Discussant: Evidence of Protection

L. Pereira da Silva

To introduce a discussion on evidence of protection, the first logical thing to do is to define protection. In fact, this term has been employed with different meanings. At an epidemiological level, it has been used to define an immune status of some human groups; in endemic areas, i.e., adults who develop immune resistance mechanisms upon repeated clinical and parasitological episodes of malaria in the past.

However, these protected individuals often present parasites in the blood, especially gametocytes, and represent the source of infection for maintenance of malaria transmission in the area.

Would this be the aim of a vaccine against falciparum malaria? It would be certainly useful to induce in the risk population, children, migrants from non-infected areas, visitors, soldiers, etc., the same "immune status" of protected adults from endemic areas. Such an aim can be foreseen by the results of "partial protection" already obtained by some research groups working in asexual blood forms vaccines and which have been reported and reviewed by the different speakers in our section. However, any student of a public health school would be aware of the limitation of inconsistencies of such a solution: it would probably have no impact on the transmission, it would maintain the disease in an incubated "dormant" situation, and it would expose the vaccinated population in the area (after the selection of variant strains) to epidemic outbreaks of vaccine-resistant malaria.

Consequently, we are obliged to be better than Mother Nature. We need to develop vaccines that will induce not only reduction (or elimination) of morbidity and mortality but also induce reduction of transmission. Let us call it "the ideal vaccine."

The corollary of this assessment is that we must introduce vigorous criteria of evaluation of protection. This raises a series of questions and I have selected five which have been discussed by the different speakers in the morning and afternoon sections:

1. Could we pretend to develop an ideal vaccine against only one stage of the parasite or would we be obliged to use polyvalent vaccines?
2. Have we already finished dressing the list of good candidates for protective antigens?
3. Do we dispose of the methodological tools to evaluate rigorously the protection? Which are they?
4. Do we already have a sufficient understanding of basic mechanisms of immune protection, parasite escape mechanisms, genetic polymorphism, and variability which allow us to predict eventual complications in the practical use of vaccines?
5. Have we already accumulated enough information to forsee into the near future, clinical trials in the field with one or other antigen?

Certainly, someone in the audience will argue that all the very efficient viral and bacterial vaccines (starting with the anti-rabies vaccine by Pasteur exactly 100 years ago) have been developed empiricially without answering these questions.

I will respond to this argument that we must remember that parasitarial vaccines will be obligatory "2nd generation vaccines" produced by genetic engineering or chemical synthesis and that for developing 2nd generation vaccines, virologists and bacteriologists are faced nowadays with the same kind of problems.

The outstanding scientists selected by Dr. Siddiqui and the Organizing Committee have brought to you previous information from their own labs and others concerning the current status of developmental vaccines, and it is not my purpose to repeat or to summarize their speeches. I will only try to discuss some points which have been raised.

It was very clear from what we have heard, that for the different vaccines—sporozoite, gametocytes, and asexual blood forms—the amount of information accumulated is too variable to permit us to respond to these five questions.

Let us start with the more complex, or the more confused, if you will.

Asexual blood form vaccines. If we had to answer the questions—as it is a tradition in this country, by checking the box yes or no—we would have five no's.

Being more optimistic, we moved from a phase in the past, in which every laboratory had its own candidate as protective antigens, to a period in which data starts to converge. A series of polypeptide antigens, as reviewed by the speakers, have been identified by more than one laboratory and in several cases significant immunoprotection was obtained by injecting purified polypeptides in four different laboratories. The extremely positive aspect of these results is the persistence of the immunogenic properties of polypeptides purified under denaturing conditions, detergents, and reducing agents. This is a promising situation if we remember that vaccinating molecules produced by DNA

recombinant technology or chemical synthesis will probably not have the native configuration. However, two observations reported by one of the speakers today must be recalled: a native polypeptide (purified by immunoabsorption) showed to be more effective to induce protection than the denatured molecule. Synthetic polypeptides corresponding to fragments of a polypeptide molecule were much less efficient in inducing protection than was the entire molecule. Research directed to identify the relevant epitopes of different immunogenic molecules are in progress in many laboratories. (And we had three examples in the reports we heard.)

This laborious approach is a prerequisite for the development of an "ideal" fusion polypeptide harboring antigenic determinants from different proteins and prepared by DNA recombinant technology.

Considering the complex mechanism of immune protection against the asexual blood forms of the parasite, evidence of protection cannot be deduced only from the development of neutralizing antibodies in mice or rabbits following the injection of the purified antigen, or even in primate models. The evidence of protection which is being utilized is the challenge with blood form parasites of homologous and heterologous strains or isolates. The existence of non-human primate models which had been expertly reviewed in our section is one of the privileges of the asexual blood form vaccine research groups. However, we must remember that this model has some limitations: I am not going to discuss the choice of the monkey's species, the reliability of the use of splenectomised or intact animals, which have been very well reviewed and that, for this point, I think that is secondary. The "artificiality" is given by the fact that malaria is transmitted by a mosquito bite (and only rarely by blood transfusion) and the inoculated sporozoites arise after a sexual stage which provides the cellular and molecular basis for the genomic rearrangements we know to occur in the parasite. Therefore, the challenge by injection of infected blood and the evaluation of protection has only a limited significance. Furthermore, in the absence of a primate model for reproducing the entire cycle, we persist in the ignorance of the possible consequences of immunoselection in the survival fraction of the parasites, observed in the partial protection obtained to date by different laboratories.

In conclusion, asexual blood stage vaccines, in spite of important progress reported recently, remain in a situation in which large amounts of research are still necessary in order to permit us to respond yes to most of the questions. Paradoxically, two points would facilitate the progress in this area and they concern also the sporozoite and gametocyte vaccines:

1. The development of primate model allowing the reproduction of the entire cycle of the parasite;

2. The development of effective sporozoite and/or gametocyte vaccines which would potentiate the utility of a partially active asexual blood stage vaccine, eliminating the inconveniences which have been discussed.

A few words concerning the *gametocyte vaccine*. Considerable progress has been obtained recently by two laboratories, and there was an excellent review of this progress by one speaker today. The situation is much more clear than the precedent. First, a gametocyte vaccine will be obligatorily utilized in association with others in the form of polyvalent vaccines. It would be, however, of great utility to potentiate a control of transmission of the other forms of vaccines and could eventually be by itself a guarantee in this sense. Since the antigamete, antizygote, and/or antiookinete activity is certainly dependent on neutralizing antibodies, the description of monoclonal antibodies with transmission-blocking activity is very significant. Immunochemical analysis seems to point out a limited number of target membrane polypeptides which are extremely stage-specific. The same target proteins have been identified for a series of monoclonal antibodies in different isolates of *P. falciparum*. This indicates a genetic stability of the target polypeptide antigens. The absence of a primate model has also been blocking the research on this point, since DNA recombinant methodology will certainly allow us in the future to provide a sufficient amount of antigen for immunization experiments. We would finally remark that inhibitory monoclonal antibodies seem to recognize a particular conformation in the structure of the target antigen.

I will finish my comments by a brief discussion on the *sporozoite vaccine*. It is by far the most advanced project in the area, thanks to the extraordinary work developed by the Nussenzweigs for many years. It is entirely unnecessary to summarize the excellent research work developed up to now, which was described today in our section by Ruth. I will only remark concerning the checking of the box, that the target antigen in this case is unique and perfectly well identified, cloned, sequenced, and characterized. No variants of the CSP protein have been observed up to now in natural isolates of *P. falciparum* all over the world. I'll still add that the Nussenzweigs accumulated a large amount of evidence to show that the basic mechanism of protective immunity is dependent on neutralizing antibodies: Fab fragments of monoclonal antibodies are able to inactivate sporozoites *in vitro* and low amounts of monoclonal antibodies injected in mice are able to protect the animals against infective bites. These arguments are important for the interpretation of the results obtained in the *in vitro* neutralizing tests. Such neutralizing antibodies have been raised in primate model for *P. knowlesi* by injection of synthetic peptides reproducing the epitopes representing the repeats of amino acids present in the CSP. However, the crucial experi-

ment is missing: the induction of protective immunity by injection of the antigen. In the absence of a convenient primate model, the proposed solution is the use of human volunteers to be immunized with synthetic peptides or a DNA recombinant molecule. Confirming the rumors we have been hearing abroad, we had in this meeting the announcement that the experiment is projected for the near future by two different groups: the Nussenzweigs' group and the USA Department of Defense in collaboration with NIH. It is not necessary to comment on the suspense which is now created and to the enormous expectations arising in the scientific, medical, and sanitary world about the results of such experiments. All of us deeply wish that the experiments succeed.

However, not to finish my comments with all five out of five for the Nussenzweigs, I will raise a last point. I am personally convinced that a positive result in the experiment with volunteers will be not enough to justify the immediate development of clinical trials in the field and I think that additional experiments must then be developed using animal models. In the absence of a good model for *P. falciparum*, primate malaria like *P. knowlesi* or *P. cynomolgi* could be employed. The reason for such precautions arises from our ignorance on the effect of the immunoselection pressure in the evolution of the CSP protein. If one assumes that genomic rearrangements or mutations can give rise to variants of the CSP protein, the introduction of selective pressure upon the introduction of mass vaccination, in the absence of control measures for the development of the blood cycle, could rapidly originate and spread vaccine resistant strains. This possibility cannot be analysed in experiments with human volunteers since the blood infection, if occurring, must be immediately treated.

General Discussion

LEVINE: A couple of comments for Dr Pereira da Silva's lovely discussion just now. First, not only the NIH/Department of Defense vaccine will be tested shortly, but also the Nussenzweigs' vaccine will also be tested in the near future in collaboration with the Center for Vaccine Development at the University of Maryland. Second, regarding volunteers and allowing the infection to go on to the point where gametocytes stage is reached, while that's not a high priority, that question can be answered in volunteers. It is possible to allow a breakthrough infection to continue; that is a theoretical possibility. Third, regarding mutation. If I understood the point that was being made, that point is, in fact, made with almost every new vaccine that comes along. That is, a selective pressure will be created for a mutant, and that mutant will take over. And, fortunately, throughout the years since Jenner began with vaccination, through the most recent viral and bacterial vaccines, that is a very, very rare event indeed. In fact, the three types of polio virus, for example, over 20 some odd years, we have not seen other serotypes of polio virus come along. The same for measles virus; the same for tetanus toxin in terms of mutation. It is a theoretical fear, but at least, the overwhelming experience of other immunizations tells us that what exists now as a pathogen may be the best that exists, and if one can protect against that, one has protected against what is already optimal. I think we can feel encouraged in terms of malaria vaccines as being as successful as other vaccines.

PEREIRA DA SILVA: Well, I only want to make one additional point in relation to the point that was raised by Dr. Levine. There is no comparison between a viral or bacterial vaccine and the malarial vaccine. Why? Because it is a sporozoite vaccine. We will not change the source of infection, which is represented by gametocytes. So, the source of infection will be exactly the same. We can reduce the transmission, but we are not going to reduce the rate of infection by mosquitoes, which are

going to find the same amount of infective forms. It is not the same in viral vaccines or bacterial vaccines. The immunosystem which destroys the infective form destroys at the same time the source of infection. So, it is not the same situation.

COLLINS: I want to comment on Dr. Levine's comment about allowing a patient to break through with falciparum. The biology of the falciparum parasite is such that not only does it have to break through, but it has to have quite a bit of malaria before gametocytes are even produced. Most protocols require treatment when the patient is, say, under 5,000 count. If you treat the patient then, you don't get much in the way of gametocytes at all. So, it's very, very difficult in a continuative volunteer program to allow people to "break through" to produce gametocytes.

LEVINE: I thought that the point being made about the gametocyte was that although the individual might be protected by the sporozoite vaccine, that since it doesn't affect the development of gametocytes, perhaps the genomic material that is in the gametocyte, which would lead to infection in another individual after transmission, might lead to infection with a genetically different circumsporozoite. I thought that was the point that's being made. Regarding volunteers going on to the point where they have gametocitemia, I think Dr. Clyde can speak on that. In the days before there were plasmodial cultures, all the work that went on during the years of the Vietnam War, for example, in the USA in terms of research on new drugs against malaria such as those that gave rise to mefloquine, they involved volunteers agreeing to have a prolonged malarial infection such that gametocytes did appear and mosquitoes could be fed on those individuals.

COLLINS: It wasn't up to the patient. It was up to the committees that allowed, and the ethics committees stopped every one of those projects.

V. NUSSENZWEIG: Well, I want to make two points. One practical point of a direct experiment that has not been published yet, and the second a theoretical point. We have done a series of experiments because of this point raised by Dr. Pereira da Silva in mice, using *Plasmodium berghei*. We tried to immunoselect in *Plasmodium berghei* the parasites which go through an infection in rats. Rats are very sensitive, much more sensitive than mice. Given very large numbers, in the presence of 1 milligram per mouse monoclonal antibodies, very few would go through. Then we went selectively through mosquitoes and then we went through a second selection and then through a third selection. Well, up to now we couldn't find any difference in the CS protein after this immunoselection. Of course, this doesn't prove that in nature there are no variants. It only shows that immunoselection in this particular system did not produce these variants.

But, I think the theoretical point is more important than this experiment alone. Everything depends on the function, really, of the repeats. It's very difficult to make sweeping statements and solve all the problems, but if the repeats involve a specific receptor, if the repeats are important, have an important function, then in order for a variant to occur, this variant would have to have another set of repeats, which would substitute for that function. There are so many repeats there that one mutation would not change the function, as we saw in the NIH strain. But, in order for a mutation to occur, and to change a CS protein, it has to propagate to the rest of the repeats. You know, a mutation here has to convert the other repeats and after conversion, those repeats still have to function. So, it's very complicated. It's not a simple mutation; it's not a point mutation which would dramatically alter the parasite and have it functional. We have to have a mutation and then that mutation to convert everything else into something which is also functional again. Which may not be that simple. I don't think that there is a very good argument not to go through with trials in the field if the clinical trials in man are proven successful.

PERRIN: Along the same line, I'd just like to comment that I don't think that you should foresee something will happen two or three years from now. Doing research you have to be optimistic, not pessimistic. The vaccine should be allowed to be tried before any further comments or any further speculation may arise as what may happen later on. So, we are speculating on something which may happen or may not happen maybe 3 or 4 years from now. I think we should first try the vaccine for its efficacy and immunogenicity before discussing what may happen later on.

MEUWISSEN: I also would like to comment on Dr. da Silva's remarks. I agree that there should be an animal model. We should look for these effects in mice, I would say, or in rats, in a rodent model. These studies are under way. But, on the other hand, we should also consider the biological aspects of transmission. It's not so simple that each fertilized female gamete leading to an ookinete leads to an oocyst. We should consider that only 1% of the fertilized gamete actually achieves the development of an oocyst. So, in case of selection, there is a big chance that the surviving fertilized ookinete will not achieve, actually, the rates of a second generation that would do the job. So, I cannot exclude it. It should be tested. But, I am not that pessimistic that Dr. da Silva would like me to be.

FREEMAN: This is perhaps not really to the point, but, just what Victor then Ruth said this morning, I thought that the passive transfer of 10 micrograms of this monoclonal antibody resulted in the protection of

100% of mice. And Victor just said that the passive transfer of 1 milligram did not protect the rats from challenge sporozoites. That sounds very inconsistent to me.

V. NUSSENZWEIG: I don't think there's any inconsistency there. It's very simple. The mice were challenged with 5,000–10,000 sporozoites. These rats were challenged with them between 250,000–one billion sporozoites in order to be able to be immunoselect. That was our purpose—to try select for the variant. So, we had to give a massive dose of sporozoite to select the few which would go through. And the question was, where those few would go through by chance identical to the ones which didn't go through or were they genetically different in the CS protein?

FREEMAN: That's fine. You didn't mention numbers challenging.

V. Nussenzweig: I may be opening another can of worms, but I have a question regarding pregnant women and newborn babies. Pregnant women are highly susceptible, I understand, to malaria. They have antibodies and the IgG antibodies go through the placenta. Newborn babies are highly resistant to malaria. Does anybody want to comment on this?

BRUCE-CHWATT: Well, the situation is not nearly as simple as it appears from what Victor said. Dr. McGregor has recently published an excellent paper in the *American Journal of Tropical Medicine and Hygiene*. He has pointed out a number of complex problems that have arisen. For instance, highly immune women in tropical Africa show, during part of their first pregnancy, a certain decrease of the immunity because their parasitemia increases. Then towards the end of the pregnancy, their parasitemia decreases once again, so there is a fluctuation in their immune response. But with following pregnancies, those fluctuations are much less common and the degree of protection she has and that she passes on to her baby is very much higher than that. There are a number of paradoxes when it comes to the transfer of immune antibodies from the mother to the baby. I'm talking at the present time mainly, if not only, about tropical Africa. Those paradoxes are, for instance, that throughout the whole of tropical Africa, throughout the whole endemic area of Africa, the placental infection is a very high order. Usually at least 25% and very often up to 40% of women show very high infection of the placenta. And yet, paradoxically, the newborn babies don't show the congenital malaria. It is extremely rare. I'm sure that David Clyde will be able to confirm what I'm saying. Now why is it? This is a bit of a mystery. Obviously, the malaria parasites in very small numbers do pass across the placenta to the circulation of the baby *in utero*. But there are very small numbers of malaria parasites with infected cells. But at the

same time, there is a tremendous amount of IgG that passes across the placenta to the newborn baby. The baby is, so to speak, passively protected at the moment it is born. Congenital malaria is virtually absent in tropical Africa. But the situation is very different in non-immune women. There are large numbers of cases of congenital malaria, reported in the United States and the United Kingdom, of non-immune women who have been infected in tropical countries. The whole problem of relationship, the immunological relationship between the mother and the newborn baby is extremely complex, and we still understand very little about it.

PERRIN: Just one question in relation to this problem. Did the mother breast-feed her baby or not? Of course, there're all those experiments on mice, that when you give infected mice just milk which lacked para-amino benzoic acid, they cleared the parasitemia. A European mother is not going to breast-feed most of the time, while an African is going to do it.

BRUCE-CHWATT: This is one interpretation.

CLYDE: And, of course, there is one other factor, Mr. Chairman, we mustn't forget. The part that fetal hemoglobin plays. This is non-supportive of parasite development very largely. This is an additional factor.

BRUCE-CHWATT: May I ask one question? I keep asking myself, and only yesterday I spoke to Bill Trager about it. Why is it that nobody ever tried to find out if there is any transfer of antibodies in maternal milk in Africa? We really don't know if there is any transfer of immune antibodies in maternal milk in highly immune women in tropical Africa. This has not been done.

MITCHELL: Well, isn't the conventional answer that absorption from the human intestinal tract is very transient, unlike some rodents', and that most of the action occurs through the placenta. That everything is being acquired in that way—is that not the case? I should say there is very little absorption from the human intestinal tract—it is a matter of hours, isn't it, in man? And even if you found antibodies in the milk, which you will, of course, that it is not going to do very much as distinct from those intestinal viruses and bacteria, where it can localize in the tract and do a lot of good. I don't know whether Mike Levine or someone wants to talk on this—is this your baby, Mike?

LEVINE: My interpretation would be somewhat similar to yours, Graham. Since this is a blood infection, where if antibody is important, it is serum antibody. Why look at milk, which is mainly secretory IgA, and where the protective factors are directed against organisms that invade

through the intestinal tract, or attach to the lining of the intestine? The maternal milk immunology is very much directed towards protection of gut-oriented organisms, where lymphocytes from the gut will home to breast milk and will preferentially be found in breast milk, as well as their product, secretory antibody. I think much more important would be what you discussed earlier, which is the placentally passed antibodies.

BRUCE-CHWATT: I would like not to be misinterpreted. I don't believe that antibodies are being passed. Not a great deal are being passed through maternal milk to the baby. I fully agree with Mike Levine. But oddly enough, nobody ever tried to look into it. Most of the protection of the newborn baby in Africa is from immunogobulins transmitted across the placenta.

Rapporteurs' Summary

THEODORE GREEN AND KENTON KRAMER

Antigen candidates for the development of vaccines against sporozoite, schizont/merozoite, and gametocyte stages were discussed. These antigens were characterized by their fluorescent staining characteristics, reactivity with monoclonal antibodies (McAb), and/or relative molecular weights. For a working classification of the asexual blood stage refer to the preceding section on "Asexual Blood Stage Antigens of *P. falciparum*."

Dr. Meuwissen reported that gametocytes were produced in synchronized cultures in the presence of N-acetylglucosamine and matured for 7 days. Monoclonal antibodies specific for gametocyte antigens were described.

Monoclonal antibodies against a 48/45 kd gametocyte surface antigen and monoclonals against a 25 kd gametocyte surface protein were discussed. These antigens are expressed on the gametocytes and on the surface of gametes/zygotes and ookinetes. The monoclonals specific for the 48/45 kd antigens were found to be of two types: "transmission blocking" and "non-blocking." It was shown that transmission blocking and non-blocking monoclonals recongized two different epitopes on each protein. Combination of blocking and non-blocking McAb resulted in potentiation of the blocking effect. The 48/45 kd antigens were shown to consist of no more than one polypeptide chain.

The 25 kd antigen is present in small quantities in early stage gametocytes and becomes expressed on the surface of gamete/zygotes. Attachment of McAb against the 25 kd antigen did not significantly influence the number of ookinetes formed, yet almost no oocysts resulted. Perhaps penetration of the gut wall was hampered. In the strains tested, there was no evidence of antigen diversity, as the McAb bound equally well to all isolates.

Sporozoite surface antigens, designated CSP antigens, were described by Dr. Ruth Nussenzweig for *Plasmodium berghei, P. knowlesi, P.*

cynomolgi, and *P. falciparum*. The *P. falciparum* CSP antigen was designated 58(CS). Its Mr was 58 kd and it was derived from precursors of Mr 65–67 kd. Its isoelectric point was 5.3 (5.0–6.0). All of the described CSP antigens were found to be immunodominant and protective. The McAb to CSP's are species specific (except that recognition of *P. malariae* and *P. brasilianum* were identical). The McAb were found to block the binding of specific immune sera to sporozoites. Six different geographic strains of sporozoites were found to react with the same McAb, showing only slight difference in molecular weights. This was maybe indicative of different numbers of amino acid repeats.

To summarize, the CSP antigens were found to cover the surface of the sporozoite, to be shed, to be of Mr of 40–60 kd depending on the species, and to be derived from two precursors. The antigens had a single immunodominant epitope and were synthesized by mature sporozoites. The immunodominant area of *P. knowlesi* is composed of 12 tandem repeats of 12 amino acids. Relevant findings included: 1) Fab McAb fragments inhibited sporozoites as well as complete antibody *in-vivo* and *in-vitro*; 2) CSP covers the entire surface of mature sporozoite; 3) CSP is stage specific; and 4) CSP remains in association with parasite membrane after penetration of hepatocytes. A repetitive DNA hybridization probe has been developed which is exquisitely sensitive in detecting blood stage parasites.

Dr. Perrin described the induction of protection against asexual blood stage polypeptides. These were: 1) Pf185–200 (merozoite/schizont membrane associated proteins); 2) Pf41 (low molecular weight rhoptry proteins); and 3) Pf140 (high molecular weight rhoptry proteins). These polypeptides were selected by their preferential recognition by *Saimiri* monkey immune sera or the basis of McAb inhibition of asexual blood stage growth. The polypeptides were purified by affinity chromatography and/or electroelution from SDS PAGE gels. Challenge of all monkeys was by a heterologous parasite strain. Native Pf41 polypeptide was used as an immunogen in *Saimiri* monkeys and gave evidence of good protection upon challenge. The conformation of the Pf41 polypeptide is important in conferring protective immunity since the level of protection was diminished in the animals receiving SDS denatured Pf41. In another experiment, a 43 amino acid synthetic polypeptide corresponding to a portion of the Pf200 protein was coupled to tetanus toxoid and used in a *Saimiri* monkey vaccination trial, which resulted in evidence of protection.

Several conclusions were reached concerning this work. They are: 1) polypeptides can equal merozoites as protective antigens; 2) partial protection was found to be independent of isolate specificity; and 3) structural configuration of blood stage immunogens may play a role in conferring protection.

Siddiqui et al. reported on the use of *Aotus* polyclonal defined antigens and a combination of two McAb defined antigens as immunogens in *Aotus trivirgatus azure* (karyotype VI). The McAb defined antigens were from: 1) merozoite/schizont membrane associated proteins, and 2) high molecular weight rhoptry proteins. There was one animal in each group. Each animal received three injections with FCA. By solid phase RIA, the 14-day tertiary antibody titers were identical, that is, >1/31,250, in the animals receiving either McAb defined antigens or polyclonal defined antigens. By SDS PAGE, three immunizations were necessary to induce antibodies to all sets of antigens recognized by the monoclonal or *Aotus* polyclonal antibodies. When challenged with FUP K+ parasites, none of the immunized animals developed a patent parasitemia.

Dr. Ristic reported the immunization of *Saimiri* monkeys with partially purified SP antigen containing an 83 kd protein from *P. falciparum* culture supernatants. SP antigen was prepared by cation-exchange chromatography. The 4 monkeys immunized with 2 mg of alum precipitated SP protein and challenged with the homologous Indochina I isolate gave evidence of protection. The 8 animals immunized with 2 mg of alum precipitated SP protein and challenged with the heterologous Geneve isolate showed less protection. No mortality was observed in the immunized groups, while 2 of the control animals died.

An 83 kd protein has been purified from the SP fraction by the use of conventional column chromatography, high performaece size exclusion liquid chromatography. The 83 kd protein exists as a dimer in the native state and has been identified as a parasite protein by Western immunoblotting and ELISA. The N-terminal sequence of the first 35 amino acids residues has been determined. A similar 100 kd antigen was also reported. As yet it is unclear whether the 83/100 kd purified exoantigens of Ristic et al. is identical to any of the previously described antigens. N-terminus sequence data will resolve this issue.

Dr. Collins reported on the use of monkey malarias and human malarias in primate models for the testing of malaria vaccines. Two species of monkey malaria, *Plasmodium knowlesi* and *P. cynomolgi*, are likely candidates for testing sporozoite vaccines in *Macaca mulatta* monkeys. The advantages of this model are the predictable time frame for detecting patent parasitemias and ready availability of sporozoites for challenge. For testing erythrocytic stage vaccines, *P. fragile* appears to be an appropriate choice in Rhesus monkeys. Infection results in fulminating infections and if the animals survive, the infection is characterized by its long-term persistence.

Sporozoite vaccines against human malarias using monkeys is more difficult. Only *Aotus* monkeys of karyotypes II, III, and IV are sufficiently susceptible to sporozoite inoculation to be used in vaccine trials.

In addition, only the Santa Lucia strain of *P. falciparum* has been transmitted with enough frequency to establish transmission rates. In this model system 25% of intact and 85% of splenectomized monkeys inoculated with relatively large numbers of sporozoites have subsequently developed patent parasitemias.

Erythrocytic stage vaccines have been tested in *Aotus* and *Saimiri* monkeys. The limited availability of Columbian *Aotus* monkeys has slowed the development of vaccines against *P. falciparum*. However, limited numbers of *Aotus* monkeys from Peru, and Bolivia are available. In addition, *Saimiri* monkeys from Guyana, Peru, and Bolivia have been used in immunization trials with the FCP and Indochina I strains of *P. falciparum*. Only Indochina I has been adapted for growth in all monkeys examined. However, additional strains are soon expected.

Dr. Pereira da Silva discussed the human condition with regard to protection against malaria. He recalled that immune adults frequently have blood stage asexual parasites and gametocytes circulating in their blood, but are considered protected. However, these individuals represent a source of infection for unprotected children and migrants. A reduction of transmission is also required. Partial immunity may not be a rigorous enough criterion for a vaccine. Five questions were posed for application to the candidate vaccines:

1. Can the "ideal" vaccine be developed without being polyvalent?
2. Do we have a list of good candidate proteins?
3. Do we currently possess the method to rigorously evaluate immune status?
4. Do we already understand the parameters needed for the practical use of successful vaccines?
5. Have we presented enough information to set up clinical trials?

It was argued that malaria vaccines will be "second generation" vaccines, that is, genetically engineered vaccines. It was felt that in regard to blood stage vaccines the above criteria could not be answered in the affirmative at this time, although great studies are being made and relevant antigens and epitopes are being studied in many laboratories. However, the limitations of the existing animal models must be considered and more research is thought to be necessary. A primate model which could reproduce the entire life cycle of the parasite would be ideal.

A gametocyte vaccine is needed for the production of a polyvalent vaccine. Gamete stability of the target antigens has been demonstrated, and genetic engineering should be able to provide enough antigens for testing.

Thanks to the Nussenzweigs, the sporozoite vaccine is by far the most advanced. All of the five questions posed earlier can be answered in the affirmative. Only production of CSP for immunization is still needed.

With regard to early testing in human volunteers, Dr. Pereira da Silva felt that not all the needed answers could be obtained. He felt that the question of parasite mutation under vaccine pressure needs to be seriously addressed.

In the discussion that followed it was stated that history has shown that in vaccines such as polio, measles, tetanus, etc., that vaccine pressure induced mutations have been very rare. Dr. Pereira da Silva insisted that the malaria vaccine can not be compared to viral vaccines in this regard. The feasibility of allowing breakthrough and subsequent gametocyte production to occur in human subjects was discussed and discarded as not feasible. Dr. Victor Nussenzweig stated that preliminary studies in rats with *P. berghei* had been performed to address this question of vaccine pressure induced mutations without evidence of its occurrence being noted.

Session III

PRACTICAL CONSIDERATIONS OF MALARIA VACCINES

A. PRODUCTION OF PLASMODIA ANTIGENS BY NEW BIOTECHNOLOGY

Circumsporozoite Antigen Production

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Introduction

The potential of a sporozoite derived vaccine for humans was first demonstrated in 1973.[1] In these experiments infected mosquitoes that had been irradiated to neutralize their sporozoites were allowed to bite human volunteers many times over a period of weeks. When the volunteers were then challenged with infected mosquitoes that had not been irradiated, they were protected against infection. However the protection lasted for no more than about six months. The potential usefulness of this approach was therefore demonstrated even though it was obvious that it would be impossible to vaccinate a large number of people without a different source of the antigen. Later it was shown that the important sporozoite antigen, with regard to eliciting a protective response, was a single protein which covers the surface of the sporozoite.[2] This finding suggested that there might be an alternative approach to obtaining antigen for a vaccine by artificially producing this single protein. Moreover, the discoveries that only a single epitope on this protein was involved in the host response and that this was a linear repeating epitope both indicated that even a small segment of this protein might be sufficient to elicit an immunogenic response.[3-5] Indeed, all species of malaria that have been investigated to date have a simple repeating peptide as the dominant epitope (Fig. 1).[3-7] In the case of *P. falciparum* this peptide would consist of a tandemly repeating tetramer of the sequence Asn-Ala-Asn-Pro. The repeating sequence of the dominant epitope of other human malarias is not known.

Although antigen for a sporozoite vaccine can be produced in a number of ways, the host immune response to varied products will not be identical. There are then a few considerations with regard to antigen production for a sporozoite vaccine that relate to the epidemiology of malaria. The first consideration is the dynamics of infection. The sporozoites that are injected into the blood reach the liver within minutes, where they are protected from the host immune response. If only a sin-

gle parasite makes its way to the liver, the infected individual will contract malaria. Since antibody titres must be held at a very high level to be effective, the immune system needs to be more than primed to respond; it must be continually producing specific antibodies. The hope for effective long-term protection is that vaccination will produce a lasting response and that there will be natural boosting of this response by the bite of infected mosquitoes. In terms of antigen production, these facts favor the live vaccines because host responses tend to be longer lived. Another factor which relates to a sporozoite vaccine is that a purely antibody-mediated response can be protective. Many of the standard arguments against the clean, inexpensive peptide vaccines may therefore not apply in this particular situation. For example, if only an antibody response is necessary then the peptide vaccines become attractive even though they would not elicit certain types of T cell responses. A final consideration relates to the fact that the group at highest risk is infants. Even a short-lived vaccine that had to be continually boosted would be of great value in saving lives in this age range. The ideal vaccine might take longer to develop and to approve than simple but shorter lived vaccines. A sense of urgency may dictate the development of both.

Below I will discuss the synthesis of the *P. falciparum* epitope. The production of an appropriate antigen is the first step in producing an effective vaccine. Presently four different sources of antigen are being investigated. There are chemical and microbial production synthesis, vaccinia vaccine, and avirulent *Salmonella* vaccine; all seem to have promise. We will reference work of each type as well as mention some pros and cons of each approach.

Chemical Synthesis

The chemical synthesis approach is presently being used by both our group and that of Ruth Nussenzweig and her collaborators. Some of her results are discussed in this volume. One major objection to the chemical production approach in other systems is that short, chemically synthesized peptides often don't display an antigenic site in the manner that a complete folded protein would. Initial experiments by our group with chemically synthesized peptides from the repeating epitope of the CS protein indicated that as few as two repeats containing seven amino acids weakly mimicked the antibody binding site of the complete CS protein.[6] This suggested that overall configuration of the protein was not necessary to display the appropriate antigenic site and that short chemically synthesized molecules might be useful in a vaccine. Another objection raised against peptide vaccines is that the host response to synthetic peptides is incomplete and may not be completely protective. As stated above, however, a short-lived protective response can be

totally antibody mediated in the case of sporozoite infectivity. Other advantages of this approach include the low cost and high purity of reagents. Disadvantages include the fact that appropriate carriers and adjuvants need to be found for delivery. Although there are no available peptide vaccines for any diseases, there are systems being tested which show promise. One example of this is a synthetic diphtheria vaccine.[8]

Recombinant DNA Synthesis

Microbiol production based on a recombinant DNA approach has been used by two groups. Sharma and Godson have produced the *P. knowlesi* CS protein in yeast.[9] A protein that is approximately the size of the precursor CS protein was detected. These researchers report production of 1 μg of stable CS protein per 20 mg of total protein. The bacterial fermentation approach now being used by the NIH–Walter Reed group in collaboration with workers at Smith, Kline, and Beckman has both advantages and disadvantages when compared to the yeast system.[10] The complete CS protein could not be synthesized as in the yeast system. Clearly products were rapidly broken down *in vivo*. But when only a segment of the immunodominant region was expressed in the bacterial vector, the products were stable and the yields were nearly 5% of total protein or several thousand times better than the yeast system. These researchers went on to show that antisera raised to their product block hepatoma cell invasion by sporozoites *in vitro*.[11]

Comparison of products produced by microbes with those produced chemically may favor the latter. Similar polymers to the ones produced in bacteria can be synthesized more cheaply by chemical means, although the bacterial system may be able to make much larger polymers. The quantities of CS protein produced in yeast appear to be too low at present to be very useful. Further, the purification of bacterial or yeast products to meet EPA standards for human trials may be difficult and perhaps costly. The need for adjuvants are the same as those for chemically-derived products.

Genetically engineered products have been successfully used in other systems such as a Hepatitis B vaccine. Researchers have produced a surface antigen in both yeast cells and Chinese hamster cells. The product is undergoing clinical trials and seems to be as effective as plasma-derived products in a vaccine.

Vaccinia

The use of genetically engineered vaccinia virus is discussed in this volume and elsewhere by Bernard Moss.[12] The advantages of this system are many: 1) It can be administered by untrained personnel. 2) An engineered virus could accommodate not only a sporozoite gene but

appropriate genes from other stages of the parasites' life cycle. 3) The virus stimulates both a humoral and a T-cell response. 4) The costs of production are relatively low and the virus can be mass produced. 5) The virus is stable at room temperature in a freeze-dried form over long periods of time. The most serious problem with vaccinia that has been documented is that complications have arisen in a small number of vaccinations. For example, in 1968, out of 14.2 million small pox vaccinations there were 572 that involved complications, including 9 deaths. Another conceivable problem with this type of vaccination is that many adults have a high titre of antibody against the virus. Given passive transfer of this antibody from mother to child, it is possible that vaccinations might not "take" well in young children. This supposition, however, has not been substantiated.

Work directed toward a vaccinia-derived sporozoite vaccine is already proceeding. The gene coding for the CS protein of *Plasmodium knowlesi* has been inserted into the Vaccinia genome.[13] Cells infected with these recombinant virus produced a protein which reacted with monoclonal antibodies against the CS protein. Also, rabbits infected with virus produced antibodies against sporozoites. To date, studies of successful vaccinations have not been reported. Some success in producing recombinant vaccinia vaccines for other diseases makes this system look like a promising approach for a malarial vaccine. Vaccinia-derived vaccines are now being tested for Hepatitis B, Influenza, and Herpes Simplex.[14-16]

Salmonella

Another potential "live vaccine" involves the use of nonvirulent, nonreverting strains of *Salmonella* to deliver antigens.[17] These strains of bacteria could be genetically engineered with any antigen or group of antigens. They could be grown in the laboratory but cannot grow in the host because they are dependent on media supplements that don't exist in host tissue. They apparently exist long enough to elicit an immunogenic response in the host but don't grow and divide. Vaccines could potentially be delivered orally, and there has already been a great deal of testing of the system in the veterinary field. A live bacterial vaccine, however, is another level of increased complexity over the vaccinia system. The fear of loss of control over a "live vaccine" therefore increases.

Present technology appears to have met the challenge of producing materials for a vaccine in several ways. The immediate problem for production of material for a sporozoite or other malarial vaccines appears not to be scientific as much as a question of reconciling the needs of the global community for safe vaccines and the vaccine producers need for a profit and reduced liability.

CS Protein Repeats

P. falciparum	Asn Ala Asn Pro	major
	Asn Val Asp Pro	minor
P. knowlesi	Gly Gln Pro Gln Ala Gln Gly Asp Gly Ala Asn Ala	
P. cynomolgi	Asp / Gly Gly Ala Ala Ala Ala Gly Gly Gly Gly Asn	

FIGURE 1. The Immunodominant Epitope of Three Primate Malarias.

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Production of Asexual Blood-Stage Antigens

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Introduction

Despite the ease with which *Plasmodium falciparum* can now be cultured *in vitro*, research on the antigens of *Plasmodium falciparum* has been hampered by difficulties involved in purifying parasite proteins by conventional biochemical means. Recombinant DNA technology has solved this problem by providing the means of production of defined parasite antigens in large amounts. Several different expression systems have been used to express asexual blood-stage antigens of *P. falciparum*.

Expression Systems

Systems used to express *P. falciparum* antigens are summarized in Table 1. Early work with low level expression systems, such as pBR 322 has been superseded by high level expression systems such as λgt11 and its derivatives.[1-4] Either complementary DNA (cDNA) prepared from messenger RNA or fragmented genomic DNA can be cloned in these expression systems. A genomic DNA library has the advantage that it is capable of expressing sequences from all life-cycle stages. The resultant expression product may be either a protein fragment, as usually occurs in fused polypeptide expression systems, or a full length polypeptide, as for example in libraries derived from DNA fragments generated with mung bean nuclease. Less commonly, complete polypeptides can be generated from full-length cDNA.[4,5] Blood-stage antigens have been detected in such clones using either monospecific probes such as monoclonal antibodies or rabbit antisera raised against purified proteins.[3,4] Polyvalent sera derived from patients or from experimentally immu-

TABLE 1. Systems used to express *P. falciparum* antigens. This table summarized vectors that have been used to express asexual blood-stage proteins. A bacterial promotor is used to control initiation of transcription except in the case of mung bean digested DNA in λgt11 where transcription is apparently initiated using the *P. falciparum* promotor sequences.

Vector	*Insert*	*Product*	*Promotor*	*Inducibility*	*Expression Level*	*Refs.*
Prokaryotic						
λAmp3	cDNA	Fused polypeptide to β-galactosidase	Lac	Yes	High	2
pUK270	sonicated genomic DNA	Tribrid fused protein	Lac	Yes	High	8
p31A	cDNA	Fused polypeptide to MS2 polymerase	P_L	Yes	High	14
PATtrp	cDNA	Fused polypeptide to anthranilate synthetase 1	Trp	Yes	High	13
λgt11	mung bean nuclease digested DNA	Native protein	phage or *P. falciparum*	No	Low	33
pUC9	cDNA	Fused polypeptide to β-galactosidase	Lac	Yes	High	15, 32
pBR322	cDNA	Fused polypeptide to β-lactamase	β-lactamase	No	Low	1
Eukaryotic						
pGS62 (vaccinia virus)	cDNA or genomic DNA	Native protein	7.5K late	No	Low	34
pTT5 (S. cerevisiae)	cDNA or genomic DNA	Fused polypeptide to β-galactosidase	PGK	No	Intermediate	

nized animals have been successfully used to detect antigens, but for this purpose a high level expression system is mandatory.[5–9]

Our own laboratory has worked extensively with the λAmp3 bacteriophage into which cDNA from a Papua New Guinea isolate (FC27) or a Ghanian isolate (NF7) has been inserted. Using various combinations of human antisera obtained from people living in endemic areas, we have been able to identify many antigen positive clones (Table 2). A subset of 314 clones has been analyzed by hybridization with labelled cDNA inserts to determine the relationship among clones. Not all of these clones express unique antigens and there are many examples of clones expressing overlapping portions of the same antigen (Fig. 1).

The antibodies used for screening the library can heavily bias the type of clone found. For example, antibodies affinity purified on saponin-lysed parasites selected strongly for sequences such as Ag23, Ag61, and Ag48 (Fig. 1, Array A) which were weakly recognized or not seen at all by unfractionated sera (Fig. 1B, Arrays B and C). Conversely, unfractionated sera identified antigens not found using affinity purified antibodies, e.g., Ag196, 205, 319, 394. Thus, if screening with polyclonal sera is used, as wide a variety of sera as possible should be used to maximize the diversity of antigens recognized.[10,11]

cDNA libraries screened in this manner are biased towards detection of determinants close to the carboxy end of the molecule. To look at other determinants of a particular antigen we have used these short sequences to isolate larger cDNAs or genomic clones, fragmented the DNA by sonication, and reinserted them in an expression vector. These clones are screened for expression in the usual way. This approach allows detection of determinants not in the original clone or the production of several clones each expressing a single determinant (see the section on RESA, Figs. 1, 2).[12]

Expression systems in eukaryotic hosts are less advanced. Yeast and vaccinia virus are being used as second generation tools to express

TABLE 2. Summary of antigen positive clones. λgt11-Amp3 cDNA clones expressing *P. falciparum* antigens were identified using human antisera.

Antigen positive clones	>400
Characterized gene products	25
Unassigned clones	121
Sequenced antigens	15

Note: Of these, 14 have regions of tandem repeats and the remaining antigen contains a region of degenerate repeat.

sequences previously isolated in prokaryotic systems. Vaccinia virus has been modified so foreign DNA sequences inserted into it will be expressed as native molecules, during the course of viral infection. We have inserted the genes of several asexual blood-stage antigens into the virus including the S antigen, RESA, the 195K protein, the circumsporozoite protein-related antigen and the small histidine rich protein (see Fig. 3 and Table 4). As well as a role in expression, vaccinia has the added advantage of providing a vehicle for vaccine delivery.

Expression of Specific *P. falciparum* Antigens

Some properties of blood-stage antigens expressed in *E. coli* are summarized in Table 3. Antigens from all stages of the asexual cycle have been expressed and they have been identified both with monospecific and polyspecific probes. Most examples of expression of asexual blood-stage proteins use cDNA inserts expressed as fused polypeptides. In addition to those sequences summarized in Table 3, clones expressing an additional 25 distinct antigens have been identified in our laboratory, some of which are listed in Table 4. Antigens from all stages of the asexual cycle have been identified, including antigens present on the surface of the merozoite (Ag75) and at least two antigens present in rhoptries (Ag44 and Ag394). The utility of using polyvalent serum as a screening method is illustrated by the fact that several clones identified by other groups using monovalent sera have also been identified by our procedure. For example, Ag75 encodes the merozoite surface-coat precursor protein, Ag23 encodes the putative glycophorin binding molecule, and Ag61 and Ag5.1 are equivalent.[3,4,11,12,14]

Characterization of Cloned Antigens

Cloned antigen fragments enable the preparation of monospecific antibodies capable of identifying the corresponding native proteins. Immunization of mice or rabbits with lysates of bacteria or with fused polypeptides purified from *E. coli* has been used to raise high titre monospecific antibodies.[4,6,7,13] This technique is most successful when the antigen is present in high amounts, i.e., detectable as a Coomassie staining band after gel electrophoresis of bacterial lysates. The λAmp3 system is a high level expression system which can produce up to 2.5 mg of fused polypeptide/liter of culture.[9] An alternative method is to use a crude bacterial lysate as an affinity reagent to purify antibodies specifically reacting with the clone product. The protein may be bound *in situ* to nitrocellulose filters or coupled to cyanogen bromide sepharose-activated and used to purify antibodies from monospecific or polyspecific antisera.[4,5,9] This procedure can be used even when only low

TABLE 3. Antigens expressed in *E. coli*. This table summarizes published reports of asexual blood-stage antigens expressed in *E. coli*.

Name	*Type of Antibody*	*MW (kd)*	*Location*	*Repeats*	*Refs.*
S-antigen	Polyspecific	200	Parasitophorous vacuole of schizont	Yes	6, 16
RESA	Polyspecific	155	Membrane of ring infected cells	Yes	7, 12
pPF-11.1	Polyspecific	~250	Membrane of schizont infected cells	Yes	8
Pf190 195kd	Monospecific Monospecific	195	Merozoite surface	Yes	3, 14, 15
FIRA	Polyspecific	300	Extraparasitic inclusions in host erythrocyte	Yes	9
CRA Ag5.1	Polyspecific Monospecific	23	Ring surface and parasitophorous vacuole of schizonts	No	4, 5
GBP	Polyspecific	155,130	Schizonts and merozoite surface	Yes	15

TABLE 4. Characterized antigen positive clones of asexual blood stages. This table lists a series of antigen positive clones and the corresponding *P. falciparum* protein.

Clone	*Other Names*	*Molecular Weight (kd)*	*Predominant Stage Specificity*	*Refs.*
Ag7	Glutamic Acid Rich Protein (GARP)	>250	Mature forms	36
Ag13	Ring-Infected Erythrocyte Surface Antigen (RESA), Pf155	155	Surface of ring-infected cells	7, 12
Ag16	FC27 S-antigen	200	Mature forms	6
Ag23	Pf120, Glycophorin Binding Protein (GBP)	110	Mature forms	1, 15
Ag44	Ag2	105	Rhoptry of merozoites	
Ag57	Small Histidine Rich Protein	30	Mature forms	38
Ag61	Circumsporozoite Protein Related Antigen (CRA), Ag5.1	23	All stages	4, 5
Ag63		75	All stages	
Ag75	195K molecule, Pf190	195	Schizonts and merozoites	13, 14, 35
Ag169		35	N.A.	
Ag196	Acidic Basic Repeat Antigen (ABRA), Ag359	105	Schizonts	
Ag205		96	N.A.	
Ag231	Falciparum Interspersed Repeat Antigen (FIRA)	>300	Rings and trophozoites	9
Ag319	Asparagine Rich Protein (ARP)	200, 160	All stages	37
Ag358	ARP related	210, 190, 160	N.A.	
Ag361		70	All stages	
Ag372		110	N.A.	
Ag394		N.A.	Rhoptry of merozoites	
Ag501		130	Schizonts	
Ag617	SD17	108	N.A.	
Ag661	NF7 S-antigen	80, 75	Mature forms	

N.A. = not available

amounts of the expressed protein are present. Antibodies thus produced can be used for localization studies using immunofluorescence or immunoelectronmicroscopy, immunoblot analysis, or functional studies such as *in vitro* growth inhibition (Figs. 4 and 5).

Tandemly Repeating Peptide Antigens

A striking structural feature of many plasmodial antigens is the presence of segments of extensive tandem repeat sequences (Table 5). Details of some repetitive antigens of Plasmodia are summarized in Table 5. This structural feature occurs in plasmodial proteins that interact with the immune system. For those antigens studied, it appears that much, if not all, of the naturally occurring antibody response is directed against the repeats. The function of the repeats is not known. It is possible that, in some molecules, repeats act as ligands leading to an increased affinity of binding to specific receptors. However, the very large number of repetitive molecules now identified suggest that this cannot be true for all of them. Alternatively, the repeats may be involved in immune evasion. For molecules that show great diversity in the repeat sequences among isolates, such as in the S antigens, this is most likely to be the case, but even where repeat sequences are conserved as in the RESA, the repeats may be involved in diverting the immune system from other portions of the same molecule.[12-16] Alternatively the profusion of repeat molecules may act collectively to cause generalized immunosuppression and may also interfere with the normal maturation of a high affinity antibody response.

In *E. coli* expression systems recombination events frequently lead to deletion of repeats from the DNA insert. For example, a genomic clone of the FC27 S antigen lacked over 90 copies of the 33 nucleotide repeat, accounting for more than 90% of the repeat region of the protein.[16] This instability may make these systems unsuitable for expression of vaccine molecules if large numbers of repeats are essential for successful immunization, as has been suggested by recent experiments.[17]

Because repeats may have evolved as an evasion mechanism which suppresses immune responses to other parts of the molecule, we have expressed in the λAmp3 vector repeat and non-repeat fragments of an antigen so that the immune response to these different regions of an antigen can be determined (see below).

Antigenic Differences Between Different Strains of *P. falciparum*

One consequence of the presence of multiple repeats is the ability to rapidly alter the predominant antigenic character of a protein. Unequal crossing over is an obvious mechanism that could allow the rapid spread

TABLE 5. Repeat sequences in *P. falciparum* antigen. This table lists the repeat sequences of *P. falciparum* antigens from several different strains.

Antigen	*Isolate*	*Repeat size*	*Repeat Number*	*Canonical Sequence*	*Refs.*
S-antigen	FC27	11	>100	AKASQGGLEDP	6
S-antigen	NF7	8	43	A$^{L}_{R}$KSDEAE	15
RESA	NF7, FC27	10, 11	5	DDEHVEEPTVA	7, 12
		8	6	EANVEHDA	7, 12
		4	36	EEN$^{A}_{V}$	7, 12
pPf11.1	FUP	9	22	L EEIVEEVVP V	8
FIRA	FC27	6	32	VTTQEP (extremely variable)	9
GBP	FCR-3	50	>4	A LT$^{S}_{A}$DPEGQIMREYAVDPEYRK$^{H}_{D}$LEI P F$^{H}_{V}$KILTNTDPNE$^{E}_{D}$VER$^{R}_{Q}$NA$^{D}_{E}$NKEE D A	15
GBP	FC27	50	10	Conserved compared to FCR-3	
195kd	SGE2	6, 3	2, 6	SGGSVA	14
195kd	FC27	6, 3	4, 4	SAQSGA	35
GARP	FC27	6	18	GESKET	36
ARP	NF7	8	4	NNNMNHNM	37
		4	3	NNNM	
Ag57	FC27	6	14	AHHAAN	38
		5	8	HHDGA	

of a single change in sequence throughout multiple copies of the repeat and even relatively trivial changes in nucleotide structure can lead to markedly different antigenic properties. This is most strikingly seen in the S antigen system (Table 5).[16] Conversely, antigens that lack repeats, such as the circumsporozoite-protein related antigen (CRA), show fewer differences between different strains.[4,5] One might postulate that the absence of variation within a repeat sequence among different strains suggests that the repeat is required for some purpose such as receptor binding.

Candidate Vaccine Molecules

Several of the antigens expressed in *E. coli* are potential protective immunogens:

195K PROTEIN (Pf190)

The molecule with the most convincing credentials as a vaccine is the merozoite surface coat precursor protein (195K protein, Pf190).[18,19] Homologues of this molecule from other malarial species have been demonstrated to be effective vaccines in several experimental systems.[20,21] Protection has been best against homologous challenge suggesting variability in the epitopes against which the immune response is directed. In the case of *P. falciparum*, immunization of Saimiri monkeys with purified merozoite surface coat precursor protein gave some protection but in a very small-scale study.[3] Several groups have obtained clones for this protein.[5,15,11,22,23] This molecule is antigenically complex with strain-specific and species-specific epitopes as well as epitopes that are common to several species of Plasmodium.[24-26] The amino acid sequence of the 195K protein from two different strains shows regions that are conserved as well as regions of repeat sequence that vary between strains (Table 5). These variable repeat regions presumably encode the strain-specific epitopes recognized by several monoclonal antibodies. Detailed analysis of the sequence basis for this complexity may enable the expression of a molecule that immunizes against all strains of *P. falciparum*.

RESA (Pf155)

The ring-infected erythrocyte surface antigen (RESA), the 155K molecule present mainly in merozoites and ring-infected erythrocytes, is transferred into the membrane of the erythrocyte as or immediately after the merozoite invades. Experiments using antibodies affinity purified on the native molecule have demonstrated considerable inhibition of parasite growth *in vitro*, although antibodies to cloned fragments that we have tested do not inhibit growth.[27] The repeat sequences in this

molecule are conserved in at least two *P. falciparum* strains.[12] The approach of expressing repeat and nonrepeat fragments of the molecule offers an opportunity to better define its potential protective immune response (Fig. 2).

Using clones expressing RESA fragments as targets it has not proved possible to detect naturally occurring antibodies against nonrepeat portions of this molecule (Fig. 2). Such results should be interpreted with caution, as it is possible that nonrepeat regions may possess conformational determinants that are not formed in fused polypeptides while repeat regions possess sequence determinants that are preserved. Nevertheless, data from the study of S antigens support the contention that repeats may act to camouflage nearby regions of unique sequence from the immune system as antibodies to these unique sequences have not yet been demonstrated.

Several other molecules have been suggested as potential vaccine molecules. These include the analogue of a rhoptry protein which protects in the *P. yoelii* system, the cytoadherence molecule responsible for parasite sequestration, and molecules where specific antibodies are correlated with growth inhibition.[29-31] As yet there is little direct evidence for the utility of these molecules in inducing resistance to *P. falciparum* infection.

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FIGURE 1 (opposite page). Sibling Analysis of 325 Antigen Positive Clones. This schematic depicts 325 antigen positive clones selected by antibody screening. Arrays A and B are composed of cDNA clones derived from the FC27 isolates while Array C was derived from the NF7 isolate. Clones at array A were identified by screening a cDNA library with a pool of five inhibitory sera that had been first affinity purified against a sonicate of asynchronous parasites.[2,24] Clones of array B were selected by screening with a single high titre serum from Papua New Guinea.[11] Clones of array C were identified by screening with a pool of 10 high titre PNG sera. Array D are clones expressing abundant fused polypeptides derived from re-expression of previously identified sequences (see Table 3 and Figure 2). In Arrays A-C, clones that hybridize with a particular cDNA are denoted by a common number. * - Clones producing fused polypeptide detectable by Coomassie blue staining of electrophoresed bacterial products. ‡ - Clones producing fused polypeptides detectable only by immunoblotting.

A

1	*2	*2	23	2	61	*63	7	48	48	61	61	48	12
13	*13	*13		16		48	23	*61	61	22	*23	*13	24
25		23	23	*13	48	48	48	*13	48	34	13	36	36
37	37	48	48	48	*13	36	61	*44	45	*13	*13	*48	48
49		48		52	23	48	16		*57	58	*59	*61	60
61	61	62	*63	*63	*65					70			72
73		74	75	*48		*23	78						

B

101	16	16	13	169	13			63		*16	16	16	112
113	16	16		16	63			*120	*120	*63			124
125	13	*196	13		13	13	13	13	44			44	136
137	16							*196	*196				148
149	13	13				13	231		231				160
161		*16	16						169	13	231		172
173	13	16	16	16		13	61	44			183	*183	184
185				75	196			16		16		*196	196
197	44	44	231	*13	13	*13	196	16	205	205			208
209	*16			*13	16	16		16	16	16		*13	220
221	*13	13	16	16	13	13		44	16	13	231	231	232
233	13												

C

301		13	303	196	196		307	319		307		358	312
313	*313		319	349		358	*319	358	358			358	324
325	358	358	358	196	358	358	303	358	63	358			336
337		338				319				358			348
349	349		358	352	358	354			358	*358	307	63	360
361	*361	358	358				63		358		358	372	372
373	373			376			379					358	384
385	358		319							394	349		396
397	*358			358	358		319						

D

*617	*631	*632	*633	*641	*642	*651	*652	*653	*661	*681	

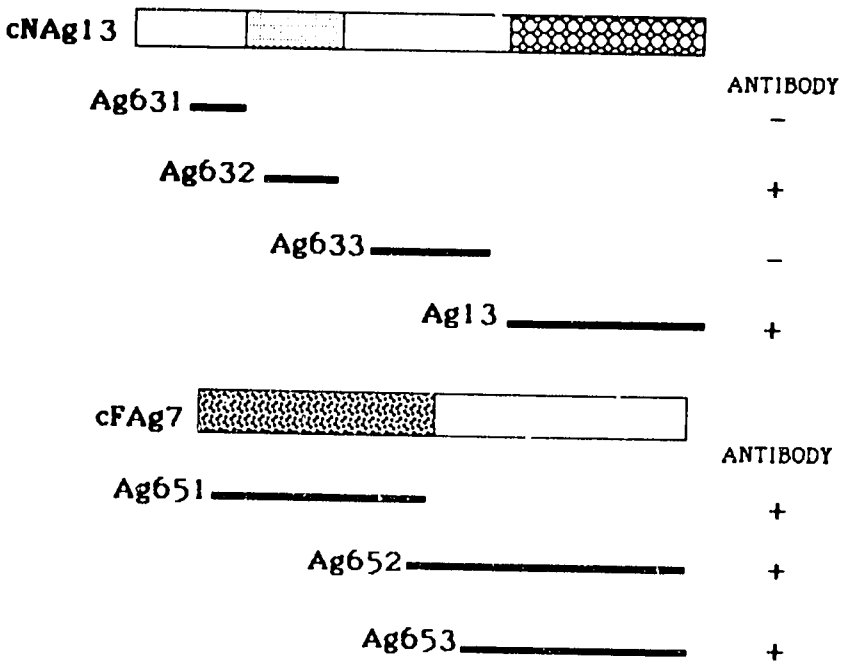

FIGURE 2. Relationship of Clones Derived by Sonication and Re-expression RESA and GARP sequences have been fragmented and re-expressed to produce subclones expressing portions of the whole molecule. In a similar manner, Ag641 and Ag642 were derived from FIRA, Ag661 from the NF7.S antigen and Ag681 from the 195k molecule. Repeat portions of the molecule are depicted by patterned areas. The subclones can be used as affinity adsorbents to isolate human antibodies reacting with different portions of the parent sequence. For example in GARP, but not RESA, it is possible to detect naturally occurring antibodies that react with both repeat and nonrepeat sequences.

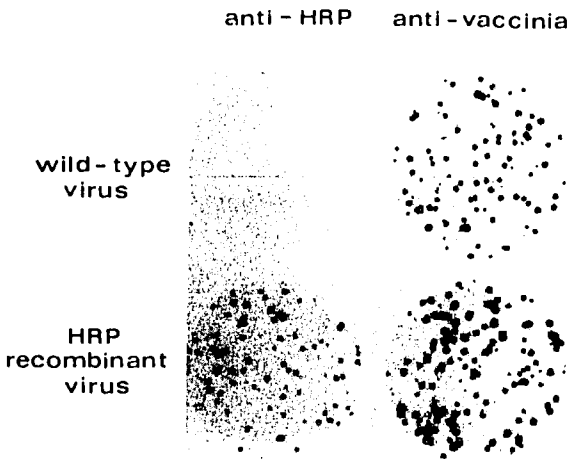

FIGURE 3. The Expression of *P. falciparum* Genes in Recombinant Vaccine Virus Infected Cells. Duplicate monolayers of BSC-1 cells were infected with either wild type or small-histidine rich protein (HRP) recombinant virus. One of each pair was probed with anti-HRP antibodies and the other with anti-vaccinia antibodies followed by ^{125}I-protein A.

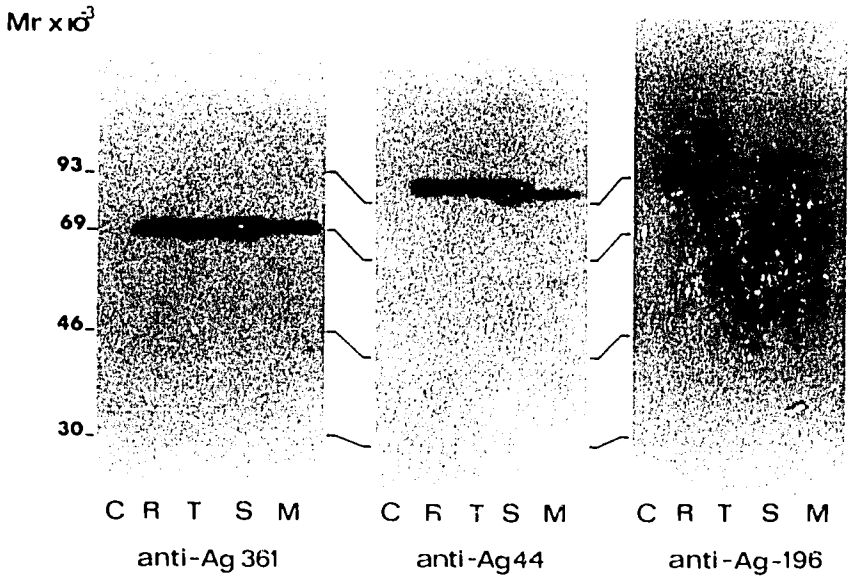

FIGURE 4. Stage Specific Immunoblots Probed with Affinity Purified Antibodies. Human antibodies affinity purified against the indicated antigen positive clones were used to probe immunoblots of detergent solubilized proteins of synchronised *P. falciparum* asexual blood stages. Each panel consists of control uninfected erythrocytes C, ring stages R, trophozoites T, schizonts S and merozoites M.

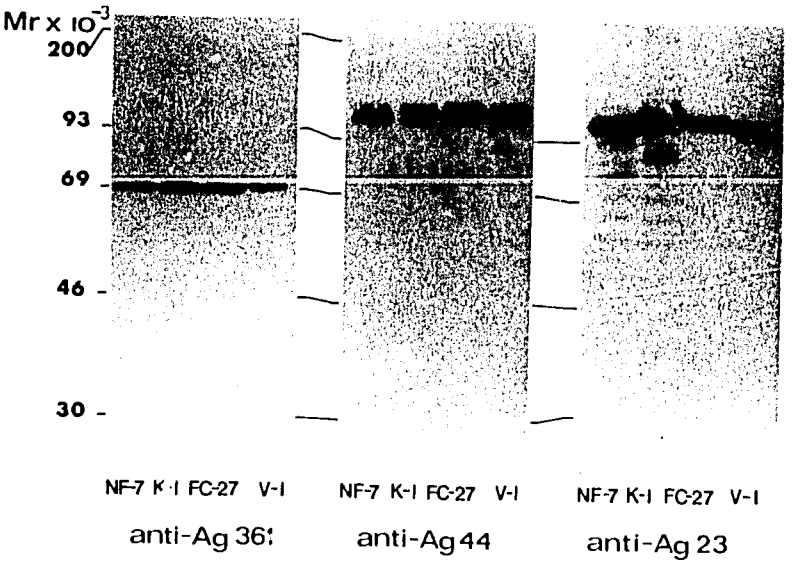

FIGURE 5. Affinity Purified Antibodies Probe Immunoblots of Different *P. falciparum* Isolates. Human antibodies prepared as described for Figure 4 were used to probe immunoblots of detergent solubilized proteins of asynchronous cultures of several different isolates of *P. falciparum*. NF7 (N) from Ghana, K1 (K) from Thailand, FC27 (F) from Papua New Guinea and V1 (V) from Vietnam.

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Use of Biotechnology to Study *Plasmodium Falciparum* Asexual Blood-Stage Antigens

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Introduction

For the purpose of this review, we will consider biotechnology as a large body of very precise techniques which can be broadly applied to many biological systems. Conceptually, this approach was pioneered with the introduction into biology of techniques from physics and chemistry. These allowed us to begin thinking of cells in terms of their component molecules and, from the physical properties of these molecules, conceive the ways in which they are likely to interact. In the very recent past, two new approaches were added to this arsenal: the use of monoclonal antibodies (MoAbs) and recombinant DNA technology. These procedures have provided the biologist with power he previously would have felt unrealistic. Now, relatively easy, reliable systems are available by which monospecific antibodies (monoclonal antibodies) can be produced to a variety of cellular components. These allow isolation and characterization, as well as assignment of a molecule to a particular area of a cell, such as its membrane.

A second group of techniques which are profoundly affecting our ability to study cells are those associated with recombinant DNA. In the most recent three to four years, increasingly efficient systems have been developed which allow expression of foreign DNA in bacteria, yeast, and mammalian cells. Thus, one can copy the messenger RNA (cDNA) which encodes a particular protein, insert it into an expression vector so that it can be cloned, use antibodies against the proteins to detect clones containing the genes of interest, and sequence the DNA which encodes these proteins (it is much faster to sequence the DNA than to sequence the protein itself). The corresponding protein sequence can be predicted

in a matter of seconds by a computer and then in specific cases, regions of interest can be made with synthetic peptide chemistry.

Because these technologies are so new and are still evolving, it is not as yet clear whether chemistry or recombinant expression systems are likely to be more useful for producing antigens for immunization purposes. At present, except in very few cases, synthetic chemistry has not been able to produce peptide antigens greater than 20–25 amino acids in length with efficiency. Recombinant systems can make much larger polypeptides than can be synthesized chemically, and large peptides are more likely to adopt a conformation that resembles the native protein. As yet, however, neither system can provide a fully processed polypeptide containing the appropriate sugars. The chemistry to do this is just being developed, and even recombinant mammalian hosts are not likely to glycosylate the foreign proteins in the way that human malarial parasites do.[1,2]

The objective of this review is to relate how this technology is being focused on defining the structures of the blood-stage antigens of the human malarial parasite *Plasmodium falciparum*. Because the relationships are not generally clear, we will omit reference to work on other malarial parasite systems.

What Parasite Antigens Should We Study?

To provide a sound basis for any infectious disease study, one must first define what aspects of infection cause the disease and, second, determine how proliferation of the organism within the host can be stopped. If an immunologic solution is being sought, it is useful to deduce how one might theoretically interrupt the organism's development and then assess what is likely to be possible in a practical sense. The pathology associated with human malaria caused by the parasite *Plasmodium falciparum* is due to proliferation of the asexual blood stages within erythrocytes. The asexual blood cycle (Fig. 1) begins when merozoites liberated from liver cells first infect erythrocytes. This extracellular merozoite stage could clearly be a focal point for immune intervention, but direct observation as well as time lapse photographic studies suggest that merozoites can achieve entrance into erythrocytes within as little as 20 seconds.[3,4]

This efficiency makes it likely that antibodies or other such serum factors (i.e., complement) would be required to interfere at this stage of the cycle since immune cells, unless already present at the local site, would probably not have sufficient time to play a major role. This, of course, assumes that most merozoites achieve entrance into an erythrocyte without having to move far from the original site where they are

released. This is a reasonable assumption since sequestration of schizonts in small vessels such as post capillary venules allows merozoites close contact with a steady flow of erythrocytes.

It has been demonstrated that antibodies in immune human or monkey serum can block the invasion of merozoites *in vitro*.[5,6] Such antibodies can passively protect humans or monkeys and can also be shown by immunoelectron microscopy to bind to antigens found on the outer surface of merozoites.[7,8] Therefore, such antisera can be used as molecular probes to identify the structures with which they interact. Similar studies have also provided evidence that immunity directed against antigens on the surface of the parasitized erythrocyte are an equally useful point of attack. Because we have dealt with this in detail elsewhere in this volume (see Reese et al., "Changes Induced in the Surface of Erythrocytes by the Human Malarial Parasite *Plasmodium falciparum*," this volume), we will not discuss it further here.

Important Merozoite Antigens

Attempts have been made in several laboratories to purify merozoites and label with ^{125}I those proteins which are located on their surface.[9-12] These types of experiments always suffer from the problem that merozoites clearly disintegrate during the collection, purification, and labeling procedures so that there is no assurance that what is labeled is truly on the surface. In addition, a major surface protein may be missed if it contains no exposed tyrosine with which the iodine can react.

In two groups of such experiments, care was taken to not only collect high quality merozoites but establish their purity and integrity by several procedures, including electron microscopy.[9,12,13] Immunoprecipitation experiments were then conducted using both human and primate sera having different *in vitro* inhibitory capacities (Fig. 2). The results suggested the merozoites had molecules of immunologic importance on their surfaces of approximately 34K, 45K, 75K, and 83K. Less intense bands of approximately 130K and greater were also observed.

Because of the limitations inherent in surface labeling merozoites, alternative approaches have been used. Merozoites have been metabolically labeled while they were still within schizonts.[14,15] They were then allowed to invade fresh erythrocytes. Determination of the labeled components carried into the erythrocyte provided a minimal picture of the merozoite. This assay system was refined by demonstrating that of the major merozoite proteins carried into the erythrocyte, molecules of 34K, 46K, and 56K were glycoproteins.[15] Since virtually all of the glycoproteins which have been studied so far have been either secreted through or are found on the surface of a membrane, these proteins are

likely to be associated with the merozoite surface. Thus, these observations regarding the 34K and 46K proteins are consistent with the evidence from the surface labeling studies mentioned above.

Monoclonal Antibodies

The introduction of monoclonal antibodies against specific parasite antigens has allowed more detailed studies to be conducted than are possible with polyvalent immune sera. These reagents have specific advantages over more traditionally produced reagents. Epitopes recognized by McAbs consist of small regions within the antigen of interest. Nevertheless, these epitopes can be used to detect the antigen, even though the total size of the antigen may change due to processing or less specific events. Using panels of McAb which react with different epitopes on the same antigen, it is thus possible to map the fine structure of a molecule, including changes which may occur in specific regions of it as it is processed. However, monoclonal antibodies have the disadvantage of being so specific that they often serve as poor probes for screening genetic libraries which are expressing unprocessed fragments of gene products (i.e., cDNA libraries expressed in *E. coli*).

Monospecific Polyclonal Antibodies

Recently, efficient systems have been developed by which parasite genes, expressed during particular points in the parasite's life cycle, can be copied into cDNA and then introduced into *E. coli* and yeast. In this way, the microorganisms are directed to make the parasite protein or the fragment encoded by the portion of the gene which was cloned (see Fig. 3 for an example). This has been accomplished for plasmodial genes by inserting the gene or gene fragment into cloning vectors carrying bacterial genes which can be efficiently turned on and controlled by the investigator. For technical reasons, usually only fragments of genes have been obtained. In the two most widely used vector systems, the same bacterial gene is used. This gene (lac Z) encodes and controls the synthesis of the enzyme β-galactosidase. With the pUC8 or pUC9 vector systems, the foreign cDNA is inserted very close to the region encoding the amino terminus of β-galactosidase so that the codons for five amino acids of the bacterial enzyme are fused to the inserted parasite gene fragment.[16-17] In contrast, the λgt 11 vector system allows almost complete synthesis of the bacterial protein before beginning to make the parasite protein.[18-20] Because β-galactosidase has a molecular weight of approximately 116K, this adds considerable size to the protein product of the fused gene. Together, these systems (pUC8, pUC9, λgt11, and, in one case, the 31A pc1857vector system) have allowed the identification

through expression of clones encoding five merozoite surface proteins and one parasite protein associated with the erythrocyte membrane.[16–22] Clones containing cDNA encoding several other parasite proteins (see Geppel et al., this volume) have also been identified. For each of the molecules we will list, monospecific antibodies, either monoclonal or polyclonal, have been used in immunoprecipitation experiments to identify the protein of interest by analytical SDS-PAGE; the relative molecular weights of these proteins were determined with standard proteins. Monospecific reagents have also been used in immunofluorescence experiments, first on fixed schizonts and segmenters and, in some cases, on the external surface of merozoites visualized by fluorescence and/or electron microscopy (Fig. 4). The monospecific reagents have also been used for affinity chromatography to allow isolation of the molecules with which they react. In this way, it has been possible to appraise some of the biochemical properties of the molecules as well as assign them rigorously to the surface of either the merozoite or infected erythrocyte.

Antigens Identified by Monospecific Antibodies

Using the approach described above (monospecific antibodies, affinity chromatography, and immunoelectron microscopy), three glycoproteins, having molecular weights of 34K, 46K, and 56K, can be assigned to the merozoite surface (Table 1).[15,23–26] Of these, all appear to be found in isolates throughout the world except for gp56. The gp56 glycopeptide appears to have a lower molecular weight analogue (approxi-

TABLE 1. Merozoite Surface Glycoproteins

$M_r \times 10^{-3}$	*Stimulates Protective Immunity*	*Lipophilic*	*In Most Isolates*	*References*
gp56*	ND	+	—	Stanley *et al.* (23)
gp41, 46	ND	+	+	Howard *et al.* (26)**
	ND	ND	+	Holder and Freeman (40)**
		ND	+	Pirson and Perkins (25)**
	+	ND	+	Perrin *et al.* (32)**
	ND	+	ND	Halder *et al.* (27)**
gp34	ND	+	+	Stanley and Reese (24)
	ND	ND	+	Howard *et al.* (15)
	ND	ND	ND	Jungery *et al.* (35)

* Gp50, which is found in many isolates, may be an analogue of gp56.

** Although the molecules studied by different groups vary in M, and have not all been shown to be glycosylated, they are all likely to be the same molecule.

ND = No Data

mately 50K) which replaces this molecule in many parasite strains.[23] Another characteristic of gp56, which is both important and so far somewhat unique, is that a major portion of gp56 molecules found in schizonts are lipophilic. This suggests that at least a population of these molecules are bound to the lipid bilayer of the merozoite.[24]

A second glycoprotein, gp46, also appears to be lipophilic.[15,26,27] This molecule does not partition like a water soluble protein when schizonts and merozoites are sonicated.[26] It is probably made as a water soluble glycoprotein and then acylated to make it lipophilic.[26,27] The acylated molecules then appear to bind tightly to one or more of the merozoite lipid bilayers. Above, we have shown a protein of 46K is associated with the merozoite surface by ^{125}I labeling (Fig. 2). This molecule appears to be important in stimulating immunity.[11] Shortly, we will discuss the origin of this molecule and return to its potential immunologic importance.

There is only one major schizont protein which can be easily labeled with ^{35}S-methionine and has an M_r of 34K.[15,24] A protein of that size is glycosylated, suggesting it is membrane bound, and a 34K protein is associated with the merozoite surface by ^{125}I labeling procedures (Fig. 2).[15,24] McAbs have now been produced against a 34K molecule which have allowed it to be rigorously assigned to the merozoite surface by immunoelectron microscopy.[24] Using McAbs which see two separate epitopes on this molecule, immunofluorescence experiments have been performed in which a much weaker signal was observed than is usually seen with most of the merozoite surface proteins. This may mean that a limited amount of this protein is present on the merozoite surface, but could also reflect low affinities of the McAbs for the fixed antigen.

As with the other two merozoite surface glycoproteins described above, a major population of the 34K molecules is apparently lipophilic.[24] This suggests a tight linkage of this subset of molecules with the merozoite membrane. As with gp46, data, obtained by radiolabeling schizonts and examining ring stage parasites for radiolabeled merozoite proteins, suggest that this glycoprotein also is carried into the erythrocyte during endocytosis of the merozoite.[15]

Electron microscopic studies examining the entrance of merozoites into erythrocytes have demonstrated the surface coat being stripped off as the merozoite moves past the junction between its surface and that of the erythrocyte.[28] If the gp46 which is in the schizont-labeled ring stage is the same molecule as gp46 on the merozoite surface, then modification of this molecule appears to occur during entrance. This is based on the observation that some McAbs which bind to gp46 on the merozoite surface no longer bind to ring stage parasites.[29] Nevertheless, all three of the merozoite surface glycoproteins, gp56, gp46, and gp34, appear to be inserted tightly enough into the merozoite membrane that they are

carried in during the entrance procedure.[15] These molecules as a group may also provide the "glue" to anchor the merozoite "surface coat" to the lipid bilayer. As all three of these molecules are likely to play a critical role in maintaining the integrity of the merozoite surface and perhaps in penetration itself, they should be carefully evaluated as candidates for components of a vaccine. Their properties are summarized in Table 1.

Merozoite Surface Proteins Which are not Detectably Labeled With ^{3}H-Glucosamine

A larger group of merozoite surface proteins appears to be nonglycosylated (Table 2). All are prevalent on the merozoite surface (as judged by immunofluorescence) and are present in all of the parasite isolates which have been examined. Although some variation in molecular structure may well occur among isolates with many of these molecules, it is also apparent that some epitopes which are expressed on the surface of the merozoite are highly conserved.[24,26,40,8,16] These molecules appear to be hydrophilic so that they might easily dissociate from the merozoite surface during entrance into erythrocytes. In Figure 5, we present a diagram depicting the composition of the merozoite surface as we know it at this point.

The two largest proteins are likely to be p140 and p130.[16,17,31] Mole-

TABLE 2. Merozoite Surface Proteins Which are not Labeled with ^{3}H-Glucosamine

$M_r \times 10^{-3}$	*Stimulates Protective Immunity*	*In Most Isolates*	*Glycophorin Binding*	*Clone Available Encoding Surface Epitope*	*References*
p83* (gp185)	+?	+	ND	+	Hall *et al.* (20)
	ND	+	ND	ND	Howard *et al.* (26);
	ND	+	ND	ND	Holder and Freeman (40)
p180, p59	ND	+	ND	+	Ardeshir *et al.* (16)
p140	+	ND	ND	ND	Perrin *et al.* (32)
	ND	ND	+	ND	Jungery *et al.* (35)
	ND	+	ND	+	Ardeshir *et al.* (16)
p130, p155	ND	ND	+	+	Perkins (31); Ravetch *et al.* (17)
p75	+	ND	ND	ND	Dubois *et al.* (38)
	ND	ND	+	ND	Jungery *et al.* (35)
	ND	+	ND	+	Ardeshir *et al.* (16)

* See "Schizont/Merozoite Antigens" by R. R. Freeman and A. A. Holder, these volumes; p83 not used for immunization studies; gp185 was therefore +?.
ND = No Data

cules of approximately 130K and 140K have been found to bind to glycophorin (see Reese et al., "Changes Induced in the Surface of Erythrocytes by the Human Malarial Parasite *Plasmodium falciparum*," this volume). Two different gene fragments have been cloned, one encoding the glycophorin binding protein p130, and the other encoding a merozoite surface protein of M_r 140K whose glycophorin binding capacity is not known.[17,46] At this point there is too little information available to determine whether these molecules are the same or different.

A protein of approximately 140K has also been isolated by affinity chromatography and used to immunize squirrel monkeys. Partial protection was achieved, suggesting a potential role for this molecule as part of a vaccine.[42] A number of groups have reported molecules having molecular weights between 130K and 160K which, by immunofluorescence on fixed schizonts, produced patterns consistent with localization on the merozoite surface.[42-45] As yet, these proteins have not been rigorously assigned to the merozoite surface by immunoelectron microscopy or even by indirect immunofluorescence on intact extracellular merozoites, and their relationship to the molecules just described above is unclear.

Antibodies against another fusion protein bind parasite proteins of 180K and 59K. The antibodies also react with the merozoite surface, implying that one or both of these molecules are localized there.[16] The p180 molecule can be distinguished on SDS-PAGE from gp185, but whether they derive from the same or similar genes has not been resolved (see below).[16,26,36]

Monospecific polyclonal antibodies have also been produced against a cloned fusion protein, which is large enough to encode approximately half of a merozoite surface protein of 75K.[16,47] This 75K molecule has been localized to the merozoite surface by using monospecific reagents in immunofluorescence and immunoelectron microscopy as shown in Figure 4.[16,47] It is highly conserved among isolates from widely different regions of the world.[47] Although there is no proof, the product of the cloned gene probably corresponds to the 75K protein which was biochemically localized to the merozoite surface by ^{125}I labeling of live merozoites (Fig. 2). As mentioned earlier, immunoprecipitation experiments using sera which could efficiently inhibit *in vitro* growth of the parasite predict it is likely to be a vaccine candidate.[11,47] When molecules of this size were partially purified from blood-stage parasites and used to immunize squirrel monkeys, considerable protection was induced.[48]

Perhaps the most studied blood-stage antigens are related structurally to a glycoprotein of 185–200K.[10,11,15,20,22,26,27,30,31,36,39-42] The variance in molecular size which has been reported for this molecule appears to be a function of the specific parasite isolate from which it derives, the gel systems used, and the markers included to determine its relative

size. Gp185 constitutes one to three percent of the total ^{35}S-methionine-labeled protein made by the trophozoite or schizont. The molecule can be thought of as having two domains: one glycosylated, the other non-glycosylated. Each of these domains contains epitopes which are shared with families of smaller molecules.[26,36] The smaller molecules may be created by processing.[26,40]

Most laboratories believe gp185 itself is not on the merozoite surface.[9-12,26] Data accumulated in immunofluorescence and immunoelectron microscopy experiments with McAbs suggest that an 83K protein and glycoproteins of 46K and perhaps 40K, all of which share epitopes with gp185, are on the merozoite surface.[10-12,15,26,36] The p83 and gp46 molecules have also been associated with the merozoite surface biochemically using ^{125}I surface labeling techniques (Fig. 2).[9-12]

The gene which encodes gp185 was first cloned by Hall et al. and more recently by several other laboratories.[20,22-41,42] A structure for what is believed to be the carboxyl terminus of gp185 has been proposed based on the DNA sequence of the cloned cDNA gene fragment.[20]

Hall et al. also used affinity chromatography to purify gp185 from *in vitro*-cultivated parasites. Squirrel monkeys immunized with this antigen were found to have considerable protection against subsequent challenge with *P. falciparum*.[20] These experiments have again suggested that the epitopes carried on gp185 may be useful in inducing immunologic protection. However, we still do not know which epitopes they are.

Monoclonal antibodies have recently been produced from owl monkeys which experience a life-threatening disease very similar to that of man when exposed to *P. falciparum*.[40] Some of these McAbs detect gp185. Among these are a group which react with five different but overlapping subsets of proteins, all of which are presumably related by sharing epitopes with gp185.[40] Such McAbs, together with molecular biological techniques, should allow us to resolve what portions of this molecule are useful in stimulating protection and which may in fact "down regulate" the immune response.

In addition to parasite-induced antigens which are associated with the surface of infected cells, those proteins which are secreted from the rhoptry are also strong candidates for stimulating immunity. For a discussion of the rhoptry proteins as well as the antigens on the erythrocyte membrane, please see Reese et al., "Changes Induced in the Surface of Erythrocytes by the Human Malarial Parasite *Plasmodium falciparum*" (this volume).

Concluding Remarks

Most of the data included in this review have been accumulated during the last four years. They are a direct outgrowth of the application of new immunochemical and molecular biological tools. It is comforting to

note, however, that the early immunoprecipitation experiments shown in Figure 2, using polyclonal sera which had varying *in vitro* inhibitory capacities, were able to predict the molecular sizes of many of the molecules we now believe are likely to be important in stimulating immunity.[11,12] Because p83, p75, as well as a series of rhoptry proteins which may be important in providing protection, all migrate in the 70–85K region, it was impossible at the time, using polyclonal sera, to distinguish one from the other or evaluate their individual importance. When nonsurface-labeled material has been examined, it has been equally difficult to evaluate the origin and immunologic importance of proteins of approximately 40K–45K. Our new tools have allowed us to dissect the merozoite and schizont and identify many, if not most, of the major structural or secretory proteins. Because many of the monospecific reagents detect epitopes on several polypeptides of different sizes, most of which are likely to derive from the same gene product, it is becoming increasingly clear that the large number of polypeptides detectable after two dimensional gel electrophoresis of blood-stage proteins can be reduced to a much smaller subset of antigens.[12,13] In fact, it is very probable that the reagents already available can account for most of the immunologically important proteins.

The Future

What can we expect in the next few years? We can expect to know the amino acid sequences of most of the major parasite proteins. These will be deduced mainly from DNA sequence data, but in some cases it is likely protein sequence will be available from Edman degradation of purified parasite proteins. Such information will be particularly useful if we are to rigorously establish an amino and a carboxyl terminus for these molecules. It has just been realized that the UAA and UAG codons which are read as "stop" signals by bacteria and mammalian cells may be read by some *Protozoa* as glutamine.[14] If this is true of *Plasmodia*, then we must carefully evaluate the meaning of the codons whenever they are seen, and we must also be alert to any other possible alternatives to typical interpretation of the genetic code.

When epitopes are identified which are encoded by small peptides, it is likely that relatively good estimates of their three-dimensional structures will be achieved using a combination of peptide chemistry, physical chemical procedures, and computer modeling systems. Thus, in the next few years we should know the structures of a series of antigens which are likely to be useful in inducing immunity. We must, however, resist the temptation to use each of these compounds independently as they become available. We already know that in some cases those molecules which compose the surface of the merozoite or are inserted into the

erythrocyte membrane vary among different isolates (gp185, gp56). From drug studies, we also know the tremendous capacity of *P. falciparum* to quickly alter its sensitivity even to such compounds as chloroquine. With its capacity for rapid adaptation, the only reasonable way of attacking this parasite with a hope for success is to wait until a number of different independent antigens can be used in concert. Not only is such a vaccine likely to be more efficient initially, but it will undoubtedly take longer for resistant organisms to evolve.

Owl monkeys provide an excellent model to test the protective capacity of many of these antigens. Thus, groups of these animals should first be immunized with single antigen vaccine candidates and a separate group with a mixture of those antigens which show potential. In these tests we should look for both positive as well as potentially negative effects. Only at this point should human trials begin. Because of the newness of the approach as well as the potentially wide use of the vaccine, there is a large opportunity for problems. Too rapid a use in large numbers of humans may result in an undeserved or negative impact on not only this vaccine but the entire approach in general.

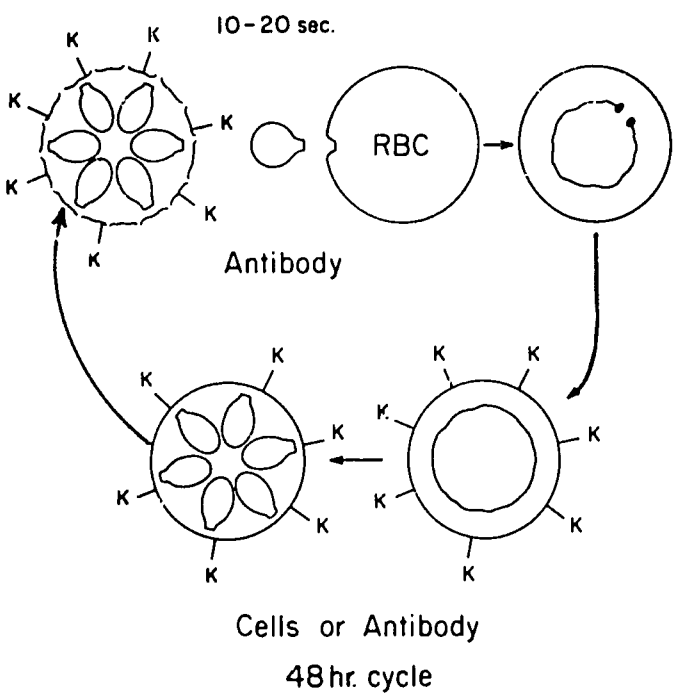

FIGURE 1. Asexual Blood Cycle of *P. falciparum* Suggesting Antibody Inhibition of Merozoite Invasion and Both Cellular and Antibody Effects During Trophozoite Through Schizont Stages.

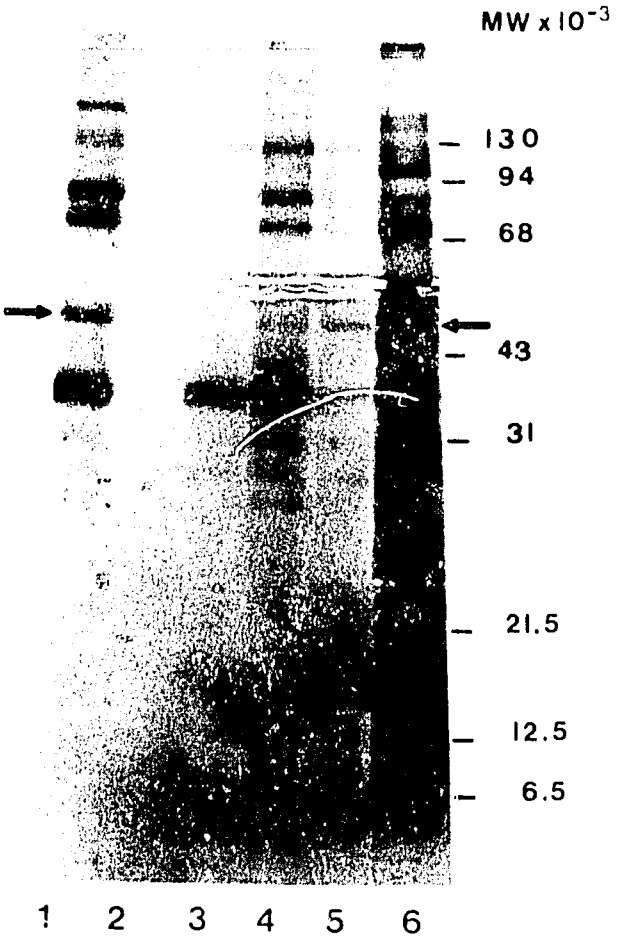

FIGURE 2. Purified Merozoites ^{125}I Labeled and Immunoprecipitated with Human and Monkey Immune Sera and then Separated by SDS-Polyacrylamide Gel Electrophoresis. Lane 1, immune owl monkey serum; > 5% growth inhibition; lane 2, human serum with 1/400 fluorescence titer to *P. falciparum* antigens but unable to inhibit *in vitro* growth of parasite; lanes 3 through 5, immune Nigerian sera, 40–60% growth inhibition; lane 6, ^{125}I-labeled purified merozoites used as antigen. Inhibition levels measured *in vitro* with 1% serum in a four-day assay.[13]

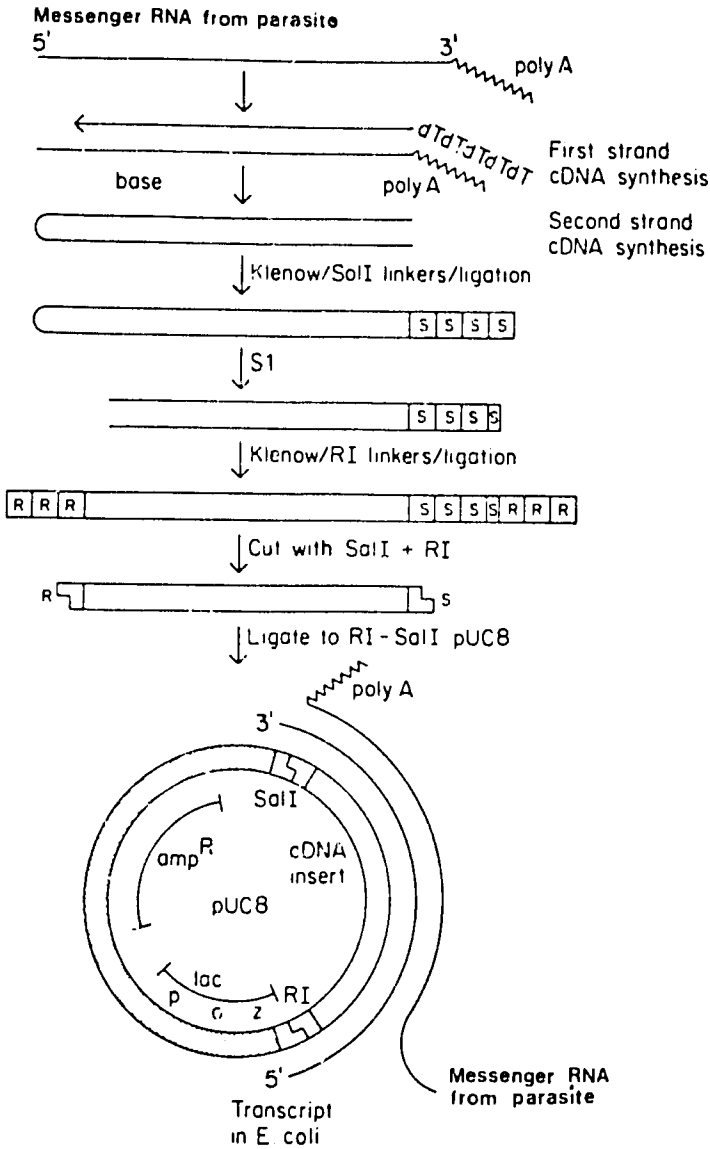

FIGURE 3. Construction of a cDNA Expression Library.

FIGURE 4. Detection of Antibodies Bound to Merozoite Surface Proteins Using Immunoelectron Microscopy.

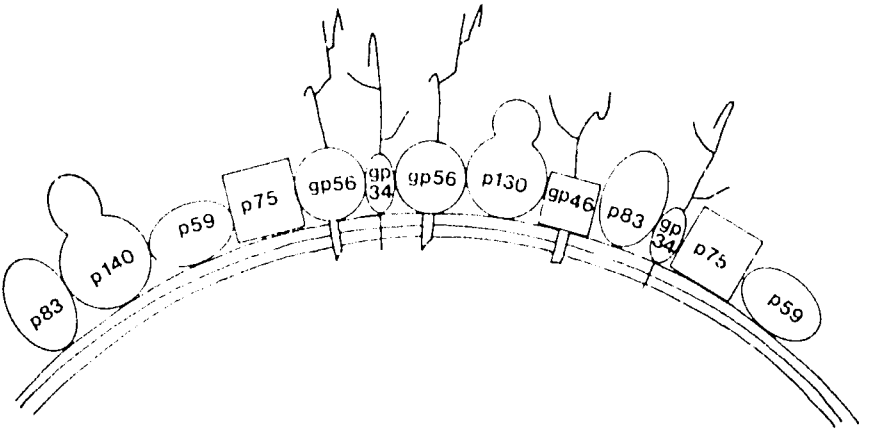

FIGURE 5. Possible Diagram of Molecules Known to Compose the Surface of *P. falciparum* Merozoites.

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Rapporteurs' Summary

RONALD KENNEDY AND KAREN YAMAGA

Dr. Victor Nussenzweig reviewed the relevant features of the circumsporozoite protein (CSP) which included the fact that Fab McAb fragments inhibited sporozoites *in vivo* and *in vitro* and that the CSP covered the entire surface of mature sporozoite. He discussed three regions of the CSP: N2 which flanked the repeats in the direction of the N-terminal; the C2 region which was situated between two pairs of cysteine residues close to the C-terminal end of the molecule; and the repeat area. The two flanking regions showed considerable conservation when compared with different Plasmodium species. Both were exposed on the molecule and were potential targets of the immune system. Antibodies raised to the C2 peptide of *P. knowlesi* did not neutralize *P. berghei* sporozoites whereas antibodies to the N2 peptide showed partial neutralization. As shown before, antibodies to the repeats did neutralize. Dr. Nussenzweig reviewed some of the properties of the repeat region: they occupied 40–50% of the molecule; the repeat sequence was more conserved at the level of amino acid than at the nucleotide level; the sequence was conserved in isolates of the same parasite species from around the world; the sequence was less conserved than the rest of the molecule. The possible function of repeats include: structural, but this seems unlikely because they varied too much; a ligand for a liver receptor; to enhance or deviate the immune response but antibodies do neutralize. The second and third possible functions were possible. It is also possible that repeats were responsible for concomitant immunity. Perhaps they were preserved to enhance the immune response to sporozoites and to prevent superinfection without affecting merozoites. If a parasite species was in equilibrium with its host, those variants bearing CSP with different repeats have a selective advantage.

A synthetic vaccine composed of the repeat sequence may be ideally suitable because it contained only three amino acids—proline is found in the bends of the sequence, and asparagine is found in beta bends. It was found that three repeats was the minimum number needed to react

completely with the McAb to the CSP. In addition all antibodies from adults or from rabbits reacted with three or more repeats. An antigen composed of three repeats coupled to tetanus toxoid in Freund's incomplete adjuvant elicited antibodies in rabbits at high titer. The rabbit antibody when absorbed with *P. falciparum* extract removed 70% of the repeat reactivity.

The arguments favoring the development of a sporozoite vaccine composed of repeats included: (1) the $(NANP)_3$ repeat was found in strains from all endemic areas of the world; (2) $(NANP)_3$ was repeated at least 10 times in each molecule; (3) McAb neutralized infectivity; (4) populations in endemic areas have a B cell repertoire capable of recognizing $(NANP)_3$. The problems were listed as well. The synthetic vaccine may not elicit sterile immunity. If it did not produce sterile immunity, it might still be useful to reduce the parasite load. It is not known if the vaccine will function with alum adjuvants. It is not known if the immune response will be boosted with natural parasites.

Dr. McCutchan reviewed four approaches to producing plasmodia antigens by new biotechnology methods. Chemical synthesis has the following advantages: it is free of biological contaminants; it is less expensive; and response can be targeted to a single region of a protein. It has the following disadvantages: appropriate carrier and adjuvant must be found, and short peptides may not accurately mimic immunodominant regions. The advantages of bacteria and yeast host vector systems are that a large insert can be incorporated and that the material produced may be very immunogenic. The disadvantages are that the cost is high; biological contaminants must be removed; and carriers and adjuvants may be required. The vaccinia system has the following disadvantages: the virus is stable; it can be administered by untrained personnel; the genome may accommodate 15–20 foreign genes; it elicits both cell mediated immunity and humoral antibody responses; and it may provide long-term protection. The disadvantages are: serious complication may occur in 1–2 per million; there may be loss of control over live vaccines; and its use may be limited in infants.

Dr. Coppel emphasized the fact that there are a large number of antigens in the asexual blood stages and that antibodies react with many antigens. However, the immune system may have more than one target to attack. Expression libraries have been generated from cDNA producing fused polypeptides or full length products or from genomic DNA using mung bean nuclease cleaved genes or sonicated genes. He reviewed the lambda gt Amp system used by his laboratory which is screened with human antisera using a colony immunoassay. Dr. Coppel reviewed the system that has been used to express *P. falciparum* antigens and noted one change. The vaccinia virus seems to express only low amounts of gene product like the lambda gt 11 system. The repetitive

sequence of *P. falciparum* seem to be a frequent feature of the antigens. Two types of repetitive sequences have been noted. Type I antigens have 4, 8, or 10 amino acid repeats in tandem (CS protein, S antigen, and histidine rich protein). Type II antigens have repeats interspersed with non-repeat sequences (RESA, FIRA, and 21K histidine rich protein). Of the more than 400 antigen positive clones, 25 yielded characterized gene products, 121 unassigned clones, and 13 of these clones have been sequenced. Of these, 12 have tandem repeats. Repeat and non-repeat fragments of RESA have been expressed and antibodies generated. Antibodies were formed against those antigen fragments containing the repeat sequences. Repeats have not been made easily in *E. coli* and have had a tendency to be unstable in bacteria. Dr. Coppel presented data that located the different genes on one of seven chromosomes found in *P. falciparum*. It was estimated using the highest expression vector possible that 5–10 people would be immunized with 1 liter of *E. coli* in broth culture medium.

Dr. Reese reviewed the surface of the merozoite and infected erythrocyte. Immune *Aotus* serum, which could inhibit the *in-vitro* growth of *P. falciparum*, had been shown by electron microscopy to contain antibodies against the surface of both the infected merozoite and schizont. These sera could passively protect other monkeys. A series of surface antigens were identified on purified ^{125}I surface labeled merozoites. Monospecific reagents, either McAb or polyclonal antibodies produced against cloned expressed fusion proteins, were then used to demonstrate first by immunofluorescence and then in several cases by immunoelectron microscopy that the merozoite surface was composed of three glycoproteins, gp56, gp46, and gp34. The sugar moities on the structures were found to be important in maintaining the appropriate conformations of these molecules. A second group of non-glycosylated molecules having molecular weights of 140K, 130K, 83K, 75K, and 59K were also shown to be on the merozoite surface. Several of these molecules have been implicated as having the capacity to bind to glycophorin and to stimulate immunity in monkeys when purified in their native states by affinity chromatography. Methods were suggested which would allow approximation of three-dimensional structures to be produced using physical techniques such as high resolution NMR and computer logarithms which will allow lowest energy states to be calculated.

Dr. Pereira da Silva presented immunogenicity studies with two fusion polypeptides (one containing a nonapeptide repeat, the other from clone 2L). When SDS treated, denatured forms were used to immunize monkeys, the antibodies were directed to the denatured forms but gave very little reactivity to the native protein. Therefore, the use of DNA recombinant gene products may not elicit antibodies that react with native protein.

B. APPLICABILITY OF INFORMATION ON MODERN VACCINES FOR THE PRODUCTION OF MALARIA VACCINES

Native Proteins for Malaria Vaccine Produced by Large-Scale *In-Vitro* Culture Systems

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The development of all types of malaria vaccines have proceeded along basically the same path. First, whole parasites and then parasite derived polypeptides have been shown to be immunogenic in small animals and then to be protective in either the *Saimiri* or *Aotus* monkey model system. The work has then expanded into identification of specific polypeptides using monoclonal antibodies or hyperimmune serum. These have then been purified and finally either sequenced and produced synthetically, as in the case of the circumsporozoite protein, or a cDNA library has been produced and inserted in a number of host cell systems. In general, the assumption has been that if the whole parasite, be it merozoite, sporozoite, gametocyte, or in the form of culture supernatent, is protective then the genetically engineered or synthesized polypeptide will likewise confer protection.

Any vaccine to be practical will have to be produced in bacteria, or yeast or else be totally synthetic. The key question will eventually be, does the polypeptide, or polypeptides, protect as well as the parasite derived "native" proteins?

There are at least two possible answers to this question; first, that the genetically engineered or synthesized polypeptide or polypeptides protect as well or better than the native proteins. On the other hand, these polypeptides may not protect at all or not as well as the native protein. If not, why not?

Let me reiterate the obvious differences between native and genetically engineered or synthesized polypeptides. First, there are numerous possible post translational modifications that may take place within the

living parasite, especially those affecting tertiary structure of the protein. These include limited proteolysis of the polypeptide, derivation of key amino acids, and addition of carbohydrate groups. These may not necessarily be duplicated in the bacteria or yeast host system and may not be reproducible in the process of peptide synthesis. These modifications may play a major part in the protective nature of the native proteins.

The importance of post translational modification was seen in developing the second generation of HbsAg vaccine currently undergoing human testing. When the viral genome fragment was placed in bacteria the resulting polypeptide did not protect as well as the native protein. When placed in yeast, however, the necessary modifications took place and the polypeptide was shown to be protective in animal systems. Whether correct post translational modifications leading to the full protective nature of the antigen in question takes place will obviously depend on the host system used as well as the unique nature of the antigen itself.

With such limitations of the new biotechnology in mind our laboratory has maintained the philosophy that at each step in vaccine development, the protection of purified or genetically engineered polypeptides must be compared directly against parasite derived antigens. Immunization studies in small animals, *Saimiri* monkeys, *Aotus* monkeys, and finally in humans using "native proteins" should precede or at least parallel studies using genetically engineered or synthetic antigens.

This has required that we develop the capacity to produce large volumes of parasite material and a methodology for isolation and purification of the crude parasite antigen using monoclonal antibodies. Our colleagues working with the circumsporozoite protein have not had available to them a means for producing sporozoites *in-vitro*. This has necessitated the rapid development of the synthetic CSP protein which they are now testing.

Methods for large-scale production of gametocytes are available and serve as the source of material for the work already described.[1-2]

In terms of the erythrocytic stages of the parasite, numerous laboratories have devised methods for the production of quantities of the parasite to meet their needs.[3-8] All these methods have been based on the original method for *in-vitro* cultivation described by Trager and Jensen.[9]

When we looked toward the prospect of producing sufficient quantities of material for full-scale immunization studies in *Aotus* monkeys and eventually a limited human trial we made the decision to undertake the development of a semi-automated culture system that would be capable of producing large amounts of crude parasite antigen.

Very briefly, the system is based on large plastic culture vessels (Fig. 1), each holding a total culture volume of 500 ml, which contains 25 ml

of packed erythrocytes. The culture medium is RPMI 1640 supplemented with 7% reconstituted human plasma. Medium is withdrawn and replaced at preset intervals automatically. The intervals between changes decrease as parasitemias rise.

Starting at approximately 1 to 2% parasitemia we obtain final parasitemias of 18 to 22% with total parasite yields of 10^{10} parasites per vessel, as shown in Table 1.

The system can handle up to 12 culture vessels. We are currently operating with only 4 vessels.

In looking toward human trials the key question will be how much material will be needed to vaccinate one human. At this point there is no data on which to base an answer. Doses of purified antigens in published experiments using *Saimiri* monkeys have ranged from 100 μg to 30 μg.[11-12] A major determining factor will be the nature of the adjuvant finally selected for use in humans.

We have arbitrarily chosen a total of 100 mg protein as a working assumption for the total amount of protein needed for a small-scale clinical trial, regardless of individual dosages. Based on that figure and using the methodology briefly described by Dr. Kan for isolating specific protein bands identified by our monoclonal antibodies 219.5 and 5.2, together with data from the immunogenicity experiment described by Dr. Siddiqui, we have made the following calculations.

In that experiment we started with 21.9 ml of saponin lysed, crude parasite antigen. This was extracted to yield 250 mg of protein which was divided into two lots of 125 mg each. One lot was used for isolation with McAb 5.2 and the other with McAb 219.5.

The yields for each were 312 μg using McAb 5.2 and 113 μg with McAb 219.5. Immunizing doses given the experimental monkeys were 80 μg of 5.2 purified antigen together with 30 μg of 219.5 purified antigen.

Each large-scale culture vessel yields 18 ml of packed, infected ery-

TABLE 1. Growth of the FUP strain of *P. falciparum* in Large-Scale (500 ml) Cultures[13]

Experiment Number	*% Parasitemia*		*Total Parasites*
	Starting	*Harvest*	*(× 10^{10})*
1	1.2	22.9	2.7
2	1.2	21.[illegible]	3.0
3	1.2	18.4	1.9
4	1.6	19.0	2.1
5	1.8	18.8	2.9
6	1.2	19.7	2.1

throcytes which when saponin lysed yields approximately 2.6 ml of crude parasite antigen. The 250 μg of extracted antigen therefore represented the production of 8 large-scale culture vessels or two weeks production with four boxes in operation. Full-scale immunization studies using a minimum of six immunized *Aotus* monkeys and the same inocula will therefore require six weeks of production.

Further immunization studies comparing genetically engineered or synthetic polypeptides against native polypeptides would require a similar amount of production time.

To produce enough material for a small human trial totaling 100 mg of 5.2 purified antigen using current methods would take approximately 1.6 months and of 219.5 purified antigen approximately 4.4 months. When expanded to 12 culture vessels the system could produce 100 mg of 5.2 purified antigen in two weeks and of 219.5 purified antigen in approximately 6 weeks.

Judging from these figures limited human trials using parasite derived antigens are feasible. We strongly believe that parallel immunization studies be carried out first in *Aotus* monkeys using both native and synthethic or genetically engineered polypeptides and that a small-scale parallel trial is feasible and should be carried out in humans.

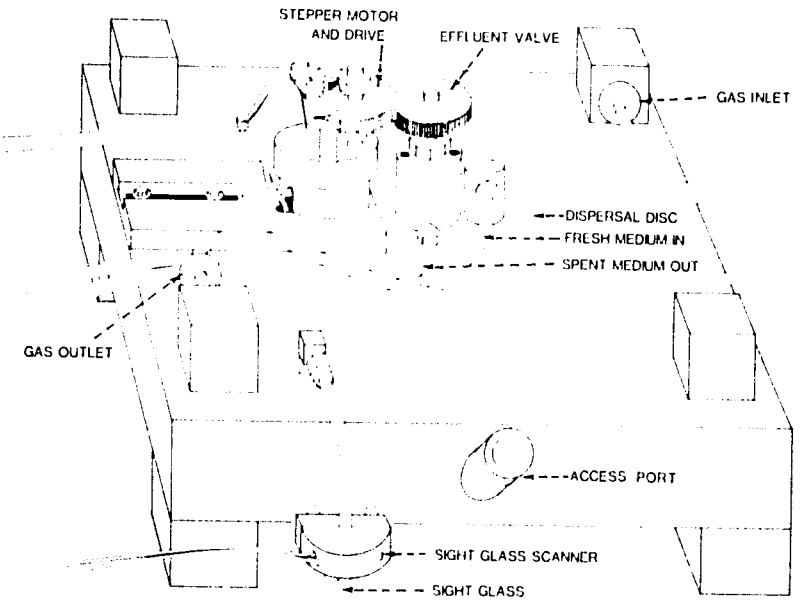

FIGURE 1. Diagram of One of the Large-Scale Plastic Culture Vessels.

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Production of Native Proteins for Malaria Vaccines

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The successful experimental immunization with *P falciparum* blood forms, crude culture supernatants, or merozoites in *Aotus trivirgatus* and in *Saimiri sciureus* monkeys and the recent availability of *P. falciparum* blood forms from continuous *in vitro* culture have encouraged research on the development of malaria vaccines for use in man. The ideal situation, as it has been pointed out by work from the laboratories of Drs. Perrin, Siddiqui, Ristic, and others, should be the use of vaccines based on defined and purified parasite antigen(s). At the moment, it remains difficult to choose and produce the best candidate antigens by DNA-recombinant technology. We feel that a pragmatic approach, for the moment, would be the use of semi-purified native antigens for preliminary field trials. Such blood stage antigens, while providing total immunity only in exceptional cases, can result in a much less severe course of infection on challenge. If total safety of these antigens can be warranted, preliminary human trials should be engaged in order to enlighten the potential of the selected blood stage candidate vaccine

After the beautiful and initial method described by Trager and Jensen, many improvements have been made by different groups. In our laboratory, we are now using large sovirel flasks which are filled with 250 ml of RPMI 1640 medium supplemented with 2 g per liter of glucose and 10% human A^+ serum. The working hematocrit is 6% and a continuous gas flow consisting of 8% CO_2, 2% O_2, and 90% N_2 is used. The medium is changed daily, and subcultures are made twice a week. Various strains have been grown, such as the Geneva I, Indochina I, FCR-3, and Uganda-Palo Alto. A yield of 220 ml per flask of crude supernatant is available each day.

Purification. Once these crude supernatants are collected, centrifuged, and filtered, antigens are purified by biochemical and immunoaffinity methods. There is now good evidence that protective antigens exist in

the crude culture supernatants. Evidence from the Illinois group under the direction of Dr. Ristic in collaboration with us was given today.

Two kinds of ion exchange chromatography were tested. With anion exchange involving QAE Sephadex, crude supernatants are concentrated 10× by ultrafiltration and then applied to the column. Protein elution and ELISA are monitored for each eluted fraction. A typical elution profile, as has been described at the Annecy Congress by Ambroise-Thomas, is shown in Figure 1. A group of fractions called "E" antigen corresponding to a specific ELISA activity were pooled, characterized, and used for monkey trials. The "E" fraction was eluted with 50 mM Tris-HCl, pH 7.6, and with a salt gradient from 0 to 500 mM NaCl.

The purification has been much more improved with cationic exchange chromatograpiny. Crude supernatants are filtered and concentrated 10×. After dialysis against 10 mM acetate buffer, pH 3.7, the supernatants are centrifuged at 3,500 rpm and filtered through 0.8 u pores. The filtered materials are then applied on a SP Trisacryl column (diapo) and proteins were eluted using 10 mM acetate buffer, pH 3.7, and a salt gradient from 200 mM to 300 mM NaCl. The peak ELISA activity was eluted at 225 mM NaCl as shown in Figure 2. This "SPE" fraction was also used in monkey trials.

Candidate antigens are also purified by immunoaffinity chromatography. Polyclonal IgG was extracted from human placentas collected in endemic areas of Western Africa and conjugated to cyanogen bromide activated sepharose. ^{35}S-methionine labeled *P. falciparum* polypeptides were adsorbed and eluded either with pH 2.5 buffer containing 10% dioxane or with 50 mM diethylamine, pH 11.5.

Quality Controls. All preparations are extensively controlled. Serologic tests for hepatitis and AIDS are conducted. Bacterial, fungal, mycoplasmal, and viral contaminations are tested for. Safety tests according to the European Pharmacopea are conducted. The purified products are inoculated into mice and guinea pigs, and tests for pyrogens are performed in rabbits. Other contaminants such as albumin, hemoglobin, blood group A and B as well as Rhesus "D" antigen are monitored. Protein is titrated and protein nitrogen quantitated. All preparations are titrated with aluminum hydroxide, which is used as the adjuvant. The preparations are compared with a standard antigen by silver staining and Western blotting with polyclonal or monoclonal antibodies.

Immunogenicity and Protection. Monkey trials have been initiated at the University of Illinois in collaboration with Dr. Ristic's group. Soluble *P. falciparum* exoantigens were used to immunize Bolivian *Saimiri sciureus* monkeys. Sera from immunized animals were monitored for antibody activity by ELISA tests and immunofluorescence. Exoantigens contained in crude culture supernatant fluids induced protective immunity against experimental falciparum malaria. The SPE and E immunogen

conferred significant clinical protection of monkeys when combined with an aluminum hydroxide adjuvant and challenge was with the homologous isolate (Fig. 3), when compared to the normal control which died.

Discussion. Do the malaria vaccines prepared with native proteins present special considerations in terms of good purity with a high degree of chemical and physical characterization? The answer in our opinion is no. The first step is to devise a production system that gives the antigens in good yield of reproducible level of purity. Conventional industrial vaccine produces are more and more used to the protective antigens being a minor component of the final product. We feel that purified preparations from naive antigens should be used in limited clinical trials just to confirm that asexual blood stage proteins are good candidates for immunization trials in humans, while waiting for DNA recombinant vaccines in the future.

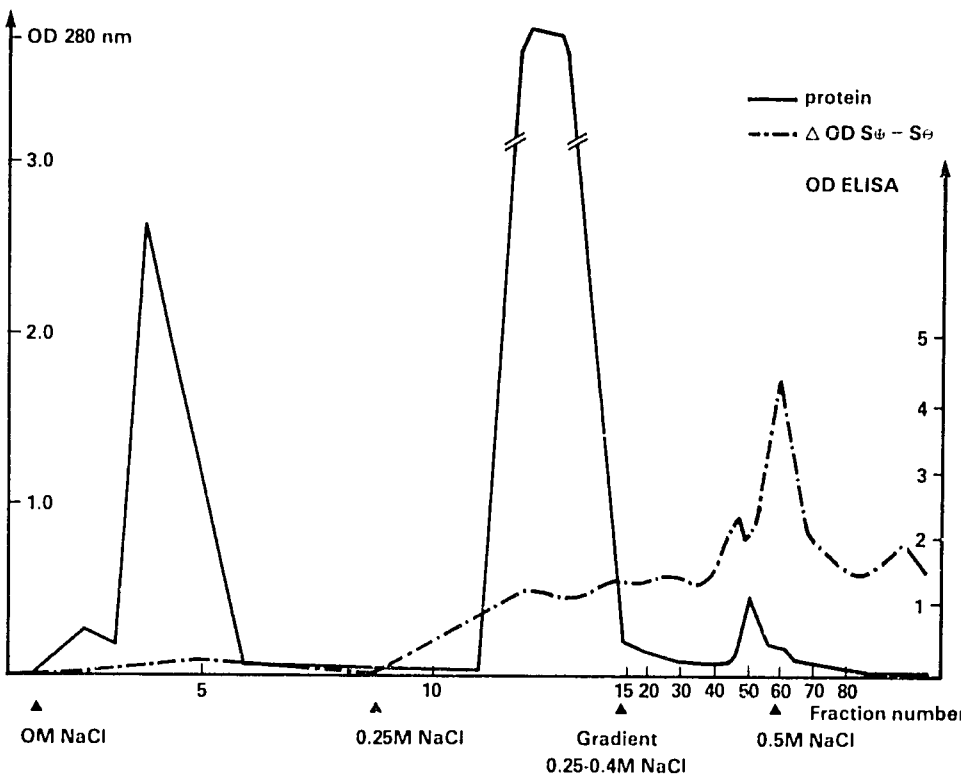
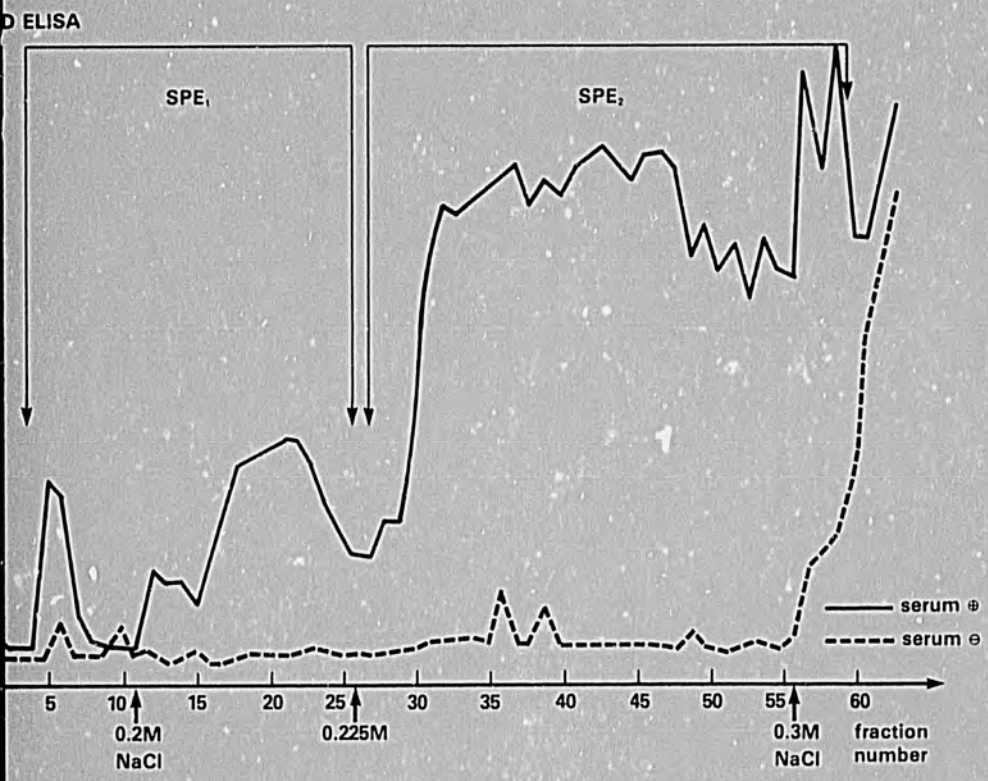


FIGURE 1. Fractionation of Crude Supernatant on Q A E Sephadex.



URE 2. Fractionation of Crude Supernatant on SP Trisacryl ELISA Activity.

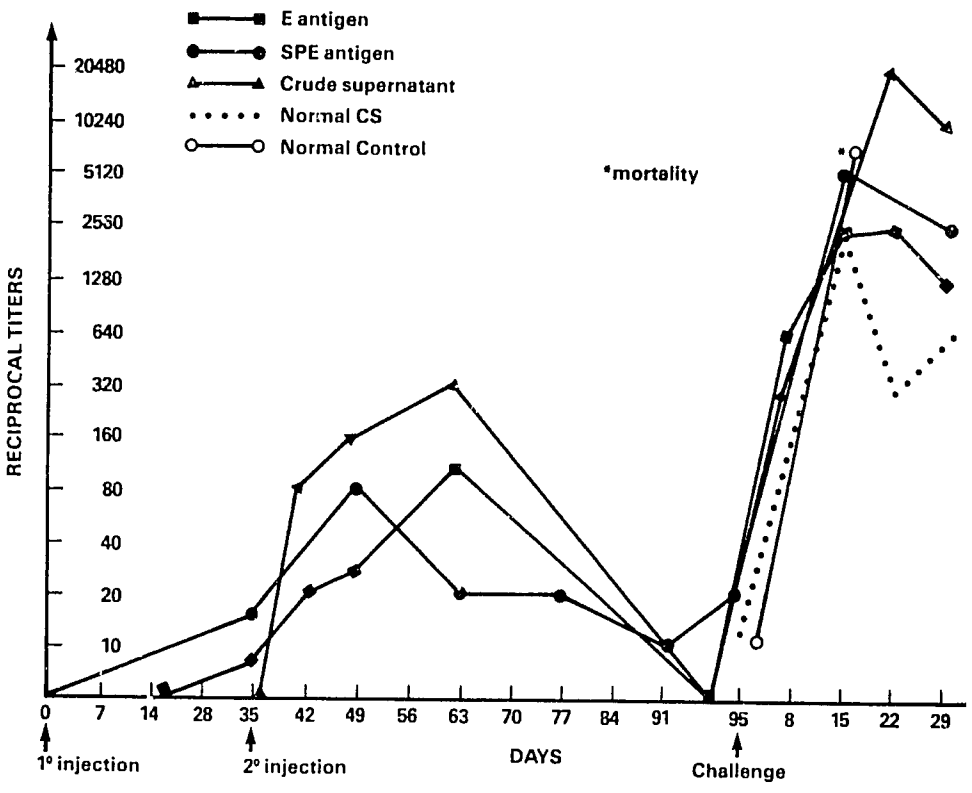


FIGURE 3. Mean IFA Titers.

Synthetic Peptide Vaccines, Carriers, and Adjuvants

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The major etiologic agent of malaria in the human host has been identified to be *Plasmodium falciparum*. Its wide distribution in tropical countries presents a major world health problem. Therefore, the need for an effective economical vaccine to control the current global resurgence of malaria is readily apparent. To facilitate the development of a vaccine, the identification and characterization of antigens associated with this complex parasite is paramount. Current ongoing investigations in this area have been summarized in the preceding chapters of this book.

Major biotechnologic breakthroughs of the past several years have facilitated the development of several methods to produce possible economically effective vaccines. These include: 1) purified subunits derived from the parasite grown *in vitro;* 2) parasite proteins derived from prokaryotic cells in which a specifically defined segment of DNA has been cloned; 3) expression of parasite antigens by genes cloned into a live virus vaccine; 4) mirror image based anti-idiotype reagents; and 5) synthetic peptide vaccines. The latter approach has recently attracted attention for virus vaccines and is the focus of this presentation.

The first task in developing a synthetic peptide vaccine is to determine the primary amino acid structure of the defined malarial proteins. Fortunately this data can be rapidly provided by the development of gene cloning techniques. More important, an antigenic site(s) on the given protein must be identified. These sites are distributed on the surface of the molecule usually at certain turns of the polypeptides.[1] In this regard, Atassi and Smith described two types of antigenic sites, continuous and discontinuous.[2] According to their classification, a continuous site consists of a sequence of residues defined by the primary structure. In contrast, a discontinuous site is composed of residues that are distant from others in the polypeptide structures but that are juxtaposed in three-dimensional space. The antigenic sites of a number of proteins

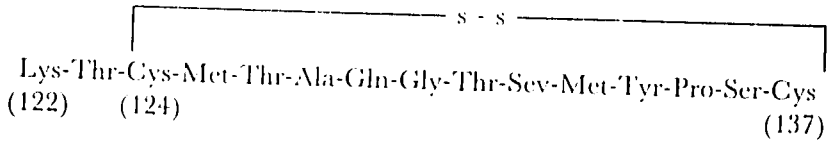
with known tertiary structure have been determined. As an illustration of the above definitions it has been shown that sperm whale myoglobin has both continuous and discontinuous antigenic sites.[8,9] All of the seven sites mapped for the protein, five continuous and two discontinuous, coincide with turns in the molecule.[1]

Synthetic peptide analogues with possible vaccine potential for a large number of infectious agents have been described. In the first part of this presentation, a series of experimental observations in reference to the ongoing work with the lipoprotein surface subunits of hepatitis B virus (HBV) will be summarized. In the closing parts of this presentation, an attempt will be made to apply the experience gained in the ongoing research involving HBV synthetic peptide vaccines towards the development of an effective synthetic peptide vaccine for malaria.

It has been shown that antibodies against hepatitis B surface antigen (anti-HBs and HBsAg, respectively), the surface or envelope material of HBV, are protective against infection. Formalin-inactivated HBV subunit vaccines have been produced and licensed that utilize HBsAg derived from the plasma of healthy persons chronically infected with HBV.[6,7] Compared with more conventional vaccines such as polio or measles, the hepatitis vaccine is unique in that HBV cannot be grown in tissue culture. The immunizing antigen consists of noninfectious, 22-nm subunit particles of HBsAg, formulated in an alum adjuvant. This vaccine has undergone extensive field trials devised to evaluate its safety, immunogenicity, and efficacy (for review, see 8).

Three major developments opened the way for the production of HBsAg synthetic peptides as putative vaccine candidates. First, my laboratory demonstrated that the major HBsAg polypeptides derived from HBsAg, P25 and GP30, contain an antigenic determinant capable of eliciting protective antibodies in susceptible chimpanzees.[9] Second, the cloning of the HBV DNA genome in bacterial and eukaryotic cells made it possible to establish the amino acid sequence of P25.[10] Third, my laboratories and two additional groups reported the antigenic sites of HBsAg were dependent on conformation secured by disulfide bonds.[11-13] Therefore, the major antigenic site(s) of epitope(s) of HBsAg can be classified as discontinuous.

We selected for synthesis residues 122–137 from the reported amino acid sequence for HBsAg P25 and confirmed by sequence data reported by Peterson for HBsAg/*ayw*.[14]



This region was originally selected for synthesis because: (1) computer analysis of the complete amino acid sequence of P25 indicated that these amino acid residues comprised a hydrophilic region of the polypeptide containing two potential β turns;[15] (2) the predicted secondary structures were distinct in this region for three published sequences for P25;[14,16-18] (3) the amino acid sequences in this area had a relatively high degree of amino acid variation for different HBsAg subtypes, thus indicating a potential antigenic hot spot;[10-19] (4) this sequence contained numerous cysteine residues and previous studies indicated that intact disulfide bonds were necessary for the maintenance of antigenic integrity;[11-13] and; (5) a cyclic disulfide could be formed between Cys_{124} and Cys_{137}. The peptide 122–137 was synthesized and cyclized by introduction of a disulfide bond between the cysteine residues at amino acid positions 124 and 137. Thus, by locking the conformation of the potential β turns at 129–132 and 135–137, this peptide theoretically should mimic the native structure of HBsAg.[10]

The observations noted in my laboratory have established several important parameters in the development of a synthetic peptide based HBV vaccine. We have clearly shown that our cyclic peptide can induce an antibody response that has specificity for native HBsAg.[15] Based on studies using a number of different methods for antigen presentation, it appears that the peptide must either be coupled to a protein carrier or be aggregated with liposomes or micelles.[20] In the latter case, the immunogenicity of the micelles was significantly enhanced following adsorption to alum. It was of note that 35 days after a second injection, the anti-HBs titers were essentially the same in groups of mice inoculated with either native HBsAg or peptide 1 conjugated to tetanus toxoid. The antibody titers were similar when the anti-peptide sera were tested against *ayw*, *adw*, and *adr* subtypes of HBsAg. This indicated that the immune response was directed against the common group *a* specificity of HBsAg.

Our latest observation in reference to the utilization of dimethyl dioctadecyl ammonium bromide (DDA) is of particular interest. DDA can be used to induce cell mediated immunity (CMI) while priming the T-cell dependent antibody response and has been shown to be an effective potentiator of the immune response to peptides and haptens.[21-23] A vigorous anti-HBs response was elicited in mice by incorporation of free cyclic peptide with DDA and alum.[25] Hopefully, adjuvant vehicles such as DDA or other synthetic adjuvants will be proven to have the potential to enhance the immunogenicity of synthetic peptides. In this regard, it should be noted that Hopp demonstrated a significant enhancement of the anti-HBs response when rabbits were immunized with a HBsAg synthetic peptide conjugated dipalmityl-lysine moiety.[24] The peptide

utilized in this study contained amino residues 138–149 and induced a 27-fold or greater anti-HBs response than had been reported by a number of other workers studying either this or overlapping HBsAg peptide sequences which had been conjugated to a variety of protein carriers. Unlike the covalently linked dipalmityl-lysine conjugated moiety, DDA was not covalently attached to peptide 1 in our experiments.[25]

The above observations suggested that cyclic peptide 1 contained an *a* cross-reactive determinant of HBsAg. The next series of experiments were designed to confirm these observations and more fully define the epitopes (antigenic determinants) associated with peptide 1. This was performed by using a panel of monoclonal anti-HBs reagents. Peptide 1 was analyzed for its potential capacity to inhibit the reaction of 14 individual monoclonals with anti-*a* antibody specificity.[26] Preincubation of cyclic peptide with six monoclonal anti-*a* preparations inhibited subsequent reaction with intact HBsAg. This inhibition was not observed by preincubation of the same monoclonal antibodies with linear peptides preparations. However, the reactivity of the eight remaining anti-*a* hybridomas binding to HBsAg was unaltered in the presence of peptide 1 as no inhibition was observed. This study demonstrates that the group *a* specificity associated with HBsAg contains two or more distinct antigenic determinants and that peptide 1 contains one of these epitopes. In addition, a continuous *y* epitope was also associated with peptide 1 in that three anti-*y* monoclonal antibodies reacted with both cyclic and linear forms of peptide 1.[26]

A tool that could aid in determining the ability of peptide 1 to elicit a protective antibody response resides with the idiotypic specificity of the anti-HBs antibodies produced by naturally HBV-infected humans. We had previously demonstrated that humans infected with HBV generate anti-HBs which contains a common idiotype (CId).[27] The CId was found to be associated with the group *a* determinant(s) in that purified 22-nm particles of *adw*, *ayw*, and *adr* serotypes inhibited the CId-anti-idiotype reaction to the same degree.[28] Of particular importance to this study was that peptide 1 efficiently inhibited the reaction. The major role of conformation was emphasized in that reduction of the intrachain disulfide bond (Cys between residues 124 and 137) and alkylation of the free thiol groups destroyed the capacity of peptide 1 to block the CId-anti-idiotype reaction.[29]

A series of linear synthetic peptides containing amino acids of the P25 HBsAg polypeptide have also been described by other laboratories.[1] Each has succeeded in raising positive, albeit low, anti-HBs responses in animals immunized with each respective synthetic peptide conjugated to either protein carriers or to red blood cells. These reports illustrate that a number of HBsAg epitopes appear to be associated with the amino acid sequence 110–155 of P25. This is in keeping with the obser-

vation that only about 50% of our panel of murine monoclonal antibodies with anti-*a* group reactivity reacted with cyclic peptide 1.[26] In a recent study, Brown et al. reported that human antibodies reacted avidly with a cyclic peptide containing amino acids 139–147.[30] Therefore, a second *a* epitope appears to be associated with this region of P25. Milich et al. studied yet another set of peptides containing amino acids 95–109 and 47–52 of P25.[31] These investigators noted that their synthetic peptide preparations reacted preferentially with T-cells. In this regard, we have shown that anti-HBs activity can be boosted with cyclic peptide 1–tetanus toxoid conjugate in a chimpanzee which has preexisting anti-HBs activity (Dreesman, Hollinger, and Burk, unpublished data). However, this increase in antibody activity was not obtained until a second inoculation of peptide conjugate was administered. Analysis of the antibody from the second inoculation showed that it contained both IgM and IgG anti-HBs. This emphasizes the role of the carrier effect originally described by Mitchison.[31] It appears that the epitope responsible for the induction of T-helper cells required for the generation of an anmestic response was not associated with cyclic peptide 1. Therefore, no cross recognition of the carrier afforded by native HBsAg and tetanus toxoid would be expected by the T-helper cells.

The ongoing molecular and immunologic studies with malaria antigens that were summarized during this symposium clearly indicate that the synthetic peptides offer a viable alternative for development of an effective vaccine. It has been shown that irradiated sporozoites are an effective immunogen that induce protective immunity.[33–35] This protection induced by immunization with sporozoites has been correlated with a surface protein referred to as circumsporozoite (CS) protein.[36–40] The synthetic peptide vaccine approach has recently been facilitated when Dame et al. cloned and then sequenced the complete CS gene of the human malaria parasite *Plasmodium falciparum*.[41] The CS protein is composed of 412 amino acids containing a large center domain comprising of 41 tandem repeats of a tetrapeptide. Thirty-seven of these repeats are the sequence Asn-Ala-Asn-Pro and four are Asn-Val-Asp-Pro.

For illustration purposes, we have analyzed the reported amino acid sequence for CS protein into a modified computer algorithm in an attempt to predict possible antigenic determinants.[42] A plot of the average of the hydrophilic values of each residue of the CS protein is shown in Figure 1. Three hydrophilic areas were noted encompassing residues 80–120, 320 to 340, and 360–390, respectively, similar to those areas reported by Dame et al.[41] The maximum hydrophilic index was noted at residue 95. The data indicate that these regions are likely to be exposed on the surface of the CS protein. It is of note that the tandem repeat area appears to be neutral in regard to hydrophilic or hydrophobic properties.

The possible secondary structure of the CS protein as determined by the Chou-Fasman predictive scheme is depicted in Figure 2.[44] The tandem repetitive sequence appears to possess primarily beta structure, although a low probability β turn potential is revealed by the presence of a proline every fourth residue. This region suggestively contains a large amount of secondary structure. The hydrophilicity index indicates that the overall repetitive sequence is neither hydrophilic nor hydrophobic in nature, except for a small hydrophilic peak associated with repeat of four aspartic acid residues. This region represents an area where a specific amino acid substitution would be expected to alter greatly the predicted hydrophilic index and secondary structure potential. Three regions each, on either side of the repetitive sequence, possess highly predicted β turn potential, along with hydrophilicity indices indicative of a highly charged area. These include residues 9[illegible], 101, and 110 and again 371, 391, and 399, respectively. Based on these considerations, the regions between residues 90 and 112 and 368 to 400 appear to represent likely antigenic determinants of CS protein.

Two reports have recently appeared that indicate synthetic peptides, which contain the tandem repeat of CS protein, represent good immunogens. In this regard, Victor Nussenzweig and his colleagues have shown that a synthetic dodecapeptide representing a tandemly repeated immunogenic region of *Plasmodium knowlesi* CS protein contained immunologic reactivity similar to that of the native CS protein.[45] In addition, Dr. Nussenzweig's presentation at this symposium indicated that experimental animals injected with this peptide produce an anti-native CS protein analogous to a human immune response (see Session II). During the preparation of the manuscript, Ballou et al. reported that antibody to peptides from the repeating region of *Plasmodium falciparum* CS protein recognized native CS protein and neutralized the sporozoite invasion of human hepatoma cells.[46]

These recent reports are highly encouraging. However, the efficacy of a synthetic peptide vaccine must await results of human clinical trials. To establish a long-lived effective immune response, a complete vaccine may have to incorporate a number of defined regions of the CS proteins. In this regard, continued research with additional peptides may be warranted. Possibly regions predicted to contain antigenic determinants by computer algorithms may be helpful in developing a long lasting protective immunity. Certainly, as has been pointed out by our experience with HBV, it would be advisable to examine both B- and T-cell immunogens before a final synthetic peptide or combination of peptides is formulated.

In closing, we believe that ongoing studies with synthetic peptide vaccines indicate that the potential of this approach for vaccine development can be viewed optimistically. However, several important areas

must be addressed before the full potential of the approach is realized. It is apparent that both the amino acid composition and three-dimensional structure of the protective epitopes must be defined. In addition, other areas that must be investigated include adjuvant vehicles acceptable for human usage, and epitopes that elicit both T- and B-cell responses must be identified. Again, these areas are under active investigation, and we are confident that effective, safe synthetic vaccines will be available in the future.

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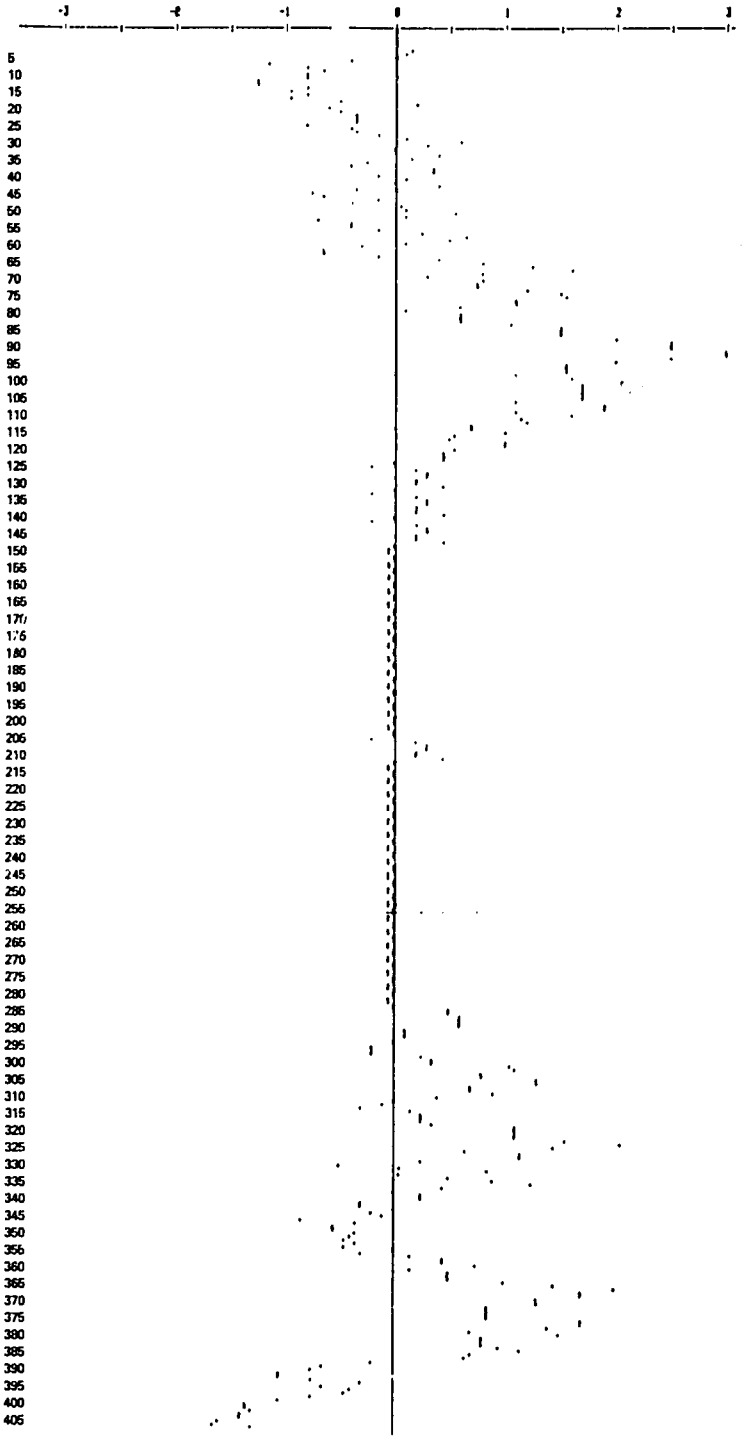

FIGURE 1. The predicted hydrophilicity profile of the CS protein is shown. This prediction was determined by a modified Hopp-Woods algorithm that was generated in this laboratory.[42,43]

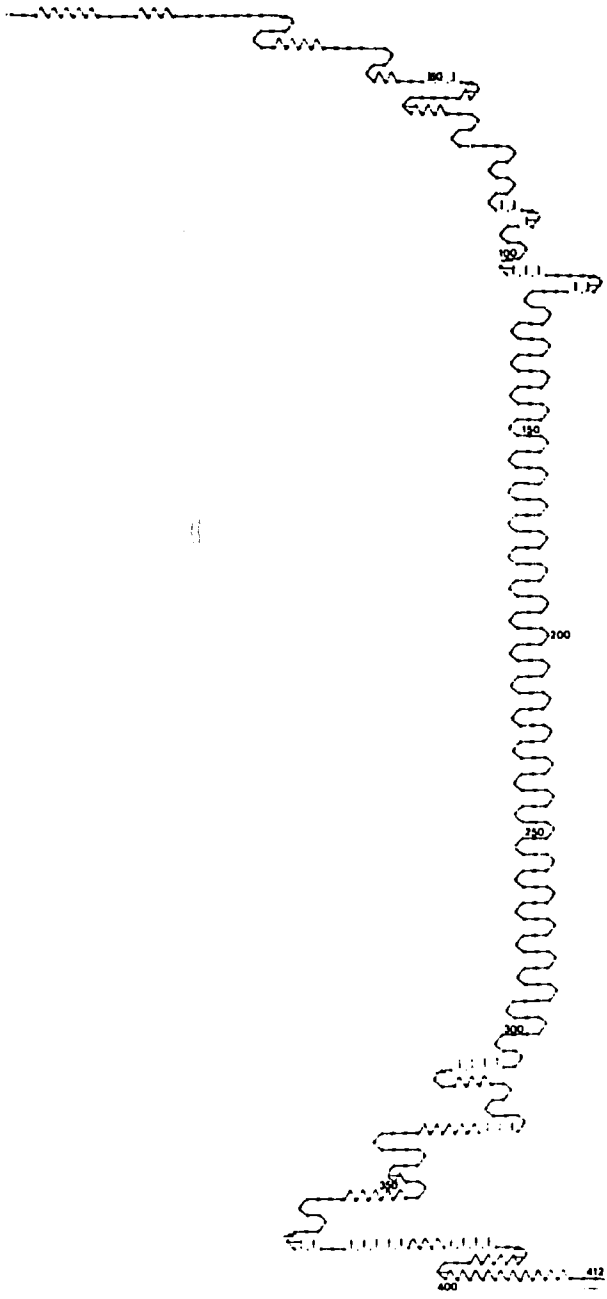

FIGURE 2. The predicted secondary structure of the CS protein as determined by a computer program developed by Chou-Fasman is depicted.[45] The following figures represent the potential secondary structure: alpha helix ( ); beta sheet (∧); beta turn (<); highly predicted beta turn ( ); and no secondary structure predicted (—). Each dot represents one amino acid and the numbers indicate amino acid positions every 50 residues.

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New Trends in Vaccine Development: Anti-Idiotypic Antibodies

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The theoretical considerations for the use of anti-idiotypic antibodies (anti-Id) as regulators of the immune response reside with the idiotype network theory initially proposed by Jerne.[1] In simplified terms, this theory states that the immune response to a given antigen can be regulated by a series of idiotype (Id)-anti-Id reactions. Id, that are located on or close to the antigen receptors for B and T lymphocytes, along with homologous anti-Id induced by the Id, represent components of this regulatory network. Numerous investigators have demonstrated that anti-Id injection can have either a positive (enhancing) or negative (suppressing) effect on the immune response to a wide variety of antigens.[2,3] Anti-Id pretreatment prior to antigenic stimulation that results in suppression of the immune response obviously has no role in vaccine development. However, anti-Id that is capable of inducing a positive (enhancing) effect on the immune response to antigens associated with infectious agents has the potential of priming the host prior to infectious challenge. It is the anti-Id which produces a positive effect on the immune response that will be the focus of this review.

The injection of a host with Id antibodies (Ab-1) under certain conditions will result in the production of an anti-Id. Similarly, injection of an anti-Id (Ab-2) can result in the generation of an anti-anti-Id (Ab-3) response. In certain cases, this anti-anti-Id has the capacity to bind the initial antigen involved in producing Ab-1. Therefore Ab-3 mimics Ab-1 in its ability to bind antigen. Alternatively, Ab-1 has the capacity to bind both antigen and anti-Id (Ab-2) at sites on Ab-1 referred to in immunological jargon as paratope and idiotope, respectively. If the paratope and idiotope are the same site, then the anti-Id bears the internal or mirror image of the antigen via their complementarity for binding to Ab-1. It was this internal image nature of anti-Id that was the basis of the possible application of anti-Id to vaccine production as proposed by Nisonoff and Lamoyi.[4] A possible application for this internal

image based antibody vaccine was discussed initially for the etiologic agent of malaria. An anti-Id which bears an internal image population that mimics the antigens of an infectious agent may be useful as a vaccine since that anti-Id can induce an immune response (Ab-3) similar to that induced by the antigen (Ab-1) (see Fig. 1).

Studies involving the use of anti-Id as vaccines against pathogens are summarized in Table 1. Each of these different systems will be briefly described with regard to the potential of anti-Id as an approach for vaccination.

The first report of anti-Id induced protection was in Balb/c mice that had been inoculated with a lethal dose of *Trypanosoma*, the causative agent of African sleeping sickness.[5] Anti-Id, produced against a mouse monoclonal antibody that neutralized *Trypanosoma in vitro*, induced protective immunity. The mechanism of this anti-Id induced immunity was not determined and subsequent studies demonstrated that the anti-Id protection was restricted to certain inbred strains of mice.[6] This restriction found with anti-Id immunity against *Trypanosoma* raised concerns about the potential use of an anti-Id based vaccine in genetically outbred populations, such as humans. However, the anti-Id preparation used in these studies did not appear to contain any internal image com-

TABLE 1. Studies involving the potential use of anti-idiotypes as vaccines against infectious agents

System	*Authors*	*References*
Trypanosoma surface glycoprotein	Sacks and coworkers	5, 6
Hepatitis B surface antigen	Kennedy and Dreesman	8, 11, 13
Rabies virus surface glycoprotein	Reagan, Koprowski and coworkers	23
Reovirus hemagglatinin	Greene, Fields and coworkers	19, 20
Sendai virus T cell clone	Ertl and Finberg	21, 22
Phosphorylcholine of *Streptococcus*	Kohler, McNamara and coworkers	24
Polysaccharide of *E. coli*	Stein and Soderstrom	25
Poliovirus VP1	Uytdehaag and Osterhaus	26
Listeria monocytogenes	Kaufmann, Eichmann and coworkers	27
Herpes simplex virus	Gell and Moss	28

ponents. This was based on the observation that injection of anti-Id without subsequent antigen exposure failed to produce any detectable anti-*Trypanosoma* response in mice. Thus, the anti-Id preparation failed to mimic the antigens of *Trypanosoma* and induce an Ab-3 response that was capable of binding to the organism.

Internal Image Anti-Id Mimics Hepatitis B Virus Surface Antigens

Our laboratories have been involved with the characterization of Id associated with human antibodies to hepatitis B surface antigen (anti-HBs).[7-14,22] Hepatitis B surface antigen (HBsAg) is the envelope material of hepatitis B virus (HBV) and is produced in an overwhelming excess by infected hepatocytes during the course of infection. Anti-HBs is responsible for protection against HBV, and serologically the group-specific determinant of HBsAg, termed the *a* determinant(s), induces this protective immunity.[15] Previously, we described a common human idiotype present on anti-HBs from individuals infected with HBV.[7] The determinants involved in the induction of this anti-HBs Id have also been characterized and the Id-anti-Id reaction recognizes an interspecies Id produced by active immunization with HBsAg.[9,10,12] The anti-Id preparation appeared to contain an internal image bearing population that recognized a specified three-dimensional conformation on the anti-HBs molecules expressing the interspecies Id. This conformation in the antibody combining site of Ab-1 is induced by HBsAg and can be detected by an anti-Id reagent that has complementarity to HBsAg.

Injection of anti-Id prior to antigenic challenge with either HBsAg or a cyclic synthetic HBsAg peptide has significantly enhanced the anti-HBs response in Balb/c mice.[11-14] These data indicated that anti-Id reagents could prime the immune response to HBV antigens. Alternatively, mice that were injected with anti-Id alone produced anti-HBs.[8,13] This anti-Id induced anti-HBs recognized the group specific *a* determinant of HBsAg and also expressed the interspecies Id. Thus, injection of anti-Id without subsequent HBsAg exposure produced an antibody response that appeared serologically similar to that induced by injecting HBsAg. These data confirmed that the anti-Id preparation contains an internal image population that mimics HBsAg. The potential use of anti-Id as a vaccine for HBV infection was demonstrated by the fact that anti-Id immunization alone induced an anti-HBs response that shares an idiotype expressed in a human anti-HBs response that resulted from an HBV infection. This anti-HBs response protects these individuals from subsequent reinfection with HBV. The specificity of the anti-HBs response produced by anti-Id injection was also directed

against the *a* determinant(s) on HBsAg which is responsible for inducing protective immunity against HBV.

Recently, we have extended the findings in Balb/c mice and have induced an anti-HBs response in both rabbits and chimpanzees by injecting anti-Id (Kennedy et al., submitted for publication). These data were consistent with the fact that internal image bearing anti-Id do not appear to be restricted with regard to inducing an interspecies anti-HBs response. Therefore, restrictions similar to those discussed in the *Trypanosoma* system may not be a limitation of anti-Id based vaccines. In addition, chimpanzees are the only nonhuman primate that can be infected with human HBV. We are testing the efficacy of an anti-Id based vaccine by challenging the chimpanzees that produced an anti-Id induced anti-HBs response with infectious HBV. It is noteworthy that the two chimpanzees received milligram quantities of affinity purified rabbit anti-Id in four sequential injections over a period of 16 weeks and no deleterious effects associated with immunization of heterologous serum proteins were observed.

Anti-Id as Potential Vaccines Against Other Infectious Agents

Anti-Id reagents have been prepared and characterized for a number of additional infectious agents. In an earlier study, anti-Id were raised against mouse antibodies specific for the hemagglutinin of reovirus, a virus capable of producing a fatal encephalitis in susceptible hosts.[16,17] These anti-Id were used to characterize the receptor for reovirus on susceptible host cells and on T lymphocytes.[18] In addition, the anti-Id preparations could be used to block reovirus infection by competitively binding to cellular receptors.[19] The vaccine potential of this mouse monoclonal anti-Id was demonstrated by its ability to induce cellular immunity to reovirus in Balb/c mice.[20]

Another study utilized a mouse monoclonal anti-Id produced against a Sendai virus specific helper T-cell clone.[21] This anti-Id was capable of inducing in mice an anti-Sendai virus immune response *in vivo*. It was of interest that the T-cell immunity induced by the anti-Id appears to be less genetically restricted to the murine major histocompatibility complex than was Sendai virus antigen-specific T-cell immunity.[22] The vaccine potential of the anti-Id was demonstrated by its ability to produce cellular immunity capable of protecting mice against a lethal infection with Sendai virus.

Studies have also demonstrated that anti-Id can induce viral neutralizing antibodies against rabies virus glycoproteins.[23] These neutralizing antibodies were induced by anti-Id injection without subsequent antigen exposure, suggesting that the anti-Id may mimic the rabies virus glycoprotein. However, the antibody titers produced by anti-Id injec-

tion were not sufficient to protect mice against a lethal intracerebral challenge of rabies virus. Although these observations suggest a potential for anti-Id vaccines for rabies virus, further studies are required to increase the immunogenicity of the anti-Id preparations.

Monoclonal anti-Id coupled to a carrier protein was also used to immunize mice against a lethal infection with *Streptococcus pneumoniae.*[24] This monoclonal anti-Id was capable of significantly increasing the resistance of Balb/c mice to a lethal challenge of *Streptococcus*. Antibodies against phosphorylcholine (PC) determinants protected mice against a lethal *Streptococcus pneumoniae* infection. The demonstration that an anti-Id can be used as an antigen substitute for the induction of protective immunity again suggested its potential use as a vaccine. An important point to be emphasized is that a carrier protein may be required for correct presentation of the anti-Id to B cells since mice immunized with the anti-Id alone failed to demonstrate a statistically significant protection upon *Streptococcus* challenge, even though an anti-PC response was produced. This study reinforces the possible role of T cells in the presentation of the anti-Id to B cells for the induction of protective immunity.

Studies have also shown that either Id or anti-Id can prime for protection against another bacterial infection.[25] The ability to respond to the capsular polysaccharides (Ps) of pathogenic bacteria does not develop until late in ontogeny. Thus, newborns or neonates are extremely susceptible to these pathogenic bacteria because they fail to produce a protective anti-Ps response. Anti-Ps specific for *Escherichia coli* or anti-Id raised against the anti-Ps were capable for priming neonatal mice, such that a significantly enhanced protection from challenge with a lethal dose of these bacteria was obtained compared with control groups of mice. This study indicated a role for Id or anti-Id administration early in life by providing immediate passive protection as well as priming for protective antibodies upon subsequent antigen exposure.

A neutralizing antibody to poliovirus was induced by injecting anti-Id into mice.[26] Monoclonal anti-Id was raised against an Id specific for a neutralizing site on type II poliovirus. This anti-Id detected an interspecies Id present on anti-poliovirus antibodies produced in rats, guinea pigs, and humans similar to that described with the anti-Id to anti-HBs. Although the anti-Id was capable of inducing polio neutralizing antibodies, it failed to protect Balb/c mice against a lethal challenge with poliovirus type II. These findings suggest that the anti-Id represents an internal image antibody which mimics a neutralizing epitope on poliovirus. Therefore anti-Id can again be used as an antigen substitute for the induction of an immune response. Recently, a clonotypic antiserum (anti-Id produced against specific T-cell clones) was used to protect mice from a lethal infection with *Listeria monocytogenes* and anti-Id has also been reported to induce a cell mediated response in mice to herpes simplex virus.[27,28]

The studies described herein give several experimental examples which indicate that anti-Id reagents represent a viable alternative for a vaccine. A potential advantage of an anti-Id based vaccine is where newborns or neonates are immunologically incompetent in their ability to respond to a currently licensed vaccine. Neonatal mice failed to produce an anti-Ps response to Ps vaccination. However, these mice would produce an anti-Ps response if primed with Id or anti-Id prior to vaccination. Two other possibilities where anti-Id represent viable vaccine candidates are situations where there is difficulty in obtaining adequate amounts of purified, safe antigen or when a conventional attenuated living vaccine has a high propensity for reverting to a virulent form. Theoretically, internal image anti-Id could be generated that mimic the antigens of the infectious agent and induce protective immunity in a susceptible host. Alternatively, anti-Id could also be produced that mimic tumor antigens, whereby a potential vaccine against specific cancers could be generated. Other possibilities where an anti-Id vaccine may be advantageous are when killed vaccines contain contaminating products that could be injurious to a nonimmune host or when immunity to a single epitope on an infectious agent is adequate for inducing protection. This is especially true when either the contaminating products or the infectious agent itself contains antigenic determinants in common with host tissue or body components. A vaccine that either contains these contaminants or is directed to the entire organism which shares determinants with the host could produce a deleterious autoimmune response. An anti-Id based vaccine would induce immunity against only a single epitope present on the infectious agent and bypass potential allergic reactions or autoimmune disease. A pool of anti-Id reagents could then be used to induce immunity against several epitopes.

Although internal image bearing anti-Id appear to represent a viable alternative for vaccines in certain situations, there still may be problems associated with multiple injections of a heterologous antibody preparation into humans. Multiple injections may decrease the effectiveness of the anti-Id vaccination or produce deleterious side effects resulting from allergic reactions or immune complex formation. It is noteworthy that four injections of a rabbit anti-Id were used to induce an anti-HBs response in chimpanzees and no untoward side effects to date have been observed in these primates due to a potential heterologous anti-globulin response. Therefore, the use of anti-Id prepared in a heterologous species for human vaccination may not represent a limitation of anti-Id based vaccines, especially if one considers the example of influenza virus vaccines where numerous egg proteins contaminate vaccine preparations currently being used in humans against flu.

Alternatively, potential heterologous and homologous anti-Id vaccines may be replaced by synthetic peptides that possess a predefined

specificity and conformation. Synthetic peptides could be produced that have a complementarity similar to that of the antigen, once the three-dimensional structure of an anti-Id bearing the internal image is unraveled. We have previously shown the importance of conformation in the ability of synthetic peptides to inhibit an Id-anti-Id reaction.[12] Therefore, if synthetic peptides are to replace anti-Id preparations as potential vaccines, they must contain the secondary and tertiary structures necessary to induce a particular conformation involved in the internal image. Hopefully, in the near future it should be possible to synthesize a peptide that may correspond to a portion of the internal image site and to use these synthetic peptides to induce protective immunity against infectious agents similar to that obtained with the anti-Id. Due to the complexity of the antigenic library of *Plasmodium*, anti-Id preparations or a pool of such reagents offer an alternative approach for developing a vaccine to control malaria. Although this infectious organism does represent a complex pathogen, anti-Id in conjunction with either synthetic peptides or recombinant vaccines may be a means by which malaria can be effectively eradicated.

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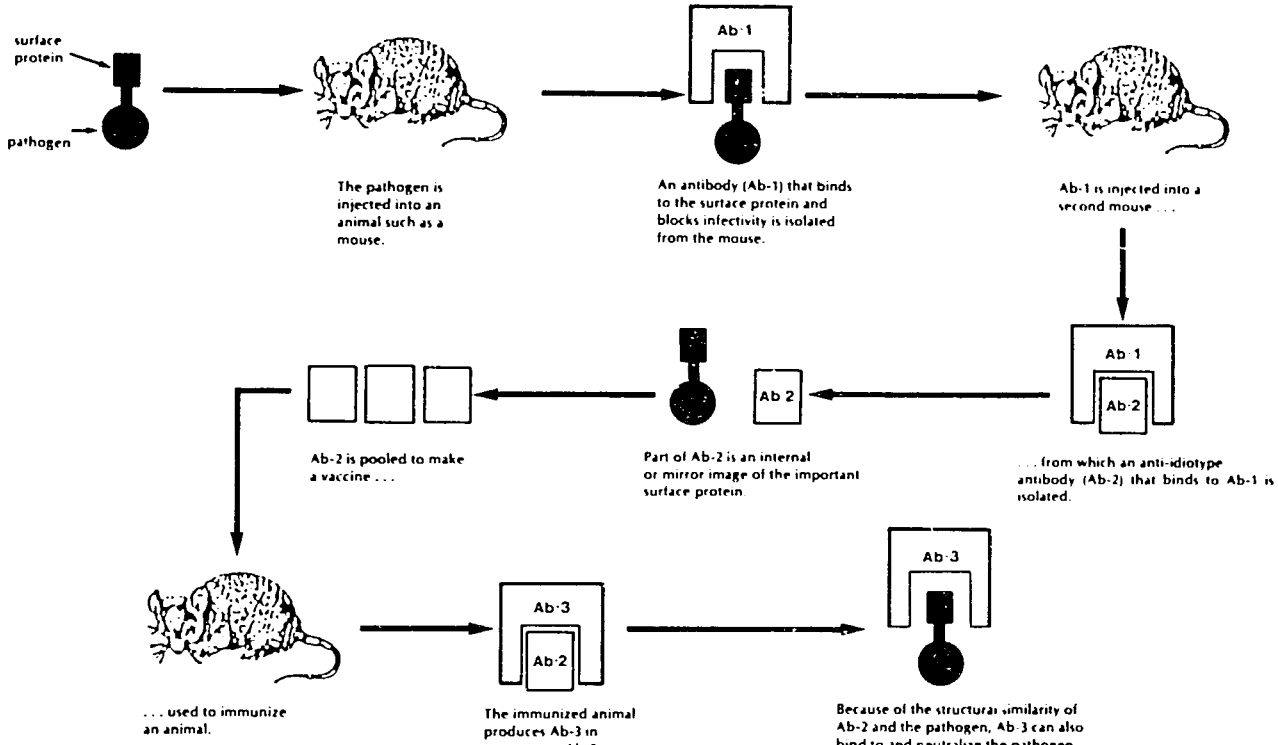

FIGURE 1. Production of an Anti-Idiotype Vaccine.

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Virus Vaccines as Examples for Development of Malaria Vaccines

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Introduction

Recent progress in the identification of genes that encode Plasmodial antigens has raised the possibility of developing an effective malaria vaccine. Choices need to be made not only with regard to the antigen but to the type of vaccine. Although the ability to protect against disease is the paramount consideration, safety, cost, and method of delivery also must be taken into account. It seems appropriate at this juncture to consider previous experience with virus vaccines as well as new emerging technologies.

Live Vaccines. The majority of virus vaccines consist of live viruses that have reduced pathogenicity. The first vaccine was directed against smallpox.[1] Initially variola virus, the causative agent of smallpox itself was found to be protective when inoculated in the skin. However, this procedure, which was called variolation, was abandoned when Jenner demonstrated that vaccinia virus effectively protected against smallpox without the risk of spreading the disease itself. Vaccination, a term originally used to signify inoculation with vaccinia virus but now expanded to mean immunization against any infectious agent, proved highly successful and resulted in the total eradication of smallpox. The development of smallpox vaccine in the late eighteenth century was based on the fortunate existence of a virus of low pathogenicity that was closely related to the disease agent. In general, however, the development of live vaccines has depended on attenuation of viruses. This has been carried out empirically by repeated virus passage in laboratory animals or tissue culture.[2,3] In this manner, safe and highly effective vaccines against poliomyelitis, measles, rubella, mumps, and yellow fever were produced. Typically, live vaccines are effective after a single dose and provide both humoral and cell-mediated immune responses of long duration. Usually, they are relatively inexpensive because of the low cost of production and small inoculum size. The stability of live vaccines varies: in some cases, such as measles, the vaccine must be refrig-

erated whereas smallpox vaccine can be stored in dried form at elevated temperatures for long periods. The production of adverse side effects particularly in immunodeficient individuals, virus spread to susceptible contacts, and reversion to virulence are possibilities that have to be evaluated for each vaccine. In some instances, e.g., poliomyelitis, spread to contacts may be considered advantageous since it contributes to the immunity of the population as a whole.

Killed vaccines. A second type of vaccine consists of killed or inactivated virus. There are important differences between live and killed vaccines and only a few of the latter type, e.g., poliomyelitis and rabies vaccines, have been used successfully on a large scale. Of practical importance is the requirement for a sufficient mass of virus particles and/or repeated immunization to obtain protective antibodies, the low stimulation of specific cell-mediated immunity, and the need for careful safety testing to ensure that the vaccine is free of infectious organisms. Occasionally, important antigens have been destroyed during the inactivation procedure leading to partial immunization that has actually increased the frequency of severe complications following subsequent infections. This may have occurred during some trials with killed respiratory syncytial virus and measles virus vaccines.

Natural subunit vaccines. Subunit vaccines, so-called because they contain only a subunit of the virus particle, share some characteristics with killed vaccines. Advantages of such vaccines include precise composition and freedom from extraneous proteins and nucleic acids. Purified proteins, however, may be much less immunogenic than intact virus particles. Repeated immunization may be required and specific immunity is predominantly humoral. At present, the hepatitis B virus vaccine is the only licensed virus subunit vaccine. The inability to grow hepatitis B virus in tissue culture prevented the development of either live or killed vaccines. However, the presence of 22 nm lipoprotein particles consisting of excess hepatitis B virus surface antigens in the blood of chronically infected individuals provided an alternative material.[4] Rigorous procedures have been used to remove or inactivate infectious particles and the vaccine is safe as well as efficacious. The high immunogenicity of this material is probably related to its particulate structure. Unfortunately, the limited quantities, requirement for multiple inoculations, and expense have thus far prevented wide use of the subunit vaccine in areas of the world where hepatitis B is highly endemic.

Recombinant DNA derived subunit vaccines. Recombinant DNA technology has provided powerful methods for the identification and expression of potential antigens to be used as vaccines. The most straightforward approach is to clone the gene of interest into a high expression vector in order to synthesize the protein subunit. Prokaryotic systems, currently based on *Escherichia coli,* are the best understood and were the first to be used. In order to obtain high expression and to ensure stability, fusion

proteins that contain both prokaryotic and viral sequences are frequently constructed. In addition DNA coding for hydrophobic regions of the protein may have to be removed. Provided the product retains immunogenicity, the bacterial vector system has great potential. Preliminary results with a capsid protein of foot-and-mouth disease virus are encouraging.[5] However, viral proteins made in prokaryotes are not glycosylated and are frequently not in a native configuration so that protective antibodies may not be induced.

For the above reasons, eukaryotic expression vectors have advantages. Yeast systems are particularly attractive since they can be readily grown in bulk. Yeast recombinants that express hepatitis B virus surface antigen (HBsAg)[6] and influenza virus hemagglutinin (HA)[7] have been constructed. Although not glycosylated, the recombinant yeast derived HBsAg forms lipoprotein particles that are both immunogenic and antigenic. Protection of chimpanzees has been demonstrated and it seems likely that this yeast recombinant HGsAg will ultimately replace plasma derived vaccines.[8] The yeast recombinant influenza HA protein is glycosylated and is antigenic, although its ability to induce neutralizing antibodies or protect has not yet been determined.

Mammalian cells also can be used to produce virus subunits that are properly processed and glycosylated. Several different approaches have been developed. The desired gene can be introduced into a defective virus (e.g., SV40, adenovirus, herpesvirus, retrovirus) which is then used in conjunction with a helper virus to infect cells. Alternatively, cells may be permanently transformed and the desired gene greatly amplified. With such systems HBsAg, herpes simplex virus type 1 glycoprotein (HSV-1 gD) and influenza hemagglutinin (HA) have been made.[9-13] Although in the past vaccines could not be made from continuous cell lines, modern methods of protein purification may eliminate concern about contaminating DNA with transforming potential.

Synthetic peptides. Chemically synthesized peptides could provide a completely defined immunogen for use as a vaccine. Synthesis and testing of peptides has been greatly stimulated by the wealth of sequence information that has been obtained by recombinant DNA methods. Evidence has been obtained that peptides corresponding to epitopes in proteins from foot-and-mouth disease virus, hepatitis B virus, and influenza virus are immunogenic.[14-16] Partial protection has been obtained in the case of hepatitis B virus.[15] More work needs to be done with regard to an adjuvant, however, if this approach is to be generally useful in man. The presence of repeating epitopes in many malaria proteins makes the use of synthetic peptides particularly attractive, as discussed in more detail elsewhere in this volume.

Live virus recombinants. Genetic engineering also can be used for the construction of live virus recombinants that may be suitable as vaccines. Such recombinant viruses would retain many of the features asso-

ciated with a live vaccine. To serve as a vector, the virus should have a DNA genome large enough to accommodate additional genes without losing infectivity. There are three major families of viruses that may meet these requirements: adenoviruses, herpesviruses, and poxviruses. Of these three, poxviruses have a number of advantages. They have the largest capacity for additional DNA, one member of this family—vaccinia virus—has been used extensively for mass vaccination, and natural infections with viruses of this family are now rare.

Initial experiments have demonstrated the feasibility of using vaccinia virus as an expression vector and methods of inserting and expressing foreign genes and isolating recombinant viruses have been developed.[18,19] One general method involved the construction of plasmid insertion vectors containing the vaccinia virus thymidine kinase gene interrupted by a segment of DNA containing vaccinia virus transcriptional regulatory elements (promoters) and multiple sites that can be cut with restriction endonuclease for insertion of the foreign gene.[20] Homologous recombination is used for insertion of the foreign DNA and recombinants are selected on the basis of their thymidine kinase negative phenotype.

With proper engineering, it appears that virtually any gene can be expressed in a vaccinia virus vector. Moreover, the proteins are generally processed and glycosylated in an appropriate manner.[21] Evidence has been obtained that vaccination leads to humoral and cell-mediated immune responses to the foreign proteins.[22] Vaccination with recombinants that express the HBsAg, the influenza virus HA, and the herpes simplex virus glycoprotein D have been shown to protect animals against challenge with the corresponding infectious agent.[23–25] Recently, the circumsporozoite surface protein of *Plasmodium knowlesi* and *falciparum* (unpublished) have been expressed in a vaccinia virus vector and vaccinated rabbits were shown to produce antibodies to the repeating epitopes.[26] With further engineering it may be possible to obtian vaccinia virus recombinants that express antigens from multiple developmental stages. If effective, such vaccinia virus recombinants may be particularly suitable for prophylaxis of malaria since the production of the vaccine should be inexpensive, refrigeration is unnecessary, and medically skilled personnel are not required for administration.

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Acyl and Alkyl Derivatives of Muramylpeptides as a Potential Adjuvant Useful for Malaria Vaccines

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I. Introduction

Since the discovery that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) can replace the mycobacteria in Freund's complete adjuvant, the most powerful immunoadjuvant so far developed, first by Lederer's group and subsequently but independently by a Japanese group, extensive studies have been made on the biological and immunopharmacological activities of MDP in a number of *in vivo* and *in vitro* assay systems.[1-5] MDP has been found to have a very wide range of effects, especially on the natural and acquired defense mechanisms, of mammals.

The immunoadjuvancy to stimulate antigen-specific immune responses of hosts, the most prominent activity of MDP, should be useful in medical application of this compound. However, the following problems must be solved before MDP can be used as an adjuvant to potentiate vaccines for humans. One of the problems is that MDP itself must be administered as a water-in-mineral oil (w/o) emulsion with an antigen to stimulate humoral and cellular immune responses, particularly the latter, as does Freund's complete adjuvant, and that the w/o emulsion produces irritative reactions at both the injection site and in the regional lymph nodes.[3,6] This makes it impracticable to use MDP in human vaccination. The other problem is that the biological activities of MDP are so multifold that some of the activities are more harmful than beneficial.

For several years, various studies have been performed to obtain a

derivative of MDP with the least deleterious effects, such as pyrogenicity, arthritogenicity, and others, and the highest adjuvant activity by chemical modification of MDP and related compounds and to find a more innocuous route of administration other than injection.

In this study we searched for a new MDP derivative that exerted high immunoadjuvancy when parenterally administered with a non-irritative vehicle and had less deleterious effects, either local or systemic, than MDP and examined whether muramylpeptide derivatives that in spite of their high adjuvancy are disqualified as an immunoadjuvant for human use by their deleterious effects, can exhibit their inherent adjuvant activities by oral administration with the immunogen.

II. Selection of MDP Derivatives Capable of Exhibiting Powerful Adjuvancy without the Use of Irritative w/o Emulsion

1. *Comparison of the adjuvancy of acyl- or alkyl- derivatives of muramylpeptides using different vehicles.* The immunoadjuvant activity of various synthetic MDP derivatives synthesized by Shiba and Kusumoto, Faculty of Science, Osaka University, and supplied by the courtesy of Research Institute, Daiichi Seiyaku Co., Ltd., Tokyo, were examined.[7-10] The test compounds were classified into: 1) 6-*O*-acyl- or 6-*O*-alkyl-phospho-MDP derivatives (Table 1); 2) N^{α}-MDP-N^{ϵ}-acyl- or alkyl-lysine derivatives (Table 2); and 3) 6-*O*-acyl derivatives of *N*-acetylglucosaminyl (GlcNAc)-β (1–4)-*N*-acetylmuramyl (MurNAc)-L-alanyl-D-isoglutamine (GMDP) (Table 3). They were examined under our standard assay conditions for their ability to elevate serum antibody level and to induce delayed-type hypersensitivity against ovalbumin in guinea pigs. In brief, five Hartley albino guinea pigs (female, weight about 300 g) per group were immunized by intra-footpad injection of test adjuvants (standard dose, 0.1 mg/animal) and crystalline ovalbumin (1–2 mg; grade V, Sigma) in Freund's incomplete adjuvant, liposomes, squalene-in-water (o/w) emulsion, Intralipid®, phosphate buffered saline (PBS), or phosphate buffer (PB). The induction of the delayed-type hypersensitivity (DTH) was examined by the corneal test and serum anti-ovalbumin precipitin level was estimated by the quantitative precipitin method, 3 and 4 weeks after immunization, respectively.[11]

Table 1 shows that unlike MDP itself, 6-*O*-stearoyl-MDP (L18-MDP) and most of the 6-*O*-α-branched higher fatty acid derivatives of MDP can work as a potent adjuvant in stimulating the primary immune responses, either humoral or cellular, against ovalbumin, without the use of w/o emulsion as an administration vehicle. Above all, 6-*O*-(2-tetradecylhexadecanoyl)-MDP (B30-MDP) exhibited strong adjuvant activity in combination with the o/w emulsion, liposomes in PBS, or PB.[6-12] Because none of these vehicles were irritative, and were

TABLE 1. Immunoadjuvant activity of MDP derivatives administered with various vehicles — I. 6-*O*-acyl- or -alkylphospho-MDP

	Stimulation[a] of Ab production/induction of DTH Administration vehicle			
Test adjuvant	*w/o*	*o/w*	*Liposome*	*PB[S]*
MDP	+++/+++	—/—	+/—	—/—
L8-MDP	+/+	—/—	—/—	
L12-MDP	+++/±	—/—	—/±	
L18-MDP	+++/+++	++/+	+++/+++	++/+
L18-MDP (Val)			+++/++	
L20-MDP	+/+	++/+	±/±	
L22-MDP	±/+	—/±	+/++	++/+
L30-MDP	+/++	+++/++	+/+	±/±
B30-MDP	+++/+++	+++/+++	+++/+++	+++/+++
BH32-MDP	+/++	+++/++	++/+++	+/±
B46-MDP	+/+++	+++/++	++/+	+/+
BH48-MDP	++/++	+++/+++	+++/++	±/±
BH48-MDP (Ser)	++/+++	+++/+++	++/+	
BH48-MDP-Lys-D-Ala		+++/++		
LA18-P-MDP		±/±		
LA46-P-MDP		+++/++		

[a] Arbitrarily, but according to the same criteria, graded with a maximum of +++ (the same in table 2 and 3).

Abbreviations: L = linear acyl; B = α-branched acyl; BH = α-branched and β-hydroxylated acyl; LA = linear alkyl; numeral = total number of carbon atoms of acyl or alkyl group; MDP (Val) = MurNAc-L-Val-D-isoGln; MDP (Ser) = MurNAc-L-Ser-D-isoGln.

TABLE 2. Immunoadjuvant activity of MDP derivatives administered with various vehicles — II. N^{α}-MDP-N^{ϵ}-acyl- or alkyllysine derivatives

	Stimulation of Ab production/induction of DTH Administration vehicle			
Test adjuvant	*w/o*	*o/w*	*Liposome*	*PB[S]*
MDP-Lys(L18)	++/+++	+/+	++/++	+/+
MDP(Val)-Lys(L18)		±/±	++/++	—/—
MDP(Ser)-Lys(L18)		±/+	++/+	—/+
MDP-Lys-NHLA18	+/++		++/+	
MDP-Lys(L18)-OMe	+++/++	+++/+		+/+
MDP(Val)-Lys(L18)-NHLA18	+++/+++	+++/++		±/+

Abbreviations are the same as in table 1.

TABLE 3. Immunoadjuvant activity of MDP derivatives administered with various vehicles — III. GlcNAc-β(1,4)-MurNAc-L-Ala-D-isoGln and its derivatives

	Stimulation of Ab production/induction of DTH			
	Administration vehicle			
Test adjuvant	*w/o*	*o/w*	*Liposome*	*PBS*
GMDP	+++/+++			
M(G)DP	+++/+++			
L18-GMDP		+/+	+++/+++	
B30-GMDP		+++/++	+++/+++	++/++
BH48-GMDP		+++/++	++/±	

Abbreviations are the same as in table 1.

easily metabolized, they were considered applicable to human vaccines. Table 2 summarizes the assay results obtained with N^{α}-MDP-N^{ϵ}-acyl- or alkyllysine derivatives. Some of them given with the o/w emulsion or liposomes stimulated antibody production significantly, but could not induce DTH. Among the GMDP derivatives, an α-branched fatty acid derivative, B30-GMDP exhibited adjuvancy irrespective of the vehicle used for administration (Table 3).

2. *Immunopharmacological activities other than the adjuvancy of several MDP derivatives.* The immunobiological activities of acyl derivatives of MDP selected by screening their definite adjuvancy when administered by any one of the non-irritative administration vehicles and those of some reference compounds were compared in several *in vivo* assay systems (Table 4). B30-MDP in PBS, unlike either L18-MDP or MDP-Lys (L18), is not pyrogenic when given intravenously and does not produce any hemorrhagic necrotic inflammation at the site prepared by mycobacteria and nocardia or MDP plus ovalbumin in w/o emulsion.[3,6,13] One harmful activity of B30-MDP the physician must be aware of is arthritogenicity. However, as shown in Table 5, the arthritogenic effect of B30-MDP for susceptible rats was noticed only after repeated injections, i.e., daily injections of as high a dose as 0.1 mg per rat for 2–3 weeks. Twelve daily, subcutaneous administrations of 20 μg of B30-MDP in PBS did not induce any detectable polyarthritis, though similar injection of B30-MDP in NIKKOL HCO-60 (a detergent for medical use) resulted in mild arthritis.[6] Therefore, we believe that the arthritogenicity of B30-MDP may not seriously disturb its use as a potential adjuvant for vaccines, because the usual vaccinaion schedule does not involve so frequent administration at short intervals of the immunogen. B30-MDP was shown to cause acute joint inflammation in BALB/c mice by one intravenous injection of 0.1 mg dose in PBS per animal. But the induced joint inflammation was very mild and com-

TABLE 4. Immunopharmacological activities of lipophilic derivatives of MDP

Activity	*MDP*	*L18-MDP*	*B30-MDP*	*MDP-Lys(L18)*
Increase of nonspecific resistance				
Slc:ddY mouse (*E. coli*)[26, 27]	+	++	—	+++
Pyrogenicity (rabbit)[12]				
intravenous	++	++	—	+
intracerebroventricular	++	++	++	ND[b]
Polyarthritis[28]				
SHR/NCrj, Lewis & SD rat; repeated	—	++	++	ND
Acute joint inflammation				
BALB/c mouse; one shot	+	++	+	ND
Epithelioid granuloma (g.p.)[14]	—	—	+	—
Hemorrhagic necrosis at primed site (g.p.)[13]	+	+	—(+)[a]	+
Ileal strip contraction (g.p.)[29]	+	++	++	ND

Activities are scored in terms of the relative potency.
[a] In PBS (in NIKKOL HCO-60).
[b] Not done.

TABLE 5. Arthritogenicity of B30-MDP in SD/NCrj rats

Vehicle	Dose (mg/kg)	Route	No. of daily injections	Sex	Polyarthritis		
					+/Total	Onset day	Severity
Intralipid®	1	i.p.	1	Female	0/7		
NIKKOL HCO-60	1	i.p.	1	Female	0/7		
PBS	1	s.c.	12	Female	0/9		
PBS	1	s.c.	12	Male	0/8		
PBS	5	s.c.	21	Female	9/9	12–15	13.8 (severe)
PBS	5	s.c.	14	Male	8/8	15–16	9.5 (moderate)
NIKKOL HCO-60	1	i.v.	12	Female	10/10	2–9	5.3 (mild)
NIKKOL HCO-60	0	i.v.	12	Female	0/10		
PBS	0	i.v.	7	Female	0/5		
PBS	0	s.c.	12	Female	0/5		

pletely subsided by the 3rd or 4th day after the injection (Toshio Koga et al., Department of Biochemistry, Kyushu University Dental School, unpublished).

3. *B30-MDP as a tentative candidate for a potential adjuvant in human vaccines.* Figure 1 shows the dose-response (induction of DTH) relationship noted with the guinea pigs receiving an intrafootpad injection of B30-MDP and ovalbumin with various administration vehicles.[6-12] Administration of B30-MDP and ovalbumin with any of the test vehicles (liposomes, o/w emulsion, Intralipid®, and PBS) caused dose-dependent induction of DTH in a way similar to that induced by B30-MDP administered in water-in-mineral oil emulsion. In combination with any of the test vehicles, among which o/w emulsion and Intralipid®, an intravenous fat emulsion for clinical use, seemed to be most effective, B30-MDP served as an effective adjuvant in stimulating anti-ovalbumin precipitating antibody production (Fig. 2). As shown in Figure 3, the swelling at the injection site in test groups was negligible when compared with the strong irritation observed in the control group receiving w/o emulsion, at all test dosages. An undesirable, local effect noticed with B30-MDP was the swelling of lymph nodes in the area of the injection site induced by high dosage, particularly with liposomes or PBS, due to the induction of epithelioid granuloma formation as an expression of macrophage activation.[11]

4. *Usefulness of B30-MDP as an adjuvant for vaccines.* Usefulness has so far been demonstrated with: 1) influenza subunit and highly purified HA-NA vaccines, Virosome fluvaccine in guinea pigs and mice; 2) *Plasmodium falciparum* antigens derived from long-term culture parasites in *Aotus* monkeys; and 3) hepatitis B subunit vaccine that was isolated and purified from a human plasma source.[6,15-17] Peltola et al., who studied local and the systemic reaction of *Aotus* monkeys inoculated with *P. falciparum* antigen with B30-MDP in liposomes, concluded that the addition of B30-MDP caused some local and general reactions as well as hematological and biological changes, but none of them were so severe that B30-MDP could not be used as an adjuvant.[18]

III. Attempts to Stimulate Host's Immune Responses by Oral Administration of MDP Derivatives with a Protein Antigen

Much effort has been made to minimize the harmful effects of MDP without decreasing its immunostimulating activity by chemical modification of the molecule, resulting in the synthesis of a few compounds such as *N*-acetylmuramyl-L-alanyl-D-isoglutamine-α-*n*-butyl ester (murabutide) and B30-MDP.[19-23] Many muramylpeptide derivatives, however, have been disqualified for use as a potential adjuvant in human vaccines, in spite of their high adjuvancy, because of their pyrogenicity, arthritogenicity, and other possible deleterious effects.

The discrimination between beneficial and deleterious effects induced by biological response modifiers such as MDP is made subjectively and consequently, it is not easy to minimize "harmful" activities without decreasing the "useful" effects of the immunomodulators in question. One of the ways to use MDP derivatives that have strong adjuvancy but cannot be used because of their "harmful" activities may be oral administration, by which most of the deleterious effects of MDP derivatives that are noticed in parenteral administration may be avoided.

Semisynthetic, lipophilic derivatives of GlcNAc-β (1–4)-MurNAc-L-Ala-D-isoGln-*meso*-2,6-diaminopimelic acid (A_2pm) (GMP_3) were tested for their adjuvancy to stimulate circulating anti-bovine serum albumin (BSA) antibody production by oral administration to mice. Test adjuvants (GM-40, -87, -123, and -133) whose chemical structures are illustrated in Figure 4, were prepared by chemical modification of GMP_3 isolated from hydrolyzate of *Lactobacillus plantarum* cell walls by combined catalysis by M-1 *N*-acetylmuramidase and AM_3 D-alanyl-*meso*-A_2pm endopeptidase as described in a separate paper.[24] A test adjuvant (0.5 mg per mouse) was incorporated with BSA (1 mg per mouse) into liposomes consisting of synthetic lecithin and cholesterol (1:1, molar ratio) and the resulting liposomes were given by gastric intubation to groups of 7 to 12 BALB/c mice on day 0 and 1. Oral administration of the immunogen in liposomes was repeated on day 27 and 28 as a booster, in the same way as the primary immunization. The anti-BSA antibody titer was determined on serum specimens taken on day 33 by the passive hemagglutination (PHA) method using sheep red blood cells treated with glutaraldehyde and coated with BSA. Anti-IgG, IgM, and IgA levels were estimated by enzyme-linked immunosorbent assay (ELISA). Details of the methods are described separately.[25]

Table 6 shows that the mice receiving oral administration of any of the semisynthetic, lipophilic derivatives of GMP_3 and BSA in liposomes

TABLE 6. Adjuvant effect of acyl derivatives of GMP_3 orally administered with BSA (1 mg/mouse) in liposomes in production of anti-BSA antibodies in BALB/c mice

Test adjuvant (500 μg/mouse)	*PHA(2^n)*	*ELISA (mean ± SE)*		
		IgG(S.I.)[a]	*IgM(OD_{405}×100)*	*IgA(OD_{405}×100)*
GM-40	4.5 ± 0.4**	6.0 ± 1.9*	164 ± 10	28 ± 6*
GM-87	5.7 ± 0.8**	46.1 ± 21.7	263 ± 42*	88 ± 28*
GM-123	5.5 ± 0.8**	32.3 ± 18.6	185 ± 24	56 ± 25
GM-133	6.2 ± 0.9**	97.6 ± 32.9**	184 ± 22	96 ± 31*
None	2.4 ± 0.3	1.0 ± 0.5	146 ± 16	10 ± 3

[a] Ratio of ELISA unit in a test to that in the respective control.

*,** Significant difference from control by Student's t-test (*P<0.05, **P<0.01).

showed definitely elevated production of anti-BSA antibodies, both IgG and IgA classes, as compared with the control group of mice orally administered with BSA alone in liposomes. Among the four compounds tested, GM-133 seemed to have the highest adjuvancy. The mice in the test groups gained body weight as did those in the control group and showed no abnormal signs macroscopically detectable during observation period of over one month, though no hematological or clinicochemical examinations have so far been performed.

Whether oral administration of the test adjuvants can induce cell-mediated immunity such as DTH and raise secretory antibody levels or not remains to be examined.

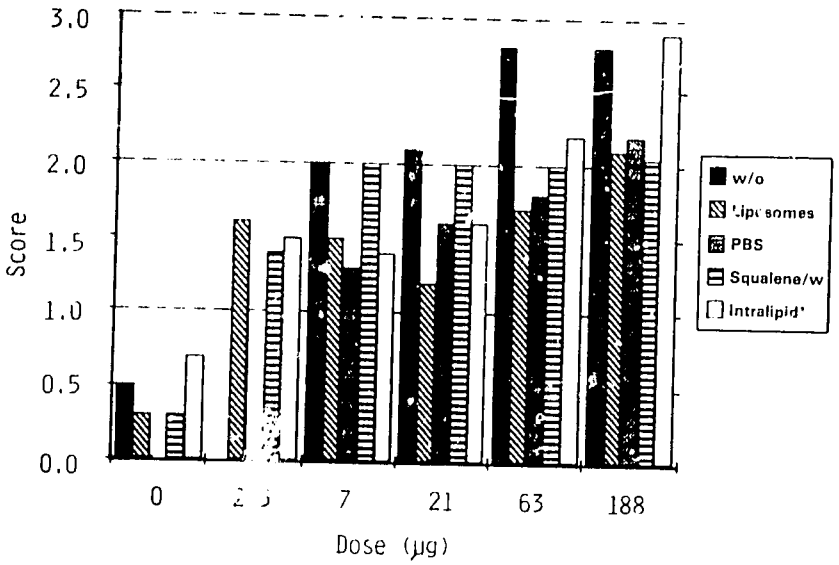


FIGURE 1. Induction of DTH (corneal reaction) Against Ovalbumin by B30-MDP in Guinea Pigs (3rd week)

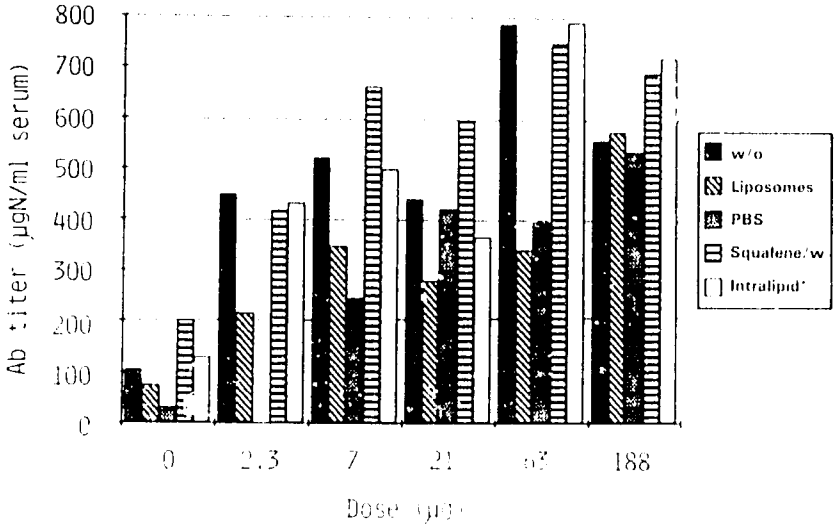


FIGURE 2. Stimulation of Anti-ovalbumin Precipitating Antibody by B30-MDP in Guinea Pigs (4th week).

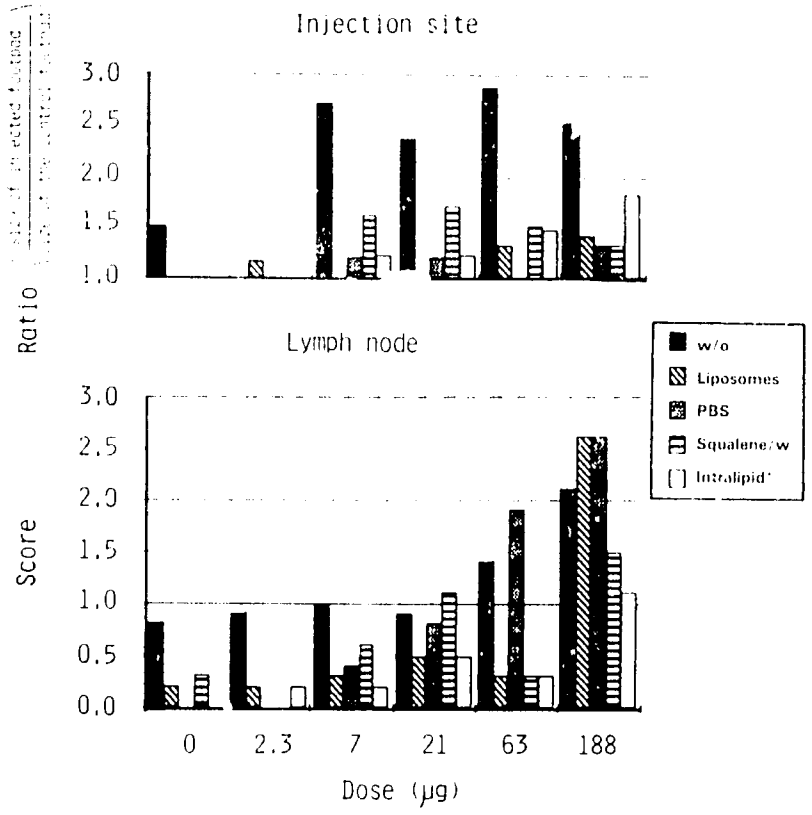
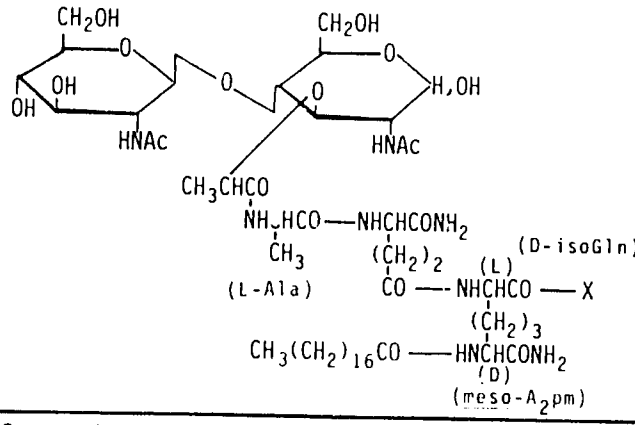


FIGURE 3. Swelling of Injection Site and Regional Lymph Nodes (3rd week).



Compound	X
-40	ONa
-87	OCH_3 [OMe]
-123	$OCH_2(CH_2)_3CH_3$ [OAmyl]
-133	$OCH_2(C_2H_5)CH(CH_2)_3CH_3$ [O-n-Octyl]

FIGURE 4. Chemical Structures of Acyl Derivatives of GlcNAc-β (1-4)-MurNAc-L-Ala-D-isoGln-meso-A_2pm (GMP_1).

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Potential Mode of Action of Adjuvants Used for Malarial Vaccines

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Introduction

Immunological adjuvants refer to compounds which, when mixed with an antigen, will enhance the immune response to the antigen either quantitatively or qualitatively. The prototype, and still the most effective experimental adjuvant, is that developed by Freund.[1] It consists of an aqueous solution of antigen emulsified in light mineral oil to which non-viable mycobacteria are added. The water in oil emulsion creates a depot effect allowing for the sustained release of antigen, while the mycobacteria activate the cellular elements of the immune system attracted to the site of antigen injection. This formulation enhances both the humoral and the cell mediated immune responses. Freund's complete adjuvant (FCA) has been used in many of the experimental studies demonstrating anti-malarial immunity in primate and non-primate species immunized with malarial antigens.[2-5] Its use in man, however, is contraindicated because of the intense granulomatous inflammatory response against the mycobacteria, leading to chronic ulceration and scarring. Other experimental adjuvants have tended to be less toxic, but also less effective, than FCA. Nevertheless, an analysis of the various activities of these adjuvants has helped provide insight into potential approaches to immune augmentation.

Overview of the Immune Response

The immune response can be viewed as occurring in three stages (Fig. 1). These stages are: 1) localization of antigen to specialized "antigen presenting cells" (APC); 2) presentation of the antigen, in association with major histocompatibility complex (MHC) coded components, to T lymphocytes; and 3) lymphocyte activation. Each of these steps is influenced by many variables which can have a profound effect on the nature and magnitude of the evoked immune response.

Localization is the process by which antigen makes contact with appropriate APC, such as monocyte/macrophages 6 and various dendritic cells in the skin and in the interfollicular and follicular regions of lymphoid tissues.[7,8] This step is generally achieved by the transfer of antigen, via the bloodstream or lymphatics, to the lymphoid tissues. Alternatively, APC may migrate to the site of antigen administration. Localization may be facilitated by accessory molecules which bind receptors expressed on certain types of APC. For example, antibody and activated C3 component of complement bind to Fc and C3b receptors, respectively. Fibronectin and platelets may also function in the transfer of antigen to APC.[9] The physical state and route of administration of antigen are clearly important in antigen distribution and localization. Ingestion and processing of certain antigens may also be required for the appearance at the cell surface of the potential immunogen.[10,11]

Presentation of cell surface bound antigen is required for the effective interaction between APC and helper T cells. T cells rarely, if ever, respond to free antigen. Rather, the antigen is required to associate with molecules coded for by the MHC. For T helper cells, the antigen-MHC association is with class II MHC molecules or Ia antigens.[12,13] For cytotoxic/suppressor T cells, the association may be with class I molecules (HLA A, B, and C coded).[14,15] The efficacy of antigen presentation is dependent on the quantitative level of Ia antigen expression of the APC and possibly also by interactive forces between the antigen and Ia molecules.[16-19] Genetic polymorphism exists among Ia molecules and certain examples of genetically determined immunological unresponsiveness can be attributed to a lack of specific antigen-Ia interaction.[19]

Lymphocyte activation can be conveniently divided into early and late phases. The initial activation of T helper cells for either antibody or cell mediated responses follows contact of T cells with antigen-bound APC. The T cells trigger the synthesis of interleukin 1 (IL 1) by the APC, possibly via the lymphokines colony stimulating factor (CSF) 20 and macrophage activating factor (MAF). IL 1, along with specific antigen, will induce the T helper cells to release interleukin 2 (IL 2).[21-23] IL 2 acts as a second signal along with specific antigen for the activation of various effector T cells.[24] These cells include cytotoxic cells which kill by direct contact with a target cell; cells which kill by releasing various lymphotoxins and/or gamma interferon; cells which activate macrophages to become cytocidal; and cells which evoke granulomatous inflammation.[25] Another class of T helper cells appear to interact directly with the Ia molecule on antigen bound B cells and to release B cell growth and differentiation factors which stimulate B cell proliferation and immunoglobulin synthesis. Both IL 1 and possibly IL 2 also act as immunoregulatory signals for B cell activation.[26-29] Gamma

interferon is another important immunoregulatory molecule. Among its many actions is the potential to enhance Ia antigen expression and stimulate IL 1 synthesis by APC.[30-32,33] On the other hand, prostaglandins, released by activated macrophages, appear to dampen the immune response, possibly by down-regulating Ia antigen expression.[34,35] Other suppressor mechanisms include antigen specific and antigen nonspecific 38 suppressor T cells, and possibly interferon induced non-T natural killer (NK) like lymphoid cells.[36-37,39] Complex reciprocal interactions also exist between the humoral and cell mediated pathways and within various components of each of these pathways. The schematic outline shown in Figure 1 is, therefore, oversimplified, but it does provide a useful framework on which to consider the potential mode of action of various adjuvants.

Potential Mode of Action of Adjuvants

The immune response may be augmented by selective manipulation of one or more of the three major stages outlined above. Thus, an adjuvant could facilitate antigen localization, promote antigen presentation, or enhance lymphocyte activation. In this section we will review the properties of known adjuvants to help identify their site of action.

Antigen Localization

Injected antigens may be cleared so rapidly from the body that the period of effective contact with the immune system is inadequate. Therefore, considerable improvement in immunogenicity may be achieved simply by providing the antigen in a form from which slow and sustained antigen release will occur.[40] Prominent examples of this approach are alum adsorbed antigen and water-in-oil emulsions such as incomplete Freund's adjuvant.[41,1] Saponin, a mixture of triterpene glycosides extracted from the bark of the Quillaia tree, is used as an adjuvant for various veterinary vaccines. It appears to work, in part, by promoting the aggregation of protein and polysaccharide antigens and facilitating their interaction with APC.[42,43]

More specific approaches to directed antigen localization have been investigated. Malarial protein antigens have been administered complexed with specific IgM antibodies to promote localization to macrophages.[44] The IgM antibody is likely to fix large amounts of the C3b component of complement and selectively bind APC via the C3b receptors on these cells. On the other hand, immunogenicity of malarial antigens may be impaired in the presence of specific IgG antibody.[45] Block copolymer surface active agents, consisting of arrays of hydrophobic and hydrophilic molecules, have also been shown to fix large amounts of complement. Antigen bound to these polymers has been shown to

TABLE 1. Conditions which Affect Antigen Localization

1. Dose & route of administration
2. Depot antigen
   Alum Adsorbed, Saponin, Water in Oil Emulsion
3. Antigen delivery
   a) Natural Opsonins
      IgM Antibody, Fibronectin, Platelets
   b) Bacterial Opsonins
      Tetanus, Cholera, etc.
   c) Block Copolymers
   d) Liposomes

localize preferentially to the draining lymphoid tissue.[46-47] The inherent affinity of APC for a wide variety of bacterial components may explain, in part, the adjuvanticity of antigen complexed to either tetanus, diphtheria, or cholera toxoids. Cholera toxin consists of two polypeptide chains; one chain binds to cell surface receptors, while the second chain confers toxicity. Isolated cell surface binding chains of cholera toxin have been coupled to antigen and shown to enhance the immune response to the antigen. Concanavalin A has been similarly used, bound to tumor cells, to enhance tumor cell immunogenicity.[48]

Liposomes have been employed to focus encapusulated materials to macrophages.[49] The composition of liposomes can have significant effect on their ability to specifically localize to the macrophages in various organs.[50] The stability of liposomes can be increased by exposure to osmium tetroxide and such osmicated liposomes have found use in experimental vaccines.[51]

The various potential approaches to providing more effective antigen localization to the immune system are summarized in Table 1.

ANTIGEN PRESENTATION

As indicated previously, a rate limiting step in antigen presentation is the density of surface Ia antigens on APC. An increasing number of substances have been shown to be capable of enhancing the expression of Ia antigens. These include gamma interferon, interferon inducers, and even simple compounds such as beryllium.[52] While adjuvants have not been specifically designed to selectively stimulate Ia antigen expression, several of the more powerful adjuvants (e.g., FCA) certainly achieve this effect.[52] Interestingly, this function is not retained by muramyl dipeptide (MDP), a synthetic analogue of the major cell wall component of the mycobacteria used in FCA.[52]

A more significant problem exists if effective interaction between the vaccine antigen and Ia molecules fails to occur. This situation might, in part, explain the adjuvanticity of strongly immunogenic molecules cou-

TABLE 2. Enhancement of Antigen Presentation MHC: AG Association

1. Increased expression of class II molecules
   a) interferon
   b) interferon inducing substances
   c) macrophage activating factors
   d) beryllium
2. Substitute carriers
   a) bacterial toxoids
   b) keyhole limpet hemocyanin
   c) influenza and vesicular stomatitis virus
   d) vaccinia viral vector

pled to a vaccine antigen. The immunogenic molecule may provide a substitute carrier capable of effective Ia interaction. Chemical coupling and even simple mixing have been employed to link vaccine peptides to tetanus toxoid, methylated bovine serum albumin, keyhole limpet hemocyanin, and either influenza or vesicular stomatitis virus. Using recombinant technology, certain carrier determinants can also be synthesized as direct extensions to the specific vaccine antigen. One basic problem with this approach is that T cell memory would be specific for the substituted carrier rather than for the actual pathogen. Immunization might, therefore, not give long-term memory or recall on re-exposure to the infection. In addition, the use of a carrier to which an individual is already immune can occasionally induce so called "epitope specific suppression" resulting in an inhibition of immunity to the attached vaccine antigen.[53,54] There is also concern that the use of a substitute carrier might adversely affect the immune response to the carrier antigen by either suppressing subsequent immunity or inducing an adverse hypersensitivity response.

An interesting approach to promoting physical interaction between vaccine antigen and both normal cell surface antigens and immunogenic foreign viral antigen is the use of vaccinia viral vectors.[55] This approach may have an additional advantage if infection results in interferon secretion, but it also carries the risk of untoward infection in immune suppressed hosts.

A summary of some of the potential approaches to enhanced antigen expression by APC is provided in Table 2.

LYMPHOCYTE ACTIVATION

The critical role of IL 1 in the early phase of lymphocyte activation has been discussed previously. Exogenously administered IL 1 has been demonstrated to potentiate priming to bovine serum albumin in mice.[56] In these studies, the timing of IL 1 administration was found to be important, with the maximum effect occurring when IL 1 was given

two hours after antigen. The more usual approach to enhancing IL 1 synthesis is the use of agents which cause the release of IL 1. Prominent is the use of various analogues of MDP, peptidoglycan and Klebsiella lipopolysaccharide. Correlations can be shown between the IL 1 inducing activity and the adjuvanticity of these agents.[57-59]

Late lymphocyte activation involves a complex network of cellular and soluble regulatory mediators. Some of the possible potentiators of this phase of the immune response are listed in Table 3. Enhancement of the late activation step could be achieved by the acceleration of antigen-specific lymphocyte proliferation. Mediators of T and B lymphocyte growth, such as IL 2 and B cell growth factor (BCGF), could be exogenously supplied, or their *in vivo* production could be induced. Pertussigen, derived from *B. pertussis*, induces lymphocytosis and has been found to enhance cell mediated immunity as measured by delayed type hypersensitivity, whereas recruitment of additional B cells can be achieved by ribonucleosides such as 8-mercaptoguanosine.[60,61-62] A wide variety of pharmacological compounds such as isoprinosine and cimetidine can be shown to have immunomodulatory activity, possibly by affecting the metabolic pathways on which lymphokines exert their effects.[63-64] Antibodies, specific for the IgD molecule on early B cells, have been shown to enhance the humoral immune response.[65] The actual requirement for antigen can also be replaced by using monoclonal anti-idiotype antibodies reactive with the internal antigen image of antibodies specific for the vaccine antigen. These antibodies can induce proliferation of specific B and T cells.[66]

An area of considerable uncertainty is how to preferentially elicit cell mediated immunity (CMI). The immunogenic requirements for CMI appear to be more stringent than for antibody production. The drug

TABLE 3. Augmentation of Lymphocyte Activation

1. Early acting
   a) Interleukin 1
   b) IL 1 inducers
      (e.g. MDP)
   c) IL 1 agonists
2. Late acting
   a) Interleukin 2 and/or B cell growth factors
   b) IL 2 and BCGF inducers
   c) Immunopharmacological drugs
      (e.g. isoprinosine)
   d) Pertussigen
   e) Ribonucleosides
      (e.g. 8 mercaptoguanosine)
   f) Monoclonal antibodies
      (e.g. anti IgD, anti idiotype)

cyclosporin selectively inhibits CMI, and the development of drugs with opposing effects is to be expected. Viable organisms are generally more potent in eliciting cytotoxic T cells (CTL) than non-viable antigens, possibly because infection allows more effective association of the antigen with class I MHC molecules. CTL can be elicited, however, using non-viable vaccines as was shown with influenza.[67]

All phases of the immune response are controlled by suppressor mechanisms. Inhibition of suppression may be a potentially useful technique for immunopotentiation. Thus, certain cytotoxic drugs, such as Ara A, cyclophosphamide and colchicine, have been shown to enhance both humoral and cellular immunity by inhibiting suppressor mechanisms.[68-70] Dextran may augment the cellular immune response by specifically inhibiting the release of a macrophage derived suppressor factor.[71]

It is clear from the foregoing that there is great potential to achieve immune enhancement to vaccine antigens. It is also apparent, however, that many areas of adjuvant activity have yet to be fully defined. The next section will identify the major issues of concern in the development of clinical adjuvants.

Practical Considerations

An ideal adjuvant for widespread clinical use with malarial or other candidate vaccines has yet to be devised. Certain basic criteria can be defined for such a compound. For example, it should augment the development of protective immune responses, whether humoral or cell mediated or both. The immunity should be expressed at the site of potential invasion of the pathogen. The adjuvant should not disrupt normal immunoregulatory pathways for either normal self antigens or unrelated foreign antigens. It should be non-irritating to tissues, easy to administer, and inexpensive. It should be structurally well characterized and evoke uniform responses in different individuals over a reasonable dose range.

Several important questions underlie the development and selection of an appropriate adjuvant for malarial vaccines. First, what type of response is most desirable? In the case of sporozoite immunity, antibody may provide sufficient protection, even in the absence of complement fixation or opsonization. On the other hand, merozoite immunity is more likely to involve CMI mechanisms which, as yet, are poorly defined. Adjuvants are often judged on the basis of their ability to enhance the primary immune response. A more important action, however, is the achievement of more effective priming leading to an increased secondary response and to greater persistence of the memory response to rechallenge.

Given these goals, the next question is: What is the rate limiting step

in the immune response to the malarial vaccine? Depending on whether this is localization, presentation, or activation will determine the most appropriate type of adjuvant. *In vitro* assays will be needed to isolate and analyze each of these steps and to define the need for intervention with a given type of adjuvant.

Our laboratory is currently investigating the potential use of a human B cell derived IL 1 72 as a means to enhance the proliferative response of cultured human T lymphocytes to various mitogens and candidate vaccine antigens. The results from an assay demonstrating the immunopotentiating effect of IL 1 on the phytohemagglutinin (PHA) response of cultured human T cells are displayed in Figure 2. Antigen can be substituted for PHA and the priming effect can be measured by rechallenging the cultures with homologous or unrelated antigen 1–2 weeks later.

In general, we feel the use of IL 1 or its agonists may offer certain advantages over the use of IL 1 stimulating compounds, such as MDP. Specifically, these compounds may have actions apart from IL 1 induction and, in particular, may induce secondary immune suppression.[73] The response to these compounds, in terms of both adjuvanticity and IL 1 release, varies significantly between mice of different strains.[74,75] Recent findings indicate that genetic factors also play a significant role in the *in vitro* response of human cells to MDP.[76] The responsiveness of macrophages to IL 1 inducers may also be greatly influenced by any pre-existing state of activation, as might be expected in individuals harboring chronic infections.

The exogenous administration of IL 1 is also, however, subject to several major concerns. The *in vivo* half life of IL 1 is short, it needs to be present at the same time as antigen, and it may induce a range of non-immunological potentially toxic effects. Thus, IL 1 has a range of activities unrelated to adjuvanticity (Table 4). For these reasons, the development of approaches aimed at restricting IL 1 activity to the immune system is highly desirable. This might be possible by the use of structurally modified IL 1 or naturally occurring IL 1 sub-species which display only immunological activities. Of interest, in this regard, is the

TABLE 4. Potential Adverse Actions of IL 1

1. Fever
2. Fibroblast and Smooth Muscle Proliferation
3. Procoagulant Synthesis
4. Collagenase Production
5. Neutrophilia and PMN chemotaxis
6. Alterations in plasma Fe++, Zn++, Cu++ levels
7. Synthesis of acute phase reactants

report that an MDP analogue elicited the production, in rabbits, of a non-pyrogenic IL 1.[77] Interestingly, the IL 1 produced by a human B lymphoid cell line, established in our laboratory, is also apparently non-pyrogenic. Compounds may also be produced which will act as IL 1 agonists.[72] Towards this goal, we are undertaking studies to define the IL 1 receptors on human T cells. We plan to test for chemical compounds which are able to induce lymphocyte activation by specific binding to the IL 1 receptor.

A potential concern with the overall activation of the immune system is that response to self antigens may occur, leading to autoimmune disease.[78] The question of concomitant delivery of antigen and adjuvant to the specific site of immune activation is of considerable importance. Among the several suggestions offered are: a) the use of liposomes with entrapped antigen and lymphokines; b) the use of antigen-adjuvant complexes such as MDP coupled to antigen; and c) the use of adjuvant-acting polymers to which antigen is attached.[47] We are pursuing the prospect of using antigen-bound platelets, pre-incubated with lymphokines, as a novel method of selective antigen delivery and lymphoid cell activation. The lymphokines will include IL 1 and/or gamma interferon. The initial test system will involve an assessment of the responsiveness of cultured T cells from either normal humans, vaccine injected human volunteers or vaccine injected primates. We plan to phenotype the responding cells, using a panel of monoclonal antibodies, to determine whether they are likely to have helper or suppressor activity. We then plan to test these cells in more specialized assays of CMI and humoral immunity. Similar *in vitro* assays should also be applicable to testing other proposed antigen-adjuvant delivery systems and in defining the rate-limiting step in immune activation to malarial vaccine antigens.

Summary

This paper has provided an overview of the pathways of immune activation and the potential mode of action of adjuvants for use with malarial vaccines. The rapid progress in the identification of various malarial proteins has outpaced the development of new forms of adjuvants. From a practical point of view, it is likely that initial clinical studies will simply involve the use of alum adsorbed antigen. However, if the levels of immunity evoked using this approach are insufficient in intensity or duration, or if the evoked response is inappropriate for protective immunity, further trials involving new forms of adjuvants will be required. The development of a safe and effective new adjuvant for widespread clinical use may be yet another benefit to be derived from the Malarial Vaccine Development Program.

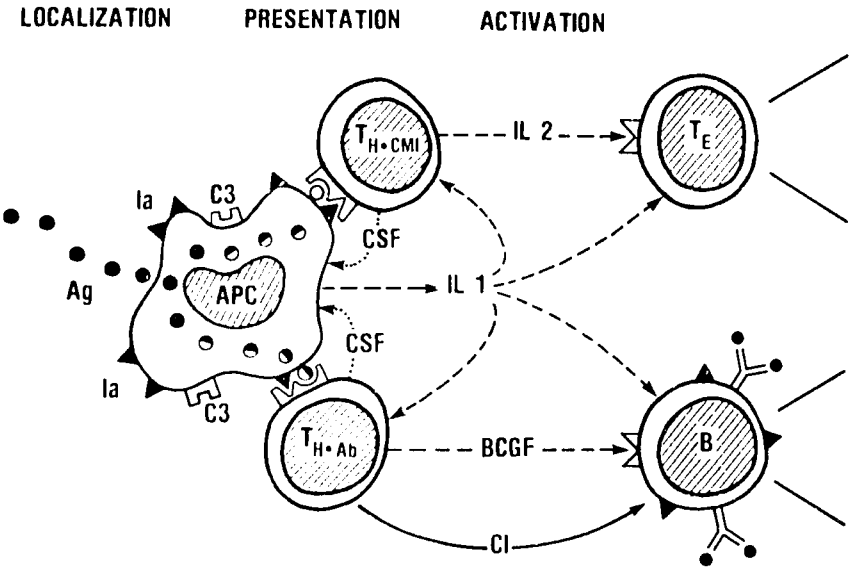

FIGURE 1. Schematic Representation of Immune Response. The abbreviations in the diagram represent the following: Ag = antigen; C3 = C3 receptor; Ia = class II MHC antigen; APC = antigen presenting cell; TH-CMI = helper T cell for cell mediated immunity; TH-Ab = helper T cell for humoral immunity; TE = effector T cell; B = B cell; IL 1 = interleukin 1; IL 2 = interleukin 2; CSF = colony stimulating factor; CI = cell interaction.

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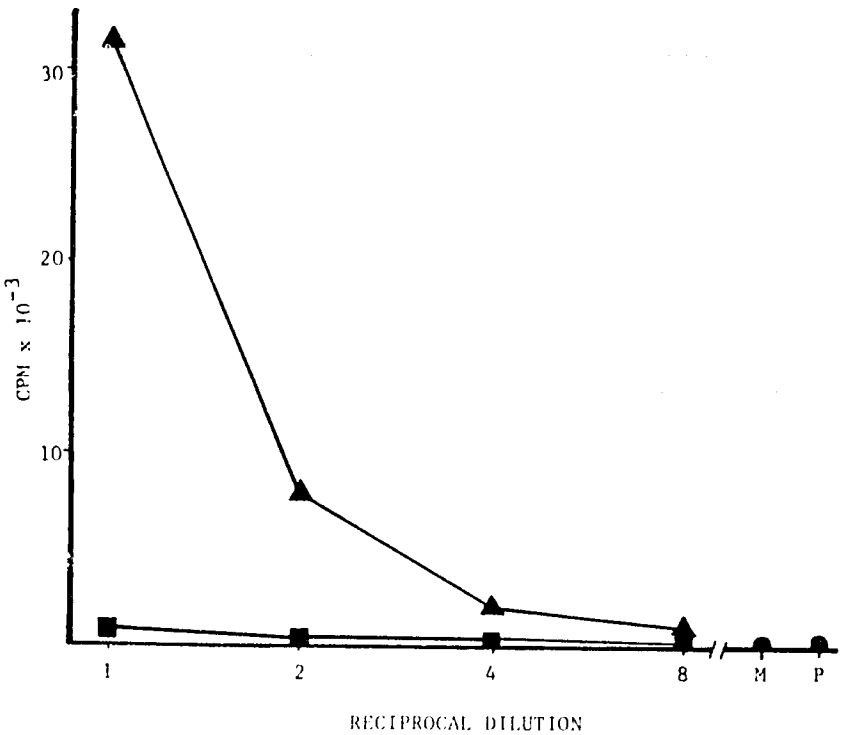

FIGURE 2. IL 1 Assay Utilizing Human Cultured T Cells (CTC). Human leukocytes were cultured in the presence of interleukin 2. After 10–14 days of proliferation, the cultured cells were aliquoted into 96 well microtiter plates at 10 5 cells/well in 0.1 ml. Interleukin 1, at various dilutions, was added to the wells at 0.1 ml/well, plus 20 microliters of either plain medium of PHA (final concentration of 0.25 μg/ml). Cultures were incubated 72 hours, with 1.0 uCi 3H-thymidine added, in 20 μl medium, for the last 6 hours. The symbols in the figure represent the following: circles = medium (M) or PHA (P) alone; squares = IL 1 alone; triangles = IL 1 + PHA.

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Discussant: Potential Role of Adjuvants for Production of Future Malaria Vaccines

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A good conventional vaccine should give an early, high, and durable response of the appropriate isotypes and also in certain cases induce cell-mediated immunity. Moreover, the immunogen should be effective in immunodeficient or immature hosts. Since such an immunogen rarely, if ever, exists an adjuvant is required to compensate the absence of one or several of the above-mentioned characteristics. An adjuvant may also be required in view of reducing the amounts of rare or costly antigens. These comments are particularly valid in the case of malarial antigens which have by-passed the conventional route of vaccine development.

Production of conventional parasitic antigens raises a number of problems. Parasites are most difficult, if not impossible, to cultivate. Moreover, demonstration of the efficacy of certain parasitic vaccines is restricted by the necessity of using very expensive and not easily available animal models such as *Aotus* or *Saimiri* monkeys for malarial antigens. It is therefore clear that the opportunity offered by modern biotechnology (monoclonal antibodies, genetic engineering, DNA sequencing, peptide synthesis, purification methods, etc.) will be taken advantage of.[1] Indeed, many other second generation antigens have already been prepared by genetic engineering, including a Hepatitis B surface antigen (HBs) produced in yeast and currently in clinical trials.[2–5] It does not seem unreasonable to expect that the following generation of vaccines will probably be immunogens produced by peptide synthesis. Interestingly, whereas most if not all these new antigens have been obtained just by improving former conventional methods of production of existing vaccines, it is expected that the first malaria vaccine will be a second or third generation vaccine. Thus, recently the protein composed of tandem copies of the tetrapeptide repeating sequence

found in the circumsporozoite protein (CSP) of *Plasmodium falciparum* was expressed in *Escherichia coli*. The genetically engineered products were found to be immunogenic and produced biologically active antibodies in mice.[6] They are considered as potential candidates for a human malarial vaccine. Simultaneously, peptides produced by chemical synthesis and copying the same structure of *P. falciparum* CSP were also found to be immunogenic. It must be added that earlier studies had shown that biologically active antibodies can be obtained after administration of a peptide of 24 amino acids copying a tandemly repeated epitope of the *Plasmodium knowlesi* circumsporozoite (CS) protein.[7]

Synthetic vaccines containing viral, bacterial, parasitic, and hormonal synthetic antigens have been described and constitute a very rapidly expanding field of research. The "synthetic" approach has certain theoretical superiorities vis-à-vis the "genetic" one. Thus, molecules containing less than 40 residues should be easier and cheaper to produce by synthesis and their safety and purity easier to control. Besides these and other economical advantages for industry (patent protection), these methods have the unique capacity of designing artificial sequences more performing than the natural molecules. Today, the success of such a strategy has been repeatedly demonstrated in the field of hormones or other natural substances.[8]

To be immunogenic, a preparation has to contain three entities: the antigenic determinants which will evoke antibodies specific for the pathogen or hormone; a carrier molecule affording suitable presentation of these determinants to the immune system; and an immunopotentiating moiety which will augment the response. All these elements are generally present in natural vaccines. However, natural vaccines also contain other structures, in most cases ill-defined, which are useless for vaccination and even are capable of inducing unwanted side effects. Thus, one of the main reasons for developing synthetic vaccines is to obtain efficient preparations composed only of these three necessary and sufficient components.

According to the degree of knowledge one has of a given immunogen, biological or structural criteria have been used to select the most performing peptide sequence. In the case of pathogens presenting, under selective immunological pressure, regions with high frequency of mutation, these regions can be assumed to represent targets for antibodies and therefore important antigenic sites (foot-and-mouth disease virus).[9] Neutralizing polyclonal or monoclonal antibodies can be used as probes for identification of the appropriate epitopes (streptococcal M1 protein; hepatitis B virus; poliovirus; or malarial sporozoite antigens).[10–13] In the case of malarial antigens following the Nussenzweigs' pioneering experiments, very elegant results using monoclonal antibodies have

been reported on the identification of major antigenic determinants of the CS protein of *P. knowlesi* and also of *P. falciparum*.[14-16] Promising results have been obtained by using a similar approach for searching antigens of the blood stage.[17-19]

Whatever their mode of preparation, most artificial peptides are poor immunogens and will probably need the help of a carrier and of immunoadjuvants. As a matter of fact, the above-mentioned peptides have been usually coupled to a large carrier molecule, such as tetanus toxoid (TT) or keyhole limpet haemocyanin, and often injected with Freund's complete adjuvant (FCA). However, other procedures have also been advocated, such as utilization of vaccinia virus recombinants or inert delivery systems.

Live vaccines for heterologous pathogenic agents have been produced by constructing vaccinia virus recombinant expressing various foreign genes. The first example was reported by Smith et al.[20] who inserted the coding sequence for HBsAg into the vaccinia virus genome. Investigators have also reported successful production of vaccinia recombinants expressing a gene coding for the circumsporozoite protein of *P. knowlesi* and *P. falciparum*, and vaccinated rabbits were shown to produce antibodies to the repeating epitopes.[21] Although very promising, more experiments will be required to evaluate the efficacy of such vaccines and to delete the virulence of the vaccinia organisms. Furthermore, it will be necessary to rule out the possibility of reversion of recombinant organisms to new virulent forms since small-pox vaccination has been abandoned in most countries.

Parallel efforts are being pursued in view of finding inert delivery systems, such as liposomes. Protein antigens have often been encapsulated into liposomes.[22,23] Such preparations elicited stronger immune responses for many reasons: "depot" of antigen, entrapment by macrophages, better presentation. *In vitro* produced *P. falciparum* merozoite antigens and lipophilic analogs of MDP were incorporated by Siddiqui into liposomes. *Aotus* monkeys immunized by these preparations were protected against challenge.[24]

After briefly reviewing various existing adjuvants, our purpose is to describe the role of synthetic adjuvants called muramyl peptides or MDPs in malaria models represented by totally or partially synthetic constructs containing one or several specificities. It will also be shown how certain problems that can be raised by the use of carriers can be corrected or avoided. All these examples should delineate various strategies allowing the use of clinically acceptable future malaria vaccines. It will also be underlined that, contrary to inert delivery systems, a synthetic immunoadjuvant intervenes actually in the reading of the epitopes and in the modulation of the antibody response.

Adjuvants

Before reporting the current status of adjuvants it should be mentioned that other procedures and other agents are also being considered.

Thus, host mediators such as interleukins will perhaps be used in the future to amplify the antibody response to synthetic antigens. It can even be assumed that more performing copies of these recently cloned endogenous molecules will also be obtained by chemical synthesis. An alternate promising approach for regulating the immune response is utilization of the idiotype network. Injection of anti-idiotypic antibodies can induce antigen specific helper T-cells and has been shown to increase the immune response to HBsAg.[23] In the meanwhile, experimental and clinical adjuvants are mainly mineral alum hydroxide, saponin, and FCA or Freund's incomplete adjuvant (FIA). FIA and other mineral oil preparations have been used for veterinary vaccines such as foot-and-mouth disease and Newcastle virus.[24] Saponin has also been used extensively in such vaccines.[25,26] Nevertheless, both of these adjuvants, FIA and saponin, cannot be used in humans, although the former has been assayed in clinical influenza trials but has been abandoned since.[27-30] The adjuvant which has been—and still is—the most used in humans is alum hydroxide administered sometimes with certain polyvalent vaccines containing *B. pertussis* which is also considered to be an adjuvant.[31] However, even these acceptable agents are not devoid of certain drawbacks. They can induce local reactions and other side effects such as enhancing the production of IgE.[32,33] Development of new agents such as muramyl peptides capable of potentiating the antibody production and devoid of side effects are therefore being pursued actively. The most common adjuvants used in the laboratory are FIA and particularly FCA.

It must be emphasized that one of the earliest models used by Freund for his adjuvant were *Plasmodium lophurae* in ducks and *P. knowlesi* in Macaccus.[34,35] These results were confirmed by Brown who used *P. knowlesi* and showed that FIA was inadequate because cell-mediated immunity was required. Further, it was also demonstrated that FCA could overcome antigenic variation.[36,37] However, the very powerful activity of FCA, which is a water-in-oil emulsion containing Mycobacteria, is not utilizable in humans or even in veterinary vaccines because of its numerous side effects: granuloma formation, lymphoid hyperplasia, potentiation of endotoxin toxicity, arthritogenicity and tuberculin sensitization.[38,39] This is a most important issue for many oily veterinary vaccines. Addition of Mycobacteria induces a positive tuberculin response and, therefore, a suspicion of previous tubercular disease.

Over ten years ago, the synthesis of N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide or MDP), representing the copy of a

peptidoglycan fragment present in bacterial cell walls, was achieved by Lederer and independently by Kotani.[40,41] Following this important step, several hundred synthetic muramyl peptide derivatives or analogues were produced in several laboratories and more particularly by Pierre Lefrancier and his colleagues at the Choay Institute. This impressive armantarium has allowed a good understanding of the relationships between chemical structure and biological activities of these molecules.[38,39] As in the case of many immunomodulating agents, studies have shown that MDP may possess a wide spectrum of properties. Besides their manifest immunological capacity, these molecules also have general pharmacological effects including some which can be considered as neuropharmacological.[42,43]

MDP has been considered as the smallest structure capable of inducing all the adjuvant activities produced by Mycobacteria in a water-in-oil emulsion including sensitization to ABA-tyrosine and recognition of auto-antigens. Molecules lacking the muramic acid moiety, such as desmuramyl peptidolipids, have been shown to enhance antibody response, yet were inactive when used in the more requiring tests mentioned above.[44,45] By adding MDP to FIA one can strengthen considerably immune responses and obtain all the effects produced by FCA, including immunity against malaria.[46] Moreover, many of the drawbacks of FCA, namely sensitization to tuberculin, would be avoided. Thus, the question raised by Freund almost 40 years ago has been answered since "the fraction which exerts the adjuvant effect of Mycobacteria has been identified" and that this "effect could be produced *without* sensitization to tuberculin."*[47] It can therefore be reasonably predicted that a water-in-oil emulsion containing MDP has the potential of enhancing several veterinary vaccines.

Although generally less active in the absence of FIA, MDP can augment the humoral antibody response to several antigens.[38,39,48] Certain lipophilic derivatives of MDP have also been shown to increase the cell-mediated immune response even when administered in saline.[49,50] By chemical modification, it has been possible to eliminate fever and certain side effects of MDP and to retain its immunopotentiating activity. In most experiments muramyl peptides were injected with classical laboratory antigens (such as albumins), however, these adjuvants were also used with conventional or even synthetic vaccines. Coupling MDP or derivatives to carrier or to antigenic materials increases certain of their biological activities.[51,52] Such procedures have been repeatedly used in synthetic vaccines. The most promising compound is presently

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*"It would be of great interest to identify the fraction or fractions which exert the adjuvant effect of Mycobacteria, and to know whether the effect could be produced without sensitization to tuberculin." J. Freund, 1947 (47).

NAcMur-L-Ala-D-Gln-α-n-butyl-ester also called Murabutide which is as active as MDP but devoid of any detectable side effects.[53,54] This compound is currently undergoing clinical trials.[55]

Problems Raised by Carriers

The choice of appropriate carriers is of primary importance for designing synthetic vaccines for human use. Tetanus toxoid (TT) has been employed in most studies so far because it has been very widely used as a vaccine for many years. Moreover, since most humans have been immunized against TT, it was assumed that the response against a synthetic peptide conjugated to TT carrier should be improved by the preexistence of an anti-carrier immunity.

However, as mentioned, earlier use of protein carriers is not without limitations for basic research and may be unacceptable for future application. The utilization as carrier of a natural molecule bearing a large number of highly immunogenic antigenic determinants renders difficult the analysis of the immune response to the conjugated synthetic peptide. For example, it is impossible to delineate the capacity of the peptide itself to trigger the different cell populations or to determine its influence on the characteristics of the antibodies produced. Carriers may also raise immunotoxicological problems if they are to be used in future synthetic vaccines. It is generally assumed that it would not be safe to administer a conjugate containing more than 100 μg of TT (the current vaccinal doses vary between 1 μg and 10 μg), making it difficult to couple sufficient amounts of synthetic malaria peptides. There is also a risk of sensitization after repeated administrations of even small quantities of toxoid. Moreover, since most humans have been immunized with TT, there could be a high probability of specific epitopic suppression. This phenomenon was first described by Herzenberg who used laboratory antigens (DNP as hapten and bovine gammaglobulin or hemocyanin as carriers).[56] Mice presensitized by one carrier and treated later with a conjugate containing a hapten linked to the same carrier did not synthesize antibodies against the hapten. On the contrary, the response was specifically suppressed even when this hapten is administered a second time coupled to another carrier. It was therefore of primary importance to know if the results observed by Herzenberg were not limited to a laboratory model and whether it could be reproduced using a synthetic peptide coupled to TT. Mice presensitized to tetanus toxoid which received two injections of synthetic diphtheric or streptococcal peptides conjugated to tetanus toxoid did not develop anti-peptide antibodies whereas their non-presensitized controls presented respectively high anti-diphtheric or anti-streptococcal peptide

antibody responses after immunization by the conjugate administered in saline with a synthetic adjuvant.[57,58]

Similar experiments were performed using a conjugate containing a *P. knowlesi* CS peptide coupled to TT.* Once again, mice presensitized to TT did not respond to the peptide contrarily to their naive controls which developed high titers of anti-peptide antibodies after immunization by the conjugate given in saline with Murabutide. Interestingly, under certain conditions the establishment of the specific epitopic suppression can be blocked by the use of adjuvants.[59] Experiments are in progress to confirm this observation in models of synthetic vaccines.

Since problems raised by carriers may represent a major roadblock for the development of synthetic vaccines, new procedures of immunization must be searched for. It is indeed possible to avoid certain of these problems by administering free peptides in mice previously treated by protein antigen and adjuvant. It is also possible to produce primary immune response by immunizing mice with synthetic antigens coupled directly to MDP synthetic peptide.

1. Secondary Immunization by a Synthetic Free Peptide in Absence of Both Carrier and Adjuvant

An important protective epitope of the M protein of *Streptococcus pyogenes* type 24 is represented by a 34 amino acid peptide S-CB7 (for synthetic cyanogen bromide fragment no.7) originally described by Beachey.[40] Polymerized S-CB7 administered in saline without adjuvant or carrier elicited a strong secondary response in mice previously immunized with the natural M24 protein. The antibodies were directed against the N-terminal of S-CB7 which contains the major epitope shared by the synthetic peptide and the natural M24 protein. Most interestingly, these antibodies were also shown to be protective.[60] The same protocol was used to immunize human volunteers. Subjects were immunized either by the M24 protein alone or by the M24 protein associated with Murabutide. They received on days 60, 90, and 120 two hundred micrograms of S-CB7 in saline. Serum antibodies were measured on days 90, 120, and 140. A comparative study was performed on the specificity of sera taken on day 45 and on day 140. A consistent elevation of anti-peptide antibody titers was observed and the best titers were obtained in the group which had received Murabutide with the priming. On day 140, a conversion from the anti-M24 protein specificity to the S-CB7 antibody specificity was observed. The sera were filtrated through a S-CB7 immunoabsorbent column which retained only anti-peptide antibodies.

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*This peptide described by Godson et al. (13) represents a tandemly repeated sequence of the circumsporozoite protein.

These antibodies were eluted from the column and were shown to be bactericidal when incubated with human leukocytes and *S. pyogenes* type 24. Such results have offered the first evidence that a synthetic peptide can induce biologically active antibodies in humans after primary immunization by a natural antigen.[61] It can be pointed out that the utilization of synthetic material for the re-vaccination of subjects already sensitized (by natural infection or by previous vaccination) to a given pathogen could find practical applications. It is, however, possible to produce both primary and secondary responses in the absence of carrier by administering certain antigens directly coupled to the adjuvant.

2. IMMUNIZATION BY A CONJUGATE CONTAINING A SYNTHETIC ANTIGEN DIRECTLY COUPLED TO MDP

Synthetic luteinizing hormone-releasing hormone (LH-RH) is a decapeptide hormone which controls the secretion of LH and FSH (Follicle Stimulating Hormone) by the pituitary and thus the development and function of gonads in males and females.[62] It had been demonstrated previously in several animal species including cattle that coupled to a protein carrier and administered with FCA, LH-RH evoked antibodies resulting in immunological castration. In later experiments, LH-RH-MDP-Lys conjugates were obtained either by coupling with carbodiimide or even by total synthesis and administered in saline.[63,64] Control groups received LH-RH conjugated to bovine gammaglobulin given in FCA or the LH-RH-MDP-Lys conjugate with FIA. Surprisingly, the most effective treatment was LH-RH-MDP-Lys administered in saline. Testosterone levels were the lowest and all the animals had severe atrophy of the testes as shown by involution of the seminiferous tubules and interstitial tissue, as well as a decrease or even absence of spermatozoa. Similar results were obtained using the molecule obtained by total synthesis. This compound of less than 2000 dalton was capable of inducing castration in males and also in females.[65] These experiments demonstrate that appropriate synthetic peptides conjugated directly to a synthetic adjuvant can evoke biologically effective immune responses. Similarly, a diphtheric peptide (SODP for synthetic octodecapeptide) and more recently a synthetic FMDV peptide copolymerized with MDP-Lys were shown immunogenic and induced protective antibodies in the absence of carrier.[66,67]

Such results are encouraging since they suggest that when the conformational requirements of conjugates will be understood better they may allow us to apply such procedures to other antigens. In the meanwhile, they cannot be generalized and we have been unable to detect antibodies in mice immunized with the *P. knowlesi* CS peptide copolymerized with MDP. There exist, however, other procedures to avoid the use of a

protein carrier or to minimize its immunogenicity and this can be utilized with a malarial synthetic peptide.

3. Immunization by a Conjugate Containing a Derivative of Murabutide and the *P. knowlesi* CS Peptide Covalently Linked to TT

Previous results showed us that this malaria peptide conjugated to tetanus toxoid and administered in saline with Murabutide produced biologically active antibodies (as tested by the CS precipitation [CSP] reaction). Interestingly, this activity was higher than in the group treated with alum or FCA.[68]

A derivative of Murabutide (ε-amino-caproic Murabutide) has been covalently linked to the *P. knowlesi* CS peptide-TT conjugate. This new construct was given to mice in saline. High levels of biologically active anti-sporozoite antibodies were obtained using a conjugate containing only 1 μg of Murabutide: titers were equivalent to those obtained with 100 μg of adjuvant mixed with the peptide-TT preparation. In contrast, very low anti-TT antibodies were observed as compared to the anti-carrier titers obtained in the control groups which received the peptide-TT conjugate.[69]

4. Immunization by a Totally Synthetic Vaccine Mixed with Murabutide

The use of tetanus toxoid as a carrier may be totally avoided by associating peptides of different specificities within the same construct. As already mentioned, S-CB7 is a structure of 35 amino acids which represent the major protective epitope of the M protein of *S. pyogenes* type 24.[10] It contains five lysyl residues allowing the attachment of several peptides. A construct was prepared containing, besides S-CB7, a diphtheric peptide, a hepatitis B (S antigen) peptide, and the *P. knowlesi* CS peptide.[65,70-73] Swiss mice received this tetravalent synthetic vaccine in saline, with or without Murabutide or with FCA. Antibodies of the four specificities were obtained. The group immunized by the immunogen with Murabutide in saline showed titers as high as those measured in the FCA group. Moreover, these antibodies were biologically active. Thus, adjuvant-treated mice survived after a lethal challenge of *S. pyogenes* type 24 in contrast to control mice immunized by the conjugate only.

Anti-sporozoite antibodies were evaluated by the CSP reaction and titers of the Murabutide-treated group were even higher than those of the FCA-treated mice. It should be added that the ELISA titers can be misleading since they were higher in the FCA-treated group. This fact is not unique and in different synthetic vaccine models it was shown

that titers measured by ELISA did not correlate with the biological activity of sera; this could be explained by the constraint induced to the antigenic structure of small peptides in a solid phase assay hiding the protective epitopes. More interesting is the observation that sera of the FCA-treated group are less effective than sera of the Murabutide-treated group, suggesting that administration of a synthetic vaccine in an aqueous vehicle provides a better presentation of the protective epitopes than administration in a water-in-oil emulsion.

Strategies for Clinically Acceptable Synthetic Vaccines

In the future, immunogens may be also produced through the manipulation of the idiotypic network. Injection of soluble anti-idiotype prior to HBsAg or synthetic peptide stimulation increased the anti-HBs response, suggesting that the immune response to HBV may be controlled by this approach.[23]

In the meanwhile, immunogens are being produced by genetic engineering and chemical synthesis.

During the past four years, a great deal of effort has been devoted to the design and production of synthetic antigens capable of evoking protective or neutralizing immune responses towards pathogens and hormones.

The use of synthetic peptides for future clinical vaccines will depend upon the feasibility of preparations suitable for human and veterinary applications. As described in this presentation, synthetic structures can be used in alternate order with natural structures to induce fully protective immunity. Such approaches would be useful in the case of rare or expensive natural vaccines. Secondary immunization by synthetic epitopes instead of conventional vaccines could be generalized in subjects already sensitized to a given pathogen by previous immunization or natural exposure. Although two other components, carrier and adjuvant, are most generally considered as essential, peptides can induce in certain cases a protective response when administered in absence of carrier but associated to synthetic adjuvants. Active antibodies producing immunological castration have been elicited in the absence of carrier by a monomeric synthetic conjugate (less than 2000 dalton) containing the decapeptide hormone LH-RH directly linked to MDP-Lys. Another possibility of avoiding natural and/or high molecular weight carriers is given by administration of Murabutide with a copolymer of several peptides representing different specificities. A construct containing a malarial peptide and administered with Murabutide has been shown to elicit anti-sporozoite antibodies producing a positive CS reaction.

Despite these examples, it can be assumed that in most cases synthetic peptides will require the use of both adjuvant and carriers such as

tetanus toxoid. Therefore, efforts should be pursued to avoid repetitive use of the same carrier and to circumvent certain hazards such as epitopic suppression.

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Polyvalent Malaria Vaccines

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Polyvalence, as applied to future malaria vaccines, could refer to several things. A polyvalent vaccine may consist of a combination of stage-specific antigens, or portions of such antigens (epitopes), from the one *Plasmodium* species. Such a combination of antigens or epitopes from blood stages, sporozoites, and gametes would be designed to effect anti-infection, anti-disease, and transmission-blocking immunity. Alternatively, the polyvalent vaccine could consist of a defined mixture of antigens or epitopes from the one life cycle stage of the one *Plasmodium* species. Finally, it could be a combination of antigens or epitopes from different species of *Plasmodium*.

At the moment, most malariologists contemplating a hypothetical polyvalent malaria vaccine would probably have in mind a falciparum vaccine consisting of invariant portions of a CS protein, a major merozoite surface antigen (e.g., Pf190), RESA, cytoadherence protein, a rhoptry protein, Pf96, and 1 or 2 gamete antigens. They would also probably express some concern about antigenic competition, a possibility that will remain until data replaces conjecture.

In this presentation, and with scant attention to the all-important specifics of human-plasmodium relationships, I want to raise several issues relevant to engineered, molecularly defined malaria vaccines. I hope that, because of the generalities, it is not too much a statement of the obvious. Particular attention will be directed to only one effector arm of host-protective immunity, namely, *antibody*, and only one type of parasite molecule, namely, *protein*.

No molecularly defined vaccine based on protein antigens exists against any pathogen of man and even the bacterial toxoid vaccines now in use are in no way defined at the molecular level. Progress towards second generation "acellular" bacterial vaccines is well advanced as is progress towards an entirely different entity—genetically engineered bacteria—that derives impetus from the proven efficacy of attenuated

live bacteria that seem to best mimic solid infection-induced immunity.[1] Meningococcal and (highly polyvalent) pneumococcal polysaccharide vaccines are chemically defined and their undoubted efficacy, at least in certain target populations, provides encouragement to pursue the same objective in parasitic diseases. Of course, all of the latter must be far more complex immunologically than bacterial pneumonias and meningitis.

Advantages of molecularly defined vaccines have been outlined previously but for this discussion, the one to emphasize is that the availability of pure substances will greatly facilitate the development of polyvalent vaccines.[2] Incorporation of much higher doses of any one ingredient in the multicomponent vaccine can be envisaged with the likelihood of inducing high levels of immunity of longer duration. Attaining comparable levels of the one ingredient as a crude extract vaccine will be impossible or prone to dangerous or unpredictable consequences.

In the molecular vaccine era, the immunologist is confronted with the same problem that bedevils chemotherapy, chemoprophylaxis, and the drug industry, namely, the selection of resistant organisms. The molecularly defined vaccine will induce a restricted immune response that will enable organisms not expressing those antigens or epitopes to evade the consequences of vaccination. Because of population heterogeneity (i.e., molecular polymorphisms; antigenic variability), the more a molecularly defined vaccine is restricted in its composition, the greater is the probability of emergence of parasites that are defective in expression of that antigen or epitope subset. Of course, the expectation that has dominated molecular immunoparasitology to date is that a *small subset* of epitopes will be found that is highly immunogenic in hosts of all genotypes, that is available to immune effector mechanisms or indispensable to parasite physiology such that function is readily inhibited by antibodies, and that is not highly polymorphic within the parasite species.[2]

Population heterogeneity must be extensive in any parasite species although some restraints, relative to the population heterogeneity of vertebrate hosts, must be imposed by genome size. High resolution methods such as 2D gel analyses of immunoprecipitates and other serological techniques, nucleic acid probes, chromosome analyses, batteries of monoclonal antibodies, monospecific antisera raised to cloned antigens, and isoenzyme analyses are only now beginning to reveal the extent, or allow quantitation, of this heterogeneity in *Plasmodium falciparum*.[3-10] Whilst it is now easy to show heterogeneity in *P. falciparum*, restraints imposed by such heterogeneity on the vaccine objective are less readily assessed. One reason for this is the lack of a good mouse model of human falciparum malaria.

For some time now, attention in immunoparasitology has been mov-

ing away from the identification of "*the* host-protective antigen" in a parasite to identification of potential "host-protective epitopes" of several antigens in a parasite. Through synthesis of an entirely new antigen construct consisting or portions of various antigens, such a vaccine could in fact be molecularly defined to a greater extent than any native molecule of the parasite. It is in this endeavour that monoclonal antibodies and cloned portions of antigens are such powerful approaches and where immunoprecipitation and immunoblotting techniques with parasite extracts and polyclonal antisera can be less informative. A constructed vaccine antigen could consist of chemically or biologically synthesized peptides and polypeptides conjugated together or to carriers such as tetanus or diphtheria toxoid. Alternatively, the vaccine antigen (as a nucleic acid construct) could be delivered as a recombinant virus or recombinant attenuated gut microorganism. Vaccinia viruses in particular provide the potential to deliver, as a soluble or membrane associated protein, relatively large numbers of epitopes as unimolecular or oligomolecular "unnatural" antigens.[11,12] Time will tell whether such living antigen-delivery systems will herald in a new era of vaccines (see Bernard Moss, "Virus Vaccines as Examples for Development of Malaria Vaccines," this volume).

This newer emphasis in the quest for molecular vaccines stems from the theoretical advantages of polyvalent versus monovalent vaccines and the increasing evidence for "suppressogenic" regions of antigens.[13,14] Though in its infancy, the identification of epitopes that preferentially induce suppressor T cell (Ts) responses should lead to means of excluding them from an antigen construct. (Of course, it could be argued that induced antibody responses to such epitopes may inhibit the capacity to stimulate Ts cells. Fundamental studies in immunoregulation are required before further speculation on this is warranted. Responses induced by portions of a [soluble] native antigen during infection may be very different from those induced by these same epitopes conjugated to an immunogenic carrier molecule or presented on a cell membrane bearing class II MHC molecule). In addition, the unnatural construct could be devoid of regions of antigens that are immunodominant, and that through whatever mechanisms are operative in intramolecular competition, inhibit high titered responses to relatively immunosilent host-protective epitopes. The constructed antigen would also be lacking epitopes that resemble human antigens with the potential for induction of autoimmunity.

The most remarkable finding from the sequencing of malaria genes coding for major antigens in blood stages of *P. falciparum* (and the use of synthetic peptides and radioimmunoassays with sera from malaria endemic areas to confirm the reading frame), is the number that shows *tandem* repeated short sequences of amino acids (see both papers by

R. L. Coppel et al., this volume). Taking all malaria antigen sequences known to date, the repeat epitope antigens are of two types. Type 1 antigens contain a single block of repeats per molecule; type 2 antigens contain at least two distinct blocks of repeats per molecule. As native molecules, and as judged by analysis of antibody levels or frequencies in sera from patients in malaria endemic areas, type 2 repeat antigens (e.g., FIRA and RESA) are usually more immunogenic than type 1 repeat antigens (e.g., S-antigen, CS-protein).

Are type 1 antigens potent stimulators of Ts cells in natural infection or are mechanisms such as effector cell blockade and B cell blockage with antigen fragments involved? Does the repeat structure reflect a strategy "employed" by the parasite to reduce the likelihood of inhibition of binding of the polyvalent molecule to a polyvalent receptor on or in the target cell be that erythrocyte or hepatocyte? Are the type 2 antigen repeats strong stimulators of irrelevant antibody production that through a swamping or smoke screen effect (i.e., antigenic competition) inhibit induction of other antibody specificities far more relevant to host protection?

Hypotheses on such matters have not yet been tested experimentally. Hopefully, studies in progress on *in vitro* responses to various antigens, repeat and non-repeat epitopes, and various combinations using limit dilution approaches will be instructive.

To supplement the multiple points of immune attack that are sought by way of a polyvalent vaccine, shared epitopes between life cycle stages are of importance. In falciparum malaria, the best example of a shared epitope is the target of the monoclonal antibody described by Hope et al.[15] This monoclonal binds to sporozoites and an Mr 23,000 protein of blood stages. The gene for this protein has been cloned and sequenced in two laboratories and the tetrapeptide Asn-Ala-Asn-Pro, the major repeat unit in the CS protein, and other closely related peptides have been identified in the Mr 23,000 protein.[16,17] The immunological importance of this finding remains unknown as yet.

If a large component of antibody-mediated protection against blood stages of *P. falciparum* is variant specific then a strong case can be made for vaccination with any invariant portions of variant antigens in a manner that sensitizes helper T cells (i.e., T_H cells). In a host sensitized for accelerated antibody production because of the presence of a primed T_H cell population, variant specific antibody may appear earlier than normal in the course of a new parasitemic wave and abort this infection before the onset of severe disease. This approach to malaria vaccination was proposed many years ago by K. N. Brown on the basis of work on *P. knowlesi* SICA variants in rhesus monkeys.[18]

Some doubts about the supposition on which the approach is based have appeared in the immunology literature (e.g., 19). Thus mice pre-

immunized with carrier prior to hapten-carrier injection show reduced high affinity IgG (but not IgM) anti-hapten responses. This contrasts dramatically with the situation following transfer of cells to irradiated recipients or if animals are preprimed to the hapten (on another carrier).[20] Interestingly, anti-carrier antibody responses are not inhibited and analogies with the phenomenon of "original antigenic sin" in response to influenza viruses are discussed by Herzenberg et al.[19] Whether the immunology of new variants appearing in malaria-infected hosts will resemble the carrier preimmunization model described above in unknown. Reduced parasitemias and accelerated responses to late variants combined with evidence for variant-specific immunity in the rhesus/*P. knowlesi* model, would seem to be readily explained by T_H cell priming. In any case, present methods to identify T_H cell-stimulating epitopes of antigens are cumbersome and even when identified and produced, it is not clear how to go about injection in order to ensure T_H cell sensitization. It is likely that studies with malaria antigens will tell us more about immunoregulation under physiological conditions, and the immunobiological consequences of vaccination, than the multitude of haptenated antigens currently in use in cellular immunology.

Once an invariant epitope or subset of epitopes of an immunogenic variant molecule are identified, questions arise as to the reasons for that invariance. Is it the sought-after portion of the antigen in which polymorphism cannot be tolerated in order to preserve function? Or is this portion of the molecule simply not available to antibodies in the living parasite or intact parasitized cell? Is it recognized well by a mouse monoclonal antibody (i.e., a heteromonoclonal) yet never recognized by human antibodies because it is an epitope that resembles a self epitope to which effective tolerance is preserved.[21]

The notion of immune responses in the vaccinee being necessary and/or sufficient is relevant in the context of polyvalent malaria vaccines. Very encouraging for the vaccine developer is any evidence that sensitization for an accelerated response of a particular type *on subsequent encounter with the parasite* is sufficient to effect solid resistance.[22] Difficulties can be anticipated in inducing and maintaining high-titered responses with molecularly defined vaccines in all individuals of the genetically diverse host population. It would be highly desirable if constant exposure to the parasite boosts and maintains protective responses in hosts whose immune system has been "locked into" that particular response by vaccination. One aspect, namely, sensitization (clonal expansion?) of T_H cells with invariant portions of variant antigens, has been discussed above. This line of reasoning also lies at the root of the anti-idiotype approach (or, more particularly, the anti-cross-reactive idiotype, IdX, approach) to vaccines.[23,24] It is inconceivable that high titered responses of sufficient polyclonality and functional activity

against *natural* pathogens in a field situation would be induced by the anti-Id vaccine; such responses may be measurable, however, after challenge of the vaccinee.

With malaria vaccines, the level of sensitization necessary to reduce morbidity and childhood mortality in human populations in endemic areas could be very different from that demanded by occasional visitors to the endemic area. In the former case, very detailed data will be required on populations prior to vaccine intervention in order to assess the overall efficacy of the vaccine. This sort of assessment is naturally very much more difficult and expensive than controlled challenge of human volunteers or *Aotus* and *Saimiri* monkeys. A very useful vaccine of value in an endemic area could be missed if profound effects on parasitemia in an acute challenge situation are considered as the only desirable consequence of a prophylactic malaria vaccine. More than ever, "control of disease" is the keypoint with "elimination of infection" being an unrealistic expectation, at least from the evidence available to date.

As a concluding comment, it is clear that artificial engineered vaccines of the polyvalent type are not just some armchair pipedream for another generation, another time. The ingredients and techniques are almost at hand, a statement that would have been fanciful just a few years ago. Agencies such as USAID, WHO-TDR, NIH, and The Rockefeller Foundation can take much credit for the fact that this optimism regarding malaria vaccines abounds. The creativity and skills of a new generation of biomedical scientists have added a new dimension to the endeavours of those individuals in tropical medicine who have pursued the vaccine objective for so long. Hopefully the polyvalence of the effort and approaches will culminate shortly in reagents of use for those most in need—the rural poor of the tropical less-industrially developed world. Population heterogeneity of *Plasmodium* spp. and their biological adaptability can be expected to be the most important stumbling blocks to rapid progress towards molecularly defined vaccines. In all likelihood, such vaccines will require modification at regular intervals.

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General Discussion

PAPPAIOANOU: I have a question that relates to the earlier session on adjuvants. We have heard that there are several antigens which are being proposed as candidates for a malaria vaccine. There is the very real possibility of using primate models. They will play a role in some aspect in the selection of the most promising antigens. The monkeys that we're looking at are hard to obtain. There has been some interest in trying to come up with some kind of standardization of protocol for testing these antigens in the monkeys. One aspect of this is the selection of the adjuvant for use. To those of you in the room who have far more experience than I do in adjuvant use and what we've come to know about the antigens themselves, if you were to run a trial in monkeys within the next couple of weeks or months, which adjuvants would you propose to use?

CHEDID: I can try and answer. I think it's a case by case situation and it depends on what's going on in the animal models with the various adjuvants. Clearly, you have the possibility of using alum hydroxide. If alum hydroxide works, you would have no problem for the time being. I think that there still is a desirability of substituting something to alum hydroxide. Actually, it's not complicated. In the animal field, which doesn't interest you, you have some good adjuvants which are saponin and mineral oil. In the human field, you have two possibilities, alum hydroxide and pertussis. There is a new possibility which is being tested currently. It is an MDP derivative. Now, in certain animal models, we can clearly see that alum hydroxide is less effective in getting protection. Moreover, we can see, and that's what I showed you under certain circumstances which are just totally unpredicted, that you can get more biological activity with a small molecule in saline than with the same antigen in Freund's complete adjuvant. So, I think all your questions are accessible to verification and ensuing strategy, but I don't think that anybody today can give you a general rule or guideline.

RISTIC: Going back to the original question. Dr. Pappaioanou asked which adjuvant should be used; which adjuvant should be using in proposed trials. It looks to me that you're talking about a three-step approach to the sporozoite vaccine. First is to find out which adjuvant gives the best immune response. Second is to go into *Aotus* monkeys to find out if that response induces protective immunity. Third is to go into man by selecting the best adjuvant shown by the previous two experiments. So I would suggest that the first step and the cheapest one, would be to take common laboratory animals, several of them, and use several different adjuvants and find out which adjuvant stimulates the best antibody production. Then go and select, based on those results, which adjuvant should be used in *Aotus* monkeys for the next experiment. Then based on the results of the protective experiments, decide how to proceed with the third experiment in man.

SIDDIQUI: I'd like to make a comment on Dr. Pappaioanou's question. This question was raised because of two factors: (1) limitation of monkeys, especially the specific type of monkeys which could be used to evaluate the efficacy of the candidate antigen, and (2) a rigorous requirement from the primate health committee. With reference to the erythrocyte vaccine candidate antigens, we have many candidate antigens. The problem is that we don't have any short cuts. We have to establish out of so many candidate antigens which we have, whether one or two or a combination of several polypeptides will show the effective immunity. Then we must use monkeys with the best adjuvant which is possible. Unfortunately none of the synthetic or semi-synthetic adjuvants can fully replace or can evoke the same kind of CMI response the way Freund's complete adjuvant does. At least in a few monkeys we must establish which candidate antigen is the best possible candidate for vaccine. Then we must have very rigorous research and development towards safe and effective adjuvant. It was very unfortunate that while this problem was recognized many years ago, not too much attention was paid to it. I think that most of the energy was diverted towards the finding of the candidate antigen. But I think that we've reached the stage where we have some very good candidate antigens, but still we are in search of a suitable adjuvant or carriers that can be used clinically. But I would be very cautious. We should not rush in and try what is available in humans. I think our evaluation has to be based on solid scientific foundation, and if there is a lack of knowledge in a certain area or a component of vaccine which is essential, I think we have to direct our attention to that.

PAPPAIOANOU: With respect to Freund's complete adjuvant, the question is that the mechanism of the immune response that we are looking at is primarily humoral rather than cell-mediated. I'm addressing the

expertise in the room. If you were to try to access the efficacy of an antigen which was believed or hypothesized to be working along a humoral response then you use Freund's complete adjuvant which would also evoke a large cellular immune response, what would that do to the interpretation of the protective efficacy of the antigen? Would it still be interpretable?

SIDDIQUI: When you give the antigen in Freund's complete adjuvant compared with giving the antigen in Freund's incomplete adjuvant, the difference is remarkable. With Freund's complete adjuvant you get much better protection.

CHEDID: The real problem in malaria is that models are difficult to have. If you have a model you can answer these questions very rapidly. Do you need Freund's complete? Do you need Freund's incomplete? If you need Freund's complete what can you do to make it better than Freund's incomplete that is feasible. I think that all generalizations are extremely dangerous. With the TGAL nonresponders and responders in mice for instance, it does not work with Freund's incomplete although in the classical sense, we are not speaking of cell-mediated immunity to get an antibody response to TGAL. Cell-mediated immunity means many more things than just the classical things that we are refering to such as delayed hypersensitivity. It may simply mean an excess of Interleukin I. As a matter of fact, you can measure an increase of Interleukin I when you give a hydrophilic muramylpeptide. We talk of muramyl peptides all the time, but I'm convinced that there must be dozens of possibilities which are about the same. I think that the best recruitment of adjuvant candidates comes from the bacterial world and copies of the bacterial world, for instance, what Dr. Kotani referred to.

PELTOLA: I'll be quite cautious to say, looking at the lists which were presented during the last couple of hours, that one adjuvant is not suitable and the other is. We really know very little about the relationships between the antigen and the adjuvant. It might be that just a little amount of a reactogenic adjuvant still could work and would not be very reactogenic in clinical practice. Regarding the question which adjuvant would be the best choice right now, I think the answer is first to concentrate on looking at various amounts of adjuvants and then to try to answer that difficult question.

MARTIN: I think the animal studies can be divided into two parts. If the question is will anti-sporozoite antibodies provide protection in a primate system, then one is at liberty to try whatever techniques are necessary to raise such a high level of antibody to prove efficacy. If, in fact, from all the data that we've heard, that point can be almost accepted or requires very little additional involvement in primates, then the addi-

tional benefit of the primate studies may well be to optimize the type of dosage that would go into humans. If that's the goal, then surely the primates should receive the human formulation to obtain that information on primates before entering into clinical studies. There are two separate questions. One is efficacy of anti-sporozoite antibody per se and the other is optimizing a formulation for potential clinical use. On the second question, it would delay progress to impose upon the malarial vaccine development all the burdens of having to prove efficacy of a new class of adjuvants.

DREESMAN: In a time when some of these vaccines are most important, is it really worth doing the experiment with Freund's complete adjuvant, or should you just select right away something that would have applicability. It seems like in so many of the hepatitis B experiments that have been done, the peptides have been crossed-linked to KLH and then given with Freund's complete adjuvant and protection is obtained. But that's rather heroic efforts which may have no application to the human situation. These are expensive and rare animals, the chimpanzees, and I really think that really is a waste of time and waste of good resources to go in with something that does not have applicability for human usage.

CHEDID: This lipid A story was a fantastically difficult chemical story. Recently, it has been synthesized in monomeric form. With MDP our luck was that it was such a simple structure and that it enabled accumulation of a lot of information. But I am sure that some day we will find molecules with properties of Interleukin molecules and synthesize artificial Interleukin molecules. After having cloned them, we will make adjuvants which are analogous as synthetic rubber is to natural rubber, in which, the synthetic rubber is better than natural rubber.

MITCHELL: My impression is that what has held back adjuvant studies is that the rules you establish in one animal species simply don't apply in others. You could get a hell of a lot of good, very active information in the rabbit, which simply will not apply to man, mouse, monkey, and vice versa. That's why we can't give a straight answer to this one. I think the strategy of doing it all systematically in one chosen species and then applying that to the next species is not necessarily the right one, because all bets are off when you go to the next species.

RISTIC: I also don't see a reason to go into very expensive animals right away and hope to accomplish everything in one experiment to get the best immune stimulation and protection. I think that you need to go step-wise.

MEUWISSEN: I have a practical question to Dr. Mitchell. We emphasized the importance of T_H cell stimulating epitopes. How do we select for these epitopes on a given protein? How would you approach that?

MITCHELL: Gordon Dreesman could also answer this because he addressed this in his talk. It's been a major problem for a long time until continuous T cell lines came along. By walking down a molecule which you've got a sequence for, you can come to a region which will preferentially induce a response in a cloned T cell line or, in a limiting dilution assay, with a non-cloned line. That will give you some idea. If you know what the phenotype of the cell is which is responding (helper, suppressor, cytotoxic, etc.) then you'll get some idea that at least this is a region of the molecule which can induce a proliferative response in that particular cell line of that phenotype *in vitro*. And that would be a good start. But, it was very cumbersome until the clonal analyses and the clonal T cell lines to really pinpoint what this T cell stimulating epitope or region was.

DREESMAN: Has anyone in the general malaria field looked at T cell responses?

MARTIN: Only a general philosophic comment. This really contrasts the life of the basic immunologist who has the luxury of dealing with simple antigens and clean assay systems and comes up with quite complex model systems, compared to this group here dealing with very complex applied systems. In one sense, I think the task is much more difficult, but unnecessarily difficult, if you deny the advances that are made by the basic immunologist. I think that one should accommodate every advance that's made by basic immunologists—every little arrow and every little box ought to be at the forefront of our thinking as we now try to tackle the applied problem. To really look at T cell recognition units, define infinite subpopulations of T lymphocytes, and to apply that knowledge and that technology to malaria is going to, perhaps, make the past approachable. To deny those advances and just to fall back on a broad black box cell mediated immunity is probably going to bode calling for any realistic advances in our understanding of what's going on or its application in terms of a vaccine development.

REESE: Perhaps this hasn't been addressed and perhaps Dr. Chedid can answer. I'm trying to remember back to another lifetime for myself when I used to be interested in various things which could modulate the immune response and we worked in inbred guinea pigs. At that time we found that the conjugation levels were really quite important in the response that we got. In one case I can recall we used a very low conjugation number and got, at an early period of time, an IgG response which was really heavily carrier related. With time the response changed, and then switched more to being a very hapten specific response. Now, are we going to have to deal with this sort of problem, when we conjugate various peptides to carriers, and have to worry about the actual responsiveness changing as a function of time?

CHEDID: I suppose you did several immunizations.

REESE: No. This was a single immunization in FCA.

CHEDID: Yes. And you got the carrier response and much later on the hapten response?

REESE: Actually, we took guinea pig albumin and we conjugated that to a low level. We put it back into guinea pigs. Initially, we got an IgG_1 response which was more carrier. It was obviously anti-hapten, but it was also related to the crossover area between the carrier and the hapten and was IgG_1. It was almost a single isotype, a spike of antibody on a focusing gel. As we waited in time and we bled sequentially, the type of antibody changed as did its specificity after a single immunization in FCA.

V. NUSSENZWEIG: A lot has been said about this carrier hapten problem. But I just want to remind two points. When you have a synthetic vaccine in which the hapten is itself a polypeptide, the hapten itself can be recognized by T cells. So, the situation there is not the same as in the situation maybe where you have a dinitrophenol conjugated to carrier. Secondly, there is already experience in humans in which semi-synthetic vaccines have been used in children using the diphtheria toxoid and haemophilus polysaccharide. As far as I know, from conversations with Dr. Robbins and from published papers, previous sensitization with the diphtheria toxoid did not, at all, suppress the response to the *Haemophilus* hapten. I don't think on the basis of a single experiment published we can generalize through all these very complex constructions. We must take each example separately and not generalize.

CHEDID: In answer to Victor Nussenzweig's remark, we must not overplay this epitopic suppression. I think that it is a hazard. I believe that it probably will be found in mass vaccination with tetanus but not necessarily so. Experiments will show if that is the case or not. But we did it with synthetic peptides which were twenty amino acids or so to make sure that it wasn't a DNP-hemocyanin story. It works the same way, but I don't think that we should overemphasize that aspect. On the other hand, I think that the amount of conjugate is going to be very, very important. In the case of John Robins, who is sometimes very enthusiastic and has been with the tetanus toxoid for a long time, he is using a very active antigen *Haemophilus* which is a T-independent antigen which he switches to a T-dependent antigen when he uses a few micrograms of *Haemophilus*. We will never have the good fortune of dealing with very small amounts, because I think that will be the limiting factor. To inject a milligram of tetanus, I don't think we will ever get away with it. I think it's as simple as that, the aircraft carrier.

MITCHELL: I agree (in response to Dr. Nussenzweig). I'd be delighted if these other observations you mentioned are the rule and these others are the exception. That's why I was interested to hear Dr. Chedid say that he has exactly the same situation. It's no longer now just Herzenberg and DNP-KLH. It is also tetanus toxoid-peptide situation as well. And I'm hoping that this experiment you refer to is more often the case than not. At the moment, the waiting is two experiments to one experiment against.

CHEDID: Just a few comments. First, we have indeed conjugated to tetanus or to N protein three peptides and obtained response to the three peptides and controlled the biological activity to the third one, being an HbS antigen which, of course, we can control. Secondly, someone was a bit anxious of genetic restriction with synthetic vaccines, which is, of course, very sensible. Most of the experiments we are doing are in outbred Swiss mice on purpose. This, perhaps, makes things a bit easier. Polyvalent vaccines will probably make it easier to avoid genetic restriction. This is the impression we are having now with inbred mice. If we just immunize with one peptide and with one adjuvant we can get poor responses, but when we put several peptides, the response is better.

MITCHELL: On that point, something like tetanus toxoid does have a little bit of genetic control, but not a lot. Does that comment still apply if you had one peptide on tetanus toxoid, the carrier, to which there is no genetic control?

CHEDID: No, I'm speaking of totally synthetic toxoids. Because with tetanus carrier, you are very favorable there. The problem of tetanus carrier, in my opinion, is what has been repeatedly said today, especially if the peptides we have are immunogens which are only going to be active at the milligram level or at the 0.1 milligram level, I really would be very surprised. Now, fortunately, there are things which can help. For instance, you do not have epitopic suppression if you give pertussis vaccine at the same time as your tetanus in the first shot. The Herzenberg's gave Freund's complete adjuvant, and it may be our good fortune that many of us have received tetanus with pertussis.

DREESMAN: I'd like to make a closing remark here as I sat here and listened to all the comments today. I think, first of all, we should thank and be very grateful to Dr. Siddiqui for going through the massive effort of having an organized a conference like this. Because I think, if an effective vaccine is to evolve for malaria, it's going to take facing the complexity of the system that I've seen this week—it's going to take a lot of open communication with groups like this. So that, at least, you can communicate your findings to one another and help one another to more effectively get to a vaccine. And secondly, because of the scarcity

of the animals that you use in your model systems, that you use these in effective ways so that a lot of experiments aren't done out of competitiveness alone. So, I think because of the scarcity of the animals that are used for these experiments, a lot of communication has to take place. Thank you very much, Dr. Siddiqui.

SIDDIQUI: Before we close, on behalf of the Organizing Committee and all the malariologists who are sitting here, I would like to especially thank the virologists and immunologists who spent some time with us and shared the experiences of their work.

Rapporteurs' Summary: Applicability of Information on Modern Vaccine For the Production of Malaria Vaccine

RONALD KENNEDY AND LESLIE TAM

Three modern approaches applied to protective vaccine candidates based on experience with bacterial and viral vaccines were discussed. First, synthetic peptides, which mimic both the amino acid sequence and potential secondary structure of antigenic determinants, were discussed by Gordon Dreesman. He discussed the role of conformation in the induction of an antibody response and the necessity for carrier proteins and adjuvants for high titered antibody responses to the native molecule. For this approach, the complete amino acid sequence of the protective polypeptide must first be determined by the molecular biologists, and this was the case for the protective hepatitis B surface antigen epitope studied by Dr. Dreesman. The characteristics of a synthetic peptide containing a 14 amino acid loop were addressed and the necessity of maintaining conformation to induce protective antibodies.

The analysis of other antigens and the location of T cell and B cell epitopes on these molecules were reviewed. B cell epitopes contain beta turns, are largely hydrophilic, and can be sequential or conformational. On the other hand, characterized T cell epitopes have been less hydrophilic, have had alpha-helix, and have been linear. These characteristics may be useful in identification of candidate epitopes in malaria antigens once they have been cloned. Dr. Dreesman presented a modified Chow-Fasman prediction of the secondary structure of the CSP protein, which indicated that the repeat sequences, which have been shown to be a strong B cell region by the Nussenzweigs, may also have some T cell potential.

In the second approach, Dr. Kennedy reviewed the potential use of internal image anti-idiotype as an antibody vaccine without the direct use of antigen. This new and novel approach was defined and the evidence for potential applicability in trypanosomes glycoprotein, the hepatitis B surface antigen, rabies surface glycoprotein, reovirus hemagglutinin, Sendai virus (T cell clone was used), phosphorylcholine of streptococcus, polysaccharide of *E. coli,* and poliovirus (8) systems were listed and the mechanisms of four outlined graphically.

The anti-idiotype approach has potential value in conformation dependent epitopes such as the hepatitis B surface virus 14 aa loop peptide. Internal image anti-idiotype may also be used potentially in the priming of immune responses for a vaccine in infants and non-responding adults. Since it is a relatively new approach, the precise use and applicability have not been defined.

In the third approach, Dr. Moss reviewed elegant work where entire genes have been inserted into the vaccinia virus, used as a vector for recombinant vaccines. He discussed successes with the hemagglutinin and neuraminidase proteins of influenza, glycoprotein D of the herpes simplex virus, the hepatitis B surface antigen, and finally the CSP gene of the malaria sporozoite. This protein was expressed as a 56K doublet in vaccinia, and it probably was not processed as it is in the salivary gland of the mosquito and is not expressed on the vaccinia surface membrane. Among many other advantages, the vaccinia vector is stable, does not require refrigeration, and can be administered by scratch alone. The stimulation of CMI, along with humoral immunity, has been documented and total efficacy of the vaccinia vaccine is known worldwide. The risk of complications looms as a disadvantage, but this may be short-sighted in the face of increasing menace of malaria in the tropics. To date the vaccinia virus system appears to be most promising for a recombinant malaria vaccine.

In closing remarks to this session, Dr. Dreesman suggested that identified protective antigens or their epitopes might well be given first in the vaccinia virus, boosted with selected synthetic peptides, and fine-tuned with selected tertiary anti-idiotypes.

In the next group of sessions, the role and mechanisms in potentiating the immune response were discussed. The first presentation by Dr. Kotani discussed muramyl dipeptide and numerous synthetic analogs as potential adjuvants boosting both cellular and humoral immunity. In particular, B30-MDP is a strong candidate with minimal pyrogenicity and other adverse side effects associated with other derivatives. A newer study is the Compound 506 related to Lipid A. It has the full adjuvant activity associated with lipopolysaccharide, but is 5000 times less pyrogenic. It is now being investigated as a second potential synthetic adjuvant.

Dr. Martin discussed the potential of Interlukin-1 in potentiating the immune response. An overview of the potential mechanism of adjuvants was also presented. It was also suggested that Interlukin-2 may also be a potential new adjuvant.

Dr. Chedid discussed the role of a non-pyrogenic derivative referred to as murabutide, which has 1000x less pyrogenicity, does not induce either acute phase proteins, slow sleep wave activity, or leucopenia, among other detrimental effects. The role of this new adjuvant in potentiating the immune response to several synthetic antigens was dis-

cussed. Increased immune responses were demonstrated with antigens uncoupled or directly coupled to the synthetic adjuvant. Increased responses to the CSP protein were obtained. Dramatically, the responses were greater than those obtained with FCA, but these were still low relative to other antigens. In addition, the quality of the antibody response to native protective epitopes was also discussed. It was noted that high antibody titers to the native protein may not necessarily correlate with protective immunity. The use of murabutide for producing a completely synthetic adjuvant with carrier function was shown by the completely chemical synthesis of a hormone linked in tandem with murabutide. This chemical synthetic product was effective in the chemical castration of cattle. This suggested the possible use of murabutide in synthesizing a malaria synthetic peptide possessing both B cell epitopes, T cell epitopes, and adjuvant activity.

The final presentation by Dr. Mitchell discussed the role of polyvalent vaccines for malaria. The ideal malaria vaccine might represent protective immunogenic proteins and/or glycoproteins formulated by the methods described earlier. The role of T cell help and potential suppression in using a polyvalent vaccine were also discussed. The polyvalent vaccine for pneumococcal polysaccharide with regards to the findings of non-antigenic competition was discussed. Potential antigenic competition would represent a problem for a putative single molecule vaccine containing several protective epitopes from different protective antigens.

The previous reports suggested the need for carrier protein that was licensed by the FDA, such as tetanus toxoid. However, the phenomenon of original antigenic sin suggested that prior immunization of the carriers might damp the induction of antibody to the desired epitope. This was also discussed with regards to the tandem repeats of CSP and a growing number of other malaria candidate antigens having a repeat characteristic. The potential problems associated with invariant hydrophilic regions along with variable tandem repeats with regards to the original antigenic sin were discussed.

In summary, CSP, pf190, RESA, cytoadherence antigen, rhoptry antigen, pf96, and the glycophorin binding protein were listed as possible candidates protective antigens. For each of these molecules it is necessary to remove irrelevant and counterproductive (suppressive) epitopes while retaining beneficial ones. The final analysis suggests that the search for putative epitopes associated with protective antigens on the malaria parasites should utilize the knowledge compiled by our colleagues working in bacterial and viral systems. In addition the success of the formulated vaccine will likely depend heavily on the development of strong but non-toxic adjuvants, which will be incorporated with the putative vaccine in the stimulation of long-lasting immunity.

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SESSION IV

STRATEGIES FOR FUTURE MALARIA VACCINE TRIALS I

A. EXPERIENCES FROM CLINICAL AND FIELD TRIALS OF ANTIBACTERIAL AND ANTIVIRAL VACCINES

Dengue Vaccine Development, Reactogenicity and Immunogenicity of Dengue 2 Candidate Vaccine (16681-PDK 53)

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Dengue hemorrhagic fever, a serious manifestation of dengue infection, remains a major cause of morbidity and mortality among the children of Thailand and neighbouring countries. Outbreaks may occur in a country where the disease may appear waning, such as in Malaysia, and new epidemics of DHF have happened in countries where the vector is available and dengue viruses have been circulating, such as what happened in Cuba in 1981. Vector control and other public health measures appear to be of limited value in most instances and vaccination against dengue infection remains an unexplored possibility. Since immunity against all four dengue virus types can be demonstrated in the majority of adults in dengue endemic areas, it is conceivable that immunization with appropriate dengue vaccines may elicit broadly protective immunity in persons at risk. This dengue vaccine which should be aimed towards the protection against and the control of Dengue Hemorrhagic Fever/Dengue Shock Syndrome in the endemic area would have to be a polyvalent one in order to protect the subjects against all four types of dengue infection and to minimise the risk of sequential infection by one of the other dengue viruses. The vaccine should be given to children six months to one year of age as the principal target population, and should not be given to the pregnant woman. Based on the experience with other viral vaccines, this will have to be a live attenuated vaccine to achieve a life-long immunity.

Development of a workable dengue vaccine rests on two major considerations: (1) attenuation of dengue viruses and (2) biological markers of attenuation.

Attenuation

There are at least two ways that attenuated virus strains could be obtained. The first way is to isolate strains from heterologous wild virus populations or mutants occurring naturally or which are induced by chemical mutagens. These mutants may then be cloned by selective or nonselective procedures. By this method, a small plaque, temperature sensitive virus was cloned and utilised to develop dengue type 2 candidate vaccine in the United States. Another way to attenuate dengue virus is based on the observation of Pasteur that prolonged passages of a micro-organism into a non-natural host results in changes of the virulence for the natural hosts. The way this is brought about is uncertain, but conceptually, the passages through non-natural host lead to selective pressures upon different virus populations. The less virulent one becomes dominant. From the biological standardization of the vaccine standpoints, the first approach to obtain attenuated candidates vaccines through cloning selection may be an ideal one because the candidate viruses should consist of a homogeneous virus population. The second, somewhat empirical method should result, theoretically at least, in a kind of vaccine with heterogeneous population of the virus which may be somewhat more difficult to standardize biologically. Yet, if one reviews the history of viral vaccine development, all of the successful, commercially available live virus vaccines for use in humans have been derived essentially by empirical method. Licensed and widely used live viral vaccines include vaccinia, thought by many workers to have been derived from variola virus which was naturally passaged in cattle; yellow fever (17D), attenuated by serial passages in mice and then chick embryo cultures; poliomyelitis vaccines passaged in monkey kidney cell cultures; Edmonston and Schwartz measle vaccine, serially passaged in chick embryo culture; several rubella vaccines, derived by serial passages in African Green Monkey (GMK) cells, rabbit kidney or WI38 cells; and mumps vaccine, passaged serially in chick embryo cells. There are evidences that dengue viruses behave like other viruses with respect to other attenuation. Sabin and Schlesinger readily attenuated dengue type 1 and 2 viruses by passaging 7–30 times intracerebrally in infant or weanling mice. Since vaccines prepared in animal brain tissue are no longer considered desirable, this early and successful model of dengue vaccines was not pursued further. We have opted for the second approach for the derivation of dengue viral candidate vaccine.

Biological Markers

A brief review of the biological markers described for vaccine virus leads to the conclusion that there is no single marker which could corre-

late the level of attenuation of a virus to the reduced virulence for man. Perhaps poliomyelitis may be the only virus to be shown that decreased ability of the virus to grow at high temperatures *in vitro* correlates with decreased virulence.

For Dengue viruses, Banerjee reported that 9 of 15 dengue viruses recovered from patients with dengue fever were temperature sensitive. Halstead and Simasathien had shown that temperature sensitive dengue 2 viruses had been recovered from patients with DSS and that temperature shut off characteristics were stable in a dengue 2 strain which was serially passaged 17 times in mouse brain. Eckels and associates have selected a dengue 2 virus from a single isolated plaque, after several passages in Primary Green Monkey cells. The cloned virus is considered genetically homogeneous. This virus produced small plaques in LLC-MK2 cells, was temperature sensitive and of reduced virulence for suckling mice and monkeys.

Currently the biological markers of attenuation and characterization for dengue virus vaccines include: (1) plaque morphology; (2) temperature restriction of growth; (3) growth in human mononuclear phagocytes; (4) virulence in suckling mouse and virulence in monkeys. There are still no absolute qualitative or quantitative parameters to define the stage of attenuation; only a comparison between the effects of the parental or wild strain and those of the serially passaged viruses on these biological markers could give an indication whether the virus is attenuated or not.

We have adopted the method of attenuation of dengue viruses by serial passage through Primary Dog Kidney (PDK) cells and have used the biological markers as presented earlier for an empirical assessment of attenuation. The vaccine has now been given into 26 volunteers in two phases. Phase Ia involved 10 subjects and was aimed towards the determination of immunogenicity and reactogenicity. The other phase, Ib, involved 16 subjects for the titration for minimum infecting doses.

The Candidate Vaccine. Dengue 2 candidate vaccine 16681 was derived from a parental strain isolated from a DHF patient in Thailand, passaged in tissue culture before inoculaton in Toxorhynchitis amboinensis twice. The virus was serially passaged to PDH cell culture at 32° C. Biological markers at passage 50 suggested that an attenuation stage had been reached. Master seed was made at passage 51, production seed at 52, and the candidate vaccine is at passage 53.

Safety Test. Microbial safety test included tests for bacterial, fungal, and mycoplasma, and inoculation of the vaccine into several cell lines and into animals. It also included tests for reverse transcriptase. Inoculation of the control fluid, parental virus, and candidate vaccine into the brain and spinal cord of two, two, and six rhesus monkeys was made. The monkeys were inspected for 14 days daily.

Inoculating Dose of the Candidate Virus Vaccine. The vaccine was given at the dose of 1 ml subcutaneously to the first 10 volunteers. Two of the ml vaccines were mixed together and 1 ml was infected while the remaining 1 ml was left in wet ice and returned to Bangkok for virus titration on the same day. The amount of virus injected ranged from 1.9–2.7 × 10^4 PFU/ml. In the second trial the vaccine was given in 4 groups of 6 volunteers in each group at 5-fold dilution—undiluted, 5, 25, and 125.

Volunteers. Twenty-six male volunteers age 15–30 were recruited into the two studies. The volunteers were screened among a larger number of inhabitants who lived in the 2 northern districts of Pasang some 700 km north of Bangkok. The volunteers were screened for the presence of HI antibody against dengue virus of all 4 types and against JE. The volunteers live in a village 35 km away from the district town; the last 10 km of road leading to the village was dirt road. The village was carefully surveyed twice six months apart before the vaccine trial, to be free from *Aedes aegypti*. The first trial involved 10 volunteers; 5 were dengue and JE susceptible and 5 were dengue susceptible but JE immune. The other 16 which were involved in the second trial were dengue and JE susceptible. After the volunteers agreed to receive the vaccine the possible consequences of the vaccination were carefully explained to them and each signed a consent form. After the immunization the volunteers were housed at the subdistrict health center in a screened house. They were allowed to go about after daily blood drawing and daily physical examination and temperature measurement. These were performed by a nurse. A physician at the district hospital also examined the subjects before the vaccination and for the first few days after. Volunteers were asked to stay at the subdistrict health station for 2 weeks and were asked to refrain from going to the district town for at least one month. They were paid subsistence living allowance and a small honorarium when they came for subsequent blood drawing.

Laboratory Study. Blood was collected for viremia and serology and clinical chemistry and hematology. Blood was drawn before the vaccine was given and the first day and every two days after that for three weeks and then weekly to the end of the month and then monthly.

Result of the First Trial

This first trial involved 10 male subjects. All volunteers showed no abnormality of their clinical laboratory profile. During the study there were no abnormal symptoms and signs and no fever. This is in contrast to the mild to moderate degree of dengue-like illness encountered in volunteers involved with the dengue candidate vaccine who showed self-limited low grade fever, headache, muscle pain, and rash.

From the laboratory standpoint, the total leukosyte count of these 10

volunteers came down from the initial level and reached the lowest around the 10th day and returned to near normal on the 14th day. There was, however, no absolute leukopenia. There was relative lymphosytosis, monosytosis, and some atypical lymphocytes were found. Giant platelets were demonstrated in the peripheral blood in 6/10 subjects but there was no thrombocytopenia in these cases.

Nine out of 10 subjects showed HI seroconversion. There were four with primary HI response, all of whom were in the Dengue JE susceptible group. The remaining 6 showed secondary response. All of the subjects showed strong PRNT response; most of these peaked about day 30 and began to drop by day 60.

Clinical Trial of the Second Group

The vaccine trial for the second group was aimed to titrate the minimal infecting dose by giving vaccine at 5-fold dilution beginning with undiluted vaccine to 1/5, 1/25, 1/125 to 4 groups of volunteers of 4 subjects each. All of these were male and were shown to be dengue and JE susceptible.

The study protocols were similar to the first group. The result was very promising. There was no untoward reaction in any of these subjects. The PRNT response was also strong even in the 1/125 dilution, even though this group may receive only 2–300 virions per ml. Laboratory study is continuing.

Based on the two trials so far, we feel that candidate dengue 2 16681 vaccine may be a safe and potent vaccine and based on a satisfactory pending result on the second trial may be moved on to phase 2 trial.

The issues involved in the dengue vaccine development.

1. There is no experimental animal model of dengue or DHF in contrast to the malaria vaccine.

2. The final form of vaccine to be deployed will have to be polyvalent —tetravalent or trivalent—to confer full protection against all 4 types of dengue viral infection.

3. In order to confer long immunity as well as the present state of the art the present choice rests on the live attenuated vaccine.

4. The molecular marker for attenuation remains unknown and even the biological markers being used are still not well prescribed and not well understood.

5. Reversion of the vaccine virus to wild strain remains a possibility both in the human host and in the mosquito host.

6. The trial of the vaccine requires volunteers who do not have flavivirus antibody, especially JE.

7. Volunteers will have to be placed in sites practically free from the

mosquito vector for some time and with little possiblity to come into contact with the vector for a long time.

Dengue 2 candidate vaccine (16681) has now been given to 26 subjects. We have completed all the safety tests designed according to the U.S. FDA for live viral vaccine safety requirements, which include the monkey neurovirulence test.

Discussion

QUESTION: Dr. Natth, when can vaccine trials begin in children, since, after all, they are the target population for the dengue vaccine?

NATTH: Based on our experience with the type 2 dengue vaccine, our present plan is to move onto dengue 1, 3, and 4. Dengue 1 is going through the final stages of safety testing and we expect to begin trials this year. Then we will move onto dengue 3 and 4. Finally, all the vaccines will be put together in a polyvalent vaccine, which will need additional safety testing to assess the possible problem of antigenic competition. If the polyvalent vaccine is successful in adult volunteers, then we will consider trying it in children. My best estimate is that this will occur some time in 1988.

Experiences from Clinical and Field Trials of Antibacterial and Antiviral Vaccines

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Since Professor Louis Pasteur started the vaccines against anthrax and rabies, many kinds of vaccines have been developed. These vaccines have been utilized as weapons to fight against infectious diseases. As we all know, the People's Republic of China is a large country with a population of one billion and with about eighteen million newborns annually. Large amounts of various kinds of antibacterial and antiviral vaccines have been used to control the infectious diseases. This may be the reason why the Organizing Committee gave me such a subject. I will try to discuss our general experience in the following points:

1. Vaccine Must be Safe and Effective

We have several national institutes to produce biological vaccines. Each lot of vaccine has to be tested by the Control Institute or Control Department, which is directly supervised by the National Control Institute. The Control Department can send examiners to any workshop of a production institute to watch the process of vaccine production and even to perform quality tests together. These tests include sterility or purity tests, toxicity and safety tests, potency tests and other required tests. The results of all tests must fulfill the requirements of the National Standard. The aim of careful tests is to be sure that the vaccine is safe and potent.

2. Vaccination Schedule

Two working systems of vaccination have been developed. One is a regular procedure and another seasonal inoculation. In large cities where immunization clinics have been established, immunizing procedure is carried out according to the regular schedule. BCG usually is the first vaccine given to the newborns, who are revaccinated at the age of seven. From the third month on triple vaccine of diphtheria, pertussis, and tetanus is given by three injections with month-intervals. A booster

injection is given in the following year. The tetanus vaccine is very effective and the effect lasting, so at the age of four, only diphtheria and pertussis are injected. At the age of seven, when pertussis is very rare, pertussis vaccine is omitted in order to avoid severe reactions, so only diphtheria and tetanus are given. Oral polio vaccine is given from the third month, first by Type I only and one month later followed by Type II and III together. At the 2nd, 3rd, and 7th year, repeat the same vaccination. This is a different schedule from that of the World Health Organization. Recently, experimental trials of different combinations have indicated that combined vaccine of reduced amounts of three types is as effective as increasing amounts.

With monotypes of each, positivity rate of appearance of neutralizing antibodies is high, about 96 to 100%, while for double vaccine of Type II and III, only 80%. For combined three types in reduced amounts after the 3rd vaccination, it showed almost 100% positive for each type. We think that the combined vaccine with reduced amounts is effective. Another method is to continue Type I separately, followed by double vaccine Type II and III, and finally followed by one combined vaccine. This schedule gives a positivity rate above 95%.

A seasonal schedule is chosen for border regions where the cold chain has not been established. The vaccines have to be given according to seasons. That means vaccination against certain infectious diseases are carried out once or twice each year, usually one or two months before the onset of outbreak. For instance, DPT is applied from September to November, polio and measles vaccination from November to January, and BCG in the springtime from February to April.

3. Organization of Vaccination

To carry out vaccination successfully, we have to have a strong working organization. In our country, we have a central or national committee for EPI and regional sub-committees for EPI to set up the principles for immunization. Under the supervision of regional sub-committees, the Provincial Health Stations are responsible for carrying out the actual immunizing work. They have to order vaccines one year ahead and distribute the vaccines to their destinations on time. They have to direct the District Health Stations to carry out vaccination according to the correct schedule. The actual vaccination is done by medical workers, under the direction of commune public health workers. There should be at least one medical doctor or public health worker to be responsible for the vaccination. They have to carry out the vaccination planned by the District Health Stations. When seasonal immunization is planned, they have to train all the village medical workers for 1 or 2 days on the importance of vaccination, the way or route of vaccination. The village medical workers have to be familiar with the contraindications and know how to treat untoward reactions. Following the vaccina-

tions, they have to report the reactions, the effectiveness of the vaccine, the percent of protection, etc.

4. Clinical Reactions

Both general and local reactions are recorded according to the severity of the reactions. For general reactions, temperature is an important index. Each vaccinee's temperature is taken before, 24, and 48 hours after vaccination. Those with a temperature higher than 37°C will be excluded from vaccination. Those with a fever of 37.6–38.5°C are classified as medium reaction and those above 38.5°C severe reaction. From the data obtained, the percent of reaction can be calculated easily. For local reactions, the local induration, but not redness, is measured at 24, 48, and 72 hours. Those with a reaction between 2.5–5.0 cm. are called medium reaction and above 5.0 cm. are called severe reaction. Lymphangitis and lymphadenitis are also recorded.

The standard for general reaction is that medium plus severe reactions should not be more than 7%. Other general reactions such as headache, dizziness, unusual malaise, nausea, vomiting, etc., must be recorded if there is any. Severe reactions such as dyspnea, lowering of blood pressure, and shock must be treated immediately. Send the reactor to hospital if necessary. It is found that those abnormal reactions usually occur in immuno-deficient people. Once noticed, they should not be vaccinated next time. For accuracy, it is better to have a control group to exclude unnoticed undergoing infections in the study regions.

5. Epidemiological Field Trial

For the field trial of epidemiological study, first of all, we have to select a suitable field. The general requirements of field are:

5.1 From the careful analysis of previous epidemiological data, or serological study of the local persons, try to establish the possibility of epidemics. Suppose a non-infected field has had cases in the neighborhood last year, then there may be chances for having an epidemic in that field.

5.2 The field chosen for observation should have no epidemics of the disease under observation within recent years.

5.3 The population of the field should be comparatively stable. The vaccinees should have no history of such or similar vaccination or attack of the disease under observation before so that they are assumed to be highly susceptible to the disease.

5.4 Based on the expected 30–50 cases in the control group, try to calculate the number of persons to be included in the trial study.

5.5 The group to be observed is divided into immunizing and control groups at random. Be sure that the total number of both groups is comparable. Sex and age distribution should also be comparable. Criteria for the diagnosis of cases should be worked out before the trial.

6. Results of Immunization

After continuous observations for years, we have collected quite a volume of immuno-effectiveness data of certain diseases which are now under control.

6.1 Control of measles. Before the development of measles vaccine, there were epidemics after epidemics. The morbidity rate was around 1,000–5,000/100,000, and the mortality rate around 5–10%. After mass vaccination, especially after planned immunization, the disease is under control. In certain places, there were no measles cases for more than ten years, except a few imported cases.

6.2 Lowering the annual infective rate of polio. Before vaccination, the average annual case rate was between 2.84–4.32/100,000. After the introduction of polio vaccine, the disease gradually came down from 1.98 in 1966–1970 to 0.80 in 1976–1980, and up to 1983, 0.3/100,000. In certain big cities, practically no cases were encountered.

6.3 Control of Japanese B Encephalitis. Japanese B encephalitis was found to be a widespread disease immediately after liberation. Then killed vaccine was introduced. In a non-epidemic city, the neutralizing antibody of children before and after immunization was studied. The children showed no neutralizing antibody before vaccination, but after two basic injections of vaccine, the antibody positivity rate raised to about 60% and quickly dropped to original level. After a booster injection, then it raised to nearly 100% and maintained at this high percentage for four years. When the children were revaccinated again, it maintained at about 100%.

6.4 Control of epidemic meningitis. In the year 1966–1968, there was an epidemic of meningitis. After the introduction of meningococcal polysaccharide vaccine, the disease has now come under control. An example of field trial can be cited. Of 90,945 children immunized by one injection of polysaccharide, only two cases developed, while of 83,402 controls, 36 cases appeared. The protection rate was 94.90%. Now there has been practically no epidemic of meningitis (Table 1).

TABLE 1. Field Trial of Meningogoccal Polysaccharide Vaccine

	Immunizing Group	*Control Group*
Total No.	33817	33919
Male	17918	17750
Female	15899	16169
1–5 yr. old	7437	7542
6–10 yr. old	14658	14998
11–15 yr. old	11722	11378
observed cases	9	48
infective rate/100,000	26.61	141.53
protection rate	81.20 ± 13.39%	

What I have mentioned here are only some of our limited experiences. In conclusion, I would like to say once more, that in order to obtain the optimal effect of vaccination, we should plan each trial carefully and be sure to use potent vaccines properly administered.

Discussion

BUCK: Dr. Chen, since your country is so large with both temperate and subtropical areas, how are serious side reactions to vaccination, such as encephalitis, monitored and are there any differences in distribution of the side reaction among the various provinces?

CHEN: We seldom see severe reactions to vaccination, however, several have been reported. For instance, there was an American studying in Beijing in 1981 who contracted encephalitis. In northern China, encephalitis is more common than in the south. So there are some geographical differences in China. We treat encephalitis clinically with both Chinese and Western medicines.

QUESTION: Why are so many boosters needed to reach high levels of immunity?

CHEN: Because of the large numbers of persons in the target population, we sometimes fail to vaccinate a portion of the target population. So the next year, some individuals receive their first immunization while others are receiving a booster vaccination.

Discussant Experience from Two Vaccination Efficacy Studies: What Did They Teach?

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Finland, a Scandinavian country of 337,000 km² (130,000 sq.mi) with 4.9 million inhabitants, has posed for vaccination field trials for several times. One of the reasons is the well-functioning child care system: around 1,050 child health centers are located all around the country and take care of the children in the given area. Vaccinations belong to the responsibility of the centers and virtually all childhood immunizations are carried out by the public health nurses, not the doctors. During decades the nurses have gained high respect among the mothers and what is deemed justified by the public health nurses is usually performable to the children. As a consequence, the coverage of DPT vaccination, for example, well exceeds 90%.

Tight involvement in two major field studies, one concerning the group A meningococcal and *Haemophilus influenzae* type b capsular polysaccharide vaccines (about 100,000 vaccinees), the other project concerning the measles-mumps-rubella (MMR) live vaccine (over 500,000 vaccinees so far), has given an opportunity to weigh pros and cons of the performance of field studies in this country. Although the (quite favorable) circumstances in Finland are not comparable to all other countries, some lessons learned may apply to any vaccination trial, including the one using the malaria antigen.

Selection of the Population to be Vaccinated

Only seldom, as, say, in tetanus, the whole population in the area has to be immunized. Usually there are some populations that are specially prone to a particular disease. For example, the highest incidence of meningococcal disease occurs before the age of twenty (the peak is among 0–4 years old), and *H. influenzae* affects almost solely children less than five; measles (and to a lesser extent mumps and rubella) is vir-

tually a pediatric infection. Thus, all immunization programs combatting these diseases should be targeted for children who are easier to reach through the child health centers and schools than are the adults. Regarding malaria, children compose the main target population for immunization. This fact limits the number of vaccinees, reduces the amount of the vaccine needed, and constrains the activities before and during the trial.

Motivation of the Vaccinators

The successful measles-mumps-rubella elimination program required a detailed scientific follow-up focusing on the possible adverse reactions of the vaccine, coverage of immunization, potential failures of vaccination, and the epidemiological consequences of the intervention. In practice, a number of indices were to be investigated, various forms to be filled, blood samples to be taken and reports to be given. Because no monetary reimbursement for the vaccinators was feasible, good motivation of the public health nurses was the only tool to be utilized in this respect.

Prior to the campaign, all nurses (about 2,000) attended one of the 23 conferences held in different parts of the country. During the three hours that each conference lasted, the project was presented in great detail using also the audiovisual equipment. A specimen of the special forms, softened envelopes (for sending the blood samples), vaccine vials, etc., were given to the nurses in order to get them acquainted with the project.

Motivation of the Public and the Doctors

In case of an ongoing (meningococcal) epidemic it is easy to motivate the public for vaccination. The study protocol on the polysaccharide vaccines required, however, a double blind fashion so that every other child was to be immunized against meningococcal disease and every other child against *H. influenzae* infections. This approach created some problems.

A snag arose with the demand from the manufacturer that a written consent was to be requested from the parents. This requirement was specially troublesome in Finland where all vaccinations are free and given under the understanding that they are considered medically and ethically justified by the best experts; request of the signature was regarded both as questionable, irritating and annoying.

Although there was no easy solution to the problem, the majority of the parents became satisfied by literal explanation of the situation and persuasion of their own public health nurse. Even the philosophy

behind the double blindness was usually understood. Eventually, 76% of the parents let their child be vaccinated. Signed consent has not been required since then, and the vaccination coverages have been higher in later studies.

Mass media (TV, radio and press) were utilized in good time in order to warn the public about the diseases and to inform them about the opportunity of immunization. A special letter was sent to the general practitioners and pediatricians in the country.

Legal Aspects

The MMR vaccine was licensed in its country of manufacture (USA) before the use in Finland, but the meningococcal vaccine was recommended for adults only. Moreover, the *H. influenzae* vaccine had passed only Phase I and thorough negotiations were imperative before health authorities became convinced that its use was ethically and medically justified. The positive attitude was finally based on the Phase II results that guaranteed the safety and immunological efficacy of the newcomer.

All field trials in Finland have to be approved at two levels. The ethical committee of the National Public Health Institute is primarily responsible for the ethics of all vaccination studies in the country, but it represents high scientific know how also. In addition, each of the 243 local health authorities are independent in their decision whether or not a field trial is relevant in their area. These authorities were approached with a special letter explaining the advantages of the project and requesting an approval.

The study protocol was sent also to the National Board of Health. This action gave the health authorities an extra opportunity to check the ethical, medical, and logistic grounds of the study planned.

Results of the Trials—What Did They Teach?

As described in detail elsewhere[1-4] both field trials (the one using the polysaccharide vaccines, the other the MMR vaccine) proved successful and delivered plenty of new information. In summary, all the vaccines were tolerated well, the group A meningococcal vaccine proved effective in children and the finding led to a mass campaign involving over a million vaccinees and waning of the epidemic; the *H. influenzae* vaccine was 90% effective, provided it was given at the age of 18 months or later; MMR decreased in a couple of years the incidence of measles, mumps and rubella in a fraction of the level prevailing before the campaign.

The key to success was existence of the child health center system, activity of the public health nurses, good preparation of the campaigns,

and of course, effective vaccines. However, what are the lessons from these studies to the malaria vaccine?

Certainly not too many lines from the Finnish experience can be drawn to the future malaria vaccine trials as such. However, some aspects may be relevant no matter how different the vaccines and the conditions. Scrutiny of the characteristics of the new vaccine, careful selection of the persons to be immunized, good motivation of the vaccinators and the target population, and taking care of the local legislation pose the fragments of which the most informative field study is composed.

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General Discussion

REESE: It seems to me that there may be some problems in evaluating vaccine efficacy when the Phase I trials are carried out, let's say in Finland, where the caloric intake is very high and where the volunteers are in good health. However, when the trials move to another area of the world where the caloric intake is lower and the individuals have other types of parasitic diseases, a different answer might be obtained. Would anyone like to comment on this?

LEVINE: That's an excellent question and it really works two ways. There are some instances where vaccines that are highly immunogenic and are completely safe in one population in the industrialized world that when transferred to a less developed country are more reactogenic or have clinical consequences. For example, the measles vaccine is completely safe and is a very important vaccine in the developing world. Yet, when one does metabolic balance studies, one sees that there is a negative nitrogen balance for a period of time, about two weeks after measles vaccination. However, it is much better to vaccinate because of the long-term benefits even though for a couple of weeks after vaccination the live virus has a negative effect on the nutritional state of the child. The converse also occurs. Some attenuated vaccines, such as *Vibrio cholera* prepared by genetic engineering in which the cholera toxin has been deleted from the genome, cause mild diarrhea in some individuals. This is very evident when given to healthy U.S. college students, for example. When that vaccine is taken into a place where prospective studies have shown that the average child in the first year of life has 6 to 10 separate documentable diarrhea episodes per child per year.

Another example is with the attenuated rotovirus vaccine. I'm speaking of the Rhesus monkey attenuated strain that has been given to human infants. When tested in Finland, Sweden, and the U.S., infants at certain doses developed fevers but no diarrhea. When the vaccine was used in Venezuela it was absolutely non-reactogenic.

So, it works both ways. What needs to be done is to test the vaccine in industrialized countries first; in both adults and children. Answer the question what happens in a well-nourished population, then move on to other countries.

It is very important to test a vaccine in the low transmission season, if the disease shows seasonality. This will minimize the background of confounding variables. Also be very clear about the definitions of the side reactions expected. Definitions may be somewhat different between industrialized and developing countries, which may have more stringent definitions. Make certain that proper clinical specimens are collected to assess the question of reversions, etc.

COLLINS: Dr. Levine, may I have a clarification? Are you suggesting that the malaria vaccine be tested during a nontransmission season?

LEVINE: For safety, of course. I can't personally draw on examples in malariology, but there are analogies, Bill, that I think are relevant, say from experiences with a live attenuated *Salmonella typhi* strain as an oral vaccine to prevent typhoid fever. In Santiago, Chile, for instance, where this vaccine was first tested in children, one would not want to give a live strain vaccine in the middle of typhoid season. That's asking for trouble. What if a fever occured in a child who was in the second or third day of having ingested a real virulent *S. typhi* strain? This happened to us in a nontyphoid season. We were able to isolate the organism from the blood and show that it did not have the markers of the vaccine strain. In vaccinating against typhoid, one wants to get in and finish the vaccination about a month before the onset of typhoid season. Not having the background problems of incrimination of the vaccine causing cases and also to give a few weeks or a month for immunity to build up. Then you maintain surveillance during the typoid season.

BRUCE-CHWATT: Thank you very much Dr. Levine. This is a facinating subject and is probably a problem we're going to have to cope with in our clinical trials with prospective malaria vaccines in developing countries.

PAPPAIOANOU: Once you get to the stage of taking the vaccine to the developing country for testing, some of the ways and the methodology in setting up the design of the study become extremely important, such as, how you go about recruiting and enrolling the subjects in the trials and the randomization and masking, blinding the people who are evaluating the outcome. Those are the most fundamental ways of trying to control all the potentially confounding factors.

CATTANI: I think that even prior to looking at the trial itself, it points out the importance of doing pilot studies to assess intercurrent infections

and endemicities. It would be a mistake to go into an area and just look at the epidemiology of malaria. We will need to know much more about the health of the study population than malaria infections.

BRUCE-CHWATT: I couldn't agree more. I would just like to point out that things are little more complicated with malaria. In tropical Africa, for instance, there is the problem of current infection and past malaria experiences of different degrees and intensity. We don't know, of course, how a current malaria infection might immunosuppress the response to the vaccine or whether there is a need for treatment before giving the vaccine. Also, we need to know about immunocompetence in relation to vaccination and to its age distribution. We do not know yet how a repeat experience, in this case with natural malaria and with the vaccine agent, might influence the situation.

RISTIC: I'd like to bring to your attention a recent experience in animal health in veterinary medicine in regard to foot and mouth disease vaccine. The U.S. government has been developing better and improved vaccines against foot and mouth disease in the Palmyra Laboratories on the east coast of Massachusetts. These vaccines have been tested extensively in the U.S. and work beautifully, producing good neutralizing antibodies and good protective immunity. When these vaccines were recently tested in Colombia, South America, in cattle, some cattle did not even respond immunologically, did not even show neutralizing antibody to the three strains of foot and mouth disease vaccine. Subsequently, studies to find out what is the cause of the difficulty showed that most cattle have been severely immunosuppressed by hepatrophic diseases like Babesiosis. Most cattle contained large spleens saturated with all kinds of fragments of blood parasites and completely suppressed the response to the foot and mouth disease vaccine.

B. PHASE I, ..., AND III CLINICAL TRIALS: GENERAL CONSIDERATIONS

Definition, Classification, and Problems of Clinical Trials of Malaria Vaccine(s)

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I. Definition and Classification of Clinical Trials

Although it is known that already in the 15th century the Chinese were using dried and powdered crusts of smallpox pustules for inhalation by persons who desired protection against this dreaded disease, the demonstration of the value of such heroic methods came much later.

One of the earliest known clinical trials carried out on humans in Europe took place in London in 1725, when Lady Mary Wortley Montague, wife of the British Ambassador to the Sultan of Turkey introduced the practice of variolation for prevention of smallpox. She attempted to persuade King George I to apply this method at the Royal Court. The King insisted on a trial of this risky practice. And so, Dr. Richard Meade, the King's physician, was ordered to inoculate six condemned criminals at Newgate prison, with the promise that if they survived, they would be reprieved. The inoculations of pus from a mild case of smallpox were successful and the six compulsory volunteers were pardoned. Thereupon the King allowed the inoculation of the royal children but declined the offer for himself. Variolation then became quite fashionable in some English higher circles, although it was a chancy procedure and a number of people died, if the source of infective pus had a high virulence.

Edward Jenner's observation of the fact that the milkmaid Sarah Nelmes's accidental (or rather occupational) infection with cowpox protected her from smallpox was the starting point of his scientific inquiry. It led to the famous clinical experiment on the eight-year-old James Phipps in 1796 and to the publication in 1798 of Jenner's series of trials, which established the value of cowpox (hence: vaccine) for prevention of smallpox. The next major step was the introduction by Pasteur in 1885 of an effective immunization against rabies, by means of emulsified portions of infected spinal cords of rabbits, after the cords had been

dried for a time necessary to attenuate the virulence of this material. The first human patient to receive this vaccine was a nine-year-old boy and the only previous trials of this heroic experiment were carried out on dogs.

From then on the advance of immunization for protection or treatment of infectious diseases of man was spectacular. Its chronology traced by Cockburn was as follows:[1] 1892, Haffkine's cholera vaccine; 1898, Wright's typhoid vaccine; 1913, Behring's toxin/antitoxin diphtheria immunization; 1921, Calmette and Guerin's tuberculosis vaccine (BCG); 1923, Madsen's pertussis vaccine; 1927, Ramon's tetanus toxoid; 1937, Theiler's yellow fever vaccine (17D); 1949, mumps vaccine; 1954/57, Salk's poliomyelitis inactivated vaccine followed by Sabin's live oral vaccine; 1960, Enders' measles vaccine; 1962, Weller's rubella vaccine; 1968/71, meningoccal A and C polysaccharide vaccine.

This list has been greatly extended during the past decade by a number of new vaccines such as the hepatitis B vaccine introduced in 1976 and now vastly improved, as well as the vaccines against Japanese B encephalitis, tick-borne encephalitis of eastern U.S.S.R., foot and mouth disease, and several more.

However, when it comes to a real impact on public health in tropical developing countries no victory has been greater than the success of smallpox eradication achieved within 15 years, thanks to a series of technical improvements of the vaccine itself, method of its administration, but also good strategy, based on sound epidemiological principles as well as courage and determination of its promoters.

One should perhaps remember that together with its remarkable successes the history of immunology contains also pages of its failures or even tragedies. Sir Graham Wilson, the grand old man of British microbiology, wrote in 1967 a study of the hazards of immunization.[2] He stated, "No vaccine or serum can be regarded as completely safe. . . . the complications or accidents are the price we pay for the protection. There is no insurance without a premium." Some of the major incidents of the past 50 years were due to avoidable hazards, related to faulty production or administration of contaminated vaccines, but others were caused by unexpected inherent hazards such as neurological or other sequelae. Thus, live poliomyelitis vaccine, although one of the safest and most effective, may still cause paralysis in between 1 to 3 million and 1 to 50 million vaccinations, depending on the type of the poliovirus mixture. Not all such tragic cases can be fully explained; the development of both killed and then live polio vaccine was a triumph of modern immunology and the clinical trials of killed vaccine in the U.S. were a model of perfection, yet soon after that there were over 200 cases of paralytic poliomyelitis in children given imperfectly made vaccine. The use of live oral vaccine poses a new problem in tropical areas, since the anti-

body response to this trivalent vaccine is poor, while killed vaccine given by injection is more dependable. There are also some problems with the production of large amounts of it in monkey kidney cells and better methods are too expensive.

To achieve success in the control of a disease it is not enough to devise an effective vaccine. One must also have an effective policy for its use and surveillance of success or failure. Diphtheria immunization was not adopted in pre-1949 England, despite the fact that the development of the vaccine had been attempted and perfected there in the 1920s. It required the war to stimulate the government to launch the vaccination campaign 20 years too late, and to overcome eventually the worst effects of this disease.

Finally, two recent episodes: one is that of the massive swine influenza vaccine campaign in the U.S. in the 1970s, which caused a wave of radiculoneuropathic symptoms of peripheral paralysis, known as Guillain-Barré syndrome, and led to a stoppage of the campaign. The second problem is that of encephalomyelitis after whooping cough vaccine; the frequency of this complication averages 1 to 300,000 vaccinated children in Europe and causes much concern.[4]

While the acceptance of immunization as the best public health preventive measure is rapidly endorsed by most of the medical profession, at some stage this attitude may change. This has happened now with regard to typhoid and cholera vaccines, the value of which in developing countries is contested in comparison with permanent improvements of water supplies and better sanitation. At the other end of the picture stands the measles vaccine, which was met with lukewarm reception in Europe, where the disease still continues to cause every year at least 1 million cases with about 1,000 deaths, while in the U.S. it is now nearly eradicated thanks to a superb immunization campaign.[5]

An attempt to give a balanced view of immunization as a public health measure with its changing risks and benefits suggests the following conclusions:

First of all, that the discovery and progress of immunization made an immense contribution to the prevention of many communicable diseases and especially those that affect children.

Second, that the technique of vaccination and immunization, when properly applied as in the case of smallpox, may eradicate some communicable diseases from the world, or at least from large inhabited parts of it.

Third, that the successful control of many communicable diseases seen in advanced countries has not or has only marginally touched the developing tropical countries.

Fourth, that like all biological substances, vaccines and sera may have certain rare, unexpected and serious adverse effects on some persons

and that strictly controlled field trials must precede the introduction of mass immunization. Moreover, the quality of vaccine products must be subject to the highest degree of control and to long-term monitoring of their effects.

Fifth, that while some inherent hazards of vaccination exist, the community has a right to receive the relevant information based on clinical trials which will indicate any small potential risks in comparison with the expected benefits of vaccination.

Sixth, that in the forthcoming development of one or more vaccines against at least one species of human malaria parasite, in order to obtain an active, usable, effective product, applicable to the vast populations of the tropical world, an unprecedented cooperation will be needed between scientists, bio-medical and other related professions, generously supported by international agencies, national governments or institutions, and private foundations.

There have been many definitions of what constitutes a clinical trial. Perhaps the most acceptable as well as most concise is that adopted by the United Kingdom's Committee on Safety of Medicines: *Administration of a medicinal product, based on the evidence that this product may be beneficial, but for the purpose of ascertaining whether and to what extent has this product any other effects, whether beneficial or harmful*

II. Problems of Clinical Trials

For the past 30 years we have been trying to control, if not to eradicate, malaria from most of the world. We had a fair degree of success at the periphery of tropical areas, but made no impact on malaria in Africa for reasons well known.

Lately, even in parts of the world where malaria had receded, a resurgence of this disease took place. A number of new developments are taking place, but by far the most glittering promise is offered by the phenomenal blossoming of immunology and molecular biology. It seems that an attempt at developing a malaria vaccine is justified, even if it would not become the ultimate weapon for mankind's renewed fight against this age-old disease. Problems of vaccination against malaria are of particular complexity because of the involved structure of this parasite and its multistage cycle of development in the human and invertebrate hosts. During the sequence of changes from the sporozoite, to the exoerythrocytic liver schizont, then to the erythrocytic blood forms, followed by sexual differentiation into gametocytes and then, after fertilization in the stomach of the mosquito vector, into the zygote-ooki-nete-oocyst and finally into a large number of sporozoites, the malaria parasite generates a great many antigenic components. Some of these stimulate important protective responses in the human host, while

others are irrelevant or may be detrimental. The concept of developing an active malaria vaccine is based on the identification and characterization of the main antigens which are immunogenic, specific to the protective response of the host. Following that the molecular biologist aims at cloning of the genes that code for the selected antigen, in bacteria or other microorganisms, there should follow the analysis of the nucleotide sequence of the encoded molecule and its production in quantity by genetic engineering methods or by synthesis of the relevant peptides.

Much of this work has already been achieved with regard to the three main stages of plasmodia of primates, including that of man. Sporozoite, asexual blood stages (merozoite), and gamete related antigens have been characterized and recent research showed that some of them contain repetitive sequences of short chains of amino acids, relatively easy to synthesize chemically. But to be immunogenic they will require suitable carrier molecules and adjuvants applicable to humans. This will pose a number of practical problems which must be solved before any clinical trials can be undertaken. Nevertheless, there is little doubt that, thanks to the rapid progress in this and other fields, such trials will start in some, not too distant future.

No one will disagree that by far the greatest experience of clinical trials of new antimalarials has been gathered by clinical pharmacologists and much guidance may be obtained from their studies, old and new.[4,5] There are now some well-established principles of a wide range of clinical trials.*

Methodology of clinical trials is probably one of the most important disciplines of modern medical science. It stands at the interface between original research of bodily ills and the application of techniques to alleviate the distress to which they give rise. Without proper assessment of the efficacy of such procedures, medical practice would not be able to advance beyond a primitive trial-and-error stage.[6,7,8]

These trials are usually classified into five classes or phases. Phase O

*Recently, the development of drugs and biological products has assumed greater than ever importance and the responsibility of the medical profession working in the pharmaceutical industry has much increased. The wide use of potent new products as well as their monitoring and marketing falls under many government regulations; it is also critically assessed by the public and the media. Before the first clinical trial begins, the medical director of a pharmaceutical company must certify that all the relevant evidence fulfills the recognized criteria of safety. The basis of the clinical trial is the step-by-step assessment of the effects of the new product on animals. The legal responsibility of the medical director is to ensure that the factual evidence complies with the regulations and is accurately assembled for presentation to the regulatory authority. There must be an established procedure for checking, coding, computer storage, and analysis of all reports, a task that demands considerable resources, as well as expertise for internal audits. All this led to the formation of a new specialty of pharmaceutical medicine, with the establishment in the United Kingdom of an academic degree in some universities, publication of a textbook, and creation of a quarterly journal under the title *Pharmaceutical Medicine*.

refers to basic pharmacological, toxicological, biochemical, parasitological, and other pre-clinical researches on experimental animals. This Phase O is followed by four sequential but closely inter-related other phases involving human beings. Phase I refers to trials corresponding to the initial administration of the drug (or any biological product) to humans, to determine tolerability and expected effect. This can be done on healthy volunteers or on small numbers of selected volunteers from among the target population in the malarious area.

Phase II consists of clinical trials further to demonstrate the effectiveness and safety of the product as also to determine its best usage schedule. Moreover, the value of the product for causal prophylaxis, radical cure, gametocytocidal, and other effects are assessed.

Phase III refers to extended trials on larger groups of population, including patients at particular risk; activity of the new product is compared with that of some standard drugs.

Phase IV trials are those carried out after registration of the product, when it is in wide clinical use. The most important data to be collected at this stage concern the side effects, especially the rare adverse reactions occurring after prolonged use, under special conditions, or in certain categories of patients.

A number of points related to each phase of clinical trials of antimalarial drugs have been recently endorsed by the WHO at the February 1985 meeting. They deal with the type of the compound, special characteristics of the area and target population, selection of participants in the trial, selection of trial centres according to the epidemiology of the area, type of laboratory facilities, monitoring the progress of investigations, tolerability studies, risk factors, etc.[9]

Trials of forthcoming malaria vaccines are classified in the same way although some of their aspects have different aims and slightly different, perhaps more complex approaches when it comes to using the response of human subjects to the new biological product.

Trials on deliberately infected human volunteers whether prisoners or military personnel or patients undergoing malariatherapy in special hospitals were widely used between 1930 and 1950 in the U.S., Australia, Romania, France, U.K., and elsewhere. These studies were invaluable and the scientific information thus provided could not have been obtained in any other way. Although it is true that since that time we have a number of mammalian animal models, such as several species of rodent plasmodia, some plasmodia of lower monkeys *(P. cynomolgi)*, South American monkeys (e.g., Aotus, Saguinus) infected with human plasmodia and even occasionally chimpanzees, yet in the final account no animal response to human plasmodia will be the same as that of the human host.

Malariatherapy for neurosyphilis has been completely given up today

as better methods of treatment have been introduced. This type of clinical trials of antimalarial drugs seems to have come to an end.

The problem of scientific investigation on human subjects is loaded with emotional overtones, in addition to real and serious moral issues involved. It appears that at the present time public opinion in most countries will not allow such investigations to be carried out on prisoner volunteers, not even under conditions of highest supervision.

Those in prison are by definition unable to make free decisions; the argument is that inducements by privileges, or by remission of sentence, or even by relief of boredom are so powerful that they constitute an unacceptable degree of pressure.[10]

This is the situation today and we must accept it. And yet as one who observed some 35 years ago a few malaria investigations on prisoner-volunteers in two malaria research centres in the U.S., I can bear testimony to the strict observance by the authorities of the prisoners' individual consent freely given and free to withdraw. I have also been impressed by the dignified and conscious pride of these men who felt that they contributed to science and humanity. I believe that such well-designed, carefully supervised, compassionate drug trials on healthy, well-informed, carefully selected and well-treated subjects are probably ethically more justified than some investigations carried out surreptitiously or in secret on hospital patients without their knowledge.[11a]

Clinical trials on naturally infected subjects with malaria are morally more justified, even though the immune status of these patients will be unknown. Several problems may arise with regard to the selection of the subjects and their follow up in endemic areas: non-compliance with medical advice, poor recording of side-effects, non-collection of blood samples, and high proportion of drop-outs and defaulters. Planning these trials of prospective malaria vaccine trials in these conditions has been one of the topics of the recent meeting on Immunology of Malaria and Applied Field Research held in February 1985 in Geneva.[9]

Ethical aspects of clinical trials have been often discussed in many reports or books.[11b-14] Among the most important of these are the code of the World Medical Association, sometimes called Nuremberg Rules or Declaration of Helsinki, the directives of the Medical Research Council, a report by the Royal College of Physicians, and a report by the World Health Organization.[15-18] There are also statements by several medical scientists and one by Pope Pius XII.[19] Ethical considerations often have to weigh the eventual benefit of community against that of individuals and vice versa. In clinical trials the first is important, because the community needs protection through knowledge, but, on the other hand, our moral stance does not allow the neglect of the individual for the sake of the community.

A clinical trial of a new treatment or product has much in common

with any other treatment. Whenever we prescribe any medicine, we often expose the patient to the risk of discomfort or of ill effects in order to help him. The risks of treatment may be small or great in relation to the risk of leaving the disease untreated, but only ineffective treatment can never go wrong.[20]

The basic question in clinical trials of both medicaments and biologicals should be whether the patients in the trial are likely to benefit from it. If there is good evidence that a new product has advantages over existing ones, then there can be no objections against its use. There must be satisfactory evidence of safety in relation to probable efficacy, but nowadays any new medicine is thoroughly tested on animals and volunteers, and often more is known about its toxicity and metabolism than about the established treatments with which it is compared.

The major issue, which one has to consider with both volunteers and patients, is that of consent. Without the fully informed consent of those taking part we cannot justify any clinical trial, and yet such consent is required for any routine therapeutic procedure. Before a patient can give consent he or she needs the necessary facts on which a decision can be formulated. Whether or not this consent is truly reached without prejudice, coercion, or ignorance of relevant information has been debated many times and is an issue upon which opinion is still divided.

Certain persons cannot volunteer on principle; those who are mentally ill and children below 14; for them the parents may take the responsibility, if the trial is likely to benefit the children.

It has been argued that doctors omitting to mention any remote risk would not invalidate any consent given by the individual concerned. Although this view has not gone unchallenged, most people recognise the situations in which it would be valid.

It is also true (though not always emphasized) that the person can withdraw consent at any time, and there is a continuing duty on the part of the doctor to disclose any relevant facts, which may arise in the course of the treatment or study, so that continuing consent is valid. Such new facts might be an unexpected characteristic of the drug itself or an unusual body reaction. It is doubtful whether this duty is always observed. Some patients are children, unable to express any valid opinion, or people from rural areas of developing countries, whose understanding of our motives and actions is different from our own views. We may think that we fulfil our moral duty to other human beings, but ethical aspects can easily be overlooked in our enthusiasm for the aims of our studies, or in our commitment to the scientific design of the work we are doing. Ethical problems arise most commonly when clinical trials are carried out on persons who do not benefit from them, whether they be healthy people or hospital patients. There are several issues which cloud any attempt at an explicit statement of ethical principles. In clinical work—especially in drug evaluation—we must take into

account the benefits which may accrue to society and the benefits and risks to the individual: it is important that we strike the right balance. It is true that the rights and benefits of the individual are protected by the concern of the law. "But ethics and the law are not the same." Some things are unethical but not illegal, others illegal but not unethical. Often the law enforces ethical principles!

Several legal aspects of medical experimentation have been recently analysed and discussed because of the present tendency of the public to question the motives and ethical standards of the medical profession and of pharmaceutical companies. There is a distinction to be drawn between the individual as a patient and the individual as a volunteer, and the law tends to be more lenient in a therapeutic situation. Any form of treatment, by drugs or by surgery, carries some risk which is usually acceptable in the context of serious illness, but whether that same risk is also justifiable in a study involving healthy volunteers is debatable. In general we must be more stringent in assessing the risks when administering drugs or new biologicals to a volunteer than in normal clinical practice where the hazards can be set against therapeutic benefits.

To summarize, the justification of a clinical trial is determined by its definition as a carefully and ethically designed study conducted on human subjects (patients) with their full consent and cooperation, aimed at answering some precisely framed questions. It is implicit in this definition that clinical trials can contribute to medical knowledge and improve the prevention of a disease or its treatment by an unbiased assessment of the value of a given product or procedure.

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An Overview of FDA Requirements for Vaccine Development

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Introduction

The development and utilization of a safe, effective malarial vaccine will require the collaborative and cooperative efforts of researchers, pharmaceutical companies, and governmental organizations. Within the United States, the Food and Drug Administration (FDA) reviews and regulates the clinical testing of experimental vaccines and decides if and when a product can be licensed for commercial use. The regulatory decisions of the FDA are based on its assessment of the potential benefits versus the potential risks of the proposed vaccine. Certain well-established principles, which in essence reflect a consensus of the scientific community, apply to this assessment. Awareness and adherence to these principles can greatly facilitate the process of vaccine development by helping to ensure the validity and relevance of preclinical and clinical data. This paper will highlight some of these principles. The opinions expressed are those of the author and are intended as general guidelines rather than any specific requirements or official policy.

General Principles of New Drug Development

I. The Product Tested in Preclinical Studies Should Be Equivalent to that Used for Clinical Trials

The decision to approve the use in humans of an experimental vaccine follows a thorough review by the FDA of the available preclinical information relating to the vaccine. It is obviously important that the vaccine material used in the proposed clinical trial is comparable to that with which the preclinical data was obtained. To help insure this requirement, detailed specifications on the composition, manufacture, storage, and stability of the vaccine must be adhered to with each lot of vaccine

produced. Standards of potency, purity, and stability need to be defined and lot to lot consistency established. This is especially important as one proceeds from small-scale to large-scale production and begins to distribute the material between various institutions.

2. *In Vitro* AND *In Vivo* ASSAYS SHOULD ACCURATELY REFLECT VACCINE POTENCY

It is difficult to envision that the FDA or other comparable regulatory agency would license a vaccine if there were no reliable test to determine whether individual lots were equally potent. The ultimate proof of efficacy of a vaccine is the demonstration of resistance to *in vivo* infection. Unless the mechanism of protection is clearly understood it will be difficult to establish reliable *in vitro* assays to correlate with efficacy. This issue is of major concern with merozoite vaccination where cell mediated immunity (CMI), rather than humoral immunity, may be necessary for protection. The capacity to evoke CMI may not correlate with either the capacity to evoke, or to bind, specific antibody and appropriate additional assays may have to be developed.

3. ANY POTENTIAL CONTAMINATING TOXIC CHEMICAL OR INFECTIOUS AGENT SHOULD BE SPECIFICALLY ELIMINATED BY THE MANUFACTURING PROCESS

The question of safety is especially important in the proposed widespread use of a vaccine in essentially normal individuals. All biological products are requested to undergo General Safety Testing as prescribed by the FDA. More exacting tests are also required which are based on potential chemical or microbial contamination of the vaccine. A thorough review of all of the components utilized in vaccine manufacture can help identify potential sources of contamination. Obviously, there is greater concern for infectious agents than for seemingly inert molecules, but even the latter could cause toxicity, e.g., by inducing an anaphylactic response on repeat immunization. If it is not feasible to remove all known or potential contaminants, then specific tests will need to be developed to quantitate the level of contamination in each lot and to evaluate the possible adverse consequences of their injection into humans. Certainly, the animal testing should closely parallel the type of exposure anticipated in humans and the possibility of genetically determined idiosyncratic responses considered.

4. CLINICAL TESTING SHOULD ADDRESS SPECIFIC QUESTIONS

The clinical testing of vaccines usually proceeds in three phases: Phase 1 testing is primarily designed to demonstrate a lack of toxicity and involves a gradual escalation in dosage; Phase 2 trials seek evidence of efficacy and are designed to optimize dosing protocols; Phase 3 trials

are more broadly based efficacy and toxicity studies and, if successful, can lead to licensure of the vaccine. The demonstration of efficacy with anti-sporozoite vaccines is relatively more straightforward than is the situation with merozoite or gametocyte vaccines. Resistance to infected mosquito transmission of malaria in normal volunteers and correlation of the resistance with levels of specific antibody are clearly pertinent. These studies will need to be extended to define the duration of protection, individual variability of response, and the need for, and timing of, re-immunizations. It will be more difficult to design acceptable studies on the efficacy of merozoite immunity on live challenge. Volunteers would be subject to the initial hepatic phase of infection, and extensive and complex testing of CMI may be required. It is likely, therefore, that merozoite vaccines may be introduced as an additional measure of efficacy to that provided by a sporozoite vaccine. In all the clinical studies, appropriate safeguards for the health of volunteers, the need for informed consent, and the quality of the clinical assessments are critical issues which need to be addressed.

5. All Pertinent Preclinical and Clinical Data Must Be Available for Detailed Scientific Review

The FDA has the legal authority to inspect and review all experimental data submitted to it in support of an IND or NDA application. To enable it to carry out this function and to help ensure the quality of the data, the FDA has defined certain requirements for the conduct of laboratory studies. These are known as the Good Laboratory Practice or GLP Regulations. They mandate that detailed records of results, procedures, instrument function, and qualifications of personnel be maintained. Similarly, the conduct of clinical trials must be monitored carefully for adherence to protocol and accurate evaluation of results. The FDA has the right, if not the obligation, to assure itself of the validity of all of the preclinical and clinical data on which it is required to pass judgement. In actual practice, detailed preclinical and clinical information is often presented to the scientific community for their input before the FDA makes a final decision on a vaccine.

Summary

The intent of this overview has not been to provide detailed instructions on how to file an IND or NDA, nor to discuss specific regulatory requirements such as GLP and GMP. Rather, its purpose has been to highlight some of the more general principles by which regulatory decisions are likely to be made. The FDA plays an important role as a focal point in U.S. medicine by helping to establish standards of high quality clinical research. Among their many contributions are specific guide-

lines relating to the proposed use of recombinant DNA techniques for vaccine production, pre-IND meetings with interested investigators, and expert scientific evaluation of submitted data. I trust that the various issues discussed in the paper will facilitate the constructive interactions between investigators and the FDA and assist in realizing the goal of a safe and effective malarial vaccine.

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Malaria Vaccine Clinical Trials: General Considerations from the Manufacturer's Viewpoint

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Mr. Chairman, Ladies and Gentlemen, I am pleased to be here to present my viewpoint before this important conference on Malaria Vaccines and Clinical Trials. I hasten to point out that I am no longer actively working in the area of malaria vaccines and I sincerely regret that fact because this is a tremendously interesting and exciting time for those of you directly involved in the research, development, and clinical evaluation of these candidate vaccines.

I am presently engaged in administrative work involving research planning and coordination for a new Health Care Division at the Monsanto Company in St. Louis, Missouri. However, any views which I express at this conference are wholly my own and not neccessarily those of my company. They are based on over twenty years of experience in the U.S. pharmaceutical industry researching, developing, and clinically evaluating vaccines primarily at Parke-Davis, now a division of the Warner Lambert Company.

Most frequently in the development of a drug or biological, the manufacturer is the entity who initiates a clinical investigation and, hence, is designated as the sponsor. However, this situation is not always the case. At the present time, the role to be played by the U.S. pharmaceutical industry in the clinical evaluation of malaria vaccines is unclear. It seems certain that the pharmaceutical industry should be the producer of vaccines, because with few exceptions this is where the expertise resides. It is possible that malaria vaccines will be produced under contract to a government or an agency, in which case the manufacturer would not be the sponsor of the clinical studies.

I will describe some of the concerns which must be addressed by the traditional manufacturer/sponsor position, but which also pertain regardless of who sponsors the investigations. I will also approach the discussion presuming that manufacture will be in the United States and, hence, under U.S. regulations. Again, this may not be the case,

but the concerns expressed generally apply, regardless of the country where manufacture may occur.

If the vaccine is manufactured in the U.S. (regardless of where it is to be tested), the process must begin with the filing of a Notice of a Claimed Investigational Exemption for a New Drug (IND). This document must be filed with the Food and Drug Administration (FDA) prior to beginning clinical trials of any new drug or biological. Information must be provided on the chemical structure, components, composition, source, preparation, methods, facilities, and controls used in the manufacturing process. Additional information regarding safety must be provided from laboratory and animal testing of the experimental product.

I have written before about the importance of the researcher understanding the need of the manufacturer to provide the detailed information required in the IND. Attention to strain selection, freedom from adventitious agents and extraneous agents, and good record keeping must be done early as well as later in the manufacturing stage. The development of potency tests and reference standards is also essential. Issues of purity and safety are of as much concern with the new genetically engineered or synthetically produced vaccines as they are with the more traditional methods of manufacture. These points will not be dwelled upon further in this paper.

In addition to the above a plan for the studies in humans, the clinical protocol(s), as well as the training and experience of the clinical investigator must be provided. This data is reviewed by the FDA, and the sponsor must wait at least 30 days from filing before commencing the first clinical trial.

The stated purpose of this conference is the review of practical consideration on malaria vaccines and clinical trials. For the past two days we have heard reports on the status of candidate vaccines. All of these candidates share at least one common feature; they are newly discovered and untried in man. Many, if not all, will be produced by manufacturing methods which are also new and untried. Considerable attention must be paid to the issues of purity and safety which are addressed by the IND before we can move to address the issues of safety, potency, and efficacy in man. These issues are always with us in the development of new drugs and biologicals, but are of less concern when there is a body of scientific information and experience to draw from. With the malaria vaccine candidates which we are discussing, that body of knowledge does not exist, thus prudent caution and care must be exercised.

The preclinical data provided in the IND is reviewed by a committee which the sponsor designates solely for the purpose of determining if there is adequate, sufficient, and appropriate data available to recommend that the drug candidate be used in man. Similar review is pro-

vided by the FDA, and frequently some of this data is reviewed by the Institutional Review Board.

The first use of a drug or biological in man (Phase I) is usually performed in a small number of normal or high risk, informed, volunteer subjects who are closely observed. The object of the Phase I study is to determine the safety of the experimental product.

Frequently, these studies are carried out by investigator/employees of the manufacturer or by contract laboratories or clinicians whose primary occupation is conducting such trials. Because of the potential risk associated with first evaluations in man, the numbers of subjects entered into such studies is small. Normal, healthy, young male volunteer subjects are usually used, but if the risk is presumed to be high, then subjects who may obtain potential benefit are sought. In the case of vaccines, these would be individuals who are likely to be exposed to contracting the disease; e.g., laboratory workers, medical personnel, military personnel, or other high-risk target populations.

In vaccine studies, human tolerance and side effects are usually evaluated at various dosage levels of antigen and/or adjuvant. Information is gathered on the antibody response of the subject (which may relate to efficacy of the vaccine) and to the duration of this antibody. Depending on perceived risk of side effects, multiple dosages may be evaluated simultaneously in different individuals or a rising-dose schedule may be employed, assessing tolerance, before moving to the next higher dosage level. Frequently booster immunizations are part of these Phase I studies. Phase I studies are often open studies, but they may be single, double-blind, or placebo controlled.

From the point of view of the sponsor, Phase I studies are relatively easy to conduct because of the tight controls which prevail. The primary concern is for full written informed consent, prior protocol review and subsequent protocol adherence, close clinical observation, appropriate laboratory testing, and good record keeping.

Phase II studies extend the safety evaluation to a larger population. In the case of drugs, Phase II studies are performed in patients with the disease for which the drug is intended and, thus, some evidence of efficacy may be expected as well as determination of the proper dose and dosing schedule. With vaccines, the distinction between Phase I and Phase II studies is less clear. The objective is to extend human safety and tolerance data before going into the large Phase III trials.

Early Phase II studies often are made up of a series of pilot studies with the objective of answering specific questions regarding safety or efficacy. For instance, a vaccine trial may be conducted in a population who has had the disease in order to assess the impact of existing levels of immunity on tolerance, side effects, and antibody response. Another Phase II study may be conducted in a population at high risk of con-

tracting the disease in order to access efficacy following a natural or artificial challenge.

The second part of Phase II clinical testing often involves pivotal, well-controlled studies that establish the efficacy of the investigational new drug or biological, as well as provide additional safety data. These trials are likely to be single or double-blind placebo controlled studies.

In designing the pivotal studies one must balance the need to prove safety and efficacy beyond any doubt which can be achieved with double-blind or placebo studies versus the delay frequently caused by the complexity of such studies. This decision involves many ethical questions and may also involve legal issues.

Late Phase II studies are often the place for the manufacturer to begin proving the very important point regarding consistency of manufacture. Here multiple lots of vaccine are evaluated side by side to assure that they have the same level of side effects and immunological potency. This evaluation of the manufacturing process may continue into Phase III studies. Regardless of when this is done it introduces a burden of exact record keeping and complicates the task of the field investigators and the logistics of the study.

With Phase II studies, because of the larger numbers of subjects and because the trials are likely to be multicentered, the concerns of the sponsor are broadened. The responsibility of the sponsor to establish the monitor function may occur as early as Phase I; however, it is almost always required in Phase II studies. Obligations and responsibilities of sponsors and monitors were described in proposed regulations published by the FDA in September, 1977. While these proposed regulations have not yet become law, however, the FDA uses them as guidelines for conducting their inspections under the FDA Bioresearch Monitoring program.

One of the sponsors' responsibilities involves the selection of the clinical investigators. This duty is important in the conduct of domestic studies but is even more important in the conduct of studies overseas. Investigators must be chosen who are experts in their field. They must have a reputation and experience in conducting clinical research. Obviously the investigators must have access to the appropriate patient or target populations and they must have appropriate, accredited, or approved facilities for conducting the required laboratory and clinical tests.

The sponsor must assure that the investigator has obtained Institutional Review if he is associated with an institution and it is required by the regulations. The Institutional Review Board (IRB) must be constituted in accordance with the regulations and must be kept informed by the investigator of the process of the study and the occurrence of adverse reactions or any changes in the protocol which might bear on the safety

of the subjects. The IRB's primary responsibility is to protect the safety and welfare of the subject.

One mechanism by which the IRB protects the subject is by review of the Informed Consent statement which is prepared by the Clinical Investigator. The contents of this document are defined by the Code of Federal Regulations under part 46, Protection of Human Subjects. Time does not permit me to elaborate on the components of the informed consent; however, inadequate patient informed consent was the single most frequently noted deficiency found by the FDA Bioresearch Monitoring program, accounting for 44% of all deficiencies. Achieving adequate informed consent is difficult in developed countries and is clearly a major issue of ethical and moral concern which must be addressed in studies conducted in developing countries.

The sponsor's single most important responsibility is the development of the protocol. I will not discuss the scientific aspects of protocol design for this will be discussed by others at this meeting and the many meetings which will follow. What I would like to emphasize is the need for the development of fool-proof reporting forms which are both easy to understand and to complete in the field and which enable the sponsor to analyze. This task requires time, thoughtful contemplation, understanding, and experience. The statisticians must be involved in this effort but so must the individuals familiar with working in the field.

The most carefully designed protocol can produce totally inadequate data for a license application, if insufficient care and attention are given to the reporting forms.

The sponsor must assure accountability for the experimental drug. This is also the responsibility of the investigator. While both the sponsor and the FDA are concerned that experimental drugs are not improperly used and that the drug or biological has actually been used in accord with the protocol, the sponsor must further make certain that the drug at the time of its use has been handled in a manner which assures its suitability for use when administered. This issue of stability and storage is important in every study but will be especially difficult to achieve with malaria vaccines in late Phase II and in Phase III studies due to numerous local conditions.

The last two sponsor responsibilities which I will discuss are intimately related. The sponsor shall establish written procedures for monitoring the clinical investigation. These describe how the sponsor will carry his responsibilities and includes all of the items mentioned as well as those assuring the safety of subjects and compliance with FDA regulation. Via the monitoring procedure the sponsor must assure prompt reporting of serious and/or unexpected adverse reactions to the FDA.

The monitor process also should assure the completeness, accuracy, and availability of the investigators' records for review by the FDA.

Again this is true for domestic studies but has been specifically cited as one of the requirements for acceptability of clinical data generated outside of the U.S.

In closing, I just want to make a few brief comments on Phase III studies. These are by definition investigations which are conducted under the conditions and in the population in which the drug will ultimately be used. Large numbers of subjects will be used in controlled and uncontrolled trials, in order to obtain additional short-term and long-term safety and efficacy data

With a malaria vaccine being studied in large numbers of subjects in endemic and epidemic areas the task of the sponsor in monitoring these trials will be enormous. To be successful will require the application of highly skilled project management techniques and the recruitment of numerous and varied human resources. The logistics must be meticulously planned and the execution must be carefully controlled and monitored. Numerous government and health agencies will want to be involved but prudent judgement must be exercised to select only those that can do the job of providing unquestionable data which will support licensing. The FDA will be much more receptive to carefully controlled well-conducted studies which yield usable information than many large careless studies which raise more questions that they answer.

FDA acceptance of the clinical data is the bottom line for the manufacturer and this is the only way to assure the availability of the vaccine to those who need it.

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The United States Department of Defense Malaria Vaccine Program

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Introduction

In addition to its specific role as a major global public health problem, malaria as a military medical problem goes back into antiquity. In fact, the French discoverer of malaria parasites (Laveran in 1880) and the British scientist who demonstrated the involvement of mosquitoes in their transmission (Ross in 1897) were both military officers.[1] The U.S. military first encountered casualties due to malaria during the Revolutionary War.[2] In every major conflict since, there have been large numbers of cases of malaria among military personnel. During the U.S. Civil War there were more than ten thousand malaria related deaths.[2] By the time of WW II medical care was greatly improved, resulting in many fewer deaths (323), but there were still almost a half million cases of the disease.[3] Research to prevent and control this disease has been a major concern of the American military for more than two decades.

A central theme of control efforts has been the development of drugs. By the end of WW II, drugs had been produced which were extremely effective in curing and preventing malaria.[4] The subsequent development of resistance of *Plasmodium falciparum* to the 4-aminoquinolines, however, is well documented.[5] During the last two decades the Walter Reed Army Institute of Research (WRAIR) mounted a major program resulting in the development of mefloquine which is still effective against most isolates of *Plasmodium falciparum*.[6] However, resistance to mefloquine has already been described.[7]

"The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense" (para 4-3, AR 360-5).

As an alternative to drug development, the U.S. military has, in the last two decades, mounted an effort to investigate vaccine feasibility as well.

In the area of blood stage immunity, emphasis has been on the induction of immunity with whole parasites, the culture of parasites, immune mechanisms, and more recently, the immunochemistry of blood-stage antigens.[8-11]

By 1970 the fact that sporozoite preparations could be used to induce active immunity to plasmodial infection in rodent and other animal malarias was well established.[12-13] Extension of these findings to man was undertaken by investigators at the Naval Medical Research Institute and the Army Research Project at the University of Chicago.[14-15] Human volunteers were exposed to multiple bites from irradiated mosquitoes infected with *P. falciparum*. Immunity to subsequent challenge was found to be species specific and limited in time to 3 to 6 months. Similar findings were reported by scientists at the University of Maryland Medical School and their collaborators.[16] Thus it was clear that the sporozoite stage of the major human malarial parasite contained antigens capable of immunizing naive subjects against infection. These promising results led to an intensive effort in several laboratories to design a suitable vaccine preparation for widespread use in susceptible humans.

By 1982, continued advancements in the area of sporozoite immunity made it seem likely that a vaccine based on the sporozoite approach would be the first to be fielded and work on this phase of the problem was accelerated in the Department of Defense (DoD) laboratories.[17-19]

In 1984, a National Institute of Allergy and Infectious Diseases (NIAID) WRAIR collaboration was undertaken in which bacteria containing malaria DNA produced at the NIAID were screened using monoclonal antibodies developed at the WRAIR. This collaboration resulted in isolation of bacterial clones producing antigen reactive with the monoclonal antibodies. The gene was subsequently sequenced and synthetic peptides prepared based on the gene sequence which were shown to be immunochemically reactive.[20] This isolation of the gene for the circumsporozoite protein of *P. falciparum* laid the groundwork for a synthetic vaccine against the parasite.

Development of Vaccine Candidates

Although the expression system which allowed detection of clones producing antigen reactive with monoclonal antibodies produced ample material for analysis, it was not capable of producing enough material for production of a practical vaccine. Furthermore, an extremely large number of possible alternatives with respect to the design of a vaccine

were available. The DoD laboratories and their collaborators, including the Smith Kline and French Laboratories, set out to choose among the many possibilities. Vaccines based on the conjugation of synthetic peptides with high molecular weight carriers were considered to be a likely method for the production of vaccines in the future, but it was considered that construction of a vaccine through genetic engineering and expression in a microbial system offered the most direct route to a first generation vaccine.[21] A series of modified genes were engineered which when inserted into bacteria produced a variety of molecules of varying structure all based on the repeating tetrapeptides (ASN-ALA-ASN-PRO and ASN-VAL-ASP-PRO) found in the naturally occurring circumsporozoite protein. These products were analyzed in terms of their stability, level of expression, ease of purification, and immunogenicity in small animals. Immunogenicity was assessed through enzyme linked immunosorbant assays, circumsporozoite precipitin tests, and inhibition of sporozoite infectivity assays. All these tests were positive with a majority of the several candidates produced, suggesting that they might be viable vaccine candidates for use in humans.[22] Candidates are currently being prepared under conditions which will allow for human trials.

Plans for Malaria Vaccine Clinical Trials

Clinical evaluation of the candidate malaria vaccine currently under development will follow the typical pattern which has been used by DoD for vaccine development. After appropriate scientific and ethical protocol evaluation and IND submission, Phase I studies, designed to evaluate safety and immunogenicity of a candidate vaccine, will begin with the immunization of a small number, probably three, of volunteers with a very small dose, probably 10 μg of candidate vaccine. Assuming the degree of reactogenicity is tolerable, escalating doses, increasing the amount in half log increments, will be given to additional numbers of volunteers at daily or longer intervals until the maximum dose, probably 1 mg, is reached. These volunteers will most likely be DoD scientists working at the WRAIR and the NMRI. Sequential serum samples from these volunteers will be used to evaluate immunogenicity.

If these initial Phase I studies indicate safety and immunogenicity, they will be followed by Phase II studies involving experimental challenge. These studies will be conducted at the ward facilities of the U.S. Army Medical Research Institute of Infectious Diseases and those of the Laboratory of Parasitic Diseases of the National Institute of Allergy of Infectious Diseases. Approximately 15 volunteers will be vaccinated and another 15 given a placebo in a double-blinded fashion. All volunteers will be challenged by mosquito bite using mosquitoes which have

been infected from cultures of parasites. Volunteers will be monitored at the appropriate time after challenge on a daily basis for development of parasitemia. Volunteers developing malaria will be treated promptly with a drug known to be active against the strain of parasite used. Current plans are to use a strain of *P. falciparum* which is sensitive to chloroquine and other commonly used antimalarials.

If the Phase II study proves to be efficacious, this will be an indication for expanded Phase I studies. In these studies larger numbers of volunteers, perhaps a hundred or more, will be immunized and studied for reactogenicity and immunogenicity through serial bleedings. These expanded Phase I studies should provide information sufficient to allow field trials.

The preferred first field trial, if feasible, will involve young, healthy non-immune volunteers. The volunteers should be immunized or given placebo in a double blinded fashion as described for the Phase II studies. They should be placed in an area of intensive transmission area without chemoprophylaxis. This will necessitate daily parasitological and clinical surveillance. The numbers of individuals to be used in such a trial is dependent on the estimated transmission rate. Accordingly, preliminary studies will be necessary to estimate the frequency of biting of man by the appropriate vector(s) and the proportion of vectors infected by *P. falciparum*.

A successful first field trial of a malaria vaccine would open the way for more extensive field evaluation. At this point, a whole variety of complex questions will be posed. Among the most important are those related to the duration of immunity. The possibility of the occurrence in nature of multiple strains with different antigenic structures cannot yet be dismissed, although the available evidence would argue against this possibility. In addition, the extension of vaccination trials to population targets other than those of immediate interest to the DoD would be appropriate at this stage. The information gleaned from studies carried on under the auspices of the U.S. DoD are confidently expected to be of great usefulness to the global problem of malaria at large.

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Discussion

BRUCE-CHWATT: We have time for recommendations on the last day of the meeting and I see no reason at all why a properly formulated recommendation of this kind couldn't be prepared and I would suggest that Dr. Ruth Nussenzweig should prepare it together with Dr. Victor Nussenzweig or separately because they seem to be sitting separately all the time. And this should be discussed and formally recommended or otherwise.

DIGGS: I certainly concur with the need for every effort to be made to assure comparability of results in these trials and I think that we need to work together toward that end.

SIDDIQUI: I also agree that the two types of vaccine should be compared. In that regard, I would like to ask Dr. Diggs a question. Before human trials begin, wouldn't it be better to compare the immunogenicity of these two products which are produced by different biotechnologies?

DIGGS: I neglected to indicate that we are doing immunogenicity studies in *Rhesus* monkeys without any opportunity for efficacy, of course.

COLLINS: I have a comment on that. I think chimpanzees, eventually along the line, might serve as a useful animal for such studies. Mine is a historical comment. In the 1940s there was an effort to make vaccines in the United States directed by Dr. Michael Heidelburger. In about 1946, he wrote a letter to Martin Young in Columbia, South Carolina, saying, "Give me a gram of sporozoites and we'll have a vaccine to malaria."

Looks like we now have the gram of sporozoites and we'll have a vaccine for malaria. It took us 40 years, though.

R. NUSSENZWEIG: In collaboration with CDC, we are doing some experiments in *Aotus* monkeys. The initial data indicates that, at least as far as immunogenicity, the antibody response goes, that these animals do respond and produce good titers of antisporozoite.

V. NUSSENZWEIG: One of the main purposes of this study is to have really quantitative data on protection. That is now possible by using a DNA probe to measure the number of EE forms developed in the liver. Therefore, it is not unreasonable to say that, in truth, vaccines can be used and compared in a quantitative way, at least for EE form development in the liver of *Aotus* monkeys.

PEREIRA DA SILVA: I don't want to disturb this more or less solemn atmosphere that was created, but I would insist on the point that I raised in my talk because I think that these clinical trials must be conducted simultaneously with some animal experiments to see which would be the consequence of introducing selective pressure on the development of immunized people. Dr. Nussenzweig, yesterday, answered to this point and said that he had already performed one experiment in mice and looked for the appearance of different epitopes in the survival fractions. If you suppose that the efficiency of the vaccination would be 90%, it would take 6 generations of cycles to get 10% of the parasites with different epitopes. If we start with the rate of mutation in the beginning of 10 to the minus 6, so this is the only level in which common methodology would be able to detect the variant in a population. And, of course, in the volunteer experiments, people will be obliged to treat the volunteers that acquire malaria. So, we cannot follow the cycle at this point. But with animal experiments that you plan to conduct simultaneously, we could see, after the six or seven cycles of entire reproduction of the parasite and the immunoselective pressure, if this problem has some reason to be rated.

REESE: Would either of the two groups who are considering these experiments consider one other possibility? Would you consider taking the antigen and going to a population which has antibody, affinity-purifying out the antibody and doing at least a passive protection experiment with human antibody, so that we could first get some idea of what protective levels of antibody are in a human and second get around the potential problems of what is the best mode of stimulation, i.e., the adjuvant effect so that when you went in and wanted to stimulate with an adjuvant you'd know what you have to achieve? I'm essentially asking for a human repeat of the mouse studies but with a real close look at what levels of antibody are necessary for protection.

DIGGS: My first impression is that the difficulties technically, perhaps ethically, or at least in terms of ethical review, would retard so much as to make it not cost-effective in that sense. However, the point that by very careful epidemiological studies in the field one can make the correlation between antibody titers and protection is a very good one and certainly needs to be done.

SAVANT: I think if one looks into Professor Paik's paper, it will be seen that protective efficacy for *P. falciparum* will last for about three months and for *P. vivax* about six months. So, if one happens to have sera taken at two different times, then one would be able to measure and compare what level of protective antibody would be necessary in order to control protection not only against *P. falciparum* but also against *P. vivax*—if you have those sera.

LEVINE: Scientifically, Dr. Reese's question is a very appropriate design to answer the question of whether anticircumsporozoite antibody of human origin can protect humans and perhaps that answer was too summarized in terms of why it would be difficult. (That kind of trial design collecting sera in an endemic population followed by various *in-vitro* chromatographic and other procedures and then putting it back into humans would be a protocol extremely difficult to pass human volunteer research committees.) Probably at this point the most frightening aspect of protocols to committees is anything that involves human blood products of an experimental nature going into human volunteers and this has to do with viruses such as the retrovirus, HTLV III, that causes AIDS that cannot always be identified in a product because of this kind of constraint. The other would be unknown hepatitis virus that cannot be detected (non-A, non-B) in such products. So it's extremely unlikely that such a design would go through and that is why it can't be done.

PAPPAIOANOU: It seems that several vaccines that currently are widely used, when one goes at least to publish studies to look at toxicity safety issues that much of the early data is collected in healthy men. In much of the discussion this morning, with regard to Phase I trials, again we're hearing about carrying out safety and toxicity studies in healthy men or even when we go to developing countries, that they, again, be carried out in men. Yet, one of the principal high-risk groups for malaria are pregnant women. I was wondering if, in fact, there would be an assurance that safety and toxicity would be assessed in a small number of women, women of child-bearing age, before progressing to Phase II or Phase III trials.

BRUCE-CHWATT: Thank you very much. This, once again, is a very pertinent comment and a very good observation and once again, I wonder

if this should not be presented as a formal recommendation at the end of the meeting.

LEVINE: In answer to Dr. Pappaioanou. One of the CS vaccine candidates that Dr. Nussenzweig's peptide toxoid conjugate will be tested in healthy young adult women as well as young men. The problem, though, in testing any vaccine in the United States with respect to pregnant women is that one cannot test a vaccine in Phase I studies in pregnant women with respect to fear of unknown complications to the developing fetus. That, a priori, is unethical and with the very best tests we have in testing young adult college women, to make sure they're not pregnant, there still remains a window of vulnerability of a couple of weeks and a great fear of every clinical vaccine tested is that, God forbid, he or she gives a vaccine to someone and something happens to the fetus unrelated to the vaccine. Then, a potentially good vaccine will be incriminated; there will be litigation, etc. Therefore, extreme caution should be used when testing a vaccine in healthy young women.

WERNSDORFER: In relation to the special risk groups, of course, there has to be consideration of under which circumstances in which populations are the vaccines expected to be used, who are the target groups and so on. Of course, there has to be testing of the vaccines in all those groups. That is a basic issue, of course. Are we going to use live vaccines or not, because that would set very different standards with regard to pregnant women. We have at present a clinical trial of a particular drug going in pregnant women, and I can tell you in the beginning it was kind of a nightmare. It is a question, that background noise has to be assessed very carefully before ever starting to give anything to target groups of this kind. There are also other target groups which are particularly vulnerable and I am thinking they are of malnourished infants, of people who are suffering from different conditions but who cannot be exempt from vaccination in the long run. So these issues have to be addressed in the form of Phase II or III or IV. That means postregistration of the vaccine. So that has to be really assessed on a case by case and priority basis.

REESE: Prophylactic cause I might be at risk, and I don't see how that experiment puts a person any more at risk than a "well-designed experiment" where you're going to challenge them with something and you don't even know the level of antibody that you have to achieve. . . .

LEVINE: That's a fair question. And I think, in all honesty, if one knew about AIDS, if AIDS existed at the time that the studies were done with the high titer Hepatitis B, honestly, they would not have been carried out. Stop the science for a moment, put yourself on the committee, make believe it's your relative dealing with a viral disease for which

there is no cure with proven diagnosis, 100% fatality; would you rather not answer this in another way? I would turn it around and say the most ethical way to answer this question is to actively immunize individuals with a peptide toxoid conjugate or with a purified protein from the genes being cloned in *E. coli* to get the antibody and answer it that way. I think that for the safety of the volunteers that's a much more reproducible and clear-cut situation.

BRUCE-CHWATT: This is a fascinating discussion and we seem to be finding more and more skeletons in our cupboards.

BRACKETT: I had one question which has to do with manufacturers, but I'd like to comment on Dr. Levine's comment. I don't think there is any evidence for concerns about transmission of AIDS from globulin products and if one were going to do that experiment and use Cohn's fractionation method, there would be no risk from this theoretical concern. Primarily, one worries about materials that have not gone through the alcohol precipitation steps involved in making commercial gammaglobulin so I don't think, Bob, that you're going to be at risk of AIDS when you recieve your Hepatitis B globulin material. The other point that I'd like to really raise is the question of the manufacturer. I was asked to comment on the concerns of the manufacturer and I've heard nothing really about the real manufacturers of these candidate vaccines. I wonder, if the manufacturers would not speak on this subject, if at least Dr. Diggs or Dr. Nussenzweig might enlighten me as to who's producing the vaccines and what their role in this is.

ETLINGER: I'd like to make a brief comment on the point that Dr. Brackett just raised. It necessarily has to be brief because of limited time constraints. I've only been working with Hoffman-La Roche for a few months and I'm not terribly informed, at least as well informed as I would like to be to be able to answer the question satisfactorily. Hoffman-La Roche has made a commitment to the development of malaria vaccines and I find that rather interesting from at least one standpoint because I think that if you push it to the extreme, ultimate success would mean the elimination of anti-malarial drugs. I think the prospective which Dr. Brackett expressed from an American, or as he put it "a personal standpoint," probably refers to us in general. The American viewpoint is similar to the one being expressed by Hoffman-La Roche. We are involved in collaboration with the Nussenzweigs on development of the sporozoite vaccine, but I don't know if Victor or Ruth would like to comment a bit more on that. But that's about all I can say.

LEVINE: When we come to challenge the Phase II studies in volunteers of Carter Diggs or elsewhere to assess vaccine efficacy if mosquitoes in captivity are going to be feed on *Plasmodial* cultures maintained with

human blood, committees are going to be very concerned with the source of that blood. We see this as an area that has to be very carefully monitored in terms of the source of the blood and will raise the same kinds of problems that I alluded to previously, particularly since some individuals, some epidemiologists, have suggested that in Central Africa both Hepatitis B, in theory, as well as HTLV III viruses can be transferred in mosquitoes as in the act of going from one individual to the next.

REESE: One can solve this problem easily by pre-treating the individual, prior to serving the blood, infecting it and using their own blood to challenge it with.

LEVINE: And that's what we'll probably do.

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C. TECHNICAL ASPECTS OF PHASES I, II AND III CLINICAL TRIALS

Technical Aspects of Phase I and II Clinical Trials of Malaria Vaccine

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Advances in basic biological research and biotechnology have set the stage for major further progress in combating infectious, neoplastic, degenerative, and other disorders. New potentialities on the horizon include vaccines to prevent a wide range of diseases including malaria.[1-13] Clinical trials directed at initial appraisal of the safety and efficacy of new modalities in man form a key part of the modalities' development.[14,15] This report focuses on such trials as they relate to malaria vaccines.

The terms Phase I and Phase II as applied to clinical trials are used differently by different persons. For this discussion, Phase I embraces the introduction of an agent into man to gain *preliminary insight* into safety and into certain pharmacologic or other data that may help to justify Phase II studies with a particular dosage or regimen, and Phase II embraces further assessment of safety and, in particular, initial direct assessments of *efficacy*. Phase III involves expanded clinical trials on efficacy and safety. Some workers view Phase I as including initial studies of efficacy. Uniform definitions of Phase I and Phase II would be of value. In the absence of definitional standardization, we will need clear understanding of what the terms Phase I and Phase II do or do not mean in individual instances.

General Considerations

Needs or requirements that generally apply to the circumstances in which Phase I and Phase II studies are conducted include:

1. An appropriate, on-site principal investigator.
2. An appropriate project monitor.
3. Clear delineation of the reporting requirements, and of other ele-

ments of the responsibilities and expected working relationships, between the principal investigator and the project monitor and between the institution conducting the studies and the sponsor(s)

4. Suitable facilities.
5. Adequate medical, nursing, and technical personnel.
6. A sufficient number of appropriate subjects.
7. Requisite laboratory and other supporting capabilities, including those essential for around-the-clock observation and emergency care when needed.
8. Adequate information management systems.
9. Appropriate institutional review board or ethics committee review and approval of all protocols.
10. Rigorous adherence to generally accepted principles for enlisting informed consent.
11. Adequate written documentation of that consent.

Needs that generally apply to protocol design include:

1. A clear, succinct statement of objectives.
2. Participation by an experienced biometrician or statistician.
3. Explicit criteria for evaluating and selecting (accepting or rejecting) subjects.
4. Appropriate plans, based on the preclinical data, that set forth the baseline and follow-through parameters to be assessed, the methodology to be used, the timing of assessments, the duration of study, and the anticipated numbers subjects needed to meet the stated objectives.
5. Appropriate controls, a method to randomize subjects, and use of a double-blind schema when possible.
6. Criteria that will underpin conclusions, including decisions to discontinue study with individual participants and decisions that data warrant proceeding to a subsequent phase of study.
7. Explicit formats and systems, consistent with the study design, to collect, record, and document the observations and maintain, retrieve, analyze, and transmit data.
8. Protocol design, methodology, and data formats that, as much as possible, allow for pooling of data from multiple studies.

Selected Malaria Vaccine-Related Considerations

The principal investigators and institutions selected for Phase I studies should be ones experienced with Phase I trials. Those selected for Phase II malaria vaccine trials should be experienced both in Phase II studies and in the diagnosis and treatment of malaria. Expertness in basic and applied immunology pertinent to vaccine evaluation and to diagnosis

and treatment of patients experiencing adverse immunological reactions will be key in both phases.

Initial sites for the studies preferably should be in non-endemic areas with facilities, personnel, supporting capabilities, and prospective subjects that can meet needs for short- and long-term trials. The subjects for initial Phase I and Phase II trials should, if possible, be non-immune men between 21 and 45 years old in good health as gauged by a thorough medical history (including no history of allergic reactions), physical examination, basic laboratory tests (complete blood count, urinalysis, normal indices of hepatic and renal function, and normal electrolyte, glucose, calcium, phosphate, uric acid, albumin, and globulin levels), normal electrocardiogram and chest x-ray, negative tests for factors that may confer a protective effect against malaria (G6PD deficiency, hemoglobinopathy, etc.), and normal laboratory indices of immune system function.

The facilities for Phase II studies should include insectary capabilities for breeding suitable anopheline vectors and for safely obtaining and handling infective mosquitoes. Initial Phase II studies should involve a strain of *Plasmodium falciparum* highly sensitive to chloroquine and with which the investigators have had considerable experience. The medical and technical staff should be experienced in examination of thick and thin blood smears for early microscopic diagnosis of malaria and for quantification of levels of parasitemia. The capabilities should include ones for appropriate, state-of-the-art appraisal of immunologic responses to the candidate vaccine preparations and of possible adverse immunologic or allergic responses.

The principal investigator(s), the sponsor(s), a biometrician or statistician, and experts in immunology should have responsibility for jointly determining the details of proposed Phase I and Phase II protocols. The preclinical data will be key in determining the beginning dose for initial Phase I studies, for deciding which tests will be used to assess immunological responses, and for designing assessments of multiple dose regimens. The type of vaccine preparation will influence protocol design. One general Phase I protocol schema that may prove reasonable would entail double-blind studies involving groups of four subjects, half in each group to be control subjects, with a very low initial dose and decreasing step-wise increments in the dose level in new groups of subjects at 2-week intervals if previous studies indicate safety of the lower dose. Vaccines having multiple components would necessitate separate evaluation of each component and likely would require increases in the size of the groups studied.

The main objectives of the first Phase I trials will be to assess safety of single doses and then of a multiple dose regimen and to demonstrate immunologic responses that furnish rationale for initial Phase II stud-

ies. The first Phase I trials should afford preliminary insight indicating that the vaccine preparation is in fact immunologically active (although insight into responses relating to protective impact may not be forthcoming), into subject-to-subject variation in responses to the same dose or regimen, and into the safety of a particular regimen such that there is reason to believe that the regimen may have a protective effect in Phase II studies and is likely to prove safe in subsequent trials.

The number of subjects required for initial sets of Phase I studies with particular vaccine preparations to obtain data that warrant Phase II studies in the same target group may be in the range of 20 to 80. The design and conduct of the Phase I studies should yield data required for Phase II studies as expeditiously as possible while minimizing prospects that the dose or regimen selected for Phase II studies will prove far from optimal. If the initial Phase II studies in the first target group afford direct evidence of efficacy, then Phase I-type studies can be expanded to additional target groups (to set the stage for Phase II-type studies in additional groups including women, older subjects, children, subjects previously exposed to malaria, and subjects not in good health), to assess possible drug-vaccine interactions, and to bolster the base of dose/response/duration data, and Phase II studies with the initial target group (and later other target groups) can be expanded to obtain information about the duration of protection against the homologous strain of parasites (reexposure), possible cross-protection against heterologous strains, and possible cross-protection against other species. Similar sequences of interactive Phase I and Phase II studies may be needed for trials of multivalent vaccine preparations.

One schema for Phase II protocols involving sporozoite-inocula would entail a long-used "multiple bite technique" in which batches of ten infective mosoquitoes bite each of a group of four subjects (three having received the agent under study and one control)[16] at specified times following vaccination (for example, single sets of exposure of different groups of four subjects each 1, 2, 4, 8, 12, and 16 weeks after a single dose of vaccine or after the initiation of a multidose vaccine regimen). Improved methods for inoculation of sporozoites and for quantitating the inocula are under development and may be available.

If it is necessary to conduct initial Phase II trials in endemic areas or with subjects previously exposed to malaria, maximum effort should be made to limit the potential for unintended exposure to infective mosquitoes; arriving at firm conclusions about efficacy in such circumstances may require more subjects than with a non-immune (not previously exposed) target group in nonendemic areas. Baseline immunological data may be important in such instances to identify or screen out subjects who have significant pre-study malaria antibody levels. The numbers of subjects needed for Phase II trials of a particular vaccine in

different target groups and to assess the duration of protection, cross-protection, and drug/vaccine interactions may be well over 100 if the candidate vaccine preparation exhibits protective efficacy; the number will vary substantially depending on the extent of the Phase II trials required.

For trials relating to protective efficacy, blood smears should be examined quite frequently at the outset to detect and treat patent infections as promptly as possible, and follow-up should be as long as is practicable. Radically curative regimens of antimalarial drugs should be administered when the studies with a particular group of subjects near completion. One temporal schema for blood smears would be twice daily from day 5 to day 21 after exposure, daily for the next 14 days, and then twice weekly until radical cure.

Concluding Comments

One pitfall to avoid is unduly abbreviated Phase I studies that lead to Phase II studies of a far-from-optimal dose or regimen and that can thereby result in unwarranted conclusions about a lack of efficacy. On the other hand, overly extensive Phase I studies prior to initial Phase II studies can also result in a needless use of time, energy, and resources. Phase I studies should be pursued with an eye to initiation of Phase II trials as soon as the Phase I data clearly or solidly justify Phase II studies in the initial target group.

Phase I studies may afford relatively few if any substantial difficulties. Phase II trials, however, may prove more difficult than is initially expected. Obtaining initial evidence of a lack of efficacy with an inactive agent is relatively easy. Documenting degrees, durations, or extents of efficacy with an active or partially effective agent may present sizeable challenges.

It will be important to keep in mind that studies with one strain are just that. Broadly based data on efficacy and on safety will be necessary to justify conclusions relating to general potential utility.

Temptations to conduct uncontrolled studies or to short-cut some of the relatively laborious elements of Phase I or Phase II documentation of safety and efficacy should be forcefully resisted. There is simply no substitute for well-designed, well-controlled, carefully executed and documented, scientifically conclusive studies and data.

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Discussant: *Sine Qua Non* Conditions for Phases I and II of the Malaria Vaccine

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The first human trials using any new vaccine pose major problems to the researchers. Principles of the studies are always the same, but special characteristics are specific to each vaccine.

Regardless of which type of antigen one is going to start with, the first point as to the malaria vaccine is to define the phases. In my mind Phase I should concentrate on three aspects:

1. Evaluation of the conceivable adverse reactions including those possibly of a toxic or an allergic origin.
2. Estimation of immunogenicity.
3. Preliminary estimation of the clinical efficacy on grounds of the previous animal studies, i.e., what is known about the correlation between immune response and protective efficacy.

Phase II should focus on the following four points:

1. Search for the optimum vaccination technique, age, site, number, and amount of the doses and timing.
2. Placebo-controlled vaccination on the double blind basis.
3. Challenge with a heavy but highly chloroquine-sensitive dose of *Plasmodium falciparum*.
4. Continuous follow-up of potential risks.

Table 1 lists the *sine qua non* conditions requiring fulfillment before the first clinical trials are at hand.

Aim and Reasons for the Study

In contrast to, say, varicella, this basic question is easy in malaria. Malaria is a potentially fatal disease, rampant everywhere in the developing world and, in fact, is deemed the single most important disease of mankind (at least as far as the infectious diseases are concerned). It is our common responsibility to fight against it with all the modern facili-

TABLE 1.

Sine qua non conditions of Phases I & II	
1. Aim and reasons for the study	
2. Detailed plan	— whom (age and sex)? — how many? — controls (double blind fashion) — vaccine (antigen & adjuvant) — vaccination technique, site — dose & timing — challenge (natural *vs.* artificial) — clinical & laboratory follow-up — long time monitoring of potential risks
3. Precautionary measures	— where? — who?
4. Ethics	— declaration of Helsinki — informed consent — legal responsibility
5. Schedule	

ties including the immunological weapons. The final goal is to eliminate malaria, as smallpox was eradicated from the world, and measles is being eliminated in some industrialized countries.

Detailed Plan

A scrupulous study protocol has to answer at least the following specific questions:

Whom to vaccinate? The very first vaccinees should be preferably young, *healthy males*. The volunteers may represent personnel of the laboratory, but any person motivated for the trial is acceptable as well. Full understanding of what is going to be done is a prerequisite for participation. Health of the volunteers should be checked with the same clinical and laboratory indices that will be used in the postvaccination period.

Inclusion of *female vaccinees* in the studies might become relevant in Phase II, whereas *pediatric vaccination* is hardly justified before Phase III. In that stage children—who are most prone to malaria—represent the age group that requires special attention.

How many vaccinees are needed? Primarily, Phase I requires a few persons only. After learning (hopefully) that the vaccine is seemingly safe, the number of vaccinees is to be increased; still some dozen people may meet the demands of the first trials.

Number of the volunteers in Phase II depends upon the results obtained from Phase I. If the vaccine proves highly immunogenic, some

dozens of volunteers suffice to provide data for reliable statistical analysis.

Placebo-controlled study on the double blind basis is a prerequisite in Phase II, where the primary goal is focused on clinical efficacy. Number of the controls may equal the "real" vaccinees, albeit there is no absolute need to do so while scrutinizing the technical details (such as searching for the optimum vaccination technique, etc.).

Nature of the antigen and, obviously, the adjuvant—when dealing with the merozoite vaccine—should, of course, be the same as in the animal studies that gave the most promising results, taking into account the safety of each of the components in the vaccine. Freund's adjuvant, for example, is thus not conceivable although immunologically it is the best alternative when combined with the merozoite antigen.

Vaccination technique and site of injection pose numerous alternatives. It is likely that reactions caused by subcutaneous versus intramuscular vaccination do not differ considerably, but use of an intradermal technique might prove beneficial. In many cases, such as in typhoid and cholera vaccinations, intradermal technique lessens adverse reactions to a great extent when compared to the traditional subcutaneous route.[1-3] Moreover, intradermal injection uses only 0.1–0.2 ml of the vaccine, which is a substantial advantage in shortage of the antigen.

Very little is known about significance of the vaccination site on adverse reactions. In tetanus vaccination it is claimed, however, that an injection in the thigh is less reactogenic than in the upper arm.[4] In order to minimize reactions the optimum site is to be sought also for malaria vaccination. Unfortunately, studies of this kind are laborious and require quite a few vaccinees.

Dose and timing of vaccination(s) are factors highly relevant to practice. Again, a reasonably high number of vaccinees are required. Dose (or doses), i.e., amount of the antigen, and other constituents of the vaccine, should be determined in order to find equilibrium between optimum immunogenicity and minimum reactogenicity of the vaccine. In addition, the volume in which the vaccine is administered plays a role as to the reactions.[5]

Timing of vaccination—how many doses, how long apart, at what age, etc.—is a crucial question. However, these variables pose so many alternatives that one has only to start with "the best guess" and may vary the choice in future.

Challenge of the vaccinees (including those who received placebo injection[s]) is the clue of the game and is possible artificially or *per vias naturales*, i.e., by the mosquito bites. Natural challenge is certainly better in Phase III but, for logistic reasons, may not be achievable in Phases I and II.

Challenge should be performed with a rather heavy dose of infecting

plasmodia, otherwise clear-cut results might not be obtained. The strains used must be highly sensitive to chloroquine so that all parasitemiae can be terminated easily and rapidly.

Clinical and laboratory indices are to be followed in detail to find every conceivable harmful effect at its earliest stage. It is of paramount importance that the placebo controls are followed identically because many of the symptoms or signs which vaccinations are accounted for are not in a causal relationship with the vaccine. The only way to distinguish a true immunization-induced reaction from the spontaneously developed reaction is by careful prospective follow-up.

A problem in choosing the laboratory indices is the fact that we do not know what to check except the basic malaria parasitology. Spleen, liver, the blood forming system, and other immunologically active organs are, however, the most likely targets of the potential toxic, allergic, or other inadvertent effects, so that their function has to be investigated primarily.

Long-time monitoring of the potential risks. Not all troubles may rise right after vaccination. Therefore a system is to be established that finds potential nuisances in the long run also. The system should not lie on spontaneous reporting but on an active query system where the vaccinees are approached regularly. In addition, the vaccinees should be encouraged to contact the researchers at any other time as well, if desired. Wide experience from vaccinations tells us that spontaneous reports reflect the true incidence of reactions only vaguely.

Precautionary Measures

Where should Phases I and II be performed? Phase I may be achievable easiest in a medical center or a hospital ward closely affiliated to the research laboratory, unless legislation of that country makes the study too complicated. Performance of Phase II is well justified (if needed) in another country where an appropriate group of motivated people is available. In any event, the facilities should be of the highest quality so that adequate medical aid is at hand immediately and appropriate treatment can be instituted without delay.

Who should perform the vaccinations? Researchers who developed the vaccine and vaccinators who administer the injections are naturally responsible for the trial. Immunization should, however, be carried out by a medical person experienced in this field. This attitude guarantees appropriate asepsis, technique, and precautionary measures of the vaccination procedure. Adrenalin (epinephrine), a steroid for intravenous use, antihistamine, atropin, and oxygen should, at least, be available immediately. I would also prefer to have an intravenous route opened prior to the very first injections being administered.

Ethics

The Declaration of Helsinki with later appendices stipulates the ethical conditions under which all clinical trials are to be performed. One of the prerequisites is the *consent* of the persons to be immunized. The consent has to be signed specially in U.S., whereas in Scandinavia, for example, demand of the signature is easily regarded as conveyance of the *responsibility* to the vaccinee. Under these circumstances it is preferable to give detailed information of the procedure orally and literally instead of demanding signatures as it is done in many juridical acts. Good psychology is half of the victory also in the first human malaria vaccination trials.

Schedule

The schedule of Phases I and II depends upon factors that are not fully defined at the present time. Therefore the phases cannot be scheduled precisely as yet. Anyway, fulfillment of Phases I and II takes several years.

As easily understood, rapid progress in the Phase I studies accelerates also performance of Phase II. Not of minor importance is the economical support for the malaria vaccine studies. If the same sum of money that is invested—rather unnecessarily—for production of a new antibiotic or antihypertensive drug would be given to the malaria vaccine development, we perhaps would reach our goal remarkably sooner than under the present circumstances.

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Discussion

COLLINS: How do you assess the effect of a vaccine in which you do have some clinical malaria before the vaccine takes effect? For the sporozoite vaccine there is no problem. You have to use a sporozoite challenge and you have the criteria of no infection. But on the blood-stage vaccine, the immunity in monkeys thus far has indicated that the animal has a week to ten days of parasitemia before you can measure the effect of the vaccine. In volunteer studies in which you have a cut-off point of say 5,000 or less to treat the patient, you don't have time to see if the vaccine is effective.

LEVINE: I am going to ask Dr. David Clyde to comment on that.

CLYDE: Well, of course there will be initial parasitemia if you are dealing with a merozoite type vaccine and it will take several cycles before it reaches 5,000. I would say we have to work on this. I couldn't give any hard and fast answer. But again, if I could kick this football a little further, I am sure that Robin Powell would also have some very good suggestions on this.

MODERATOR: Well the ball is in your court, Robin.

POWELL: O.K. Your assumption is that the merozoite stage vaccines that would be tested would have those anticipated efficacy characteristics? I guess I have not yet bought that assumption. It seems to me very possible that vaccines with a greater degree of protective efficacy could be forthcoming, in which case this would not necessarily be an issue. I think it is fair to say that I personally would be very comfortable with conducting such studies provided that the investigators involved had had considerable first-hand personal experience with falciparum malaria, gauging its management. They knew the strain, they knew their volunteers, and they knew conditions and had to comply with certain criteria such as—and I'm not sure finite level parasitemia is the criteria I would necessarily select—I think I would go mainly on clinical judgment, and as to how sick the individual is, how they are in fact doing, and watch them with the understanding that if in the judgment of the clinician, this volunteer simply is not tolerating this reasonably well, no matter what the level of parasitemia is, medication would be brought into the picture. It would complicate interpretation as to statistics, but as far as I am concerned, that study could properly, safely, and ethically be conducted, even if the vaccine had that type of anticipated effect.

COLLINS: In the past we used to manage infections with small doses of quinine, but towards the end of the drug trial period, they set rigid criteria of treatment. That is something that really has to be built in. If you have to treat a patient at a certain level, then the clinician on site cannot make that decision and cannot wait for the effect of the vaccine.

WERNSDORFER: Now, of course, as previously stated non-immunes with 5,000 parasites can be very, very ill, but we should also for the benefit of the parasite immunologists say 5,000 parasites represents a parasite density of only 0.1%. When we were going through certain antigens yesterday, we saw that the turning point was at 10–20% parasitemia, which were between 500,000 and 1,000,000 parasites per cubic millimeter, and this is an absolutely high danger level which comes close to the conditions for exchange transfusion. So, in actual fact, I think we have to share the optimism of Dr. Robin and say we hope that vaccines become available which are really putting the turning point below that possibly and hopefully 5,000 parasites per cubic millimeter level—the 0.1%.

MODERATOR: When I was in Nigeria, I had a *P. falciparum* infection—a very severe one—with so few malaria parasites in the peripheral blood that I wonder whether it is malaria or not.

POWELL: Let me just add one footnote. In the Gambia, the same kind of problem would, in fact, come up when one was carrying out the very first therapeutic studies with an agent apt to be active, but for which there were no cure. I would visualize that, in all probability in at least some centers, they will be using mosquito transmission for some of their sporozoite studies. They will need, therefore, to have some volunteers who become gametocyte carriers and who, in the course of those studies, become partially immune. That group, early to midway in their acquisition of clinical partial immunity, happens to be a very good group to study with, I think, increased safety complete unknowns such as the type of preparation Dr. Collins has referred to. So that is an additional approach that could be considered that has been used in the past and might further tend to modify some of the fears and concerns.

COLLINS: I disagree. All of the mosquitos would probably be infected off culture. I don't think anyone would use people.

LEVINE: I guess we can also hope that the first blood-stage vaccine that comes along is from vivax and that this won't be such a problem. Perhaps the immunological question can be answered without there being a significant clinical danger.

TRAGER: I think if you are going to wait for a blood-stage vivax vaccine, you might have a long wait. However, in line with Dr. Powell's optimism, as I remember a lantern slide of Luc Perrin's on Tuesday with his 41K antigen, he had levels of .01%—is that right?

PERRIN: Yes.

TRAGER: So perhaps this optimism may be partially justified.

The Technical Aspects of Phase III Malaria Vaccine Trials

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Subsequent to the successful completion of Phase I and Phase II trials of a candidate *Plasmodium falciparum* malaria vaccine, the product should be ready for trial in endemic areas where challenge of the vaccine will be through the risk of acquiring the infection under natural conditions. Such field trials will undoubtedly be highly complex and difficult—perhaps more so than the field evaluation of any previous candidate immunizing agent—but must be expertly planned, executed, and evaluated to provide the basis for ultimate approval of the product. In view of the current absence of definitive knowledge of the characteristics of the several vaccine types, early planning of Phase III evaluations, and indeed of Phases I and II, will be difficult and somewhat speculative. Nevertheless, recognizing that basic information on safety, efficacy, duration of effect, and reliable procedures for immunogenic evaluation will be established during earlier pre-clinical and clinical studies, general guidelines for the Phase III evaluation can be discussed. These guidelines will require considerable flexibility to compensate for unknown factors which may emerge during earlier product development and testing, and, at this point in time, will frequently be in the form of questions rather than recommendations.

In Phase III trials assessment of the efficacy and safety of the vaccine will be principally the assessment of individual response. Phase III trials can be roughly divided into two "sub-phases"—initially in small, well-defined, manageable populations, and later in larger more diverse population groups. Epidemiologic assessment could be included in the later trials, but most prefer to postpone these evaluations to Phase IV. Primary objectives of the Phase III trials would be to assess the capability of the vaccine to prevent or modify the naturally acquired infection and to assess the safety and tolerance of the product and vaccination proce-

dures. Secondary, but also highly important, would be the evaluation of procedural feasibility and the estimation of effects on other health parameters.

Although three major *P. falciparum* vaccine types are currently under development, only the two protective types—the sporozoite and the asexual blood-stage vaccines—will be included in this discussion. The third type—the transmission blocking or "anti-gamete" vaccine—is unlikely to require Phase III testing, except in combination with one of the others, and will not be further considered. Appropriate study populations and trial designs will differ for the two protective vaccines, and each will be discussed separately. Common to studies on both vaccine types will be the need for a thorough background evaluation of the candidate study areas.

This *background evaluation* should include the following:

1. Parasitologic studies, through surveys, in addition to appropriate active or passive case surveillance, will determine the prevalence of symptomatic and asymptomatic parasitemias in the community by age, sex, occupation, and season. Special attention should be given to the identification of groups at high risk, such as young children, pregnant women, migrants, etc. Determination of parasite species prevalence would be highly important.
2. Entomologic studies will determine vector identity and seasonality and specific seasonal sporozoite rates as a measure of risk of infection in the area. These studies must be continued and repeated at regular intervals through the study period to provide an estimate of risk modification as a factor in vaccine evaluation. While the technology of qualitative and quantitative sporozoite identification is well advanced, the availability and reliability of this technology for routine field use must be assured.
3. Immunologic studies should include serologic methods to determine age- and species-specific profiles of malaria antibodies, using a predetermined appropriate method, e.g., IFA or ELISA. After the specific antigen to be used for immunization is identified it would be highly important to determine any background presence and titer of antibody to this antigen in the population by age and other population characteristics. Because simple field tests for cellular immunity may be included in the assessment of vaccine efficacy, these tests should be used to determine background responses in the population.
4. Demographic and general public health studies should include a census of residents in the trial area; an estimation of population stability; meteorological characterization; observations on the general health status of the population; and a full exploration of protective

measures against malaria in use in the population or area, including the extent and manner of drug use, the use of screening, bednets, repellants or other measures for self-protection, and the use of vector control measures.

In addition to these pre-trial investigations, this period would also allow for establishment of the clinical safeguards necessary to the vaccine evaluation. These would include appropriate clinical facilities for detection and treatment of cases in the control populations and in vaccine failures and for evaluation and treatment of vaccine side-effects. An important issue would be a thorough investigation of the drug sensitivity of malaria parasites in the area.

Such background preparation as a prelude to the vaccine trial introduces at an early stage a quandary which will be common throughout the trial period. The high level of intensity of efforts to characterize malariometrically the area and the population may by itself result in a substantial reduction of endemicity or epidemicity. Certainly, ethical considerations cannot allow known symptomatic malaria cases to go untreated, and some schools of thought would extend this to include all parasite positive cases. Thus, through continuing elimination of malaria cases, the reservoir of parasitemias infective to mosquitoes may be significantly reduced, resulting in a lower transmission rate and reducton of risk to both vaccinated and control subjects. Careful thought must be given to this factor in the design of methods for evaluation of vaccine efficacy.

Vaccine Trial Design: Sporozoite Vaccine

In evaluating a vaccine designed to interfere with the successful establishment of infection through sporozoites, the assumption must be made that the endpoint is absolute, i.e., the prevention of any parasitemia. It is also assumed that the anti-sporozoite vaccine has no direct effect on asexual blood stages. There is at present no firm evidence to suggest that even substantial reduction of sporozoite numbers would have a significant effect in reducing the risk to a non-immune recipient, other than a variable extension of the prepatent period.

The *selection of sites* for Phase III field trials would be dependent on prior knowledge of malaria endemicity and seasonality, on the accessibility to field teams and investigators, on the presence of national resources for collaboration, and on the presence of adequate and accessible diagnostic and treatment facilities. Studies should initially be done in highly endemic areas. Later Phase III trials might be done in areas of seasonal or moderate endemicity.

Study populations for early Phase III trials should be small, but of satisfactory statistical size, and to the extent possible should be non-immune or with little immunity. Several types of populations have been proposed:

1. Non-immune migrant populations (e.g., military or labor groups) entering highly endemic areas.
2. Small villages in areas with predictable seasonal epidemics.
3. A partially immune population in an area of highly endemic stable malaria.
4. Young children (e.g., 2 to 4 years of age) with minimal immunity in a highly endemic area.

Normal criteria for exclusion from vaccination, such as current acute illness or severe malnutrition, would be applied. In addition, in the early trials it seems likely that pregnant women and infants under 2 years of age would be excluded, at least until carefully controlled studies have been done on these sub-groups. Depending on the findings of earlier phases, later Phase III trials in larger population groups may be done in the entire population, including younger children and pregnant women.

The *trial design* for early trials would be that of a randomized controlled double-blind study including the use of a placebo in the controls. Because evaluation of vaccine efficacy will rely principally on the detection of parasitemias in possible vaccine failures, and because the presence of parasitemias at the time of vaccination might in some way interfere with immunogenesis expected from vaccination with a sporozoite antigen, it is assumed that all subjects—both vaccinated and controls—will receive curative antimalaria drug therapy prior to vaccination. This would of course not apply to migrant groups entering an endemic area from a non-malarious area. This antimalaria treatment introduces several questions which must be considered:

1. The amount of effect this would have on the level of endemicity and the malaria risk in the test area. This factor can be minimized by the use of only a relatively small portion of the population in the vaccine trial, thus ensuring a continued high level of transmission risk.
2. The question of possible immunosuppressive effect of some antimalarials (e.g., chloroquine). This question continues to emerge and should be resolved during earlier phases of vaccine evaluation.
3. The possibility of treatment failures which would obscure the true effectiveness of the vaccine. The evaluation of parasites in the area for drug sensitivity and the selection of the proper drug, dosage, and timing of administration would be of importance in minimizing this problem.

Trial design for later Phase III studies may vary somewhat, with the possible elimination of placebo controls, utilization of village to village comparisons, and, depending on earlier findings, the elimination of prevaccination curative treatment.

The *methods for vaccine* evaluation will be considered for both the smaller Phase III trials as well as later trials in larger population groups.

I. Early Phase III Trials

A. Assessment of vaccine efficacy

1. *Parasitologic*

Blood films for detecting parasitemia must be taken frequently on all subjects and examined within 24 hours of collection. In addition to such surveys, active case search must be instituted on an appropriate schedule and readily accessible diagnostic posts must be available for early detection of symptomatic cases of malaria reporting to these posts. Here again blood films must be examined promptly and treatment of symptomatic cases provided without delay. The quality of the blood film examinations is of great importance, making possible quantitation of the parasitemias and differentiation of the several species of *Plasmodium* which may be found in the population. It is likely that treatment will be given to all persons with parasitemia, regardless of presence or absence of symptoms. Because the tests may involve populations with little or no immunity and will include placebo groups, the diagnostic and treatment systems must be highly organized and efficient.

2. *Immunologic*

All vaccinated and control subjects should have serum samples collected at appropriate intervals for determination of humoral antibody. This should include one or more of the standard antibody tests (e.g., IFA or ELISA) and also testing for antibodies specific to the vaccine antigen. In addition, appropriate tests for cellular immunity should be included. The identity and timing of all of the immunologic tests should be available through appropriate studies conducted during the earlier phases of vaccine evaluation.

B. Assessment of vaccine safety

Although most of the problems associated with vaccine side-effects will have become apparent and will have been resolved during earlier phases, introduction into the field will introduce a number of additional variables which may be of importance. These will include variable states of nutrition in the subjects, the presence of inapparent intercurrent infections or disease conditions, genetic variants, ideosyncratic sensitivities, unsupervised use of various drugs, and others. Thus, in early field trials there is

a need to carefully scrutinize the vaccinated population for unforeseen side-effects.

1. *Local reactions*

All recipients of the vaccine and the placebo must be examined frequently during the first week after inoculation to determine incidence and severity of local reactions at the inoculation site.

2. *Systemic reactions*

During frequent visits to the recipients during the first week after inoculation, inquiries must be made—ideally with a pre-designed questionnaire/checklist—to determine any adverse systemic side-effects. Recipients must be encouraged to visit the clinical posts to report any severe side-effects, systemic or local, without delay. In so far as is possible, symptomatic treatment for foreseeable side-effects should be available for both field and clinic administration. It should be remembered that in a double-blind study, neither the field nor the clinic staff will be able to differentiate between vaccinated and placebo subjects.

3. *Rare or late side-effects, including evidence of immunopathology*

Long-range follow-up of trial participants must be assured, with periodic visits and with continuing encouragement of participants to visit clinical posts for evaluation of possible late reactions. Prior studies during earlier phases should dictate the types of studies which might be necessary to detect the potential enhancement of malaria-associated pathology after vaccination. These might include tests for proteinuria, rbc counts, hematocrit, platelet counts, identification of circulating immune complexes, and others.

C. Assessment of vaccine acceptability

While not a primary objective of Phase III trials, there would be opportunities to determine social attitudes and responses which will reflect individual and community perception and acceptance of vaccine use as a method of malaria control in terms of efficacy and side-effects and in comparison with other methods (e.g., drugs) which may have been available. Well designed questionnaires and interviews should be constructed to elicit this information. The results would be most useful in preparation for larger Phase III trials and the eventual wider use of vaccination procedures.

D. Evaluation of feasibility

The determination of logistical feasibility will also be of importance as a guide to the requirements for larger field trials and eventual operational use of a successful vaccine. Most of the requirements for vaccine stability, for cold-chain handling, and other logistical factors will be established during earlier phases,

but additional information and confirmation of these requirements must be acquired during the early Phase III trials under actual field conditions. The administrative/management structures must be tested for efficiency prior to moving into larger population groups. Also of importance will be information of probable operational costs of vaccination programs, although the costs of early Phase III trials may have little direct comparability with those characteristic of later phases involving larger populations.

E. Evaluation of general health effects

Information on possible improvement in the general state of health of vaccinated individuals may contribute to the evaluation of the vaccine. For this purpose, if feasible, vaccinees and controls (or a sampling of these groups) may be subjected to periodic simple physical examinations, including measurements of health indicators such as hemoglobin, hematocrit, growth rates, nutritional status, spleen size, temperature, etc. Obviously, any deaths which occur in the study groups would be thoroughly investigated with determination as accurately as possible of the cause of death.

II. Later Expanded Phase III Trials

A. Assessment of vaccine efficacy

1. *Parasitologic*

The same principles apply in the expanded trials as were followed in the smaller groups. Obviously, the intensity and coverage of parasitologic observations cannot be as great, and some system of statistically sound sampling may be required. It is conceivable that the use of placebo controls may be abandoned in the larger scale trials, relying on vaccination of entire communities with similar nearby non-vaccinated communities serving as a control. Because in larger trials there will be a reduced amount of active case surveillance, either through surveys or active search for symptomatic cases, it is of particular importance that the entire population have ready access to clinical posts where persons who become ill can receive rapid diagnosis and treatment. This will provide virtually the only safeguard against vaccine failure and the diagnostic/treatment system must be efficiently organized and publicized.

2. *Immunologic*

Tests for antibodies and for cellular immunity will continue to provide important information but will not be feasible for total coverage of large test populations. A selective sampling procedure should be adopted to provide statistically sound data without overwhelming the available resources.

B. Assessment of vaccine safety

Side-effect detection will continue to be of importance in the

larger field trials, following the same principles discussed for the earlier trials. Again, a system of sampling would probably replace the total coverage of the population in the smaller trials. The availability of easily accessible clinical posts, where vaccine recipients can report for evaluation of severe local or systemic reactions, will be highly important.

C. Assessment of vaccine acceptability

The larger Phase III trials will provide the best opportunity for determining individual and community attitudes toward the vaccination program. Health education programs to enhance acceptability of the vaccine can be designed and tested, and sampling of attitudes with questionnaires and interviews will elicit information which will be most useful in the eventual use of a successful vaccine.

D. Evaluation of feasibility

The larger Phase III trials will provide the opportunity to refine and test most of the logistical factors which will be key in the eventual use of an approved vaccine. These larger trials may in many ways simulate operational vaccine use, and very careful records must be maintained on all administrative/management procedures. Cost estimates derived from these larger trials will also be highly useful.

E. Evaluation of general health effects

The larger trials may provide the first opportunity to develop firm information on the effect of vaccination on malaria endemicity and on the general health status of a community. Most of the information on malaria endemicity will be obtained through the routine procedures for evaluating vaccine efficacy and through ongoing entomologic studies (sporozoite rates, etc.). Additional parasite surveys, spleen surveys, and measurement of a number of other health indicators may be desirable to determine the effectiveness of the vaccination program.

Vaccine Trial Design: Asexual Blood-Stage Vaccine

This type of vaccine would be expected to induce an immune response which would operate by inhibiting the unrestricted multiplication or growth of asexual blood-stage parasites. The main function of this vaccine would not be to prevent infection but to significantly reduce mortality and morbidity due to malaria. It might be expected that the development of an erythrocytic infection after vaccination would in itself provide a booster effect on the induced immunity. The overall objective of Phase III studies would be to quantitate the reduced morbidity in vaccinated persons and to characterize any adverse side-effects of vacci-

nation. As in sporozoite vaccine trials, early Phase III studies should be small, with progression to larger and larger populations.

The *selection of sites* would consider the same factors as for the sporozoite vaccine.

Study populations for early Phase III trials should be small, but of satisfactory statistical size, and to the extent possible should be non-immune or with little immunity. Several types of populations have been suggested:

1. Initially, small groups of children (e.g., 2 to 5 years of age) in highly endemic areas.
2. If justified by the above study, expansion to larger groups of children living in areas of high risk.
3. In areas of moderate endemicity, larger trials in the entire population.
4. In areas of seasonal epidemics, the entire population could be included.

Criteria for exclusion and for eventual inclusion of special groups (e.g., younger infants and pregnant women) would be similar to those for the sporozoite vaccine.

The *trial design* for early trials would again be that of a randomized controlled double-blind study, including the use of a placebo in the controls. The question of pre-vaccination antimalaria treatment again arises. The malaria infection itself may be immunosuppressive and interfere with the vaccine-induced immunity. On the other hand, certain of the antimalaria drugs (e.g., chloroquine) may have an immunosuppressive effect. It is to be hoped that answers to these questions may be forthcoming during earlier phases and that rational decisions on use of prevaccination treatment will be possible. In the absence of such definitive information, the present tendency would be to treat all participants in the early Phase III trials. Depending on the findings of earlier phases, the design of later Phase III trials in larger population groups would be similar to that suggested for the later sporozoite vaccine trials.

The *methods for vaccine evaluation* will be considered for both the smaller early Phase III trials as well as later trials in larger population groups.

I. Early Phase III Trials

A. Assessment of vaccine efficacy

1. *Parasitologic/clinical*

Evaluation of the asexual blood-stage vaccine will differ substantially from that of the sporozoite vaccine. Patent parasitemias may not be prevented in vaccinated individuals, and the protection derived will be more toward modification of parasitemias and clinical attack. Thus, determination of parasite densities and

characterization of severity and persistence of malaria symptoms will be critical. Measurement of vaccine effect will be primarily by frequent (perhaps weekly) fever surveys, with measurement of temperatures, and by blood film examinations of fever cases to determine parasite species, stages, and density. Along with this enhanced active surveillance, there must be a strengthened passive surveillance system, with easily accessible clinical posts where participants can report for diagnosis and treatment of illness suspected to be malaria. Diagnosis must be rapid and accurate and treatment must be available within 24 hours. When appropriate, treatment should be offered presumptively without benefit of specific diagnosis. The search for fever cases should be supplemented by periodic surveys of the participants for detection of asymptomatic parasitemias. The timing of these surveys should be based on information available from earlier trial phases. In addition to parasite surveys, periodic spleen surveys (perhaps before intervention and at 3-month intervals) would be useful in comparing malaria experience in vaccinated and control groups. The question of necessity for treating asymptomatic parasite carriers is one which will have to be resolved on the basis of prior findings and on ethical considerations. It is conceivable that such parasitemias might have a beneficial effect on the maintenance and enhancement of immune status, although there are many factors which would have to be considered before a decision is made.

2. *Immunologic*

As with the sporozoite vaccine, serologic profiles will be monitored periodically, utilizing the standard species specific tests as well as tests which will detect and measure antibody to the specific antigen(s) in the vaccine. In addition, appropriate tests for cellular immunity should be included. The identity and timing of all of the immunologic tests should be available through appropriate studies conducted during the earlier phases of vaccine evaluation.

B. Assessment of vaccine safety

In the main, for early local and systemic reactions, this assessment will be identical with that proposed for the sporozoite vaccine. In regard to rare and late side-effects, particularly those related to immunopathology, it seems possible that the character of protection offered by the asexual blood parasite vaccine suggests a somewhat greater risk. The continuing presence of asymptomatic parasitemias, in the face of a substantial amount of induced and acquired immunity, may suggest an increased risk of immunopathologic consequences. Thus, for this type of vaccine, it may be necessary to enhance and extend laboratory studies which would assist in identifying potential problems.

C. Assessment of vaccine acceptability

This evaluation would be similar to that proposed for the sporozoite vaccine. Because of the expected development of parasitemias and even some malaria symptoms in vaccinated individuals, it might be predicted that acceptance initially may not equal that of the sporozoite vaccine. However, the persistence of protection may be longer, and it would be important to extend this assessment for a longer period

D. Evaluation of feasibility

This would be identical to that for the sporozoite vaccine.

E. Evaluation of general health effects

Identical to that for the sporozoite vaccine.

II. Later Expanded Phase III Trials

The same principles applied to the expansion of the sporozoite vaccine trials would apply to the asexual erythrocytic parasite vaccine. It must be emphasized, particularly for this type of vaccine, that there must be high quality diagnostic and treatment posts readily available to all participants in the vaccine trials to ensure rapid treatment of symptomatic cases. The use of presumptive treatment of symptomatic cases found in the field or reporting to the clinic may be necessary and should be considered.

Summary

It is apparent that the field evaluation of malaria vaccines will indeed be a difficult and complex task, requiring a well planned and executed multidisciplinary effort. A wide variety of expertise will be involved, including parasitologic, immunologic, epidemiologic, clinical, entomologic, statistical, and administrative. Well-developed resources and highly trained and motivated technical staff will be required both in the field and in the laboratory. Great effort should be made to assure that the early field trials are conducted only after thorough preparation, so that there can be no question of scientific validity of the results. A poorly organized, conducted, and evaluated field test could be disastrous to the future development of the optimal method of immunization. In view of our present state of knowledge—or lack thereof—relating to the identity of the vaccine elements to be tested, rational field testing seems to be some years away. However, with the apparent rapid advancement of immunologic technology, and with great hopes that the early pre-clinical and clinical studies of the candidate antigens will provide answers to some of the still unanswered questions, one can be hopeful that the delay will be somewhat less. In the meantime, planning for the tests cannot be initiated too soon. As earlier phases develop the needed information on vaccine identity, efficacy, and safety, this infor-

mation can be included in the planning process, so that, hopefully, when a vaccine is ready the early field testing can begin without further delay.

Predictions of a malaria vaccine have been with us for many years, sometimes overoptimistic, but always greeted with much interest and anticipation. In a period when the world-wide problem of malaria is progressively worsening, and when more and more doubts are emerging as to the capabilities of our currently available drugs and insecticides, the malaria vaccine must appear to some as our last great hope for dealing with this immense problem. Few would believe that a vaccine alone can conquer the disease in the near future; it will most likely be used in association with other antimalaria measures, and the temptation to abandon research toward the improvement of other malaria control methods must be resisted.

Discussant: Technical Aspects of Phase III of Clinical Trials of Malaria Vaccine(s)

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Introduction

Phase III clinical trials applied to chemotherapy of malaria aim at confirming that the drug is effective and safe when administered to all infected subjects, including pregnant women and children, whether immunes or semi-immunes. These studies must be based on a good knowledge of the appropriate dosage of the drug and its side-effects, as well as on the metabolic fate of the drug, its excretion, and some special problems encountered in malnourished people or in subjects with impaired renal or liver function.

Design of Phase III trial of malaria vaccines is based on the same general principles as that for chemotherapeutic studies, but it would differ in more than one way from them.

As fully described in the report of the UNDP/World Bank/WHO meeting the main objective of Phase III malaria vaccine trials is the study of efficacy, immunogenicity, safety, tolerability, and acceptability of a vaccine in groups of population, living in their usual malarious environment within an area with a well-defined epidemiological type, parasite composition, and degree of transmission.[1] The general consensus of that meeting was that a successful clinical trial should give the right answers to the questions that were posed. This depends largely on the specific points covered by its protocol; the latter must include all the steps of the trial, as well as all or most of the anticipated eventualities. The time and effort expended at this stage will be rewarded later, during the execution of the trial.

The tests for immunity in the context of vaccine evaluation have several different requirements of specificity, sensitivity and technological difficulty. In Phase I and II trials, monitoring of different immune responses requires test systems measuring these immunological parameters at an advanced level. In Phase III trials, the test systems will have to be suitable for screening of larger groups of subjects. They will

include only a few immunological parameters and the choice of test systems will partly depend on the results of Phase I–II trials. Obviously, the technological feasibility is also taken into account.

In drawing up a protocol, the aim of the trial should be stated in detail. It is essential that the proposed study is approved by the responsible administrative authority. Usually the most successful clinical trials are those that do not attempt too much. Every clinical trial creates its own problems, whether due to the product evaluated or to the human subjects involved or to the conditions of the study. Nevertheless, in the immense majority of clinical trials carried out during the past 20 years they were successful and contributed to the progress of biomedical sciences and were of benefit to the people concerned. What type of vaccine will eventually be used remains to be seen but some guesses are justified.

Sporozoite Vaccine

It is most likely that a sporozoite vaccine against *P. falciparum* will be the earliest. It is hoped that Phase I and II trials would confirm that we have such a vaccine, whether obtained by DNA manipulation or through synthetic peptides but effective, stable, sufficient, reasonably safe, and of known dosage. Also that: (a) we can carry out this trial in an endemic malarious area with the approval and support of the government and with full cooperation of the local population; (b) that we may have available for the trial not less than 2,000 indigenous inhabitants, divided into the usual age-groups, with very little movement in and out of the area, not subject to undue malnutrition or extreme poverty, and provided with a rudimentary but adequate rural health service, capable of some expansion, if necessary; (c) that we have our own, small but well trained technical and auxiliary team, speaking the local language and fully aware of local customs and mores; (d) that we have the necessary laboratory facilities, transport, accommodation, and communications; and moreover, that (e) we are not pushed to produce rapid results but have enough time and money to do things properly. If so, then there is little doubt that our Phase III trial promises either to confirm the expectations of Phases I and II or to find out why there is a discrepancy.[2]

The target population in Phase III trials will be subjected to the natural challenge of malaria infection prevalent in the area where they normally live. Thus, there will be no need for an experimental challenge as in Phase II of the trial. However, it would be of great interest to find out if malaria infection supervenes in those individuals who, after travelling to different parts of the country, were exposed to other strains of malaria parasites.

As emphasized at the Geneva meeting the quality of the vaccine and

its stability are most important factors, which would influence the outcome of the trial and improve the reliability of serological evaluation carried out in Phases I and II and repeated in Phase III.[1]

It is possible that freedom from malaria infection at the time of vaccination plays some role in the outcome of Phase III trials, and one way to solve this problem would be to use groups of subjects from non-malarious areas entering areas of high risk, to eliminate possible immunosuppression of pre-existing disease, the possibility of relapses, etc. Military units or groups of labourers may offer acceptable opportunities for such studies, if these subjects could be tested and vaccinated before entering malarious areas. As in Phase II, it may be preferable to start Phase III trials with adult males and non-pregnant females and then proceed to younger age-groups. However, in the long run these trials will have to cover all population groups. Since Phase III trials will be carried out in areas with a range of epidemiological conditions of transmission, it is evident that a preliminary survey of such areas should be carried out or that the detailed information on it should be collected and assessed.

The total number of subjects required to ensure a successful trial is of great practical importance. The statistician will usually be able to indicate the theoretical number necessary to provide significant results on the basis of the type of trial being undertaken and the response rates anticipated. But the length of time required for this number of patients to be entered into the trial on the basis of incidence rates may be such that continued interest in the study would not be maintained or possibly that variations in external factors might become significant. If this is so, the design should be changed and an acceptable compromise agreed upon. It is difficult to be dogmatic about the total time which a trial should take, as this is very dependent on many variables, but in the majority of cases two years or three should be enough.

The final questions to be answered are: How can the best use be made of available resources to conduct a study that is still within practical limits? Is the study ethically justified and is the outcome likely to be worth the effort? Perhaps the greatest temptation is to be too ambitious in the size, duration, and scope of a trial. One should never be too optimistic about the number of patients likely to complete the trial.

Asexual Erythrocytic (Merozoite) Vaccine

In Phase III trial of an asexual erythrocytic (merozoite) vaccine would show that it can modify the course of natural infection and be an alternative to antimalaria drugs. Antibody mediated immunity against merozoites of *P. falciparum* has been extensively studied but the information on the antigens involved is still insufficient for the development of an early vaccine, although some defined polypeptides have immunogenic activity in monkeys and against which specific monoclonal anti-

bodies of *P. falciparum* are directed. Other non-antibody mediating factors are also involved and their role is little known.

The eventual malaria vaccines of asexual erythrocytic type suitable for Phase III will have to be evaluated on the target populations in their normal environment and with their usual exposure to malaria transmission. This would demand studies in groups with various degrees of naturally acquired immunity. Evaluation of vaccine efficacy will be measured by the assessment of individual functional immunity and of the epidemiological effect of the vaccine within the target population/area. Phase III vaccine trials may be situated wherever adequate clinical, laboratory, and epidemiological services would be available and where adequate facilities can be organized for the testing or for the transport of blood/serum samples to collaborating institutes. The problem of using or not using an effective chemoprophylaxis of malaria in any groups during the Phase III trial presents a difficult issue with no simple answer. True enough the non-vaccinated group may be placed at increased risk of natural infection but it would be justified to withhold chemoprohylaxis if continuous medical surveillance and immediate treatment are assured.[2]

The Phase III trials may provide the following information on individual functional immunity: (a) Is vaccination equally effective in uninfected persons as compared to persons currently harbouring a malaria infection? If there is a reduced effect in malarious persons, how does vaccine response change as a result of chemotherapy? (b) How variable is the vaccine response in relation to age and sex (pregnancy)? (c) Does causal protection against infection or a vaccine-induced reduction of transmission result in a relative increase of severe malaria manifestations?

Initial Phase III trials of asexual erythrocytic vaccines should be done in small populations with high infection rates. Since vaccines can be expected to modify as well as prevent manifestations of disease, evaluation of the clinical severity of malaria cases in vaccinated and non-vaccinated groups is of importance. Clinical studies of malaria in vaccinated subjects may also detect any potential immunopathological problems. Good medical surveillance and epidemiological studies will be essential.

Immunosuppression of the effect of the vaccine by existing malaria infections, malnutrition, or intercurrent disease is a possible problem which deserves thorough investigation. In early Phase III trials this possibility may be minimized by treating potential subjects with antimalaria drugs prior to vaccination. Failure or relative failure of the vaccine to protect should be foreseen and the protocol so designed that any failures can be evaluated in some patients and treated urgently. Whether there should be a comparison group to receive another unrelated vaccine to assess its non-specific effect on vaccination against malaria is a moot point that demands an eventual decision at a later stage.

Since the number of variables in Phase III field trials will be high, and as this affects the collection of the data and the accuracy of their interpretation, every effort should be made to carry out these trials with the simplest possible design and in well followed up populations for at least 2-3 years. This should apply to the trials of all three types of malaria vaccines.

Gamete Related (Transmission Blocking) Vaccine

Although much progress has been made recently in identifying the antigens reactive with the monoclonal antibodies to *P. falciparum* gametocytes and in blocking their infectivity to Anopheles and the development of an appropriate vaccine may be successful, the clinical trials of such a product will present considerable difficulty. It is doubtful if Phase III trials could be satisfactorily carried out for testing the gametocyte related vaccine, as this vaccine would not confer any individual protection against malaria and the serological parameters would be only indirect. It seems that such a test of efficacy may be carried out by measuring the specific blocking antibody response in humans, or by a study *in vitro* of the effect of antibodies on experimental transmission. The latter test raises an ethical problem since the measurement of infection rates from either induced or natural infections might produce clinical symptoms requiring medical intervention, and this would have an effect on transmission.

One way for evaluating the transmission blocking vaccine would be to combine gamete related antigens with a proven anti-sporozoite vaccine. Then by vaccinating whole villages, it might be possible to compare the effect on transmission of the combined vaccine. In such a trial, the safety and acceptability of the combined vaccine could also be tested, but the results related to the transmission potential are bound to be blurred, even if we can measure the infection rates in human population and sporozoite rates in vector mosquitoes. However, when it comes to studying the population, a problem arises in evaluating gamete vaccination: the level and persistence of anti-gamete antibody resulting from vaccination will be enhanced by any malaria attack and will produce the new crop of gametocytes. This might obscure the part played by the vaccine.

Factors Liable to Affect the Trial

One of the most important factors in all phases of clinical trials but particularly of Phase III is the availability of an adequate number of subjects for the field trial. Perhaps many field workers are familiar with "Lasagna's Law." This has nothing to do with the succulent Italian dish since it is derived from the name of a well-known American pharmacol-

ogist and can be represented graphically by the duration of a clinical trial. As soon as the trial begins, the supply of suitable subjects becomes a proportion of what was expected before the trial began. This gradual attrition is directly related to the duration of the trial and also to its complexity. Moreover, the design of clinical trial often contributes to the operation of "Lasagna's Law" if the homogeneity of groups, their comparability, or an excessive number of required tests will seriously interfere with the normal life pattern of the sample of the population.

At the very earliest stage of the preparation of the clinical trial one should consider how many subjects will be needed and how to secure their cooperation. Furthermore, the trial organiser should be prepared to deal with the problem of trial attrition, to discover reasons for it, and to find some, at least partial, solution. Such drop-outs may be caused by many people busy with their work on the land during the planting or harvesting season, by those attending markets, by periodic political mass gatherings, church services, school holidays, etc. If this occurs in a random manner through all treatment groups it may not distort the intergroup differences, although sensitivity of the trial will decrease. More difficult problem will be if the data are based on unequal numbers in various sub-groups and so will make the statistical analysis biased. Even more serious would be a sharp increase of the drop-out rate due to side-effects of the prospective vaccine or to the excessive number of tests which require blood collection or other samples. There are also many people in rural areas who expect direct medical help from the staff entrusted with the trial and if they are not satisfied, may rouse the whole village against the team. This should be kept in mind and while some medical assistance ought to be occasionally given, such semi-permanent involvement ought to be avoided as it creates excessive obligation and may even prejudice the results of the trial. Kindness, tact, and firmness (if necessary) will dictate the appropriate action.

There are various ways of reducing the drop-out problem, especially if a long-term surveillance is needed. The trial may be based on an out-patient clinic, to have better control over the group, but this may create problems to the health staff. One may anticipate the drop-out trend and arrange for evaluation of response before it is likely to occur. Finally, one may be tempted to offer to the people concerned some small gifts or certain privileges for continuity of staying on, but this is a chancy procedure that may lead to abuses and complications.

Side-Effects

The recording of side-effects, consisting of local or systemic character, is of obvious importance in Phase III trials, although some information of their likelihood should be available from Phase I and II trials. Neverthe-

less if the prevalence of some uncommon adverse effects is generally low, they may emerge only when relatively large groups are observed.

Firstly, a distinction should be made between side-effects volunteered by the patients and those elicited by direct questioning. Where practicable, questioning with regard to side-effects should be withheld until the end of the trial, though this is obviously impossible in studies of long duration. When direct questioning is used, this should take the form of a pre-stated question put to all patients. "Has the medicine (or injection) disagreed with you in any way?"

Secondly, it is usually assumed that "side-effects" occurring during a trial are due to the medication. But these complaints may have been present before the trial started and in the initial assessment a question should determine what other complaints, if any, the patient has suffered from before the start of the trial.

Variables

A large number of other variables may enter into field trials. Factors relevant to the population behaviour, intercurrent infections, immune status, etc., affect response to vaccines. They also affect methods of data collection and therefore affect the usefulness and reliability of the data derived from the trial. The effects of these variables on efficacy will need to be evaluated. If possible they should be excluded from the early Phase III trials or their cumulative effect should be evaluated.

A tentative list of sources of variability of immune response among subjects of clinical trials in Phase III would be as follows:

Factors	Examples
Personal	Age, sex, ethnic origin, social class, education
Bio-medical	Genetic abnormalities (blood dyscrasias) pregnancy, prior or present illnesses, malnutrition
Bio-physical	Diet (food shortages), type of work, size of family, presence of domestic animals
Environmental	Climate, housing, sanitation, water supply, community structure
Socio-economic	Poverty, tribal relationship, religious belief, availability of basic health services, cultural adherence, acceptance of national medical services, psychological resistance
Trial-related factors	Type of study, length of trial, sample collection, type and frequency, placebo reaction, opposition to trial, observation bias, expectancy effects, fraudulent data

It is up to the investigator to assess the importance of these and other variables and to determine which can be disregarded, which to be taken into account, and which to be corrected if possible.

Critical Path Analysis

The steps in carrying out a clinical trial are usefully shown by means of a flow chart which presents graphically the various steps involved and also allows for an analysis of events so that if time is to be saved the critical events determining this can be shortened. In Figure 1, the lines represent activity, the circles or nodes represent an event, and the figures below the activity show the length of time in days for that activity.

The next step is to design the trial so that it answers the precisely framed question. In an ideal trial design there should be incorporated various practical considerations, such as statistical advice, ethical considerations, costing, technical feasibility, clinical facilities available, pharmaceutical problems, the competence of the proposed investigators, and legal aspects. When these have been incorporated into the initial design, one or more final designs are drawn up.

Critical path analysis in clinical trial planning is defined as a method for attempting to determine the completion date for a task, taking into account all the activities which are involved.

The critical path in the figure given is indicated by the thick line, which shows the time required to complete the trial. Other activities in parallel are completed in less time, and to shorten the overall duration it is obvious, in this example, that the delivery time for the recording apparatus must be improved. If this is reduced, then the next limitation will be the time taken to manufacture the test medication.[3]

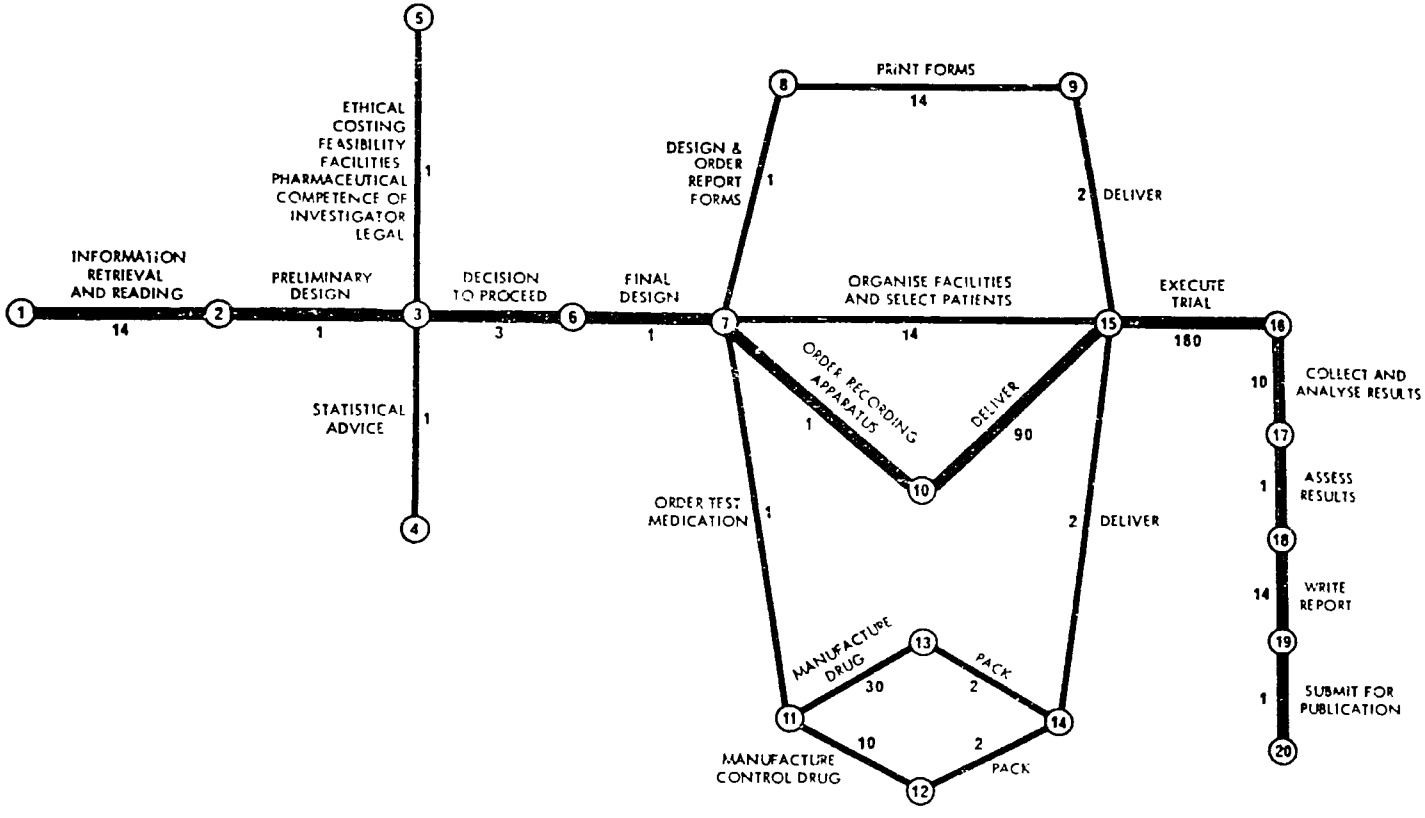

FIGURE 1. Example of Critical Path Analysis for Clinical Trials. (Adapted from Harris, 1970)

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Rapporteur's Summary

HEIKKI PELTOLA

Professor Natth Bhamarapravati described development of a live vaccine against dengue 2 virus. To date, 36 people have been involved in the trial. All showed good antibody response, and no serious reactions have been observed. Clinical efficacy will be evaluated during the five coming years.

Dr. Chen Zheng-ren outlined the general immunization policy and results of the efficacy trials using the meningococcal capsular polysaccharide and measles vaccines in the People's Republic of China. As to the vaccination studies, the two trials using the meningococcal vaccine in 250,000 vaccinees or control showed an efficacy rate of 81% and 95%, respectively.

Dr. Heikki Peltola presented the strategy and results of two vaccination studies in Finland. First trial used the group A meningococcal and *Haemophilus influenza* type b polysaccharide vaccines in a double-blind fashion, whereas the second project aimed at elimination of measles, mumps, and rubella from the country by using an appropriate live vaccine. Both studies proved successful owing to thorough motivation of the vaccinators and people, an existing good child care system, and, of course, an effective vaccine.

Professor Leonard J. Bruce-Chwatt presented lines for clarification of clinical trials of the potential malaria vaccine. Much can—and has to be—learned from the studies with the new anti-malaria drugs that cover the spectrum from initial laboratory experiments to mass campaigns.

Dr. W. John Martin overviewed the requirements for vaccine development of FDA, whereas Dr. Robert G. Brackett brought a view of a vaccine manufacturer. Both emphasized the absolute need of a detailed protocol which takes into account an especially well-functioning and realistic reporting system.

Dr. Carter Diggs reported on the successful animal studies using the vaccines carrying the same major and minor tetrapeptide sequences as does the circumsporozoite molecule. The antibodies thus elicited have

already been shown to bind to the circumsporozoite protein, and testing of the vaccine will shortly take place in humans.

Professor Robin D. Powell outlined requirements for the circumstances of Phase I and II. Also, the need for an especially scientifically solid study plan was underlined in his speech.

Phase I should concentrate on three major aspects: potential adverse effects; immunogenicity; and preliminary estimation of clinical efficacy. Phase II should focus on four points: optimum vaccination technique, age, and schedule; placebo-controlled fashion; challenge of the vaccinees; and continuous follow-up of conceivable risks. In addition, the *sine qua non* conditions for the trials were outlined.

Dr. Geoffrey M. Jeffery dealt with the technical aspects of Phase III trials. Assuming that a sporozoite, or an asexual blood stage, and a transmission-blocking vaccine would be available one day, the latter alternative might not require Phase III testing, except perhaps in the combination with one or two other antigen(s). Every effort should be made to plan the studies so thoroughly that no question of the scientific value may remain after the exhaustive maneuvers in the field.

Finally, Professor Leonard J. Bruce-Chwatt discussed the same topic "*moderato cantabile*," as he said, meaning that the theme has been highlighted in so many speeches and in the expert committee in WHO, Geneva, in February 1985, that some plagiarism is almost unavoidable. Anyway, the following sources of variability of the immune response are obvious and may be a snag in front of the researcher: personal, biomedical, biophysical, environmental, socioeconomic, and various trial-related factors. Only after taking into account all these aspects a most informative study may be achieved.

The working papers of Thursday sparked the most extensive and vigorous general discussion in the conference. The points stressed included:

1. need to obtain, as soon as possible, definite data on specific antibodies and levels that in fact reflect functional protection;

2. extent of still unanswered questions on adjuvants, carriers, multivalence, and how relevant data on those questions can best be obtained for rational decisions;

3. need to take into account not just the FDA regulations, but also the regulatory agencies and stipulations of other nations;

4. significance of ethical concerns and issues that directly influence what specific types of clinical studies can or cannot be conducted, in particular in relation to (a) studies involving transfer of blood and blood products and (b) evaluation of merozoite vaccine preparations that may not afford complete protection but may modify the course of malaria, and with the evaluation of transmission-blocking or gamete-blocking agents;

5. importance of coordination for clinical trials to maximize comparability of design and of interpretation;

6. appropriateness of continued studies in animals during or before clinical trials to evaluate the possibility that selective vaccine pressures may result in expression of new difficult parasite epitopes;

7. desirability of but limited current practicability of clinical passive transfer studies with new immunologic methods to define protective antibody levels; and

8. immense additional need for conclusive, basic preclinical data in animal models, including *Aotus* monkeys and, possibly, chimpanzees, to resolve fundamental questions prior to the initiation of clinical studies.

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General Discussion

PEREIRA DA SILVA: There is a terrible contradiction which has been disturbing me very much since the beginning of my work in malaria vaccine, which was not satisfied in the discussion of Dr. Jeffery. My question is the following. Of course we are going to treat the placebo and the vaccinated if they develop malaria, but how can we not treat the people in the area if in this area we will have the highest concentration of specialists in malaria, including the discoverers, as proposed by Professor Chen Zheng-ren, if not physically, but intellectually or spiritually? How are you going to not treat babies or pregnant women when we, of course, know that there are good specialists in malaria in the area? This is a very important ethical problem that I don't know how to answer. This is really not well answered by Dr. Jeffery in this report, so I would like to know about his personal feelings in relation to this problem.

PELTOLA: I think we have observed the same problem in other trials previously. One of the examples which right now comes to my mind is the study on interferon in sarcoma. Everybody knows how fatal a disease sarcoma is and still it has been important that the researchers from Sweden, America, and Finland have been very strict not to give this potential drug, interferon, for the control—controls which were actually selected arbitrarily before the trial was begun. So we don't have any easy way except to decide whom we are going to treat and whom we are not; the controls are not to be treated.

BUCK: I think if you do Phase III trials inevitably you will run into the normal epi-vaccination schedules. For two years I think will be DPT, there will be PCT vaccinations, there may be menningitis, there may be menningitis pills. Would it be necessary because of the interference known from acute malaria or tetanus toxoid responses, for menningitis vaccine to also evaluate the immune response in relation to the other vaccines? Would there be interference, and vice versa? I think this is a very important question and will be in any area in which malaria exists and Phase III trials will be carried out.

WERNSDORFER: I would like to say that I appreciated very much Dr. Jeffery's and Dr. Bruce-Chwatt's presentations. Regarding Phase III, we have to ask ourselves all the time—what are we going to learn new, because every phase and practically every trial gives us something new. The new thing in the Phase III is the study in the natural setting of malaria. You have to be ready and there is where we get the proof whether it works or whether it doesn't work and what role possibly continuing inoculation might play in boosting vaccine induced immunity. That is something we can also learn in a Phase II trial in an endemic area with natural transmission if we have sufficient host vaccination observations (which we ought to have anyway, because we want to learn about immunopathological long-term results). We want to learn about auto-immune phenomena in this connection and we want to know about duration of immune protection in the individual. It will also, in the Phase III trials, most probably already tell us in what way the clinical manifestations of malaria in that particular community might be modified which would influence our criteria for intervention by drugs, etc. That might have major consequences for the future.

Just to come to the problem of gametocyte vaccines, which has been elucidated and has been touched upon. There is practically no Phase II and III really possible because it is not linked with personal protection. But, Phase I, a very extensive Phase I, would be the necessity which is, of course, safety, durability, possibly even acceptability and there is of course functional immunity which could be assessed alongside. That is a relatively simple thing because one could use the criteria of infectability to mosquitos. That is an absolutely functional test which is comparatively easy to do. One could go then to a Phase IV on this extended Phase I, basic studies in different age groups of the target population. Thank you very much.

MEUWISSEN: I would like to extend that. I think that for gamete vaccines, the test is available in a biological setting, you have your mosquito, you have culture-derived gametocytes, and you can test the serum of the immunized people for transmission-blocking capacity. So, at that point I think I differ with your opinion. I think it is much better to evaluate than, for instance, with an asexual vaccine.

The second point that I would like to make is that the vaccines should aim at the population level. Let me talk about control of malaria. To that point I should like to emphasize that in the pre-vaccination era, there should be much more emphasis on the new study with new methods on the epidemiology of malaria. We do not understand the epidemiology of malaria. I think we have new tools to try to get a better insight in malaria epidemiology. It is not major endemicity and high endemicity in large areas. Malaria is a disease determined by local pre-

vailing circumstances. We should go down to the village level with very thorough studies again on the epidemiology of malaria with new tools to assess not only asexual antibodies—antibodies against the asexual blood stages, but as well against the sporozoites, as well potentially against the sexual stage immunogens. In addition, I think we should get a better grip on the transmission capacity by going through good density of mosquito, especially the infection rate of the mosquito that can be assessed more thoroughly. This should be extended, I would like to say, at least to Phase III in the assessment. We should not only look to the parasite rates in the individuals, we should also look at the transmission capacity that prevails after vaccination in the mosquito population.

Coming back to the epidemiology, I should like to have, before a field trial, an idea of the local transmission capacity actually assessed by one or another mathematical statistical model that will say before how efficient our vaccination should be carried out in the population in order to predict whether we will be able to achieve a reduction in the basic reproduction rate that locally prevails. In order not to give false hopes to the population that we approach for a vaccine trial, I think that it is a very important thing that should be considered before we start with vaccination.

BRUCE-CHWATT: Well, I am very glad that this temporary gloom that I noticed in this audience after Dr. Jeffery's presentation and my own has disappeared under the influence of Dr. Meuwissen's courage and determination. I can only say that we are going to face a number of problems, but these problems can be solved and what is needed is courage and determination.

PAPPAIOANOU: I think in listening to the speakers this afternoon, certainly the challenge of trying to assess the efficacy of a vaccine and in looking at all the potentially confounding factors that will be present underscore that this is a multi-variant problem. Dr. Powell's suggestion that statisticians be incorporated early in the design of the trial really relates to more than simply just the initial set-up of the design of the study or to a comparison of proportions or of a T test at the end of the study. There are available some fairly sophisticated multi-variant statistical techniques which can be employed to help sort out the effects of these confounding factors individually and in combination with one another if appropriate statistical expertise is brought in at the very beginning of the design of the study. That is, definitely, one means of going about collecting the right information as the trials are carried out so that those types of analyses can be employed at the end in assessing the efficacy of the vaccine.

MODERATOR: I think that this is a very good point and I wonder if this point should not be incorporated in our recommendations, that while

we fully realize the difficulty, the problems that we may be facing in Phase III clinical trials can be overcome. Perhaps there is some reason in wanting the field trial very early—not too early—in order to maintain the impetus, in order to maintain the drive that we have at the present time. I fear that if we delay field trials too long, this alarm may be lost. I think in saying that I have understood what you wanted to say.

COPPEL: I would like to extend some of the points of the last two speakers and discussants and just address the point of where it is thought that Phase III trials may be held. We've heard that it is going to require an extensive period of epidemiological evaluation in whatever area is chosen and I think it is unrealistic to expect that that period of evaluation may extend to 1–2 years at a minimum, even before you can go in and try immunization trials. I asked a question on the first day of the conference whether any of the workers in the field thought that they had such areas within their country, areas that have been well studied epidemiologically, entomologically, and by the various demographic criteria that they require for setting up a trial. I think I would like to return to that question, or at least make the point, that now is the time that we really need to be identifying those areas that would be considered for Phase III trials, now even before the first Phase I trial has begun.

MODERATOR: This is a very important statement. I would like to be guided in my decision at the present time, in our decision, of how to deal with it. I would like to have the guidance from two people—from Dr. Siddiqui and also from Dr. Wernsdorfer—as to what extent can we here at this conference discuss today or tomorrow or in other recommendations the proposal for selecting a suitable field area? Could we get this guidance from those gentlemen?

SIDDIQUI: I think we should wait until tomorrow after some more discussions on, especially, the criteria for the selection of sites. I think we can reserve judgment until tomorrow when we will talk about it.

WERNSDORFER: I should wholeheartedly agree with that because we have, first of all, to discuss the criteria, as Dr. Siddiqui has indicated. I think we are too early at this very stage. We have to recognize that there is the need for strengthening the expertise and the services and facilities which are to undertake—I think that is a very important recommendation which also should be taken here—that the strengthening, the building up of expertise and facilities is important because the quality is of utmost importance. But for the selection, I would also rather delay until tomorrow.

POWELL: A very brief question that comes back to a point made earlier, and I would like to address it to Dr. Wernsdorfer. I believe I heard you mention that you did not visualize Phase II trials with the transmission-

blocking prospective agents. Perhaps I misunderstood. I had visualized that as a possibility.

WERNSDORFER: We cannot visualize a Phase II or a Phase III in the definition of the trial with a non-protecting agent, because it is essentially a non-protecting agent. The gamete or transmission-blocking vaccine, Phase I, that is safe and reliable can be done and also functional immunity could be assessed. But in actual fact what we are doing with the merozoite vaccine, for instance, and the sporozoite vaccine, in Phase II and III, functionally is efficacy testing—that is the thing we cannot really do with the gamete vaccine because the efficacy as such has to be assessed on the community.

JEFFERY: The question asked a few minutes ago relative to the ethics of withholding of treatment in an area in order not to effect the ailment of transmission risk. I think it has been pointed out that in the early Phase III trials an effort should be made to utilize only a small portion of the population in any given village or area under consideration in order to leave as undisturbed as possible the element of risk. Now obviously, when activities are being increased in an area one cannot deny treatment to people who have malaria and we would expect them to be detected in a passive system—not actively sought, but at least have available treatment for these people if they became ill and wish to go to a clinic and receive treatment. I think this would still leave an adequate amount of risk in an area to evaluate a trend—a relatively small vaccinated as compared to a placebo group. But, certainly, the ethics would not allow for ignoring entirely the population that was not included in the vaccine trial.

CATTANI: I would like to further comment on Dr. Jeffery's observations. We have been doing studies now in a hyperendemic area for the past three years, and we have found in serving 3,000 people in our 2-1/2 month period that over 50% of children and from 30-40% of adults have asymptomatic parasitemias. We hesitated whether we could ethically not treat people who had positive slides, even though it is a couple of months after the relative infection and they may no longer be positive. We tried to explain this to the people, because, after all, they should have some input in this, and they understood after several explanations at the village level that you can have parasitemias, and as long as you don't feel sick that provides you some protection, and this was never raised as an objection in our study. There are adequate health care facilities in that area. There is a good system of rural health clinics so people could get treated when they became symptomatic, but this certainly didn't interrupt transmission.

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Session V

STRATEGIES FOR FUTURE MALARIA VACCINE TRIALS II

A. METHODS FOR EVALUATION OF THE IMPACT OF VACCINATION ON THE CONTROL OF MALARIA

Methods for Evaluation of the Impact of Vaccination in Control of Malaria: Innovative Techniques

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Detection of Malaria Infected Mosquitoes

An important parameter describing the epidemiology of malaria is the prevalence of female anopheline mosquitoes infected with malaria sporozoites. This prevalence (sporozoite rate) has been determined by dissection and microscopic observation of the salivary glands of individual mosquitoes, which is an extremely time-consuming and laborious technique. Moreover, through microscopic observation, it is impossible to determine the species of plasmodium infecting the mosquitoes, presenting a serious limitation to this technique.

We have recently reported a new technique, based on monoclonal antibodies, that can be used to determine the prevalence of naturally infected mosquitoes, as well as their sporozoite load, and which also identifies the plasmodial species.[1] In this report, we will briefly review the immunological basis of this immunoassay, recent improvements of the technique, the results of its first field applications, and its potential use.

IMMUNOLOGICAL BASIS

The two-site immunoassay is performed by using monoclonal antibodies against the circumsporozoite (CS) proteins of malaria sporozoites. As reported elsewhere these monoclonal antibodies (mAB) are species specific.[2] The antigenic determinant, or epitope, which these monoclonal antibodies recognize, seems to be an invariant structure expressed in the CS proteins of a series of strains isolated from many of

the endemic areas of the world; namely, Southeast Asia, East Africa, West Africa, Central and South America.[3] Within the same plasmodial species, all the sporozoites tested thus far show that they share the same antigenic determinant.

The epitope recognized by these monoclonal antibodies is repeated several times within the same molecule allowing the binding of two or more antibody molecules to a single molecule of antigen.[4] This peculiar characteristic of the CS proteins was used to standardize a two-site immunoassay. This assay consists of the binding of sporozoite antigen to immobilized monoclonal antibody and the detection of this bound antigen by using a radioisotope or enzyme-labeled monoclonal antibody. An important feature of this assay is that it can be performed with dried mosquitoes, since the antigenic reactivity of the CS proteins remains stable for at least four months.

The monoclonal antibodies can be labeled with ^{125}I for the two-site immunoradiometric assay (two-site IRMA) or with horse-radish peroxidase for the two-site enzyme-linked immunosorbent assay (two-site ELISA).[5] When both assays were performed simultaneously, using the same batch of sporozoite antigen, the results we obtained were quite similar. However, some differences have been observed. The IRMA can detect as few as 40 sporozoites while the limit of the ELISA seems to be around 100–150 sporozoites. On the other hand, the ELISA assay seems to have less discriminatory power and tends to reach saturation with a smaller amount of parasites. In spite of these differences, both assays are sensitive enough to be used in the field or with experimentally infected mosquitoes.

FIELD APPLICATION

The two-site IRMA was recently subjected to a field trial in the Gambia (West Africa).[6] Anopheles mosquitoes were caught in different villages of this endemic area, and the prevalence of naturally infected vectors was determined by both microscopic observation of salivary glands and the immunoassay. By dissection, a sporozoite rate of 5.5% was obtained. However, using the IRMA, this rate was determined as being 7.3%. The higher rate obtained with the immunoassay may be due to the detection of CS protein in sporozoites present in the mosquito midgut, which expresses low amounts of this antigen 24 to 48 hours before migrating to the salivary gland. The IRMA also allowed an estimation of the sporozoite load per mosquito, revealing that more than 70% of the anopheles had between 500 to 10,000 sporozoites. The maximum load detected was around 100,000 parasites per mosquito. The figures on sporozoite load we obtained were strikingly similar to those obtained in a study performed by dissection techniques, in East Africa, several years ago.[7]

Our overall results indicate that the IRMA was sensitive enough to detect naturally infected vectors and to estimate their sporozoite load in addition to determining the species of parasites.

Potential Applications

The determination of the prevalence of infected mosquitoes and of their sporozoite load are important parameters for entomological and epidemiological studies, since it permits investigation of the relationship between inoculation and incidence rate, the two direct measures of transmission.

Vectorial capacity is another important aspect that needs to be studied since in several endemic areas these vectors have still not been clearly determined. In fact, very little is known on the vectorial capacities of different species of anophelines or about their relative local and seasonal importance in transmission of different species of human malaria.

It should be pointed out that by using this technique, a single mosquito can be screened simultaneously with different mAb. In this way a more comprehensive picture on the relationship between different vectors and different malaria species can be easily obtained. This information can become useful for the design or evaluation of mosquito eradication programs or other such programs directed toward malaria transmission blocking.

Experimental research on basic biological aspects of the vector-parasite relationship should also benefit by the use of this technique.

Detection of Anti-Sporozoite Antibodies

We developed an immunoradiometric assay using as antigen the synthetic peptide H-(Asn-Ala-Asn-Pro)$_3$-OH [(NANP)$_3$] to study the specificity of antibodies to *P. falciparum* sporozoites found in the sera from humans living in an endemic area (the Gambia, West Africa). This peptide represents the repeated epitope of the circumsporozoite (CS) protein *P. falciparum*. The same human sera were tested by indirect immunofluorescence (IFA) using glutaraldehyde-fixed sporozoites, a method which reveals antibodies to the parasite surface membrane. We found a highly significant positive correlation (r_s=0.0087$p<0.001$) between the results of the two assays, and showed that most antibodies detected by IFA were in fact anti-(NANP)$_3$, since the IFA of every serum was strongly inhibited by the synthetic peptide. The proportion of sera containing antibodies to (NANP)$_3$ was age-dependent, and reached 84% in samples from adults older than 34 years, who are known to be more resistant to malaria infection than younger individuals.

The findings highlight the immunodominance of the repetitive epitope of the CS proteins in man and confirm previous similar laboratory findings in experimental malaria systems.[1] They also demonstrate the presence in humans of a B-cell repertoire recognizing the *P. falciparum* repeat, which can potentially respond to a synthetic peptide vaccine containing this epitope. Because adults living in the endemic areas are already primed for the $(NANP)_3$ epitope, a synthetic peptide vaccine may boost their natural immunity and perhaps increase their resistance to malaria infection. On the other hand, it is conceivable that inoculation of sporozoites by mosquitoes can also boost the immune response of individuals primed by a synthetic peptide vaccine.

One question raised by these and previous findings is whether the increasing levels of antisporozoite antibodies in the serum of adults and the well-known development of resistance to malaria with age are causally related. Epidemiological studies using the IRMA with $(NANP)_3$ as antigen may help to clarify this important issue, since it is now possible to correlate more precisely the risk of acquiring malaria during the seasonal transmission of the disease, with the individual levels of antibodies to $(NANP)_3$. Such studies were never attempted in the past because of the difficulty in obtaining sufficient numbers of sporozoites to perform IFA reactions and because of the uncertainties as to the nature and specificity of the parasite antigens recognized by the serum antibodies.

Finally, it should be pointed out that *P. falciparum* malaria vaccines, produced either by recombinant DNA techniques or containing synthetic peptides, are likely to be tested in the near future. The precise measurement of levels of antisporozoite antibodies in endemic areas, immediately before and following the immunization, will be necessary to evaluate and compare different vaccine preparations, to determine the effects of dosage, adjuvants, etc. The immunoradiometric assay here described seems ideally suited for this purpose provided that the candidate vaccines contain the *P. falciparum* CS protein or the repetitive epitope.

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Entomological Techniques to Assess the Efficacy of a Malaria Vaccine Trial in a Community

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If and when a malaria vaccine fulfills the criteria of safety and efficacy in clinical trials, it will have to undergo Phase III trials in natural populations where its efficacy would be tested by entomological, parasitological, and immunological techniques.

Entomological Parameters to Be Considered

A successful field trial of an effective malaria vaccine should decrease significantly or possibly cease the transmission of malaria in the particular locality, which may be indicated by the absence of plasmodial infections in vectors during the usual transmission season. But success of a vaccination program would not be proved unless there is an evidence of active transmission in a comparable "control" locality during the same season. However, malaria transmission potential may inherently be different in any two localities because of the local ecological conditions. It is a common observation that malaria transmission is usually focal in nature because transmission potential varies in different localities. Therefore, vectorial capacities of vectors in the two localities (trial locality and control locality) have to be considered. The vectorial capacity (VC) defines the average number of potentially infective bites that will ultimately be delivered by the vectors feeding upon a single host in one day. In short, it is a daily rate of potentially infective contacts caused by the vectors. The total vectorial capacity at a given time and place is the sum of the corresponding vectorial capacities of all the vector species and reflects the transmission potential in the area. The vectorial capacity is a useful parameter which, depending upon the situation, may be used to:

1. compare the transmission potential in different areas;
2. compare the transmission potential of various species in an area; and
3. identify the peak transmission season in a locality.

Vectorial capacity is a characteristic of a mosquito population related to the ecological conditions and may be assessed irrespective of whether any cases of malaria are present at the given place and time. A decrease in sporozoite rates in a vaccine-trial locality relative to a control locality can be interpreted as the vaccine effect only after adjustment for vectorial capacities in the two localities.

Detection of sporozoites in mosquitoes has been facilitated by a recently developed method based on immunological techniques.[1,2] But the classical method for estimation of vectorial capacity (VC) of a mosquito species is not satisfactory. The classical method is based on the assumption that survival/mortality of mosquitoes is not related to age. Data gathered in the present study indicated that the probability of daily survival in mosquitoes decreases with age, rather than being constant as thought by earlier workers. Based on this revised concept, a new method has been proposed to estimate survivorship in a natural population and thereby the malaria vectorial capacity of a species.

Mortality Pattern of Mosquitoes

Review of the relevant literature indicates that there is a difference of opinion as to whether survivorship or death rate in a mosquito population is independent of age or varies with age. The classical experiment of Russel and Rao (1942), on *An. culicifacies* under semi-natural conditions in Madras (India), constitutes the main argument in favour of a constant death rate hypothesis.[4] Macdonald (1952) recognized some of the limitations of their experiment but remained convinced about the constancy of survivorship or death rate over age, an assumption which formed the central column of his extensive quantitative epidemiological analyses and mathematical models.[4] On the other hand, a number of workers have found evidence indicating that the probability of survival in mosquitoes decreases with age; the phenomenon has been noticed in a number of species including *An. gambiae* Giles, *An. stephensi*, *An. culicifacies*, and *Cx. quinquefasciatus*.[5-8]

An effort has been made in the present study to sort out the long-standing controversy concerning the mortality pattern, and, if necessary, the existing procedures for estimating survivorship and vectorial capacity of mosquitoes should be revised accordingly. The survivorship or mortality pattern of *Anopheles* females was studied under two different sets of food conditions: (1) blood feeding without sugar solution and (2) blood feeding with sugar solution. Females feeding exclusively on blood

exhibited a pattern of constant survivorship/mortality rate with respect to age. The survivorship curve followed a curvilinear regression pattern —the population declined along a geometric progression. The mortality pattern was similar to the one noticed by Russel and Rao (1942), which needed logarithmic transformation to make it linear.[9] On the other hand, females feeding on blood plus supplementary food (sugar solution) exhibited a mortality rate that increased as a linear function of age (Figs. 1 and 2). Both the test species (*An. culicifacies* and *An. stephensi*) exhibited similar patterns of mortality curve. The survivorship curve followed a linear regression pattern, implying that the probability of survival decreased with age. The comparison of the data in the two experiments clearly demonstrated that the survivorship or mortality pattern of mosquitoes was influenced by the type of food consumed.

These results are in agreement with Leclerq, who stated that "most of the biting flies, when they are fed exclusively on blood, rapidly die of thirst. They must always complete their blood meal by drinking water derived from various sources, by sucking in animal secretions (plant lice, Coccids) or water of vegetable origin (plant juices, the nectar of flowers). Examples of blood sucking insects which rob flowers are numerous notably among the Ceratopogonidae, the Culicidae, the Simulidae, the Rhagionidae, the Tabanidae and the Muscidae (Stomoxys)."[9]

Clements also emphasized the requirement of plant juices and nectar as supplementary food for mosquito females.[10]

A constant survival or mortality rate in *An. culicifacies* observed by Russel and Rao could be due to either or both of the following reasons: (1) obvious difficulties in catching hundreds of mosquitoes individually by inverting a test tube on each mosquito, a limitation already pointed out by Macdonald, where with a decrease in population, catchability was likely to be improved toward the end of the cohort life; (2) mosquitoes were perhaps not served adequately with a supplementary food since a few petri dishes with sugar solution in a vast field cage (40 × 20 × 10 feet) might not be enough.[9,4]

Because of this conceptual change in the understanding of the mortality pattern of mosquitoes, the existing procedure for estimating probability of survival and expectancy of life over time (Macdonald, 1952) has to be revised according to the following expressions, which apply because of the linear relationship between l_x and age (days):[4]

Maximum life or age of individuals in a cohort = 1/b days, where b is a negative slope of the regression.
When $l_0 = 1$ (i.e., when survivorship is taken as proportions), then:
Life expectancy at the beginning of a cohort: $e_0 = 1/2b$;
Proportion surviving through "n" days: $l_n = 1 - bn$;

Life expectancy after surviving thru "n" days: $e_n = (1 - bn)/2b$.
(derivation of the above expressions is illustrated in Appendix I).

Estimation of Age and Survivorship of Mosquitoes in Nature

The epidemiological significance of the adult longevity of a malaria vector can hardly be overemphasized. But correct estimation of age and survivorship in a natural population of mosquitoes is by no means an easy task. A number of indirect methods have been proposed, but each has some limitations (Suleman, 1985).[11]

The direct method of Polovodova was adopted in the present study because it seems to have the potential of providing a fairly reliable estimate of the age-structure of a population at a given point in time, though it is a time consuming and laborious technique requiring numerous dissections and countings of ovariolar dilatations.[12] This method involves the direct recording of the number of ovarian cycles (gonotrophic cycles) undergone by the individual female. The number of gonotrophic cycles completed by the female is really a measure of its physiological age, which can easily be translated into calendar age by relating to the durations of successive gonotrophic cycles in days.

The procedure is illustrated using *An. culicifacies* data collected in a rural locality in Punjab during 1982–1983. A representative sample of females dissected each month was classified by the number of ovariolar dilatations (gonotrophic cycles) resulting in a frequency distribution according to physiological age (Table 1). The frequencies were accumulated following a cohort approach, assuming that all age-groups were representatively sampled and the population age structure remained relatively constant over a month (i.e., additions and losses were approximately equal). It is assumed that mortality during the first two days of adult life in the warm season, and the first three days in cold season, was negligible. Such an assumption facilitates the computational process without much loss of accuracy in results, as it is in agreement with direct observations on survivorship under semi-natural conditions (Figs. 1 and 2). To each dilatation class was assigned a chronological age corresponding to the mid-gonotrophic period in days (X_1 and X_2). The procedure for estimating age at mid-gonotrophic periods is given in the footnote of Table 1.

Two alternate estimates of survivorship were made: (1) median age and constant daily loss in population, considering that survivorship decreases as a linear function of age; and (2) probability of daily survival (p), assuming that it remains constant over age.

The observations and results are summarized in Table 1, which consists of four blocks as follows:

- The first block contains the frequency of females dissected for age

TABLE 1. Age-grading and survivorship of females of *An. culicifacies* by month based on physiological age structure judged from number of ovariolar dilatations in representative group of females collected and dissected at fortnightly intervals

No. dilatations	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.
	No. females dissected by month, classified by number of ovariolar dilatations observed									
	11	*44*	*56*	*46*	*54*	*53*	*103*	*49*	*57*	*42*
0	7	20	37	26	24	16	10	23	11	18
1	4	17	18	20	27	32	45	15	23	3
2	0	4	1	0	3	4	12	6	8	5
3	0	1	0	0	0	1	3	3	7	2
4	0	2	0	0	0	0	1	2	5	8
5	0	0	0	0	0	0	1	0	2	5
6	0	0	0	0	0	0	1	0	1	1
7	0	0	0	0	0	0	0	0	0	0

	X_1	*Cumulative frequencies (Lx) by No. dilatations and mid gonotrophic period in days (X)*[a]										X_2
0	0	11	44	56	46	54	53	103	49	57	42	0.0
0	2	11	44	56	46	54	53	103	9	57	42	3.0
1	5	4	24	19	20	30	37	63	26	46	24	7.5
2	7	0	7	1	0	3	5	18	11	23	21	10.5
3	9	—	3	0	—	0	1	6	5	15	16	13.5
4	11	—	2	—	—	—	0	3	2	8	14	16.5
5	13	—	0	—	—	—	—	2	0	3	6	19.5
6	15	—	—	—	—	—	—	1	—	1	1	22.5
7	17	—	—	—	—	—	—	0	—	0	0	25.5

	Survivorship (Sd_1)[b]									
$b_1(-)$	.154	.090	.157	.150	.129	.110	.067	.059	.046	.042
$(r^2)_1$	.93	.90	.95	.92	.93	.90	.81	.92	.92	.96
Median age ($0.5/b_1$)	3.2	5.6	3.2	3.3	3.9	4.5	7.5	8.5	10.9	11.9

	Survivorship (Sd_2)									
$b_2(-)$	.345	.304	.493	.506	.454	.403	.299	.204	.167	.141
$(r^2)_2$	.84	.95	.90	.74	.85	.89	.96	.95	.94	.86
P	.71	.74	.61	.60	.64	.67	.74	.82	.85	.87

[a]X = chronological age at mid-gonotrophic period in days corresponding to the number of ovariolar dilatations/ovipositions. Gonotrophic cycle (gc) varies with temperature, and the first gc is longer than the subsequent ones. During the warm season (Apr.–Oct.) the duration

determination, by month and number of ovariolar dilatations observed. In other words, this block summarizes the monthly frequency distribution of females, by physiological age.

- The second block shows cumulative frequencies (l_x) classified by month, number of dilatations, and the corresponding mid-gonotrophic periods (X_i). In other words, this block summarizes the monthly cumulative frequency distribution, by physiological age as well as calendar age, on a cohort basis.
- The third block consists of results on monthly estimates of survivorship (Sd_1 of the species, as obtained by linear regression of $(l_x)_i$ on (X_i), where $(l_x)_i$ = the proportion surviving, and $X_i = X_1$ for warm months (April–Oct.) and X_2 for cold months (Nov.–Jan.). Included in this block are the regression slope (b_1), coefficient of determination $(r^2)_1$, and median age (0.5/b or 1/2b). Median age (which equals mean age in this case) is the principal estimator of survivorship, and here survivorship is considered to vary as a linear function of age.
- The fourth block contains results on monthly estimates of survivorship (Sd_2), if the probability of survival is assumed to be constant with respect to age, as obtained by curvilinear regression between the same variables (i.e., regression of y_i on X_i, where $y = \text{Log}_e)(l_x)$ and $X = X_1$ or X_2, depending on which month is being considered). Included in this block are the regression slope (b_2), coefficient of determination $(r^2)_2$, and probability of daily survival (P). The value of (P) is derived by back-transformation (into the original scale) of the slope, i.e., $P = e^b$, where e = base of natural logarithms and b = slope of fitted regression equation.

Although the present study does not support the assumption of a constant probability of survival over age, it is included here for the purpose of comparison, in order to analyze how it would influence the estimates of malaria vectorial capacity of a species. The method adopted in this

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of the first gc is on the average 4 days and each subsequent cycle takes approximately 2 days; therefore, the calendar age at mid-gonotrophic period is 2, 5, 7, . . . days corresponding to ovariolar dilatations (gc's) 0, 1, 2, . . . and so on. In the winter (Nov.–Jan.) the first gc takes about 6 days and each subsequent cycle approximately 3 days. Therefore the age at the mid-gonotrophic period is 3, 7.5, 10.5, . . . days for ovariolar dilatation (gc's) 0, 1, 2, . . . and so on.[14-15-31] $X = X_1$ for the period Apr.–Oct. and $X = X_2$ for the period Nov.–Jan.

[b]Estimation of survivorship considering (P) not constant, but decreasing with age, using linear regression of y_i on X_i; where $y_i = (l_x)_i$, taken as proportions, and b_1 = slope of regression; $(r^2)_1$ = coefficient of determination. Slope (b_1) which is constant over age is a measure of daily decrease in population and its reciprocal ($1/b_1$) gives the maximum life of females in a cohort and the expression ($0.5/b_1$) or (1/2b) gives a measure of median age of a cohort.

[c]Estimation of survivorship if it is assumed that the probability of daily survival (P) remains constant over age (a popular conventional assumption, first made by Macdonald in 1952, by applying linear regression of y_i on X_i where $y_i = [\log(l_x + 1)]$, and X_i as defined above). Probability of daily survival (P) was calculated by back-transformation of slope (b_1) i.e. e^{-b}; $(r^2)_2$ = coefficient of determination.

study to estimate the probability of survival (P) involves an improvement upon the one used by earlier workers in that the regression analysis is based on cumulative frequencies, instead of original frequencies, a procedure to be desired in a cohort approach.[14-14]

Vectorial Capacity Estimates

Monthly estimates of the vectorial capacity (VC) of *An. culicifacies* were made, using two alternative methods as follows:

$$VC_1 = \frac{ma^2(1 - bn)(0.5)}{b} = \frac{ma^2(1 - bn)}{2b}$$ New approach posed in this study.

$$VC_2 = \frac{ma^2 P^n}{-\log_e P}$$ Commonly used formula, after Garrett-Jones (1964).[16]

Estimation procedures for VC_1 and VC_2 differ only with respect to the survivorship/mortality pattern of mosquitoes. The procedure for VC_1 has been developed in the present study, considering that the probability of survival in a natural population of mosquitoes decreases with age. The procedure for VC_2 is borrowed from Garrett-Jones, who extracted it from Macdonald's basic reproduction rate, and is based on the assumption that the probability of survival does not change with age.[16-4] The individual variables were estimated as follows:

m = No. female mosquitoes/person—based on mosquito population estimates by pyrethrum spray method, and human population by census.

a = HBI/gc, where HBI = human blood index (anthropophagic index) estimated by precipitin tests, and gc = duration of gonotrophic cycle in days.

n = Duration of sporogonic cycle for *P. vivax*, extracted from Macdonald's temperature vs. extrinsic incubation period curve (1957), against the average figure of mean daily temperature records maintained in the study village.

b = Constant daily decrease in population (= b_1 in Table 1).

P = Constant probability of survival for one day (= P in Table 1).

The component $(1 = bn)/2b$ for VC_1, and $P^n/-\log_e P$ for VC_2 are the two alternative estimates for expectation of life after surviving through "n" days, as discussed earlier.

Estimates of the vectorial capacity in this study are made in terms of *P. vivax* only; there is no need to give a parallel series of VC estimates for *P. falciparum*, because estimates for one species would serve as a base for the other. Naturally, the vectorial capacity (VC) estimates in terms of *P.*

falciparum would be slightly on the lower side (sporogonic cycle of *P. falciparum* being slightly longer than that of *P. vivax*) but the differences, at all levels, would be in the same direction and proportionate in magnitude to those of *P. vivax* estimates.

The two alternative estimates of vectorial capacity (VC_1 and VC_2) of *An. culicifacies* by month, along with the component variables, are set out in Table 2. Similarly, vectorial capacities for the remaining four species of the common anophelines in Punjab were also estimated, but only the summary information is given here for the sake of brevity (Table 3). For all the species, except *An. annularis*, VC_1 estimates came out higher than VC_2 estimates. A comparison of VC_1 and VC_2 in different species, considering both the monthly as well as the yearly total estimates, indicates that the two series of estimates differ more widely in *An. annularis*, *An. pulcherrimus*, and *An. subpictus* than in *An. culicifacies* and *An. stephensi*. These results are in line with the relationships between the two sets of monthly survivorship estimates in different species on which the VC estimates are based.

The traditional method for estimation of vectorial capacity (VC_2) gave a positive value even when the mosquito density in relation to man (m) was very small, except in very cold months when "n" became excessively high. In other words, this method gives relatively less weight to the factor of mosquito density (m) as compared to survivorship, with the result that monthly estimates of VC_2 do not reflect (m) to the extent it is reflected in estimates obtained by the alternative procedure (VC_1). Because of the underlying assumption of a constant daily survival rate (P), and thereby an exponential relationship between survival and age, a small fraction of a mosquito population is always thought to survive beyond "n" days, irrespective of environmental conditions. The traditional method gave a positive value for VC even under extreme dry-hot conditions like the one prevailing during June–July. For these reasons, monthly VC_2 estimates over the year followed a distribution with a depressed peak and a broad base, an effect which makes identification of the active transmission season relatively difficult. Under the traditional method it is taken for granted that a small fraction of the population will always survive through "n" days, no matter how severe environmental conditions may be.

Moreover, small fractions of small populations (e.g., in Mar.–April or in June–July), which may survive for a period longer than the length of the extrinsic incubation period in that particular season, can hardly be of any epidemiological significance, especially when the vector species under consideration are predominantly zoophagic in habits. Therefore this method tends to overestimate the VC during any period with severe climatic conditions and tends to underestimate it during any period with suitable environmental conditions.

TABLE 2. Vectoral capacity (VC) of *An. culicifacies* by month, estimated using two alternative estimates of survival rate of females: VC_1, using survivorship estimated as a linear function of age expressed as a constant daily decrease in population (b_1 in Table 1); and VC_2, using survivorship estimated as an exponential function of age expressed as a constant probability of daily survival (P in Table 1)

Month	*m*	*gc*	*HBI*	*a*	ma^2 ($x10^{-3}$)	*temp.* (*C*)	*n*	b_1 (—)	*P*	VC_1 ($x10^{-3}$)	VC_2 ($x10^{-3}$)
Mar.	0.00	—	—	—	—	—	—	—	—	0.000	0.000
Apr.	0.13	2	.011	.0055	.0039	26.4	9.0	.154	.71	0.000	0.001
May	0.57	2	.011	.0055	.0172	30.9	7.0	.090	.74	0.035	0.007
Jun.	1.12	2	.011	.0055	.0339	33.4	6.4	.157	.61	0.000	0.003
Jul.	5.91	2	.011	.0055	.1788	32.3	6.9	.150	.60	0.000	0.010
Aug.	10.11	2	.011	.0055	.3058	31.4	7.0	.129	.64	0.115	0.030
Sep.	6.01	2	.011	.0055	.1818	30.2	7.3	.110	.67	0.163	0.024
Oct.	0.83	2	.011	.0055	.0251	26.1	9.2	.067	.74	0.072	0.005
Nov.	5.03	3	.011	.0037	.0676	22.8	12.5	.059	.82	0.150	0.029
Dec.	1.58	3	.011	.0037	.0212	19.7[1]	20	.046	.85	0.018	0.005
Jan.	0.65	3	.011	.0037	.0087	15.5	>40	.042	.87	0.000	0.000
Feb.	0.00	—	—	—	—	—	—	—	—	0.000	0.000

f = mean for the first fortnight only.
m = No. female mosquitoes/person.
gc = duration of gonotrophic cycle (= frequency of biting).
HBI = human blood index (anthropophagic index).
a = (HBI/gc) (= frequency of human biting).
temp. = mean daily temperature (average of the mean daily maximum and the mean daily minimum).
n = duration of sporogonic cycle in days for *P. vivax*. Thus vectorial capacity of various anophelines has been estimated in terms of *P. vivax*.
b_1 = daily decrease in population of females (from Table 1).
P = probability of daily survival (from Table 1).

$$VC_1 = \frac{ma^2\,(1\text{-}bn).5}{b} = \frac{ma^2(1\text{-}bn)}{2b} \qquad VC_2 = \frac{ma^2\,P^n}{-\log_e P}$$

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TABLE 3. Summary of the vectorial capacity estimate ($x10^{-4}$) of the common anophelines, by species, month and method of estimation (VC_1, when probability of survival is considered to decrease with age and VC_2, if probability of daily survival is assumed to be constant)*

	An. annularis		*An. culicifacies*		*An. pulcherrimus*		*An. stephensi*		*An. subpictus*		*Total*	
Month	VC_1	VC_2	VC_1	VC_2	VC_1	VC_2	VC_1	VC_2	VC_1	VC_2	VC_1	VC_2
Mar.	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.02
Apr.	0.00	0.07	0.00	0.01	0.00	0.15	0.00	0.01	0.00	0.00	0.00	0.24
May	0.00	1.11	0.35	0.07	2.60	3.35	1.06	0.37	0.00	0.00	4.01	4.90
Jun.	0.00	2.08	0.00	0.03	1.57	1.83	0.01	0.05	0.00	0.00	1.58	3.99
Jul.	0.00	0.00	0.00	0.10	0.95	3.74	0.00	0.21	0.00	0.02	0.95	4.07
Aug.	0.29	3.47	1.15	0.30	0.00	0.59	0.25	0.10	0.00	0.08	1.69	4.54
Sep.	0.55	2.88	1.63	0.24	23.47	5.74	0.08	0.09	0.00	0.10	25.73	9.05
Oct.	0.00	0.46	0.72	0.05	0.00	0.22	0.00	0.06	0.00	0.08	0.72	0.87
Nov.	0.00	0.07	1.50	0.29	0.00	0.20	0.00	0.01	0.39	0.09	1.89	0.66
Dec.	0.00	0.03	0.18	0.05	0.00	0.02	0.00	0.00	0.00	0.01	0.18	0.11
Jan.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Feb.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	0.84	10.17	5.53	1.14	28.59	15.86	1.40	0.90	0.39	0.38	36.75	28.45
Ratio VC_1/VC_2	0.08		4.85		1.80		1.56		1.03		1.29	

Relative vectorial capacity of the species

VC_1	2.15	14.18	73.31	3.59	1.00
VC_2	26.76	3.00	41.74	2.37	1.00

* figures for VC_1 and VC_2 are taken from Table 2, transformed to $x10^{-4}$.

On the other hand, the vectorial capacity estimates according to the new approach proposed in this study (VC_1) are based on proportionate weights for mosquito density and survivorship. The transmission potential of a species (VC_1) therefore has a positive value only when its density is relatively high and survivorship is above a certain threshold level, determined by the duration of sporogonic cycle in that particular season. VC_1 estimates did not give a positive value for any species during the hot-dry summer season (June–July) when survivorship was too low, or during the cold winter season (Jan.–Mar.) when mosquito density was low and survivorship threshold high. This procedure does not assume that a part of the population is going to survive beyond the length of the extrinsic incubation period regardless of climatic conditions. For these reasons, estimates of vectorial capacity (VC_1) followed a seasonal pattern with a well-marked peak and narrow base, an effect which corresponds very well to the seasonal pattern of malaria transmission in the Punjab.

It is well-known fact that malaria transmission in Punjab is a seasonal phenomenon. Therefore, those VC estimates will be more reliable which would better correspond to the seasonal pattern of malaria incidence in the area. Relative merit of the two methods may be judged on this basis.

It seems appropriate to concentrate on *An. culicifacies*, which is a confirmed malaria vector in rural Punjab, in order to check the relative merits of the two alternative procedures for estimating vectorial capacity (VC_1 and VC_2). Monthly estimates of VC_1 gave low level positive values in May and higher values from August through December. VC_2 estimates gave low level positive values throughout the year, except during January through March, and no clues as to peak transmission season (Table 3). According to VC_1, this species is capable of transmitting malaria to a slight extent during late spring (May) and to a greater extent during the postmonsoon and autumn (Aug.–Dec.) but has zero potential of transmission during the hot summer (June–July) and cold winter or early spring (Jan.–April). These observations are in full agreement with previous reports, based on monthly mortality and morbidity records and monthly parasitological observations on infants regarding the malaria transmission season in Punjab.[17,18] Thus the present study has provided the entomological evidence and mechanisms to explain earlier, non-entomological findings concerning the seasonality of malaria transmission in Punjab.[17,18]

Monthly estimates of malaria vectorial capacity by revised method but not by the classical method (Table 3) correlate well with seasonal incidence of malaria in the Punjab as determined by parasitological observations (Table 4). Data shown in Table 4 reflect the monthly incidence of malaria. The years 1969 and 1979, where all age-groups are

TABLE 4. Prevalence of malaria in Sheikhupura district, as recorded by the district malaria control department, Sheikhapura, by month (pv = *Plasmodium vivax*; pf = *P. falciparum*; Exam. = No. examined; +ve = No. positive; PR % = parasite rate %)

	1982 *Infants (pv + pf)*			*1981* *All ages (Pf)*			*1982* *All ages (pf)*			*1969* *All ages (pv + pf)*			*1979* *All ages (pv + pf)*		
Months	*Exam.*	*+ve*	*PR %*	*Exam.*	*+ve*	*PR %*	*Exam.*	*+ve*	*PR %*	*Exam.*	*+ve*	*PR %*	*Exam.*	*+ve*	*PR %*
Jan.	25	1	4.00	4418	2	0.05	6207	7	0.11	10732	0	0.00	4396	1	0.02
Feb.	22	0	0.00	4561	0	0.00	4850	1	0.02	8951	0	0.00	3918	0	0.00
Mar.	66	0	0.00	4716	3	0.06	4614	0	0.00	7466	0	0.00	5262	4	0.08
Apr.	352	1	0.28	6180	0	0.00	7346	2	0.03	7994	0	0.03	4471	3	0.07
May	275	4	1.45	6154	0	0.00	8788	14	0.16	6768	3	0.04	4984	28	0.56
Jun.	60	2	3.33	3287	0	0.00	3741	12	0.32	5585	2	0.04	3720	20	0.54
Jul.	35	2	5.71	3532	0	0.00	3255	22	0.68	5768	1	0.02	4282	3	0.07
Aug.	61	3	4.92	3594	7	0.19	4303	14	0.33	7305	50	0.68	4148	6	0.14
Sep.	113	13	11.50	12064	45	0.37	6675	104	1.56	14282	611	4.28	6072	128	2.11
Oct.	276	27	9.78	8927	60	0.67	11485	299	2.60	17548	1359	7.74	7459	412	5.52
Nov.	218	21	9.63	5757	49	0.85	10271	384	3.74	13662	668	4.89	7369	416	5.58
Dec.	58	10	17.20	5685	18	0.32	6300	244	3.87	11732	125	1.07	5394	109	2.02
Total	1561	84	5.38	70875	184	0.26	77833	1103	1.42	117793	2821	2.39	61475	1130	1.84

The reasons for looking into monthly estimates of both vivax and falciparum malaria in all ages selectively for the years 1969 and 1979 are given in the text.

Assumption: Monthly prevalence of malaria, under the given set of conditions, followed active transmission.

Conclusion: Rise in prevalence rate from September through December indicates that active transmission starts somewhere in August and continues up to mid-December.

included, were selected because in both of these years malaria was resurging after remaining at negligible low levels for a few years, whereby most of the cases (even vivax cases) were most probably primary infections since relapses must have died out by that time.

Similarly, high VC_2 (but not VC_1) estimates for *An. annularis* seem to be misleading because they are not consistent with previous information on the species, as this mosquito is not included in the list of confirmed or suspected vectors in the region.[19]

Based on these evidences, it may be concluded that the revised method for estimation of vectorial capacity (VC_1) provides more logical and reliable estimates than the classical method (VC_2).

Conclusions

- Consideration of vectorial capacities along with the sporozoite rates is necessary for a reliable entomological assessment of the efficacy of a malaria vaccine trial. Monitoring sporozoite rates alone would not be enough.
- The revised method for estimation of vectorial capacity seems to be a significant improvement upon the classical method and would thus provide a better assessment of the malaria transmission potential in a community.
- Quantitative assessment of the component variables for malaria vectorial capacity is a laborious process, which involves monitoring several variables at regular intervals by trained persons. But experience shows that two persons can handle the job in an average village of about 1,500 population.
- Entomological data (vectorial capacity estimates and sporozoite rates) along with parasitological data, from a vaccine-trial locality and a control locality, will hopefully lead to a fairly accurate judgement about the effect of the vaccine trial on malaria transmission.
- Identification and quantification of the vaccine effect would be difficult where other control measures (e.g., insecticides) are also being used at the same time.

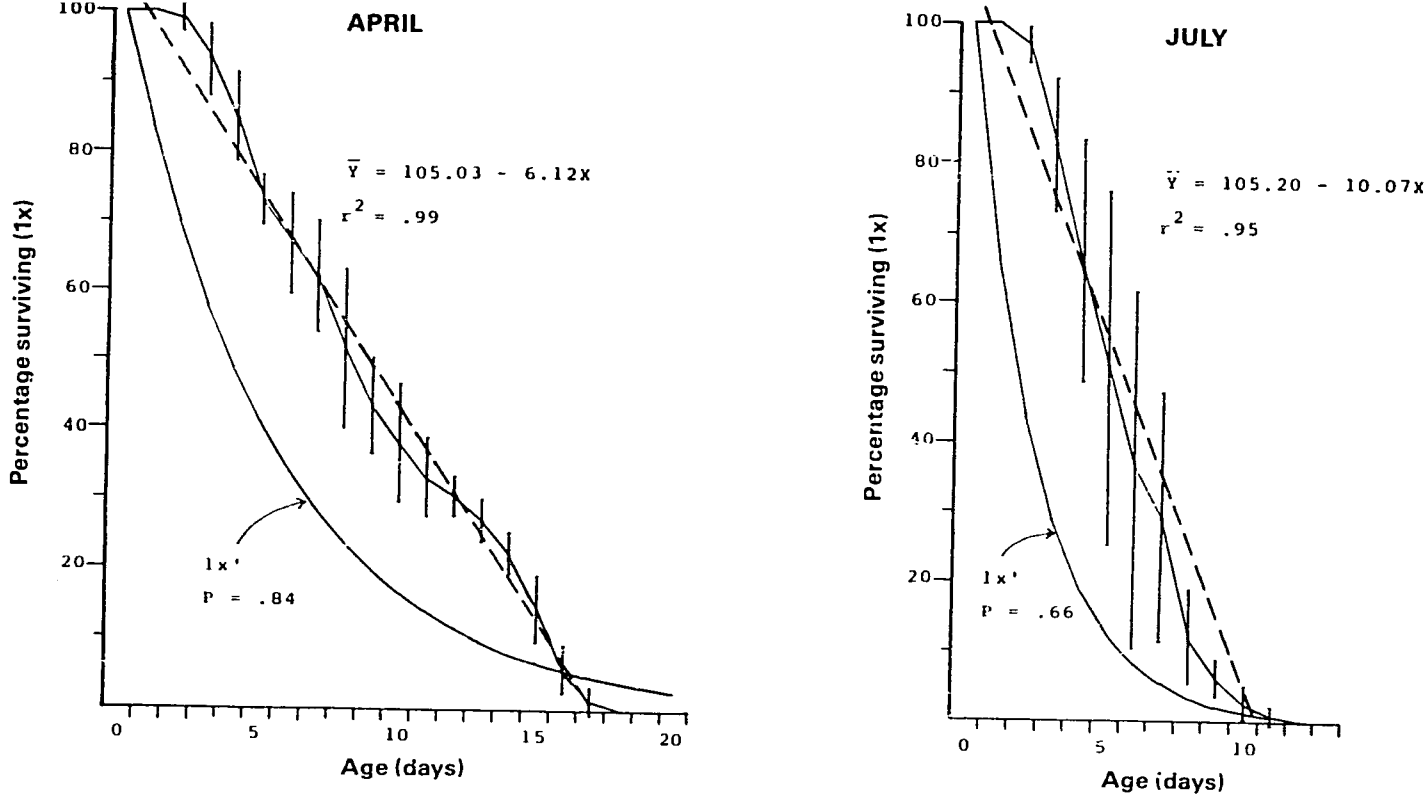

FIGURE 1. Survivorship curves (1x vs. age) of *An. stephensi* females, provided with a source of blood-meal and sugar solution, under semi-natural conditions in the study village. Broken lines represent linear regression, and the vertical bars represent 95% confidence intervals. 1x' represents expected curve if probability of daily survival (p) were constant with respect to age. (P was calculated by back-transformation of the slope of the regression of log(1x) on age.

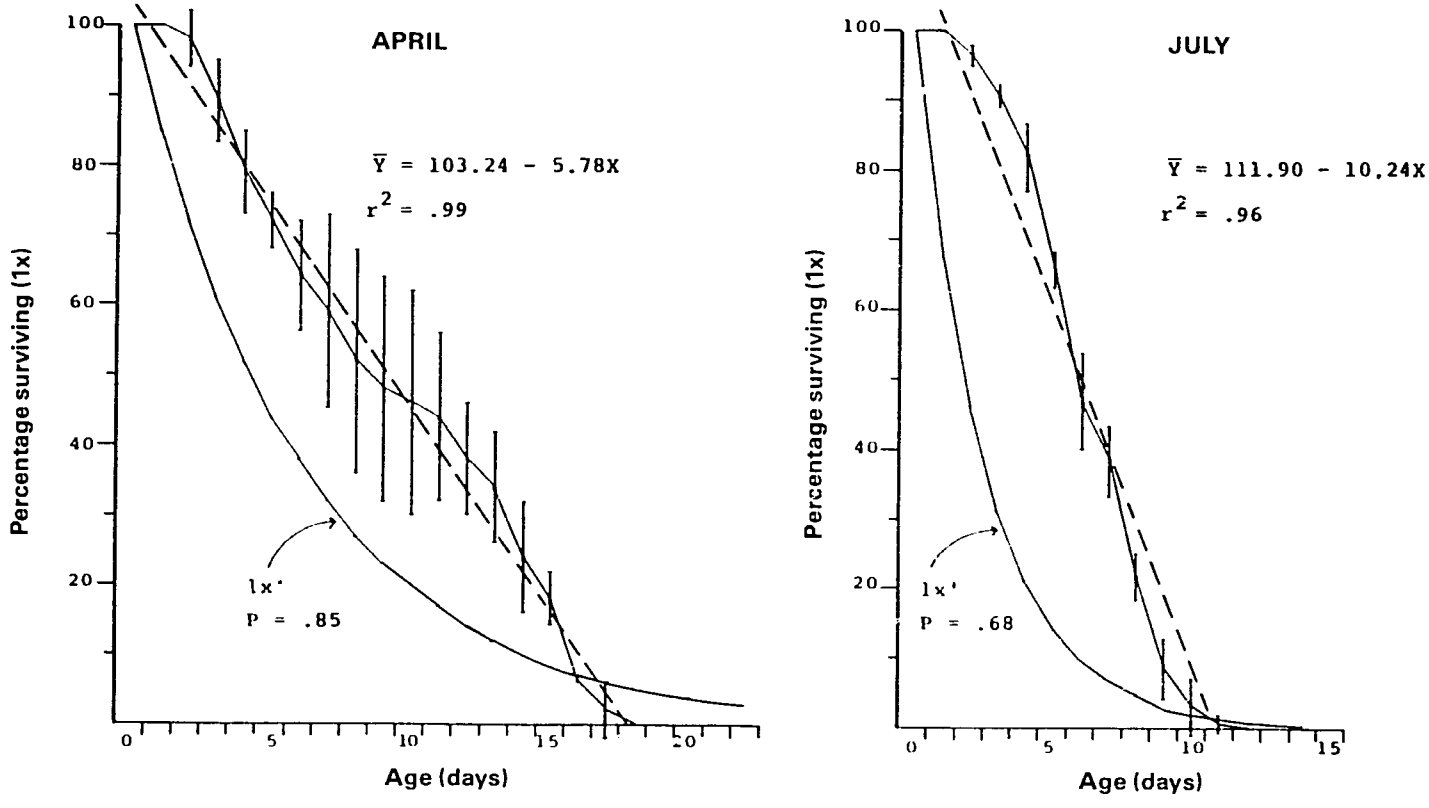

FIGURE 2. Survivorship curves (lx vs. age) of *An. culicifacies* females, provided with a source of blood meal and sugar solution, under semi-natural conditions in the study village. Broken lines represent linear regression, and the vertical bars represent 95% confidence intervals. lx' represents expected curve if probability of daily survival (p) were constant with respect to age. (P was calculated by back-transformation of the slope of the assumed curvilinear regression.

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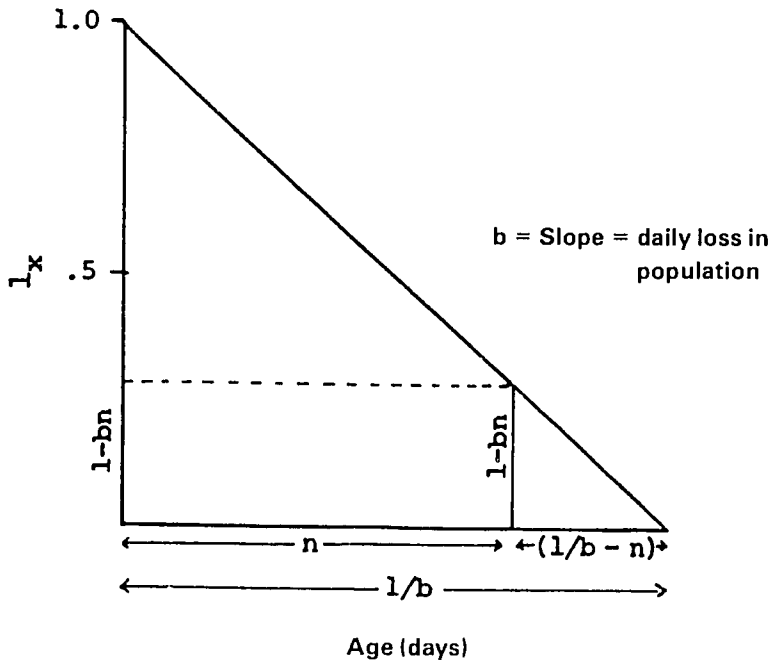
APPENDIX I

Revised Formula for Expectation of Life after "n" Days and VC

Given a linear relationship between l_x and age, and using a cohort approach, the simple linear model ($y = a + bx$) may be applied to estimate the proportion surviving through "n" days and expectation of life after surviving through "n" days. The procedure is illustrated below:



Maximum life in a cohort = 1/b days (b being a negative slope).
Expectation of life at the beginning of a cohort (adult emergence)
($e_0 = T_0/l_0$; i.e., total time lived/No. in the beginning) = 1/2b because $T_0 = (1/b)(1)(1/2)$ and $l_0 = 1$

Proportion living through "n" days = 1 - bn
(because $y = a + bx$; in the present situation $a = 1$ and $x = n$).

Expectation of life after "n" days $= \frac{(1/b - n)(1-bn)(1/2)}{1-bn} = (1-bn)/2b$

So the expression $p^n/-\log_e P$ is replaced by $(1 = bn)/2b$ and the revised formula for the vectorial capacity VC_1 differs from the classical formula (VC_2) as under:

$$VC_1 = \frac{ma^2 (1-bn)}{2b}$$

$$VC_2 = \frac{ma^2 P^n}{-\log_e P}$$

Seroepidemiology of Malaria

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1. Introduction

The need for seroepidemiological surveys of malaria has been well appreciated. It is in general agreed that this type of survey provides information on period prevalence data concerning the total infection with malaria of an individual in a given community.[1-4] The information derived therefrom is different from that obtained by parasitological examination used in the mass blood survey which provides mainly point prevalence data, i.e., the frequency of people having parasitaemia on the day of blood collection. The absence of patent parasitaemia in an individual does not entirely negate that such individual has not been exposed to malaria, since patency is influenced by the immune status and by the use of anti-malarial drugs and often occurs only intermittently during malaria infections. Furthermore, seroepidemiological surveys can be applied even in an area with low level of malaria transmission, especially where the transmission cycle has been disturbed by the use of insecticides or drugs. They can also provide information on malaria transmission in a given area using relatively small numbers of representative samples from a given community by determination of relationship between the geometric mean reciprocal titer (GMRT) and the percentage of seropositive population. Serological surveys have nevertheless some limitations. They cannot give information on parasite density and gametocytaemia, which are important parameters in epidemiology. In addition, an individual's immune response is affected by several factors, such as age, immunologic competency, cumulative exposure to malaria antigen, and the kind and amount of specific therapy. Thus a person who should become serological positive after receiving infective bites from mosquitoes may fail to do so. It is therefore important that interpretation of serological data requires detailed knowledge of the local epidemiology of malaria.

The use of seroepidemiology of malaria was summarised in 1972 by the World Health Organization as follows:[5]

In malaria endemic areas

a. For the establishment of malarial endemicity rates including species prevalence (in particular age-specific indices).

b. To assess changes in the degree of malaria transmission, usually during or after malaria eradication or control operations.

c. To permit specific epidemiological assessment of malaria—e.g., to delineate malarious areas; to show the altitude delineation of malaria; and to identify and follow up foci of malaria.

d. To identify areas or individuals requiring action with regard to malaria, especially during the later stages of malaria control programmes.

In non-endemic areas

a. For case-detection and identification, in some instances, of the species of malaria parasite responsible for the infection.

b. For the screening of blood donors.

c. To exclude the diagnosis of malaria in patients with symptoms such as pyrexia of unknown origin, hepatosplenomegaly, anaemia, and nephrotic syndrome.

2. Collection of Blood Specimens for Use in Seroepidemiology[5]

Usually finger-pricked blood specimens collected in capillary tubes or filter papers are used. Venous blood can also be collected, but it should be done with caution in view of the possible unwillingness of the community to participate in the programme.

The capillary-tube method is in general recommended for the collection and transport of samples. One capillary tube with a diameter of 1.1 mm and a length of 75 mm can take up to 70 μl of whole blood. If a larger volume is needed, more capillary tubes or a larger tube like Tatleson tube with capacity of 250 μl can be used. They should be centrifuged within a few hours and cut at the plasma-cellular interface. The plasma section is sealed at both ends and stored.

The filter paper technique can be done by allowing a precisely measured quantity of blood, e.g., by means of a Drummond microcap pipette on the filter paper, or simply by allowing blood to soak on the precise area, e.g., a circle of 12 mm of filter paper, and dry.[6]

3. Tests Used in Seroepidemiology

3.1 Serological tests for blood stages.

3.1.1 Tests indicative of malaria experience but not related to protective immunity.[7]

3.1.1.1 *P. falciparum*

3.1.1.1.1 Indirect fluorescent antibody test (IFA).

Both thick and thin blood smears can be used, and homologous species of parasites are recommended. For preparation of *P. falciparum* slides, cultured parasites using a candle jar technique can be used.[8] Before the era of *in vitro* continuous culture of parasites, other species of plasmodia could be used as a substitution, e.g., *P. fieldi* and *P. knowlesi*, but they are no longer popular.

The IFA test has merits and limitations as follows:[2]

Merits of the test

a. The preparation of comparable batches of antigen is relatively simple.

b. The whole infected cell, morphologically identifiable, is used as antigen.

c. The test can reflect experience of malaria by an individual or community.

d. The test is adequately sensitive.

e. At higher titers, the test is virtually always specific for malaria and sometimes can be used to indicate species prevalence.

Limitations

a. The reading of results is subjective at present.

b. Malaria parasite carriers can occasionally give negative reactions. This has been observed especially with children.

c. The necessity for an expensive fluorescent microscope and trained personnel as to limit its use to well-equipped laboratories.

3.1.1.1.2 Indirect haemagglutination (IHA) test.

The antigen is prepared from parasited cells mainly in schizont or merozoites stages. Parasitized cells can be concentrated by Percoll gradient centrifugation and then freed from contaminating red blood cells by saponin lysis or hypotonic lysis with triton X-100.[9] According to Kagan, treatment with distilled water containing 0.01% triton X-100 produced the highest recovery of the parasites.[2] Soluble antigen from the parasites can be obtained by several techniques including pressure disruption, repeated freezing and thawing, tissue grinding, and sonication.

The antigen is then used to sensitize glutaraldehyde fixed human group "O" cells, or sheep red blood cells with or without pyruvic aldehyde followed by the prescribed procedures.[10-1] The non-specific antibody can be removed during the test performance by mixing 50 μl of plasma or serum sample diluted 1:10 with an equal volume of control red cell suspension in double strength in the first row of the microtiter plate, followed by a brief shaking and incubation at room temperature for 1 hour. The control cells will then sediment to the bottom of the well,

and a 25 μl aliquot can then be taken to initiate a two-fold serial dilution followed by the usual procedure of the test. The IHA test has merits and limitations as follows:[5]

Merits

a. The test is simple to carry out, and no specialized equipment or highly qualified personnel are needed.
b. The test is adequately sensitive and reproducible, and it rarely gives false positive reactions.
c. Large numbers of sera can easily be handled in this test.
d. The test reagents can be prepared in a control laboratory and can be used in many areas, thus achieving a considerable degree of comparability.
e. The test is eminently suitable for field use and action can be taken on the results the day the sera are collected.
f. The reagents can be stored in a small space and can be transported easily.

Limitations.

a. Malaria parasite carriers, especially children or individuals tested during the initial attack, can show negative reactions to the test.
b. Small variations in the test procedure can drastically affect the results. The extract used contains a large number of different antigens of unknown physicochemical nature, and variations or modifications of the technique may lead to a preferential absorption of certain antigen to the red cells.
c. There are important differences in the preparation of reagents and in the performance and reading of the tests in different laboratories.
d. Antigens are available from only a few centres.

3.1.1.1.3 ELISA

Application of ELISA for diagnosis of malaria was first reported by Voller in 1975 using *P. knowlesi* antigen.[11] Subsequently the antigen used was prepared from *P. falciparum* infected blood of Aotus monkeys with parasitaemia over 20%. Antigens from cultured parasites can also be used.[13] Parasitized cells can be concentrated by Percoll gradient centrifugation and contaminating red blood cells removed by saponin lysis or by hypotonic solution in the presence of detergent. The test is performed using the standard technique. ELISA has merits and limitations as follows:

Merits

a. The test is very sensitive, being close to that of RIA, and specific.
b. The test is simple to perform, and thus can be carried out in the field. No highly qualified personnel are needed.

c. Large numbers of sera can easily be handled.
d. Only small amounts of antigen are required per test.

Disadvantages
a. If a single dilution is used and the result is expressed as ELISA value it is necessary to read the results using an ELISA reader which is expensive, and the equipment is too delicate to tolerate traveling in the field. If several serial dilutions are to be made, the results will be expressed in titers, reading of which can be done with naked eyes. Nevertheless it is rather difficult to read the end point without an ELISA reader.
b. The antigens are available only from a few centres.
c. Inherent problems related to plate to plate variations.
d. The extract used contains a large number of different antigens of unknown physicochemical nature, which have different binding properties to the plate. Thus small variation in the test procedure can affect the results drastically.

3.1.1.1.4 Other tests.

Gel diffusion test, counterimmunoelectrophoresis, and radioimmunoassay have been used, but their inherent disadvantages have precluded their extensive uses in seroepidemiology of malaria. Published reports using these tests are limited.

3.1.1.2 *P. vivax*.

IFA, IHA tests, and ELISA have been used for detection of antibody against *P. vivax*. Because of the unavailability of the continuous *in vitro* culture technique for these parasites, the antigens used in the published reports were derived from the blood of patients with vivax malaria, from experimental animals, e.g., Aotus monkeys with *P. vivax* infections, or non-human plasmodia, e.g., *P. cynomolgi*, and *P. knowlesi*.[14-19] Recently, IFA test using *P. vivax* infected cells from short-term culture has been developed (Brockelman et al., 1984, Faculty of Science, Mahidol University, personal communication). It is foreseeable that in the near future, antigens from the short-term culture of *P. vivax* or from parasites enriched from peripheral blood from malaria patients will be available so that improvement of techniques like IHA and ELISA will be made for a better detection of antibody against *P. vivax*.

3.1.2 Candidate tests for "protective" immunity.

3.1.2.1 Test interacting with extra-cellular merozoites.

The antibody can act by inhibition of merozoite invasion, merozoite clustering, and inhibition of merozoite dispersal.[20-23] Merozoite invasion inhibition has been shown to be species specific, not complement-dependent, and is mediated by IgM and IgG and its (Fab')2 fragments but not its Fab fragment.[24] It is most active against homologous strain

than against the heterologous strain.[25] Merozoite inhibitory activity correlates with clinical immunity in 70–80% of human or monkey sera studied.[26]

3.1.2.2 Test of intra-erythrocytic death of malarial parasites.

Addition of human immune sera from Sudan to the culture of *P. falciparum* induced "crisis" forms.[27] This parasitocidal effect of immune sera appears to be confined only to certain geographical locations since immune sera from Flores, Indonesia, could not cause intra-erythrocytic death even though they possessed merozoite invasion inhibitory activity.[28]

3.1.2.3 Reversal of cytoadherence test.

Erythrocytes infected with trophozoites or schizonts of *P. falciparum* are not normally present in the peripheral blood but sequestered along capillaries and vascular endothelial cells.[29] It has been shown recently that 96–100% of amelanotic melanoma cells bound infected erythrocytes much better than endothelial cells of which only 4–59% were bound.[30] Immune sera revert cytoadherence, and this reaction was strain specific.[31–32]

3.1.2.4 Tests for combined action of antibody and cells.

a. Opsonization. The value of opsonization in protection against malaria has been of some doubt. Passive transfer of immune serum in rodent malaria failed to enhance the *in vivo* clearance rate of ^{51}Cr-labelled parasitized cells.[33] Administration of silica which destroys phagocytic cells in vitro does not interfere with the recovery from malaria of vaccinated mice.[34] Moreover, recovery from non-lethal *P. yoelii* infections coincided with decreased anti-bacterial activity of spleen, liver, and peritoneal macrophages.[35]

In human malaria, it has been recently demonstrated that polymorphonuclear leukocytes (PMN) from normal blood donors phagocytosed *P. falciparum* infected red blood cells *in vitro* to a greater extent than normal red blood cells, the phagocytic activity was greatly increased by immune sera but not by sera from individual recovering from a first acute *P. falciparum* infection. The phagocytosis enhancement was mediated by IgG and was independent of complement.[36,37]

b. Antibody dependent cell-mediated cytotoxicity (ADCC). The observation that K cell activity towards chicken erythrocytes is increased in malaria infection is taken as an indication that ADCC may be important in immunity to malaria.[38] Lymphocytes West African infected children and immune adults were found to [illegible] *P. falciparum* in the presence of immune serum.[39] Immune sera have been shown to arm peripheral blood monocytes from normal unsensitized individuals to phagocytose free merozoites but not intact schizonts.[40] A marked difference in the level of merozoite phagocytosis was observed depending on immune status of individuals whose sera were tested, but not on the antibody

levels measured by fluorescence or by precipitation tests. The activity was mediated by IgG and merozoite recognition by armed macrophages was not strain specific.[40]

3.1.2.5 Surface indirect fluorescent antibody (IFA) test against ring infected erythrocytes.

It was recently shown that sera from immune individuals reacted in the surface IFA test, whereas sera from Swedish patients with primary infection was negative.[41] Antibodies eluted from infected erythrocytes inhibit merozoite invasion *in vitro*. It has been suggested then that this test may be related to protective immunity.[41]

Among these five tests, it appears that the surface IFA (if later confirmed to be related to protective immunity) can be adapted for work in seroepidemiology. Other tests, by their nature, are not easily adapted for serological surveys.

3.2 Serological tests against sporozoites.

3.2.1 Tests indicative of malaria exposure.

There have been relatively very limited publications on seroepidemiology of malaria based on anti-sporozoite antibody, the reason for which is obvious. It is difficult to prepare sporozoites in sufficient quantity for seroepidemiology and to overcome technical problems inherent to the tests of which IFA test using glutaraldehyde fixed cells and circumsporozoite precipitation (CSP) test are commonly used. Using these two techniques, Nardin showed that anti-sporozoite antibody against *P. falciparum* could be detected by IFA test in more than 90% of sera from adults living in an endemic area in Gambia, West Africa, whereas most of the samples from children gave low or negative reactions.[42] With CSP test, the percentage positivity was much less. Recent study in Thailand showed that anti-sporozoite antibodies against *P. falciparum* and *P. vivax* were detected by IFA but not by CSP tests in serum samples from individuals living in a village at Karnchanaburi, an endemic area of malaria with low but persistent transmission.[43] Both IgG and IgM antibodies against these two species were demonstrated, with seropositive rate for IgG antibodies in all age groups of 31.6% for *P. falciparum* and 20.8% for *P. vivax*. The seropositive rate was low in children and increased with age. Antibody response to sporozoite infection has been considered to be a better indicator of malaria exposure than that against the blood stage, because the response will be related only to time after exposure, the frequency and doses of infective mosquito bites. The response to the blood stage on the other hand could give rise to a high antibody titer even after a single exposure, because the parasites can increase in numbers through their cyclical development and provide sustained stimulation for antibody production.

3.2.2 Candidate test for protective immunity against sporozoites.

It was only recently that an *in vitro* inhibition of sporozoite invasion (ISI) was developed, and this could be used for assessment of protective immunity against sporozoites.[44,45] It was shown that monoclonal antibodies (MAB), or its Fab fragments, to CS protein of *P. berghei* completely inhibited the entry of sporozoites into cultured human embryonic lung cells.[44] The ISI test was later extended to human malaria. MABs to CS proteins of *P. falciparum* and *P. vivax* were shown to block *in vitro* invasion of homologous plasmodia to cultured human hepatoma cells.[45] The ISI activity was independent of the geographical origin of each strain. The possible correlation of the ISI test and protective immunity is evident by the finding that sera from adult Gambians and a volunteer immunized with *P. falciparum* were ISI positive when tested against *P. falciparum*, whereas sera from Gambian children, a volunteer immunized with *P. vivax*, and healthy controls were negative.[45]

4. Applications and Interpretation

Seroepidemiology of malaria had been conducted in several places in the world with the goal of obtaining data on the levels and patterns of antibodies to malaria antigens in the sera of population groups in relation to relevant variables and thus to contribute to the knowledge of the epidemiology of malaria. Seroepidemiological studies have in general been approached by three different types of surveys comprising single cross-sectional surveys, repeated cross-sectional surveys (repeated surveys of the same population, without identification of individuals), and longitudinal surveys (repeated survey of the same population with identification of individuals). The first kind of survey is more common than the second and the second more than the third. Usefulness of malaria seroepidemiology has been outlined in the introduction and will be highlighted in this section.

4.1 Establishment of rates of malaria endemicity.

In the past, malaria endemicity was rated according to splenomegaly and parasitaemia. Neither method is entirely satisfactory. Enlargement of the spleen may be caused by other common diseases, and incidence of parasitaemia alone can fail to present an adequate picture of the pattern of malaria in a population. The use of anti-malarial drugs may affect the parasitaemia rate without a proportionate reduction of transmission.[46] Introduction of serological surveys has proved to be useful in establishing malarial endemicity rates.

McGregor in 1965 determined fluorescent antibody levels in populations living in 4 villages in Gambia using a single cross-sectional survey and demonstrated the differences in serological age profiles in these villages indicating different patterns of malaria endemicity.[46]

Draper assessed the level of endemicity in an endemic area in Tanzania 11 years after discontinuation of a control programme. The vector densities and the theoretical inoculation rates had returned to the levels observed before the control programme began. But the age-specific parasite rates were much lower than expected.[1] The serological findings suggested that more transmission was occurring than was shown by parasitological findings: 84% in children one to two years of age, and 99% in the two to four year age-group. The dissociation between the parasite rates and the serological prevalences was attributed to the widespread use of anti-malarial drugs, which reduced the level and duration of parasitaemias and therefore their immunogenicity but did not reduce the malaria transmission.[1]

4.1.2 Assessment of changes in the degree of malaria transmission, usually during or after malaria eradication or control operations.

The effect of anti-malaria measures can be monitored by serological testing to observe the progressive changes of the antibody titers, or of the antibody prevalence in repeated surveys, or by cross-sectional surveys some times after implementation of the control programme.

Bruce-Chwatt in 1975 applied IFA test using thick blood film of *P. falciparum* from an infected chimpanzee in a serological survey of 4,605 individuals living in Hemathia, Greece, after the introduction of an active malaria control programme in 1946.[47] It was shown that all 2,965 individuals with the age below 20 were negative, but the antibody titers in individuals above 20 had rising antibody titer gradients with increasing age: this finding was interpreted to mean that malaria was disappearing from Greece. A similar study conducted earlier by Bruce-Chwatt in 1973 showed the virtual absence of positive IFA test in children below the age of five years in Mauritius, thus providing serological evidence of eradication of malaria from this country.[48]

Lobel et al. in 1976 carried out repeated cross-sectional seroepidemiological survey of falciparum and vivax malaria in Guyana in 1973 and 1974 using IHA test.[49] Seropositive rates (titer > 1:32) were rare in people in the age groups below 30 years, suggesting elimination of malaria transmission after implementation of the control programme.[49]

Mathews et al. applied IHA test for falciparum malaria in the repeated cross-sectional serologic survey in Tobago, West Indies.[50] The seropositive rates in samples collected from the same individuals in 1955 and 1966 dropped from 79% to 10%, indicating that the eradication programme was successful.

Meuwissen conducted a single cross-sectional IHA field survey in Surinam in areas with different phases of the control programme.[4] The seropositive rates (> 1:40) in areas in the maintenance phase or in the consolidation phase were 4 and 20%, respectively, whereas the rates in three other areas in the attack phase were much higher (71, 61, and 74%).[4]

Cornille-Brogger et al. conducted a collaborative longitudinal serological survey in conjunction with epidemiological parameters in Garki District, Nigeria, covering 8 villages consisting of approximately 3,000 persons using IFA, IHA, and gel diffusion tests for *P. falciparum* and IFA only for *P. malariae*.[4] It was shown that GMRT in individuals in protected villages was much lower than those in unprotected villages in the same age group, especially those with < 26 years of age during the 4th and 5th survey periods (50 and 70 weeks of protection). In a corollary study, Molinaeux showed that protected infants also had lower antibody titers than unprotected infants.[52]

Voller et al. carried out a longitudinal serological study of falciparum malaria in the West Africa Savanna using the ELISA technique showing a lower seropositive rate (ELISA value > 0.2) in protected individuals than that in the unprotected group of the same age.[53]

Warren et al. continued a longitudinal serological study in population in an endemic area in El Salvador using IFA test by taking blood samples of one resident from each of 268 widely distributed houses. An increase in the number of individuals with seroconversion from negative to positive or a two-fold increase in titer confirmed the continued transmission of malaria.[54]

Jacobs et al. conducted single cross-sectional serological surveys using IFA test in two districts, Sarapee and Li, in Northern Thailand covering 30 villages each.[55] The seropositive rate in children with the age of < 15 year was almost zero (0.39%) in Sarapee District and 2.9% in Li District, which correlated well with results of malariometric survey showing absence of transmission in Sarapee District and low rate of transmission in Li District.

4.1.3 Specific epidemiological assessment of malaria.

a. Delineation of malarious areas.

Serological surveys have been shown of value in delineating foci of malaria transmission. In a longitudinal seroepidemiological study in El Salvador, Central America, Warren et al. showed that foci of malaria transmission existed in coastal plains and were much reduced in the interior.[56] The seropositive rates (IFA antibody titer > 1:20) in persons under 15 years in 3 coastal villages were 2.3, 7.0, and 2.5% whereas those in all 3 villages in the interior were negative.[56]

Jeffery et al. applied IFA test in the repeated cross-sectional survey of malaria in Cuiaba Sector and Campo Grande Sector of Mata Grosso State, Brazil.[57] A higher seropositive rate (9.3–13.6%) was found in Cuiaba Sector and a lower rate (4.3%) in Campo Grande Sector. The maximum positive responses in Cuiaba Sector were to *P. falciparum* antigen while in Campo Grande, only 46% of the maximum titers were for *P. falciparum*. Furthermore, the seropositive rate and GRMT were different in different localities even in the same Cuiaba Sector. Seroepidemiology is therefore helpful in delineating malaria endemicity.

A single cross-sectional seroepidemiological study using IFA test in 6 villages on the Pacific side of Costa Rica was conducted by Warren et al.[58] There were no positive responses in individuals under 15 years in 3 villages and minimal responses in the remaining 3 villages with seropositive rates of 1.1, 2.2, and 3.2%. The result lent support to the surveillance data for the absence of transmission in these villages. Positive responses in some individuals could be due to old or imported cases rather than local transmission.

b. Altitude delineation of malaria transmission.

Marked differences in the serological responses were found in people living above and below the critical altitude of malaria transmission.

Collins et al. compared the IFA seropositive rates and GMRT using antigens of *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* in people living at high and low altitude, in Ethiopia. Thirty-six point seven % of persons living at altitude of 6,000 feet or less were positive whereas only 4.3% of persons living at elevation of 6,300 feet or higher were. Similar observation was made by Voiler and Bruce-Chwatt in the United Republic of Tanzania.[4]

Kagan in 1969 reported a high IHA prevalence in individuals living below 1,300 m in Nepal, whereas in persons living above that altitude the IHA prevalence was low.[60]

c. Seasonal changes of malaria antibody.

Mathews and Dondero in 1982 carried out a follow-up survey of malaria antibody every 4 weeks for a period of one year in 62 individuals in an endemic area in Malaysia using IHA test with *P. falciparum* antigen.[61] Serological responses were found to vary from month to month. In most individuals, titer increases occurred simultaneously with or 4 weeks after the diagnosis of parasitaemia by blood examination.

4.1.4 Identification of areas or individuals requiring action with regard to malaria, especially during the late stage of malaria control programmes.

It is important in the control of malaria to detect the delimiting foci of persisting or renewed malaria transmission, so that immediate action can be taken to prevent further spread of malaria.

Tikasingh et al. used IFA test to detect additional cases of malariae malaria during a recent outbreak in Grenada where malaria has been eradicated.[62]

Ambroise-Thomas et al. could delineate two areas in Corsica to have malaria transmission even before the cases could be detected.[63]

Using IHA test, Kagan observed the presence of antibodies in the one to four-year-old age group in Bulla Quchi, Afghanistan, but was negative in the same age group in Saidabad.[2] Such finding predicted malaria resurgence in Bulla Quchi, which was subsequently proved to be the case.

5. Critiques of Published Reports on Malaria Seroepidemiology

Though numerous reports on seroepidemiological surveys have been published, thorough readings of these reports reveal some shortcomings which could be improved using more careful planning, new knowledge, and technologies which have been accumulated in recent years so that more meaningful information could be obtained. Some of these shortcomings are highlighted below:

a. Most of the published serological works have been concentrated only in descriptive epidemiology trying to answer three kinds of questions concerning: (1) the presence or absence of malaria transmission, (2) the intensity of transmission, and (3) the change of transmission using three general approaches comprising single cross-sectional surveys, repeated cross-sectional surveys, and longitudinal surveys.[64] Repeated cross-sectional surveys provide more epidemiological information than single cross-sectional surveys, and longitudinal surveys allow answers to the same question as repeated cross-sectional surveys but have added advantage in being more sensitive.

There is, however, little work on application of serological surveys in analytical epidemiology to enable investigators to identify populations at risk, factors contributing to transmission and how they contribute. This type of study is considered important in the control of malaria in endemic areas with low but persistent transmission, so as to obviate the high cost of operation based on blood examinations which are known to be less sensitive and would yield results incommensurate with the works involved.

b. The published works are confined almost entirely to the blood stages and not on other stages like sporozoites or gametes. Demonstration of antibodies against these two stages is important in view of the ongoing development of sporozoite and gamete vaccines. Anti-sporozoite antibodies against human plasmodia have been demonstrated in sera from people living in endemic areas in Gambia and in Thailand.[42,43] For reasons stated earlier (3.2.1), anti-sporozoite antibody could be more closely related to the frequencies of malaria exposures than that of the blood stages, thus determination of anti-sporozoite antibody would help in assessing the relationship between man and infective mosquito contacts without interference by growth and multiplication factors occurring in the blood stages. Determination of anti-sporozoite antibody would help in dissecting only the immune response to the sporozoite in the future "trivalent" vaccine comprising of antigens from sporozoites, blood stages, and gametes. Likewise, determination of anti-gamete antibody will help in assessment of the role of anti-gamete antibodies in the population and thus highlighting only the immune response to the gamete in the future "trivalent" vaccine.

c. All the tests so far reported in the literature are measuring only the

level of antibodies signifying the presence and the magnitude of previous malaria experience but do not contain information of protective immunity. Putative tests for "protective" immunity (outlined in 3.1.2) should be more extensively tested and verified to be used in the assessment of the level of immunity in the population before and after vaccination.

6. Serological Methods for Evaluation of the Impact of Vaccination on Control of Malaria

As availability of vaccine for use in the field trial for malaria control appears imminent, strategic planning involving the use of serological methods has to be made to effectively evaluate the impact of vaccination in the control of malaria. Serological methods should help in providing answers to following questions:

1. Do vaccinees develop adequate levels of immunity after vaccination? How shall we assess protective immunity? When will protective level of immunity begin to appear and how long will it last?

2. Does vaccination have any effect on transmission of homologous as well as heterologous plasmodial species in reducing intensity (a decrease in proportion of seropositive) or causing changes in intensity of transmission (reducing the proportion positive or reducing in GMRT)? Or has the vaccine no beneficial effect at all?

3. Will vaccination induce any untoward reactions including hypersensitivity?

In order to answer the above questions, steps have to be taken as follows:

6.1 Choice of tests.

6.1.1 Tests reflecting malaria experience. The tests detecting antibody against the blood stages include IFA, IHA, and ELISA for *P. falciparum* and IFA for *P. vivax*. IHA and ELISA for detecting antibodies against *P. vivax* can be developed in the future. The merits and limitations of these tests have been discussed in section 3.

In selecting the tests, Kagan has laid down some guidelines as follows:[6]

a. The test has to be simple to perform.

b. The interpretation of results must be free of subjectivity.

c. The test must be rapid.

d. The cost must be minimal.

e. The test results have to be sufficiently sensitive and specific.

6.1.2 The tests reflecting malaria exposure. The tests will be used to detect antibodies against sporozoites. Two tests comprising IFA against glutaraldehyde-fixed sporozoites and CSP have been developed,

among which IFA is most widely used, because it is more sensitive and by technique more practical to perform. Recently a solid-phase immunoassay using synthetic peptide (NANP)$_3$ has been developed (Zavala, F., New York University Medical Center) which makes possible determination of antibody against *P. falciparum* sporozoites and thus obviates the need for the supply of infected mosquitoes. Hence IFA and solid phase immunoassay using (NANP)$_3$ should be selected for use.

6.1.3 The tests for "protective" antibodies. Candidate tests for "protective" antibodies against blood stages and sporozoites have been given in 3.1.2 and 3.2.2, respectively. Among the tests for blood stages, it appears that the merozoite invasion (parasite growth inhibition) and the surface IFA test against ring infected erythrocytes can be used. Another test, the immunoblot, should be considered as a candidate test even though it is not strictly related to protective immunity. The immunoblot technique could be considered as a substitute to the radioimmunoprecipitation (RIP) test, which has been used to demonstrate quantitative and qualitative differences of some precipitating bands occurring in immune sera and sera from non-immune persons after primary malaria attacks.[69,70] The immunoblot technique has the following advantages over the RIP test: (1) Proteins which are deficient in methionine, e.g., Mr 155 glycophorin binding protein, or high molecular weight S antigen, can be demonstrated by the immunoblot technique but not by the RIP test.[70] (2) The procedure for immunoblotting is less complicated than that of the RIP test. (3) The cost to perform immunoblotting is less than that of the RIP, because ^{125}I used in the immunoblot is less expensive than ^{35}S-methionine used in the RIP test. Besides, the non-radioactive technique can be developed for use in the immunoblot but not yet for the RIP test.

6.2 Use of serological surveys before vaccination.

Serological surveys have to be conducted along with other descriptive epidemiology surveys to gather base line information, and at the end of the survey period, the following relevant information is expected:

a. Important epidemiological factors (hosts, parasites, vectors, and their environment) related to malaria transmission will be known.

b. Population at risk will be identified.

c. Background immune status will be known.

6.3 Decision making before launching vaccination programme.

The base line information collected in 6.2 may help in the decision making for vaccine field trial as follows:

a. Selection of place for vaccine trial.

Only the places within the flying ranges of mosquitoes from their breeding places are good candidates. In the case of *A. minimus*, the dis-

tance of approximately 2 km from both banks of the running streams should be selected. Selection of housing beyond this perimeter is likely to be non-productive.

b. Selection of vaccinees.

Vaccination should be given to population at risk (identified during the base line surveys). In the study conducted by the Department of Tropical Hygiene, Bangkok Faculty of Tropical Medicine, at villages in Karnchanaburi, the population at risk were those under 30 years of age. The study in the non-risk group is unlikely to yield fruitful results.

6.4 Follow-up period after vaccination.

All population at risk in the same village should be divided into two groups, one receiving vaccine and the other receiving placebo. Serological surveys should be conducted at monthly intervals by taking finger-tip blood samples into 1 - 2 Natleson tubes each if possible, if not collection in several regular-sized capillary tubes is done instead. The plasma should be separated from blood elements within 6 hours after collection and stored in a freezer compartment of a domestic refrigerator. Attention should also be paid to the unvaccinated controls, among them some will develop malaria and the other do not. Comparison of serological findings between these two subgroups alone may provide information related to development of natural immunity in the populations under study.

After the completion of the study, comparison of serological responses between the vaccinated and unvaccinated groups will be made. It is expected that the outcome would provide at least some answers if not all to the questions addressed at the beginning of this section, and whether or not the vaccine is effective against malaria.

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Evaluation of the Impact of Immunization on the Control of Malaria

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During the past five years we have witnessed the truly dramatic research effort that will lead to a powerful new tool, that which will induce immunity to one or several stages of human *Plasmodium*. The status of the various potential vaccines has been well summarized during this conference and in several recent publications.[1,2,3] These vaccines will become available during an important phase in the renewal of conceptual, administrative, and financial commitments to malaria control. Coincident with the enthusiasm for dealing with malaria with innovative epidemiologic and operational approaches is a continuing erosion in the efficacy of available interventions—specifically of insecticides and malarial drugs. The advent of vaccines represents a major opportunity generated by the hope that the weapons available for malaria control are not hopelessly limited.

All of us, immunologists, genetic engineers, health planners, as well as malaria program staff, must understand the current challenges and evolving strategies for malaria control. No malaria vaccine is going to radically change the issue of malaria control nearly as much as the challenges of malaria control in the 1980s–1990s will define the eventual impact of vaccines.

Malaria control has evolved as a public health strategy, sharply contrasting with the concepts and methods of malaria eradication. Inherent in malaria control strategies are several factors:

1. Recognition of the great heterogeneity in malaria from both technical and operational standpoints requires operational efforts tailored to the local needs.
2. Control efforts in most areas of the world need to be formulated with the assumption that malaria will not be eradicated; consequently, major emphasis has to be placed on developing long-term commitments.
3. In the future, public health policy will deemphasize disease-specific

programs and favor integrated health-care delivery and preventive efforts, with a strong focus on local health-care initiative.

In the 1970s a general framework of tactical variants for malaria control was proposed by the World Health Organization.[4] These tactical variants define four increasingly more inclusive objectives, ranging from initiatives to control the health consequences of the disease malaria to programs that in addition incorporate efforts to limit or eliminate malaria transmission. Conceptually, the tactical variants have been important because they have affirmed strategies for malaria control that focus on the disease, that support malaria control in areas where vector control is not feasible, and that suggest a phased inclusion of more technical and expensive interventions as resources and epidemiologic knowledge permit.

Malaria vaccines, as we currently understand them, should adapt well to malaria control strategies; that is, certain vaccines may be particularly suited for blocking infection or limiting the risk of complicated disease, while other vaccines might supplement malaria transmission control strategies.

In this paper we will review the broad epidemiologic situations that malaria vaccines will confront. We will then consider the evaluation methods required to plan and assess malaria control and discuss whether these methods will be appropriate when vaccines are incorporated into the malaria control interventions.

The first epidemiologic setting to consider occurs in areas of the world with high levels of transmission where organized health services are limited and malaria control efforts are rudimentary. A sizable portion of sub-Saharan Africa, Papua New Guinea, and the Amazon Basin has uncontrolled, high levels of transmission; *P. falciparum* constitutes a high proportion of the malaria and malaria is a major contributor to mortality and morbidity. The most prominent feature of malaria under these conditions is a lack of knowledge about its epidemiology.

In such circumstances, transmission cannot soon be interrupted, and it has been proposed that controlling the more severe health consequences, malaria-related mortality and morbidity, should be the priority.[5] Malaria drug therapy to high-risk groups—notably young children and women—must be delivered at the community level. Diagnosis of malaria is based on local definitions and symptoms, generally without parasitologic confirmation. Effective implementation of this basic strategy requires that (1) patients or parents must recognize malaria as an illness, (2) there must be a well-supplied source of appropriate therapy and health education at the community level, and (3) there should be a referral system for malaria too severe to be treated in the community. The stage for primary health care is community-based health programs using simple technology to deal with a major health threat, focusing on the development of a sustainable health care infrastructure.

The practical limitations to the effective implementation of this strategy are:

1. many areas lack even this basic health care infrastructure with trained staff, and
2. the best intervention, drug therapy, may be compromised to varying extent by drug resistance.

As we consider the evaluation of malaria control aimed at limiting mortality and morbidity, we encounter several major problems. Foremost among these is the lack of uniformity and adequate sensitivity or specificity in the malaria diagnostic procedures used to decide which symptoms, disease, or even parasitemia require therapy. There is a pressing need to systematically reassess criteria for the case definition of malaria at the local level; these criteria are needed to systematize the delivery of therapy and to evaluate the interventions, including vaccines. A related issue is the persistent problem encountered in epidemiologically defining and measuring the magnitude of malaria-related mortality or morbidity. Particularly in areas such as sub-Saharan Africa, where the prevalence of *P. falciparum* infection is high, the association of a febrile illness leading to death with a coexistent parasitemia may be an extremely inaccurate estimate of the contribution of malaria to overall mortality. In a sizable proportion of deaths, malaria may be only a contributing factor along with other infectious or nutritional diseases.

The WHO Expanded Programme on Immunization (EPI) has developed formats for rapid and accurate assessment of the coverage of immunization programs; the proportion of children adequately immunized is used as the principal evaluation statistic for national programs.[9] Similar methods for malaria control, in which the program objectives stress extending services to entire populations (e.g., for chemotherapy), need to be developed.

How might malaria vaccines enhance our ability to control malaria-related mortality and morbidity in areas of the world with high levels of transmission and limited resources? Clearly a vaccine that could induce disease-protective immunity in segments of the population at risk of severe *P. falciparum* illness would have greatest attraction. Vaccines that could completely block infection would accomplish the same end, yet such absolute protection may be difficult to achieve or sustain in many rural areas of the world.

The assessment methods used for drug-distribution strategies must apply to malaria immunization. It will be important to identify groups at highest risk, since restrictions in finance and manpower mandate targeted use of even inexpensive malaria control measures. We must learn more about the epidemiology of malaria—the factors that predispose to morbidity and mortality. The new generation of antigen-specific serological tests will permit us to understand malaria as never before.

Much of the assessment required for rational malaria control, however, will continue to depend on tools of basic epidemiology as well as on more sophisticated diagnostic methods.

The second epidemiologic setting to consider is the malarious areas where programs to control malaria transmission can be instituted or strengthened. In the framework of current malaria control strategies, the malarious areas in which long-term commitment to vector control can be considered are generally where malaria prevalence and disease consequence are considerably less than in circumstances we have discussed in Africa. *Plasmodium vivax* is frequently the predominant malaria species. Basic diagnostic and therapeutic services extend to many malarious areas, largely because of malaria surveillance and operational activities. The disease consequences of malaria, such as the retardation of socioeconomic development, are real, yet have proven to be difficult to measure or assess epidemiologically.

The areas where transmission control appears possible are in the Americas and some areas of the Indian subcontinent and Southeast Asia. In these countries malaria eradication efforts had an enormous impact, even though they proved not to be totally successful. Whether sufficient political, financial, and manpower resources can be mobilized for widespread *Anopheles* control is unclear. Certainly the problem of insecticide resistance makes vector control a less attractive strategy than it was during the early years of the eradication campaigns in the 1950s–1960s.

How will malaria vaccines fit into these epidemiologic circumstances? We can ask the question in another way: Can vaccine-induced immunity be a sufficiently potent epidemiologic tool to be cost beneficial in areas where the program objective is to limit the transmission of malaria? Such immunity would need to either dramatically and rapidly eliminate asexual replication prior to gametocyte formation or specifically attack gametocytes or parasite development in *Anopheles* vectors of malaria.

Where transmission control programs are focused on special epidemiologic situations, such as urban areas, vaccines that reduce the probability of infected individuals infecting mosquitoes, combined with mosquito control efforts, might be a major adjunct to malaria control. Immunization on a broader scale, with the objective of completely halting transmission—eradication—would have to be considered with great caution and more knowledge than we currently possess.

In areas where *P. vivax* transmission is a major problem, its importance in a malaria immunization program is worth considering. We believe that *P. falciparum* infections carry a real health threat and can understand how even a relatively expensive intervention, as immunization well may be, would be supported. If immunization could contribute substantially to interrupting *P. vivax* transmission, then I suspect

that the support would be there. Would support be there for a *P. vivax* vaccine that limits the illness (a blood-stage immunity) without measurably diminishing transmission? We must know more about the clinical epidemiology of *P. vivax* infections before we can answer that question.

The entomologic components of malaria control and assessment programs were defined largely by the objectives of the WHO malaria eradication campaign. Variables such as the human-mosquito contact rate, the mosquito survival rate, and the human blood index were components of transmission models based on the concept of vectorial capacity.[7] With such models it was theoretically possible to predict levels of intervention that would completely interrupt transmission. These variables and others, such as the sporozoite rate, also provided direct measures of the ongoing level of transmission.

When vaccines are incorporated into malaria control programs, epidemiologic variables such as the sporozoite rate and human-mosquito contact rate, which reflect ongoing transmission rather than transmission potential, will continue to be of value. The information of most value in helping decision-makers to choose a vaccine strategy will be measurement of the frequency with which a person is exposed to a new infection. Thus newer methods of measuring this entomologic inoculation rate (such as more sensitive or simpler sporozoite detection assays) will be important.

Limiting transmission by immunizing the human host as opposed to direct attacks on the anopheline vector would allow more selectivity in our control application. Field studies have indicated that not all members of a population in malarious areas are contributing equally to infecting mosquitoes. Several investigators have found that children are the principal gametocyte carriers in some areas of Africa.[8] To learn more about this potential association, we need to investigate not only gametocyte carriers but also the ability of individuals to infect the coindigenous *Anopheles* vectors. This goal can be accomplished by well-designed studies of mosquito infection by humans and also potentially by *in vitro* assays.[9]

If the subset of the human population responsible for most mosquito infections could be identified, then immunization could be targeted. This would be particularly important if children, the likely candidates for blood-stage vaccines to control disease, could be immunized with effective multistage vaccines. Finally, we must recall that, despite the problems of insecticide resistance, the principal impediments to sustained attacks on malaria transmission have been the administrative and financial problems encountered in sustaining the reductions in the entomologic inoculation rates. The lesson of malaria eradication should not be missed: for achievements to be sustained, disease control programs must be incorporated into health services. Where adequate health services do not exist, we must assist in developing them with the

same commitment—technical and administrative—that we have to developing malaria vaccines.

The third epidemiologic setting that confronts us today is in areas where there is either a time-limited or geographically focal risk of malaria infection. In general there are two common examples of this epidemiologic setting. First, laborers or other individuals who frequently move from non-malarious areas may for defined periods live in areas of malaria transmission. A notable recent example occurred in Southeast Asia, where Khmer refugees who crossed western Kampuchea on their way to Thailand passed through highly malarious areas. High mortality rates of *P. falciparum* infections occurred in all age groups.

The second example of transient malaria exposure is the special problem of non-immune tourists or military visiting malarious regions. In general, this is a time-limited exposure. The risks are experienced by all age groups, and any of the human *Plasmodium* may be present. Under these conditions, the objective is optimally to block infection or at least to substantially decrease the risk of disease should the person become infected. Currently, antimalarial drugs used either prophylactically or, on occasion, to presumptively treat infections, are the most efficient methods of eliminating the risk of malaria. When prophylaxis is complied with and the drug is effective and safe, malaria can be effectively prevented.

The major impediments of effective drug prophylaxis are well known: drug resistance in *P. falciparum*, toxicity of some antimalarial drugs, and *compliance*. With tourists or military, education or coercion can to an extent assure reasonable compliance with malarial drug prophylaxis. For labor forces, refugees, or illegal immigrants, drug prophylaxis may be completely impossible.

An effective infection-blocking vaccine could potentially overcome most of these problems, even if the duration of protection was relatively short. Evaluation of vaccine efficacy under these circumstances would be quite straightforward. Epidemiologically, the population at risk must be clearly identified and the time period of risk must be defined. For immunization, we must know to which *Plasmodium* species protection is required. The issues of protection for these groups will be resolved in Phase III and IV vaccine trials.

In conclusion, malaria vaccines, regardless of their potential vaccine efficacy, will have to fit into evolving strategies of malaria control, which are substantially organizational and financial. Equally important is the fact that we need to know much more about basic epidemiology to effectively implement malaria control, even before we have vaccines. The evaluation of malaria immunization will be incorporated into the strategies of malaria control.

Many of our current methods of evaluation derive from malaria erad-

ication concepts and require reassessment. The phenomenal success in malaria immunology will clearly result in vaccines; perhaps equally important for malaria control will be improved tools for malaria epidemiology. Already we have seen the potential for much simpler and highly specific immunologically based tools for sporozoite detection.

The future of malaria serology lies in the use of defined antigens; soon we will be able to understand the natural acquisition of protective immunity, a necessity before a vaccine is accepted. Detection of parasitemia is a real issue—will we even have a replacement for the microscope? I suspect that we could, but the diagnosis of malaria in many rural areas will continue to be based on clinical symptoms alone.

The researchers and supporters of malaria vaccine research hope that their hard work will result in an intervention that will contribute dramatically to malaria control. Similarly, national authorities, in their daily struggle to develop effective malaria control programs, might be tempted to focus on vaccines as a final solution to their problems. We all know that malaria control will not be so simple. We do not need malaria vaccination programs; rather, we must work hard to build malaria control programs that will be able to use malaria vaccines to their best advantage.

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Discussant

ROBIN POWELL

Dr. Zavala described a new species-specific means to identify and quantitate sporozoites in the mosquito host. He also described the use and potential value of new methods to determine anti-sporozoite antibodies.

Dr. Suleman discussed methodology for calculating vectorial capacity and how this index can be of value in field assessments of transmission.

Dr. Savanat provided a very insightful critique of the limitations of previously used seroepidemiological methods and the potential that now exists for new, much improved seroepidemiological assessments.

Dr. Campbell provided a perspective on current malaria control conditions and potential roles of malaria vaccine as elements of those measures and the need for improved diagnostic methodology and for improved measures for field assessments of epidemiological and entomological variables.

These four presentations illustrate a translation of basic research progress into advances in the methodologies available for use in clinical trials and field studies. We need not only new weapons to attack malaria but also new and better methods to study malaria's epidemiology and the impact of these new weapons. We are in effect entering into a new era of research and development in field study methodologies. Time will be required to test and to learn how to use these new techniques. This changing situation with respect to study methodologies represents in itself a reason to proceed with deliberateness and no great sense of haste in field studies of malaria vaccine.

The pivotal data on the impact of vaccination on malaria control will relate to malaria's morbidity and mortality. En route to enlisting those data, though, we will need to proceed one step at a time to improve our abilities to obtain conclusive data, particularly for larger more complex field studies. While, as Dr. Bruce-Chwatt cautions, we should avoid trying to do too much in individual field studies, it may be useful to view the context of clinical trials and field studies as having two related ele-

ments—one to assess vaccine effect, the other to conduct basic and applied research on malaria's biology. We now have the opportunity to link basic and applied research in new ways. I think we should do all we can to foster that linkage. For example, if there are vaccine failures, we should try to be in a position to determine then and there, through basic studies involving the parasites that break through and their hosts, to pinpoint the reasons for the failures.

We will encounter substantial pressure to expedite vaccine trials and to apply instantly any positive results of such trials. Given the basic and applied R and D that remains to be done, we should consciously resist those pressures. We should exercise great care to set appropriate perspectives and expectations. In particular, we should court Delilah of the Press with considerable circumspection. We may inadvertently lead others to believe that a panacea is just around the corner, when in fact it seems likely that with the basic research still underway, the variety of candidate vaccines, the unanswered questions on adjuvants and carriers, ethical issues, and needs to improve study techniques, we may well be conducting Phase I and Phase II studies with different preparations or groups, and we may well be continuing to work on major field study methodologic questions for at least the next 10 years.

S78-

General Discussion

MODERATOR: I would like to now open the general discussion period. We have ranged rather widely this morning from quite specific technological advancements to some rather broad discussions of epidemiological methods. I hope that those who had comments on the earlier papers on the innovative techniques which were described by Drs. Zavala and Suleman will remember those comments and questions and will proceed to ask them.

PEREIRA DA SILVA: I would like to ask two questions to Dr. Zavala. For the affinity binding of the monoclonal antibodies against the treatments of the monomers of the peptide did you find that the antibody which binds to the mono or the repeats could be removed and then compared to the binding to the treatments? In other words, would you have perhaps some antibodies of higher affinity recognizing particular epitopes of the repeats which would be more relevant and affinity binding studies than the other which are obligatory recognizing the treatment of these peptides?

ZAVALA: We have not done much about that but monoclonals were, in general, all within the NP3—the three repeats. Some of the monoclonals did react very badly with two repeats. Now, as far as I know, I have learned that there is no relationship between the affinity of the antibody and their capacity to bind to either two or three. We simply do not exactly know what the situation is, but a very low affinity antibody can be inhibited by two repeats while the other higher affinity can be inhibited only by NANP3. There is no relationship or correlation between the affinity of the antibody and their capacity to react with NANP2. That is all that I know about that.

MARTIN: I had a question about the relative applicability of radioisotopic technique vs. ELISA technique vs. the most simple hemagglutination inhibition technique in the field. Can you comment whether the

radioisotopic technique is actually done in the field or back in New York?

ZAVALA: Yes, thank you. I didn't have time to go further on that but both techniques I spoke about you can use either RIA or ELISA. Some time ago, we did the mosquito assay by using enzyme-labelled antibody and the sensitivity was comparable. I saw that the ELISA could be used without any problem. There are minor differences, but basically the same thing. I did normally work with Dr. Robert Wirtz from Walter Reed Hospital trying to standardize further the ELISA that can be used for the mosquito assay. In reference to the antigen by using the peptide of the same thing, you can use an ELISA or RIA. There is no difference. I used radioimmunoassay because it is a little better for me, but I see no reason why ELISA will not work in both assays.

PERRIN: In relation to the question of hemagglutination, a lot of these people have relative factors. When you come to hemagglutination titer you have wrong sensitivity. In relation to the feasibility of the ELISA test, you can have an optical density you can see by the eye. For example, the surface antigen you can do by eye and have a perfect fitting. So, if you had a sensitive assay to be read by the eye, like a peroxidase assay, you could see it easier than you could the hemagglutination assay. So, it could be used in every country where the supply of material is difficult.

SHARMA: I have two questions. One for Dr. Zavala about the sensitivity of ELISA vs. immuno technique and one for Dr. Suleman about vectorial capacity concerning whether the sporozoite alone would not be enough to monitor the antibodies in the population as against the vectorial capacity.

ZAVALA: The sensitivity of ELISA has been determined by using different antibodies in our laboratory as well as in Walter Reed. It is almost the same. With the IRMA technique, you can detect sporozoites and with ELISA you can determine 100 and 150. The difference is minimal considering that more than 90% of the mosquitoes in the field have more than 1,000 or 2,000 sporozoites. So, the difference that exists is not really meaningful.

BRUCE-CHWATT: It seems to me that Dr. Zavala's presentation today represents a quantum jump in the possibilities of research in quantitative epidemiology. Those who use their years laboriously dissecting mosquitoes, losing usually one log and then exacting one remaining log and squashing it and seeing the sporozoites, will realize what a tremendous advance it is. In a way, it seems to me that this is an advance in quantitative and qualitative epidemiology of malaria that is so great that it is going to revolutionize quite a number of our concepts of

malaria epidemiology. I see that this method is going to abolish at least one test that was often used in malariology, namely, the blood precipitant test. What is the point in looking for blood precipitants of different animals, or of the *Anopheles* mosquito, if we have direct evidence of the infection and of the origin of the sporozoite?

However, that is secondary. What Dr. Zavala's data showed this morning when he showed the tremendous difference between the amount of infection actually transmitted and the amount of the infection that is calculated by traditional methods confirms even to a greater degree this puzzling difference between the inoculation rate derived entomologically and the inoculation rate derived actually from parasitological studies. You might remember that it was MacDonald who pointed out that entomologically derived inoculation rate is, certainly in tropical Africa, 100 or 200 times greater than the actual inoculation rate calculated from the infectivity of infants. Well, Dr. Zavala's data showed that it is probably not hundreds of times greater, but thousands of times greater, if not more. This alone shows the fantastic, the tremendous amount of infective material that is pumped into inhabitants of tropical countries day after day by countless numbers of mosquitoes. It also explains a number of other epidemiological data that are derived. This method will also allow us, probably for the first time, to assess with a certain degree of veracity the transmission-blocking potential of a gametocyte vaccine. This could not have been done before by the laborious method of dissection. It would be possible to do it at the present time. I would like to congratulate, in my personal capacity, Dr. Zavala on this really revolutionary change in our methods of studying the epidemiology of malaria.

I have listened carefully to the problem of seroepidemiology of malaria as discussed by our colleague from Thailand. I have a certain amount of reservation when it comes to the value of seroepidemiology of malaria in highly endemic areas. It seems to me that the information that we derive by seroepidemiology is not as great as we could derive in non-highly endemic areas. Seroepidemiology of malaria in the adult population of tropical Africa is to me nothing more, paradoxically speaking, than a glorified spleen rate because it shows the enormous impact of the infection on the presence of antibodies, IgG in particular, which remain at a very high level throughout the life-time and then may eventually decrease a bit. It is only when we start using the IgG and IgM method of distinction in the antibody that we may find out to what extent we are dealing here in focus with a relatively new infection and old infection. The same thing would apply, of course, when it comes to the assessment of the antibodies in different age groups when only the younger age groups, the infants in particular, are actually the indicator of early transmission.

I was very happy indeed to listen to all five speakers this morning. I would like to say to what extent I absolutely agree with the presentations of Dr. Campbell and Dr. Collins. To me it really was a vindication of everything that I have been saying for a number of years. That being that a tremendous value of vaccine against malaria should be realized only—and this should be understood—only as a new implemental tool. Nothing more than a tool. I was particularly glad that Dr. Campbell mentioned problems related to primary health care. You may perhaps see on page 62 of the green booklet to what extent I agree with Dr. Campbell when it comes to the assessment of the prospectives of malaria vaccine.

I would like to speak longer than I intended to at first. There is perhaps one aspect of malaria control related also to malaria vaccine which I intend to bring up at the next session. That is the problem of cost. Thank you very much.

MEUWISSEN: Concerning the point raised now by Professor Bruce-Chwatt on precipitant tests, I would like to have another view. I think we will need precipitant tests in order to assess the man-biting habits of the mosquitoes in order to assess properly the vector capacity of the mosquito population.

BRUCE-CHWATT: May I answer that? I would not like to be misunderstood. Of course, I do realize the value of lab precipitating tests for studies of the habits of mosquitoes generally, especially *Anopheles*. But is there any immediate practical importance of studying habits of *Anopheles* that do not transmit malaria?

MODERATOR: Would anybody like to comment on that?

WERNSDORFER: I am afraid that we have to understand the factors contributing to malaria transmission when we look into the possibilities of interferring with transmission contact between vector and man. Transmission is an age-dependent factor in relation to the plasmodium in the vector. We also ought to understand that if we have the means and ways of shortening the life span of mosquitoes, then we are able to interfere with the transmission. I believe the sooner we get away from the time consuming and not so exact precipitant methods for blood meal identification, the better it is. I guess there are better methods now in the last quarter of the twentieth century. Thank you.

COPPEL: I would like to second Dr. Bruce-Chwatt's congratulations to Fidel Zavala. I think he has described the test that is going to be indispensable to the setting up and assessment of any malaria trial. I would like to ask him a methodological question. It refers to the studies on the world-wide distribution of the circumsporozoite protein of *P. falciparum*

sporozoites that were also alluded to in the previous couple of days. Now, I wanted to know whether the studies were performed using the two-site assay that you described or whether there were other independent assays on species determination?

ZAVALA: The fact that the species from *P. falciparum* as well as *P. vivax* world-wide do have the same epitope is based, I would say, on three types of evidences. The most important one is the use of the two-site assay. I have tested sporozoites from Asia, Africa, Central and South America, etc., and all of them, all of those isolates, did have the repeated epitope, and recognized the same monoclonal antibody. Secondly, in several cases, though not too many, it has been possible to do immunofluorescence. Third, in some of the species it has been possible also to have an identification of molecule through electrophoresis technique. But mostly what has been used is the two-site assay to reflect different isolates from the work.

COPPEL: What concerns me a bit about that sort of study is the possibility that when you survey mosquitoes world-wide with a two-site assay which only finds the epitope and when that is the basis of your species determination, then if there existed *P. falciparum* sporozoites with a different repeating epitope, a two-site assay would be negative. Your conclusion would be then that there are different species, not that there might exist these different epitopes. Is there any sort of way you can independently verify species?

ZAVALA: That is an important question. However, you have to realize that, for the moment, the only way to classify a species by using the sporozoite would be either by injecting the human being, something that cannot be done, or by using specific antibodies. There is no other way to do it. In blood form you can make a diagnosis with differential species, but in the case of sporozoite it is not possible. The only way we have is the use of the specific antibody. Certainly, we are concerned with whether or not all the isolates all over the world are actually positive for this epitope, and that is what is being done. However, so far I have never seen placebos also negative for their antigen. But it may exist. For those purposes we need something. Most likely, we would need to work with DNA probes and culture blood forms, etc. But, for the moment, we are limited and that is the reality. Although I quite frankly do not believe that there may exist high percentages of negative sporozoite *P. falciparum* that may not have that epitope. The very fact that by dissection you have a 3% of infected sporozoite in the Gambia and that by the assay you have a 5% means that most sporozoites that are in infected mosquitoes do have the repeated epitope and that if there is any variant it would be in the very, very low frequency.

B. INTEGRATION OF VACCINE WITH OTHER MALARIA CONTROL PROGRAMS

Possible Integration of Vaccine with Other Malaria Control Measures in China

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With the recent rapid progress in malaria vaccine research, the possibility of obtaining malaria vaccine for operational use in malaria programmes in the near future is increasing markedly. But it will still take some time to solve all the scientific and technical problems encountered in producing safe and effective malaria vaccine and to complete the clinical and field trials before the vaccine will be made available. At this stage, however, the consideration of vaccine utilization in malaria programmes may be a bit too early; nevertheless, it will be of benefit to future operations and to vaccine production. In formulating strategies, it will, undoubtedly, involve consideration of the potency and the immunogenic properties of the malaria vaccine, which the vaccine producer should also take into account when producing the vaccine.

The Role of Vaccine in Malaria Programmes

Vaccine is often regarded as a preventive tool used to immunize individuals or groups of people to prevent disease. However, potent vaccines, such as smallpox vaccine, poliomyelitis vaccine, measles vaccine, diphtheria vaccine, etc., when strategically used, can interrupt the transmission of these diseases, and hence they can be used alone to eradicate these diseases. The successful global eradication of smallpox is an illustrative example. Unlike these vaccine-preventable diseases, malaria, although being a disease for which man is also the only source of spread of infection, will not be eradicated by malaria vaccine alone, because the immunogenicity and potency of the malaria vaccine are not equivalent to those of potent viral or bacterial vaccines. It is well known that at present the immunity induced by malaria vaccine is transient, species

specific and stage specific. Also it is not known yet what impact immunosuppression, which exists in current and chronic malaria cases always met in malaria endemic areas, will have on the building up of immunity of the recipient after vaccination. However, the frequent repetition of vaccination required to obtain and maintain effective immunity may not only increase the burden of vaccination operation, but also may cause failure owing to disgust, unacceptability, and poor cooperation among the target population. For these reasons, malaria vaccine should not be regarded as an alternative method to be used alone to combat malaria; instead, it should be used, on the basis of its distinct properties, integrated with other antimalaria measures to make up each other's deficiencies to seek strategic combined effect to obtain the desired result in malaria operation.

Since traditional antimalaria measures are generally effective, it will not be necessary to use vaccine extensively as is practised for the control of other diseases under the Expanded Programme on Immunization. Malaria vaccine should be used in localities where there is strict necessity and where other measures have not proved to be satisfactory.

It is conceivable that the traditional measures will be used alone wherever feasible. However, when these measures fail to achieve the desired result, malaria vaccine may be used as a reinforcement to strengthen their intervention capacities to seek a strategic combined effect in order to interrupt malaria transmission. In this context, malaria vaccine will play a distinctive role in malaria programmes, though the traditional measures will still play the major role.

In order to be successful, the combination of measures should be tactically maneuvered on the basis of the epidemiological conditions existing in the locality, so that the obstacles not overcome before may be surmounted by the new tactics in the malaria campaign.

It can be foreseen that malaria vaccine, when available, will be integrated into the malaria surveillance system, under which it will be brought into full play. The effectiveness and efficiency of malaria surveillance operations will also be improved by the reinforcement provided by the malaria vaccine. In conjunction with other measures, malaria vaccine will have application in the elimination of stubborn residual malaria foci, particularly where the malaria vectors are not amenable to control in areas under consolidation or in areas where malaria has been kept under control. Malaria vaccine will provide the condition for interruption of malaria transmission, despite the vectors not being significantly affected by the mosquito control measures. Coordinated by other measures, it may be applied in the highly vulnerable and in the highly receptable localities to raise the immunity level of their residents, so that they may deal better with the menace of imported malaria. It may find use also in the operation to suppress

malaria outbreak in areas where malaria has formerly been eradicated or kept under control, for timely immunization of the people living in the surrounding neighborhood of the outbreak area will prevent further spread of the disease and enable malaria operations to be concentrated in limited areas, whether at the original site of the outbreak or in the affected localities in the immediate neighborhood.

Possible Use of Vaccine in Malaria Operations in China

The present status of malaria is quite different from that in the preliberation days and during the early liberation period. The condition of hyperendemicity that formerly existed in many malarious areas no longer exists; malaria endemic areas are continually decreasing in extent. Except during outbreaks, the intensity of transmission in most endemic areas has been greatly reduced. Such situations, together with the well organized peripheral health services throughout the country, will provide favorable conditions for the utilization of malaria vaccine in localities where it is needed. It can be expected, therefore, when safe and effective malaria vaccine will be available for operational use, it will be employed in localities whenever necessary in malaria programmes to surmount the problems impeding its progress

P. vivax is predominantly prevalent in China. Over 95% of the total malaria cases reported in the country are due to this species and its endemic areas are far larger than those of *P. falciparum*, where *P. vivax* also coexists. Moreover, particularly in the vast *P. vivax* endemic areas in central and northern China, *An. sinensis*, which is not amenable to control, has made the malaria operations in the Huanghuai and the Jianghan plains difficult. The malaria cases reported from these two regions accounted for about 80% of the total cases in this country. Therefore, it is clear that *P. vivax* vaccine will be much more needed than *P. falciparum* vaccine. However, *P. falciparum* vaccine may be brought into full play in the localities whenever needed, for example, in the localities where chloroquine resistant *P. falciparum* strain exists, and in the localities where there is distribution of the notorious vector *An. dirus*, which is difficult to control. For convenience in operation, polyvalent vaccine will be needed in future malaria programmes.

Besides the immunization of individuals or of selected groups of people going to work or travelling in the malarious areas at home or abroad, such as frontier guards and other personnel working in malarious frontiers, constructors of dams, roads, and railroads in the malarious localities, members of prospecting teams working in malarious areas, etc., malaria vaccine will be chiefly used in conjunction with other control measures to resolve problems not solved before to achieve further reduction or suppression of malaria in these problem areas.

As malaria is by nature a focal disease, and in most parts of this country it is unstable, when malaria recedes to localities where it is firmly entrenched, an opportunity may be taken to attack these localities with the combination of malaria vaccine and other control measures to eliminate malaria from these places and to ensure the consolidation and the further expansion of the gains already achieved wherever possible.

Strategic attack on the strategic sites by this combination of vaccine and other control measures may also be conducted to clean out malarious sites in low endemic areas.

Undoubtedly, vaccine will be integrated into the malaria surveillance system and will play an important role in the later and final stages of the malaria programme and during the maintenance phase.

To sum up, vaccine may find application in the following activities in the malaria campaign in China:

1. Immunization of people from nonendemic or low endemic areas going to places still malarious, particularly in chloroquine resistant *P. falciparum* areas, to give them protection while staying in the endemic area and to prevent them from spreading the disease agent when they have left the area.

2. Integrated with other antimalarial measures, malaria vaccine may be tactically maneuvered in coordination with other control measures to attack malaria in localities where formerly traditional measures did not provide desired result. Such strategy will find application in localities where chloroquine resistant *P. falciparum* exists, or where the malaria vectors are not amenable to control, or where both conditions exist.

3. Integrated into the malaria surveillance system, malaria vaccine may be used in operations for elimination of persistent residual foci in areas where malaria has already been kept under control.

4. In coordination with other emergency measures, vaccine may be used in operations for suppressing outbreak, particularly in post-eradication outbreak or outbreak in areas where malaria has already been kept under control.

Concluding Remarks

What has been stated above does not imply that the integration of malaria vaccine with other control measures will be the final solution of the problems existing in malaria control or eradication. What has been discussed is only the possibilities of vaccine utilization in future malaria campaigns. Malaria vaccine, when available, will be one of the valuable weapons in the armamentation against malaria which should be used appropriately to win the fight against this disease. To bring malaria vaccine into full play, it should be integrated with other control measures, and most probably also, integrated into the malaria surveillance sys-

tem, to strengthen their intervention capacities and resolve problems already existing and not solved by traditional measures to achieve further reduction or suppression of malaria in problem areas. With this end in view, vaccine, when used in malaria operations, should have considerable impact on the curbing of malaria transmission to be of significant operational value in China.

Integration of Malaria Vaccines in the Health Delivery System in India

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Introduction

In ancient India malaria was known as the king of tropical diseases. Malaria was the single most important cause of the biggest misfortune degrading the life of people in every possible way. Until 1952, malaria was directly responsible for about 75 million cases and 800,000 deaths annually, with considerable increase in the morbidity and mortality due to malaria during the epidemic years. Malaria was nearly eradicated from the country in the early 1960s as a result of the nationwide anti-malaria campaign launched by the National Malaria Control/Eradication Programme (NMCP/NMEP) of the Government of India. Still, in 1965 there were at least 100,000 parasite positive cases of malaria scattered all over the country. This followed a period of resurgence for various administrative faults, financial constraints, and technical reasons.[1] Initially for several years, malaria cases started to multiply quite insidiously, and at that time NMEP and the general health services were not prepared to tackle the situation effectively. As a result, over the years malaria resurgence and focal outbreaks occurred even in areas that were at one time freed from the disease. To combat malaria the Government of India in 1977 implemented the modified plan of operations (MPO) through the NMEP, thus converting the malaria eradication programme to that of control and containment.[2] The implementation of MPO resulted in the steady decline of malaria cases from 6.4 million in 1976 to 2 million cases in 1984. It may be noted that although there was reduction in total malaria cases in the country, the incidence of *P. falciparum* has remained at about 0.5 million cases annually for several years now.[3]

Control of malaria in the country is beset with many formidable problems, e.g., insecticide resistance in malaria vectors, exophilic and exophagic behaviour of mosquitoes, escalating cost of insecticides, drug

resistance in the malarial parasite *P. falciparum*, and operational failures, etc. As a result the available methods of malaria control have had limited success. It is commonplace to find sudden spurts of malaria where insecticidal spraying was withdrawn (e.g., Haryana State), or in areas where vector species developed high degree of resistance (e.g., Shahajahanpur U.P.).[4,5] Use of replacement insecticides can control malaria for some time (e.g., malathion spraying in Kharkhoda PHC, Haryana[6]), but vectors have the ability to develop multiple resistance resulting in operational failures (e.g., areas in Gujarat and Maharashtra).[4] While bio-environmental methods to control malaria are being developed, malaria vaccines are emerging as the most powerful tools to help control malaria. The availability of three types of malaria vaccines is being contemplated, i.e., (i) the sporozoite vaccine, (ii) asexual blood stage vaccine, and (iii) the transmission-blocking vaccine. In the coming decade or so, all the three vaccines may be available, but sporozoite vaccine is emerging as the first most likely vaccine to be made available for clinical trials. Therefore, immunization against malaria is now becoming a reality. It is against this background that there is an urgency to make appropriate preparations to undertake trials, and plan for the integration of malaria vaccines with the primary health care system, to help realize the Alma Ata declaration of health for all by A.D. 2000.

Immunization Against Malaria

India is a thickly populated country. Latest census (1981) reported 684 million population with a density of 221 persons/sq.km. and 2.47% growth rate. Estimates in 1985 place India's population at about 700 million. The current estimates are that India's population would increase to 799 million in 1991 and 917 million in A.D. 2000. It may also be mentioned that 78% population lives in 575,936 villages and half of these villages have a population of 500 or less. Some villages are located in remote and inaccessible areas. There are at least 164,310 problem villages which have no facility of potable drinking water. Against this background, malaria vaccination is a formidable task. Even the immunization of children (up to 14 years) would be an uphill task as this group alone constitutes about 39% of the total population (273 million).

In India, *P. vivax* is the most dominant infection (70%) followed by *P. falciparum* (30%) with only small number of *P. malariae* cases occurring every year. *P. falciparum* is widely distributed throughout the country with great variation from one region to another. About 40% of all *P. falciparum* infections occur in one state (Orissa) alone. The entire northeastern region has very high incidence of falciparum malaria. To contain/liquidate *P. falciparum* and prevent its spread to other areas the *Plasmodium falciparum* Containment Programme (PfCP) was launched in

1977.[7] PfCP is currently working in 94 million population living in 79 districts.[8] As a result, while there is a considerable improvement in areas under PfCP, falciparum malaria is on the increase in the rest of the country (A. P. Ray coordinator PfCP, NMEP, Delhi, personal communication). Limited surveys conducted by the Malaria Research Centre (MRC) revealed that the incidence of falciparum malaria was extremely high in many areas in the north. Likely reasons for the increase in falciparum malaria have been discussed by Sharma.[8] In particular, mention may be made of U.P. terai, an area well known for green revolution. During the surveys in 1981–1982, there were only isolated cases of *P. falciparum*. In about two years *P. falciparum* has become the most dominant species in terai.[1] Chloroquine resistance, first detected in 1973 in Assam, has spread to most of the states in the country.[9] Fortunately R III level of resistance is limited in its distribution to 8 PHCs but R II is more prevalent and R I resistance is becoming more common. The problem is being tackled by implementing alternate drug schedules and an action oriented programme to liquidate the foci.[10]

During 1985, total estimated target population under the extended programme of immunization (EPI) is about 89 million (60–70% coverage) and it would increase to 140 million in 1990 when 100% coverage is aimed to be achieved.[11,12] Although NMEP is controlling malaria through vector control in areas with two or more API (i.e., 342 million rural and 13 million urban population), malaria affects all age groups and >600 million people are exposed to its risk. Therefore the task involved in the immunization against malaria is much greater than protection against other communicable diseases under the EPI. Obviously, the priorities of malaria vaccination would have to be fixed carefully. The following areas may be considered in order of priority: (i) PHCs showing up to R III level of resistance followed by R II; (ii) migratory labour population which may introduce new strains; (iii) green revolution and industrial areas; (iv) high *P. falciparum* areas, particularly 79 districts (94 million population) under the PfCP; (v) infants (in India infant mortality rate is still very high, i.e., IMR 110); and (vi) children.

Extended Programme of Immunization

Health Services in India have a long experience in the production and delivery of vaccines. A classical example is the eradication of smallpox in 1975. The extended programme of immunization (EPI) was launched in the country in 1978. At present EPI offers immunization against diphtheria, pertussis, tetanus, poliomyelitis, tuberculosis, and typhoid fever. Measles immunization may also be included in EPI shortly. The country is self-sufficient in the production of vaccines, except for poliomyelitis and measles. The major centres of vaccine pro-

duction are: Central Research Institute, Kasauli; Haffkeine Biopharmaceuticals, Bombay; Pasteur Institute, Conoor; BCG Vaccine Laboratory, Madras; several state government laboratories and private sectors. Training courses and workshops are conducted in the country at various levels as part of the manpower development programme.[11]

The EPI would make available vaccination services to all children and pregnant women by 1990, and targets of 88–100% coverage have been fixed. The EPI works through the infrastructure of primary health care system. At the PHC sub-centre level, one male and one female multipurpose worker are responsible for immunization activities for 5,000 population in the plains and 3,000 population in difficult areas. In cities, immunization services are provided through the hospitals, dispensaries, maternal and child health care centres. In addition, private medical practitioners carry out immunization. Selected rural areas are also covered with the help of paramedical health workers and voluntary organizations during the winter months. The central government is responsible for the supply of vaccines, cold chain equipment, training, health education material, and evaluation, etc. The state governments carry out immunization through the primary health care system. EPI is facing many difficulties in the attainment of its goals. These difficulties mainly relate to poor communication, as 80% of the people of India live in rural areas. Although a cold chain has been established, frequent power failures and lack of repair facilities of refrigerators are main handicaps. As a result, it is not possible to extend immunization to the peripheral areas. There is also the problem of high dropout rates, as in many areas people have to walk long distances and many people have mistaken beliefs about the vaccination.[12] The experience in production and handling of vaccines and immunization, etc., would be of immense value in integrating malaria vaccines in the existing infrastructure of the vaccine delivery system.

Primary Health Care

India is a signatory to the Alma Ata declaration of 1978: "Health for all by A.D. 2000." The primary health care is an integral part of the health delivery system in India. The main emphasis is on the preventive, promotive, and rehabilitative aspects of health care. In 1977, based on the recommendations of the consultative committee of experts, the vertical programme of malaria eradication was converted to that of containment and made more flexible, depending on the resources availability.[2] Known as the modified plan of operations (MPO), malaria control was to be carried out through the general health services and the Primary Health Care System.[13] At the central level, the Government of India through the directorate of NMEP was made responsible for policy for-

mulation, technical guidance, evaluation, and the running of special malaria control activities. At the state level, a state malariologist is responsible for the execution of all malaria control operations in his state. The state malariologist implements the programme through malaria zonal officers who are responsible for four or five districts. At the district level, the chief medical officer is responsible overall for malaria control activities. He is assisted by a district malaria officer. The programme of spraying, surveillance, and drug distribution is implemented at the PHC level. There are 5,739 PHCs in the country. While there are separate spraying squads, the surveillance and drug distribution is carried out by the multipurpose worker (MPW). In addition to other duties, MPWs are assigned the duty of blood smear collection from all fever cases through fortnightly visits and the administration of presumptive and radical treatment. A MPW male and not the female is responsible for malaria work for 5,000 population in plains areas and 3,000 in the difficult areas.[8] Since one of the objectives of the modified plan of operations is to reduce morbidity and mortality due to malaria by involving community participation, antimalarials are being distributed through voluntary agencies. Also Fever Treatment Depots (FTDs) and Drug Distribution Centres (DDCs) have been opened throughout the country at 1 FTD and 3 DDCs per 10,000 population. As of now 124,552 FTDs and 249,669 DDCs have been opened. Under the primary health care system, specific indicators have been proposed to enable monitoring the progress of malaria control. As per this indicator malaria cases in India per thousand population per year should be reduced to 4.6 in 1980, 2.7 in 1985, 1.9 in 1990, and <0.5 in 2000.[11] The progress of malaria control as monitored by the NMEP shows that in 1984 the API was 2.9, suggesting that the targets set out in the primary health care system as enunciated in the Alma Ata declaration are being met satisfactorily.[3]

Integration of Malaria Vaccines

Primary health care is regarded as the main system for the delivery of all health services in India, including the immunization programme. The primary health care service extends into the community, family, and to the individual. At the periphery, a health sub-centre has one male and one female multipurpose worker (MPW). *Inter alia* the MPW female is responsible for the immunization of pregnant women and infants, whereas the MPW male has the responsibility for all other immunizations. The EPI work of MPWs is supervised by an EPI supervisor. At the PHC level, a medical officer coordinates and executes the programme. Immunization against malaria would have to follow the same pattern as set out for other immunizations under the EPI. In

many malaria endemic areas, workload may increase considerably and therefore additional staff may be required. To strengthen primary health care a village health guide scheme is being implemented. Under the scheme it is proposed to train one health guide for every 1,000 population or for each village. Depending on the ease with which malaria vaccines could be delivered, the village health guide could also be trained and assigned the duties of malaria vaccination. The present difficulties faced by EPI in the maintenance of cold chain must be removed, if vaccination is to be ensured at the periphery. The cold chain is an essential requirement for malaria vaccines.

The scope of the integration of malaria vaccines with other methods of malaria control may be examined with reference to the control of malaria in urban and rural areas. In 1971–1972, the urban malaria scheme (UMS) was launched in the country. Under UMS the mainstay of malaria control is by anti-larval methods. At present 125 towns with a population of 43 million are covered under the scheme.[1] Rural malaria control is mainly achieved by spraying residual insecticides to interrupt transmission. In both these strategies, integration of malaria vaccines with other methods of malaria control can produce dramatic results as suggested by the observations made in the two field studies.

An integrated vector control methodology, mainly using biological control methods, source reduction, destruction of intradomestic breeding, etc., succeeded in interrupting malaria transmission in 25,000 population living in 7 villages. In these villages during 1984 there were 141 malaria cases, which were 65% less than in the control population in another village. The incidence was also very low as compared to the previous years when insecticidal spraying was used as a means to control malaria; e.g., there were 411 cases in 1983, when DDT was sprayed, and 4,040 and 2,355 cases in 1981 and 1982, respectively, when BHC/malathion was sprayed. The study is currently in progress in 0.35 million population (100 villages). In this area immunization of malaria positive cases, contacts, and other vulnerable groups can prevent buildup of secondary cases. Thus the villages could be maintained malaria free by integrating environmentally safe methods with malaria vaccines. It may be pointed out that when anti-larval methods have succeeded in interrupting transmission in highly endemic and well-irrigated rural areas, a good urban malaria scheme can definitely succeed in the control of urban malaria as the vector breeding sites are well defined. Therefore integration of malaria vaccines with the urban malaria scheme would be the best answer to the control of urban malaria in India.

Similarly, a study during 1981–1982 in Kharkhoda PHC revealed extremely high incidence of malaria, although the villages were under BHC spray for several years. During the same period, out of 9,124 *A.*

culicifacies dissected, 83 specimens were found positive for the sporozoites. In such a situation, immunization of an entire community may be very difficult and expensive. Also, if the protection from malaria is short lived, there may be very little value in immunization. In contrast, if malaria vaccines are administered in an area where transmission is interrupted, malaria can be much reduced or even eliminated. For example, in Kharkhoda villages where malaria transmission was intense, three rounds of malathion spraying (@ $2g/m^2$) in 1982 produced dramatic reduction in malaria cases. In 1982, out of 1,245 specimens dissected, only one mosquito was found positive for the sporozoites.[16] Malathion is still being sprayed in this area. During 1983 in 18 trips and 66 man-hour collections, only 6 *A. culicifacies* could be collected. Similarly in 1984 only 5 *A. culicifacies* could be collected (D. S. Choudhury, MRC Delhi, personal communication). The vector *A. culicifacies*, which was fully susceptible to malathion, has been nearly eliminated and the transmission of malaria completely interrupted. During 1984, there was no case of malaria as against a few hundred cases per thousand population per month during the transmission season. Such malaria-free areas need to be protected from the introduced malaria. In achieving this objective malaria vaccines would have immense value if applied judiciously. In such areas spraying may be terminated and surveillance strengthened to detect every possible case of malaria. All malaria positive cases and people living in neighbourhoods may be vaccinated to prevent the buildup of secondary cases. Withdrawal of spraying would drastically reduce cost and retain the susceptibility status of the vectors against the replacement insecticides, which are more toxic and many times more expensive. These insecticides could be held in reserve to tackle any sudden spurt of malaria in areas freed from the disease.

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Integration of Vaccine with Other Malaria Control Measures in Northern Thailand

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In Northern Thailand, malaria was previously the number one cause of death. In the 1930s, the annual malaria death rate sometimes reached 1,500 per 100,000 population. The malaria control program of the 1950s and the eradication program of the 1960s succeeded in lowering the death rate to its present level of 0.8–1.0 per 100,000. Malaria remains a major public health problem, because of transmission in forested and foothill areas. About 20,000–30,000 cases are detected annually. Of these, 98% come from forested and foothill areas. These are mainly forest working villagers, such as woodcutters, hill farmers, etc.

The two main vectors, *An. minimus* and *An. dirus (An. balabacensis)*, bite and rest mostly outdoors. *P. falciparum* is the predominant parasite species (60–70%). The combination of the villagers' temporary migration to the forests and neighboring countries, outdoor transmission, and drug pressure caused *P. falciparum* to develop and spread drug resistance, first to chloroquine, then to sulfadoxine-pyrimethamine (Fansidar), and now to quinine (some reported cases).

The current main control measures are residual DDT spraying in high receptive villages, case detection, and treatment. Supplementary measures include promotion of community self-protection using larvivorous fish, mosquito nets, and repellents.

Vaccination, if effective and available, would be included as an additional measure in this integrated control program. Vaccines would be used only with the high-risk group of villagers who work in forested and foothill areas. We estimate this group to comprise about 5 to 10% of the total population. By system of working, all of the malaria cases were investigated, so we got information already about who, where, and when these group of villagers go to work in the forest outside the village. The only problem that may arise is that when the number of malaria cases was reduced to a low level, it was very difficult to conclude whether it was from vaccination or from another cause from the integrated control program.

TABLE I. Malaria Cases, 1984 — Northern Thailand (Chiangmai Region)

1. Population			
	7.6 million		
2. Total cases			
	24,448 cases		
3. Species			
	P. falciparum	68.7%	
	P. vivax	30.9%	
	P. malariae	0.1%	
	Mixed	0.3%	
4. Age group			
	Below 1 yr.	0.16%	
	1–9 yrs.	7.47%	
	10+ yrs.	92.37%	
5. Foci of infection			
	—In the village		3.3%
	—In the forest		89.0%
	—Neighboring countries		7.7%

TABLE II. Measures for Control Malaria in Northern Thailand (Chiangmai Region)

	Receptivity of Area		
Measures	*High*	*Moderate*	*Low*
1. RESIDUAL SPRAY	X		
2. CASE DETECTION AND TREATMENT			
—Village Volunteer	X	X	X
—Midwifery Center	X	X	X
—Health Center	X	X	X
—Hospital		X	X
—Malaria Clinic	X	X	X
—Special Case Detection	X		
3. ADDITIONAL MEASURES			
—Larvivorous Fishes	X	X	X
—Self Protection	X	X	X
4. VACCINATION?	Only high risk groups		

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Integration of Vaccine with Other Malaria Control Programs in the Philippines

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I am for the integration of vaccine(s) with other malaria control programs with the following objectives:

1. To increase the effectiveness of malaria control programs through integration of vaccines;
2. To help improve our Global Malaria Control Program through[1] protecting man against death from malaria; protecting man against plasmodial infection; interrupting malaria transmission; improving malaria diagnosis; and improving epidemiological investigation and evaluation.

The following facts and figures will support my stand.

1. Introduction

Our population now is 54 million. Around 15.4 million live in areas still endemic more or less of malaria. Of the 15.4 million, 55% or 8.47 million are children below 14 years old; 5% or 770,000 are women of child-bearing age; and another 5% lives in developing areas such as mining, logging, irrigation, and road construction camps, and agricultural resettlement areas.

2. Morbidity and Mortality of Malaria

The number of malaria cases, deaths by age-group, sex and rates per 100,000 population in 1977 are shown in Table 1.[2] There were 7,597 (45.7%) malaria cases and 15 (27%) deaths in males below 14 years old, as compared with 5,224 (39.2%) cases and 137 (35%) deaths in females. There were also 9,028 (54.3%) malaria cases and 430 (74%) deaths in males, as against 8,106 (60.8%) cases and 252 (65%) deaths in females 15 years old and over. In all the ages there were 585 (60%) deaths in males, as against 389 (40%) deaths in the females.

TABLE 1. Reported Cases and Deaths by Age Group and by Sex, Number and Rate/100,000 Population, Philippines, 1977

	Malaria (084)							
	Cases				*Deaths*			
Age & Sex	*Male*	*Female*	*Both Sexes*	*Rate*	*Male*	*Female*	*Both Sexes*	*Rate*
ALL AGES ..	16625	13330	29955	66.5	585	389	974	2.2
Under 1 yr. . . .	382	373	755	46.9	29	30	59	3.7
1–4 yrs.	2394	1586	3980	67.0	47	46	93	1.6
5–9 yrs.	2361	1599	3960	61.9	46	42	88	1.4
10–14 yrs.	2460	1666	4126	74.6	33	19	52	0.9
15–19 yrs.	2361	1586	3947	86.8	39	38	77	1.7
20–24 yrs.	1546	1506	3052	77.1	63	23	86	2.2
25–29 yrs.	931	1239	2170	61.8	65	31	96	2.7
30–34 yrs.	964	804	1768	62.2	40	36	76	2.7
35–39 yrs.	831	746	1577	68.9	40	20	60	2.6
40–44 yrs.	632	586	1218	64.6	31	16	47	2.5
45–49 yrs.	399	493	892	57.9	29	13	42	2.7
50–54 yrs.	299	319	618	46.4	30	14	44	3.3
55–59 yrs.	316	186	502	41.0	28	13	41	3.3
60–64 yrs.	299	200	499	52.8	16	19	35	3.7
65–69 yrs.	216	199	415	65.3	15	10	25	3.9
70 & over	201	213	414	50.5	23	17	40	4.9
No. Stated. . . .	33	29	62	—	11	2	13	—

In 1984, the annual parasite incidence of malaria was 7.2/1000 population, with *P. falciparum* (65%) predominating.

3. Problems

3.1 DDT now costs US $2.80 per kilo, as against US $2.00 in 1983 and US $1.30 per kilo in 1982;

3.2 Some environmentalists are questioning DDT residual house spraying as polluting the environment;

3.3 Reluctance of the people now to DDT residual house spraying;

3.4 Spraying operation is now costly due to increased cost of freight and fuel-oil;

3.5 Difficulty of spraying operation due to lack of transport;

3.6 There is a high turnover of volunteer village spraymen under the Primary Health Care (PHC) approach;

3.7 Incomplete treatment of malaria cases due to frequent movement of population;

3.8 Preference of injectable antimalarial drugs more than the tablets by some people, especially traders to Sabah;

3.9 Widespread *P. falciparum* resistance (70%) to the 4-amino-quinoline drugs.

4. Recommendations

With this situation integration of vaccines with some malaria control programs is therefore a welcome additional approach. At present vaccination is given by the expanded program on immunization (EPI) of the Ministry of Health. Malaria control is already integrated into the Field Health Services like the Nutrition, Family Planning, and Schistosomiasis Control projects. The malaria vaccines will be given in the Barangay Health Stations, Rural Health Units, Dispensaries, and Clinics at the Primary Health Care level; in the District Hospitals at the Secondary Health Care level; and in the Provincial Hospitals, Regional Hospitals, Special Hospitals, and Medical Centers at the Tertiary Health Care level. In the event that the vaccines may not be adequate then the priority would be:

1. The children below 14 years old comprising 55% or 8.47 million with the aims to reduce morbidity and prevent mortality and to improve school attendance.
2. The women of childbearing age comprising 5% or 770,000, with the aims to improve the health of pregnant mothers exposed to malaria to reduce maternal death rates; and to reduce fetal and neonatal mortality rates and increase the birthweights of babies by protecting the pregnant women.
3. Those people living in developing areas such as mining, logging, irrigation, and road construction camps, and agricultural resettlement areas with the aims to reduce morbidity and mortality rates; to improve the labour output (increasing Gross National Product) and their earning power; to prevent losses of agricultural crops and earning power of industrial production due to shortage of labour or to decreased productivity; to promote utilization of idle lands; and to promote tourism.

For the clinical field trials to evaluate first the vaccines, we welcome and will gladly join the team to implement it according to formulated guidelines or protocol. At the moment, we are proposing either Tayabas or Palawan as possible sites. The epidemiological profile of Tayabas is shown in Table 2. In 1984, there were recorded 3,310 (50.04%) cases in the 14 years old and below, as against 3,301 (49.95%) cases in the 15 years old and over with 25% *P. falciparum* cases. Tayabas is 100 miles south of Manila and it is very accessible throughout the year by vehicle on concrete road.

The epidemiological profile of Palawan is shown in Table 3. In 1984, there were recorded 1,762 (59.56%) cases in the 14 years old and

TABLE 2. Malaria Cases, Tayabas, Quezon by Age Group, Sex, Species and Rates, 1984

Age Group		*P.f.* Male	*P.f.* Female	*P.v.* Male	*P.v.* Female	Total
0–11 mos.	N=	15	11	51	20	97
	%=	(1.44)	(1.7)	(1.8)	(0.97)	(1.47)
12–23 mos.	N=	11	15	57	46	129
	%=	(1.06)	(2.4)	(2.0)	(2.24)	(1.95)
2–9 yrs.	N=	280	207	821	655	1963
	%=	(26.9)	(32.8)	(28.4)	(31.9)	(29.68)
10–14 yrs.	N=	185	103	481	352	1121
	%=	(17.79)	(16.3)	(16.7)	(17.2)	(16.9)
15 yrs. & Over	N=	549	296	1478	981	3304
	%=	(52.8)	(46.9)	(51.2)	(47.8)	(49.95)
Total =	N=	1040	632	2888	2054	6614
	%=	(15.72)	(9.56)	(43.66)	(31.05)	(100.00)

TABLE 3. Malaria Cases, Palawan, by Age Group, Sex, Species and Rates, 1984

		Species						
		P.f		*P.v*		*Mix (Pf-Pv)*		
Age Group		Male	Female	Male	Female	M	F	Total
0–11 mos.	N=	20	25	13	19	0	1	78
	%=	(1.9)	(2.4)	(3.1)	(4.4)		(33.3)	(2.64)
12–23 mos.	N=	40	36	18	12	0	0	106
	%=	(3.7)	(3.5)	(4.3)	(2.8)			(3.58)
2–9 yrs.	N=	364	389	134	167	0	0	1054
	%=	(34.1)	(37.6)	(31.9)	(38.6)			(35.6)
10–14 yrs.	N=	194	173	76	79	1	1	524
	%=	(18.2)	(16.7)	(18.2)	(18.3)	(100)	(33.3)	(17.7)
15 yrs. & over	N=	449	412	178	156	0	1	1196
	%=	(42.1)	(39.8)	(42.5)	(36.0)		(33.3)	(40.5)
Total	N=	1067	1035	419	433	1	3	2958
	%=	(36.1)	(34.98)	(14.2)	(14.6)	(0.03)	(0.10)	(100)

below, as against 1,196 (40.5%) cases in the 15 years old and over with 71% *P. falciparum* cases. Palawan is southwest of Manila and it is one-hour flight by plane. There are very good electric, water, and housing facilities in both sites.

While the vaccines against the sporozoites, gametes, and the other

blood asexual stages (merozoites) especially of *P. falciparum* will have their respective indications, advantages, probably limitations as well as difficulties of application, our greatest constraints will be lack of funds, transport, and other logistics to support the program.

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Integration of Vaccine with Other Malaria Control Programs

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While it has been stressed that clinical trials of malaria vaccines should preferably be undertaken in the absence of other antimalarial measures, particularly chemotherapy which might confuse the evaluation, nevertheless in respect of operational use of proven vaccines there is agreement that this will complement and not replace standard control measures. Employment of vaccination together with other procedures for the control of malaria is justifiable, provided that the overall methodology is appropriate to the epidemiological and social situation in the locality and the community.

Achievement of malaria control within primary health care systems, utilizing vaccination and standard antimalaria measures, will require the support of expert malaria units of national health services. The extent of support depends on the structure of the primary health care system, on the epidemiological situation, on the aims of control strategy, and on availability of resources. Selection and definition of specific measures should be based on knowledge of the communities involved, and these measures should be adapted dynamically to the changing situation. The relationship of objectives of malaria control to selection of technological resources is interrelated, and new advances may dictate operational changes. This has been seen in recent years in many countries whose programs were adjusted from eradication to control. Thus, case detection and presumptive treatment have been taken up by primary health care workers instead of the former surveillance staff, and large-scale residual spraying has been replaced in many situations by focal spraying.

The availability of vaccines will change many tactical approaches to malaria control, but the changes will depend upon the particular characteristics of the vaccines in respect to stage and species protection and duration. An *anti-sporozoite vaccine* will be used mainly to protect visitors,

nonimmune immigrants, or particularly vulnerable groups of the indigenous community, exposed to high degrees of transmission. The *gamete-reactive vaccine* will be intended for interruption of transmission, during epidemics or at the start of intensive control programs using other methods. The *asexual erythrocytic vaccine* would be used (depending on its specificity and duration of action) as an alternative or addition to schizontocidal drugs for control of epidemic malaria or for large-scale treatment of seasonal waves of the disease.

Use of vaccines for these purposes will depend to a major extent on their cost, which will no doubt be a critical factor in deciding the feasibility of malaria vaccination in developing countries. Furthermore, only a small part of the budget relates to the cost of the vaccine itself—expenses of administration are much greater and would fall on the national organizations. The diversity in development of malarious countries (in respect of managerial abilities, public awareness, resources, and funds) will affect implementation of all malaria integration measures, including vaccination. Care should be taken that combining of several procedures and methods will not lead to an inability to implement and evaluate each component.

Among countries that have given preliminary consideration to the question of integration of vaccine with other antimalarial measures are China, India, the Philippines, and Thailand, and their tentative ideas may be outlined here.

In *China*, it is felt that vaccine will play a distinctive role in malaria programs when integrated with other control measures, although the latter are expected to continue to be the main approaches in the future. Vaccination should not, however, be viewed simply as an alternative tool, but must be utilized with other control measures to seek a combined strategic effect.

Malarious areas in China are diminishing and transmission intensity, except during outbreaks, has been greatly reduced. Such circumstances are favorable to the employment of vaccine. As *P. vivax* predominates in China, vaccine with *P. vivax* immunogens will be desirable, although *P. falciparum* vaccine may have a particular place in areas of the south where chloroquine resistant *P. falciparum* occurs. Polyvalent vaccine will be needed in the future malaria program. No exclusive deployment of vaccine is contemplated (other than for selected groups); vaccine should be used in conjunction with other measures, according to local epidemiological conditions, to curb transmission and solve problems. In China, it is expected that vaccine will be used in the following situations:

a. Vaccination of people moving from non-malarious or hypoendemic areas to places which are still malarious, particularly those containing chloroquine resistant *P. falciparum*, in order to protect the

migrants while living in the endemic area and prevent them spreading the disease agent after leaving the endemic area.

b. Integrated with other control measures, vaccination may be used as reinforcement in localities where traditional measures are proving inadequate. The combined measures should be designed as a strategic attack aiming at interruption of transmission when feasible. Such strategy may be used in chloroquine resistant *P. falciparum* areas, especially where local vectors are difficult to control.

c. Integrated into the malaria surveillance system, vaccination may be employed as a reinforcement tool to expedite elimination of stubborn residual foci. Immunization of the inhabitants of the focus and the surrounding neighborhood will provide better conditions for a quicker elimination of the focus by the other measures.

d. Vaccination may be used in conjunction with other control measures to suppress malaria outbreaks, especially post-eradication outbreaks. Vaccination of the people who live adjacent to the outbreak area will prevent spread and improve the conditions for suppressing the outbreak within its original site.

In *India*, both malaria control and the extended program of immunization (EPI) are integral to the primary health care system. It is felt that the delivery of malaria vaccines may suitably and effectively be merged with EPI. This will require additional inputs and a strengthening of existing infrastructure.

During 1985 the target population under EPI is 89 million (60–70% coverage), and this will increase to 140 million in 1990 when full coverage is envisioned. Although the national malaria program is implementing vector control measures in areas having an annual parasite incidence per thousand population of 2.0 or more (that is, 342 million rural and 43 million urban inhabitants), nevertheless more than 600 million people are at risk. Therefore the task that will be required to immunize against malaria would be greater than against communicable diseases under EPI. Group/area priorities for protection are the following:

a. PHCs serving areas of high levels of *P. falciparum* resistant to chloroquine.

b. Migratory labor populations which may introduce new parasite strains, particularly from northeast India to the central-eastern areas.

c. Areas of the green (agricultural) revolution and industrial areas.

d. Areas where the incidence of *P. falciparum* is high.

e. Special population groups, particularly infants and children.

Primary health care is the main system for delivery of health services in India, and extends into the community, family, and to the individual. At the periphery, multi-purpose workers include among their duties the immunizations included in the EPI program, under supervision of an

EPI worker and coordinated by a medical officer. Immunization against malaria would fit into this program, although in many areas the greatly increased workload would require expansion of staffing. A village health guide scheme is being developed, one guide for every 1,000 population or for each village; depending on the level of expertise necessary for delivery of malaria vaccination, these village health guides could be trained accordingly and assigned the work of malaria vaccination.

In the *Philippines*, vaccination is the responsibility of the EPI program within the Ministry of Health. Priority targets for antimalaria vaccination would be the following categories living in high-risk areas:

a. Children under 14 years of age (comprising 55% of the population in endemic malarious areas).

b. Women of childbearing age (5%).

c. People living in development areas (mining, logging, irrigation, road construction camps) and settlement areas (5%).

Malaria control in the Philippines is integrated with the peripheral health services, permitting early utilization of malaria vaccination procedures when these become available. Integration of malaria vaccination would usefully augment the existing program, because 65% of cases are due to *P. falciparum*, much of which is chloroquine resistant, there is an increasing cost of insecticides and problems in applying these insecticides, and there is considerable population movement.

In *Thailand*, despite marked successes in controlling malaria in recent years, the disease remains a major public health problem owing to transmission in the forested and foothill areas, which are responsible for 98% of all cases. *P. falciparum* is the main parasite and is almost everywhere resistant to chloroquine and to a growing extent to alternative drugs. Other problems include the outdoor transmission of malaria by the two main vectors *A. minimus* and *A. dirus*, and migrations of people throughout the country and across the borders with neighboring, highly malarious countries.

The integrated malaria control program in Thailand would form a ready base for addition of malaria vaccination. Vaccines would be used only among the high-risk group of villagers who work in the forested and foothill areas, a group estimated to comprise 5 to 10% of the population, and among certain categories of migrants and vulnerable individuals.

Discussant

LEONARD J. BRUCE-CHWATT

Before I start, will you allow me, Sir, to say a few words about my intervention of the previous meeting. I had a second thought about what I said about precipitin tests. And I realize now that while precipitin tests of the old kind may not be applicable anymore, newer methods of gel electrophoresis for finding out the origin of blood by *Anopheles* and other mosquitoes have still a very important place in entomology generally, and especially in entomology related to distributional viral diseases. And I'd like to apologize to my dear friend, Dr. Meuwissen. Thank you.

While the problem of integrational vaccine with other antimalaria measures as part of an antimalaria program has been most ably assessed by our colleagues from China, from Thailand, from India, and from the Philippines, respectively, and the opinions that were expressed by them were very close, really. Our colleague from China expressed himself forcibly, and I quote: "Vaccine should not be viewed simply as an alternative to, but must be utilized on the basis of its distinctive properties to seek strategic combined effect."

This is a very important and a very valid statement, with which few would disagree. Dr. Senqi limits the use of prospective vaccine to chloroquine resistant areas for immunization of stubborn resistant foci, or for a suppression of an epidemic focus.

The same is the theme of our colleague from India, who pointed out the situation in India at the present time and limited the same type of activity for India. I'm not sure when he said that vaccines should be used for protection of tourists, whether he meant, really, the use of vaccine for prophylaxis of tourists exposed to malaria. I doubt very much if one million tourists who come to India at the present time would let themselves to these prophylactic measures. I can't help feeling that other, perhaps, less drastic methods of protection of tourists, which is booming in India at the present time, are perfectly adequate.

In northern Thailand, our colleague who presented most ably the situation in Chiangmai area, once again proposed the vaccines in drug-resistant areas; specifically, in those areas where transmission is carried by *Anopheles dirus* and limited to high-risk groups in forested and foothills areas, which are operationally extremely difficult to cover. These groups combine 5% of the population, and, quite certainly, this is probably the best method for malaria control in those areas.

In the Philippines, once again, the use of vaccines will be restricted to areas such as mining, logging, road construction groups, and areas that are difficult of access. I cannot help feeling that those four presentations pinpointed very neatly the specific role of malaria vaccines for malaria control in particularly tricky areas. I'm referring to what they said, especially our colleagues from the Philippines, about malaria control within the primary health system. And there is not the slightest doubt that the support of health services in malaria control in this respect is paramount. This really refers to all four areas that we have heard about. This support will depend on the structure of the primary health system, on the epidemiological situation, on the aims of the control strategy, and on the availability of resources. The selection and the definition of specific antimalaria measures should be based on a good knowledge of epidemiology of the area and of the communities involved. As you will probably remember, one of the latest reports of the WHO on malaria control within the matrix or within the orbit of primary health care stressed again and again the importance of involvement of the community, and no better example of the involvement of the community on malaria control could be found today than in China. In China, this is a supreme example of very close cooperation between the vertical services and between the horizontal services of primary health care.

This study group defined and classified the most important antimalaria measures and stressed that their use should not be a study but a dynamic process continuing hand-in-hand with the development of socioeconomic conditions within those areas. While the relationship of objectives of malaria control to selection of technological resources is very closely related, very often new technological advances dictate the operational changes of malaria control. That is what I mean by stressing the dynamic relationship between the two. This has been seen during the past few years in many countries, which made adjustments of their programs in the course of transition from malaria eradication down to the level of malaria control. And once again, I'd like to say what I said a few years ago that it is very wrong to consider malaria control as a poor man's malaria eradication. Not at all. Malaria control in many, many ways is a much more difficult, and much more intelligent, and much more productive activity than malaria eradication, which

were by the rules of one book—let us pray and hope for the best. In malaria control, the situation is very different. You need people who know intimately the area involved, who know the socioeconomic, the epidemiological, the political conditions, and who know how to adjust specific malaria control to the situation of the area. So, in many ways, we are coming back, and perhaps, just as well, to the situation before malaria eradication, where much more intelligent approach to malaria control—a tailor-made approach to malaria control—is needed. And this can be done only if there is very close cooperation with the communities involved, with the national government, and, let's not forget it, with the training facilities for the new generation of both malariologists and community workers.

The availability of antimalaria vaccines will also change many tactical approaches to malaria control, but the actual trends of these changes will become fully evident only when all the characteristics of new vaccines are better known. At the present time, we really don't know what are the characteristics of new vaccines. We don't know the specificity of this or that vaccine, we don't know the duration of effect of this or that vaccine, we don't know the possibility of using them in hot countries and whether there is any need of cold chain. And these are technical problems that will have to be looked into very closely. Nevertheless, one could presume that anti-sporozoite vaccine will be used chiefly for protection of non-immune immigrant populations, or particularly vulnerable groups of indigenous community exposed to high degree of transmission, with few, if any, possibilities of control, in the same way as antimalarial drugs were used on a wide scale before resistance to those drugs became evident. The prospective gamete-reactive vaccine will find its use for hopeful interruption of transmission during epidemics or at the start of an intensive control program using, perhaps, other methods, very likely, in conjunction with some drugs or with environmental control methods or many other methods that are available.

On the other hand, the merozoite or asexual erythrocitic vaccine will probably be used depending on its specificity, on its duration of action, as an alternative, or perhaps as an addition to schizonticidal drugs for intensive control of epidemic malaria and for large-scale treatment of seasonal waves of malaria. Much of the future use of vaccines for these or other ways of attack or prevention of malaria will depend in apart from biological characteristics of these products and on their cost. This was stressed recently by Don Henderson when talking at the meeting on Expanded Program of Immunization, and I'd like to add a few words to this point.

Any reflections on the cost of the vaccine are purely speculative at the present time, and, really, they have nothing to do with practicability of its introduction. However, what I propose to say may serve as an indi-

cator of the realities. And it's based on information from the WHO Expanded Program of Immunization, which is now in its fourth year. Now, this program, as you probably know, refers to six target diseases; namely, diphtheria, pertussis, tetanus, measles, poliomyelitis, and tuberculosis. When it comes to costs, we know that none of the current vaccines are ideal.

Now, when it comes to tuberculosis, it is assessed that the TB vaccine is relatively expensive. It costs US $0.50 per dosage when bought through UNICEF in large quantities. It requires only one dose and can be administered from birth. Its inconvenience is that there must be very great care when using it, because in some cases, it induces side effects. And there is still the persisting concern about the efficacy of this vaccine in other groups and, perhaps, in some parts of the world, as India, as you probably know.

Now, let's come to measles. Measles vaccine is still the most expensive of vaccines, although its price has come down quite considerably during the last few years. The recent cost was US $0.07 per dose when purchased through UNICEF on international tender. The major drawback—in developing countries—is they need to wait until maternal antibodies have subsided before giving it to the child, while in developing countries, the average age of onset of measles is often above the age of 3 years. In developing countries, we have only a narrow window of opportunity after the age of 9 months, when the maternal antibodies have disappeared and when, during the very brief period of, perhaps, 6 months or 1 year, this vaccine can be given. A further problem is that, by the time a child in developing countries has reached the age of 9 months, the mother or other guardian might find it very difficult to come to a health facility because, as you say, the child is too young to walk and too heavy to carry. So, this very simple problem may be a drawback for measles vaccination.

The diphtheria, tetanus, and pertussis vaccine costs only US $0.02 per dose, and considering the primary immunization consists of three doses, the cost of a fully immunized child costs about the same as measles vaccine. That's about US $0.07 per dosage.

The trivalent oral poliomyelitis vaccine is very cheap. It costs only US $0.02 per dosage, and the cost, therefore, is the same as DPT. However, this is the least stable of vaccines, and it has been found in the field that it can withstand only one day at the temperature of 37°C before losing potency. Of course, there is a new inactivated vaccine against poliomyelitis, Professor Salk's vaccine, which is much more expensive. It costs at the present time US $0.50 per dosage, which is tenfold the price of three doses of the oral vaccine. I'm quite sure that the cost of it is going to decrease, and I'd like to stress what Dr. Luc Perrin has said at one of our meetings, that those costs today mean very little—the

more the vaccines are going to be developed, the more widely they will be used, the cheaper they will become.

But this brings me to another rub. While the vaccines themselves pound for pound, so to speak, are cheap, the costs of their use, the cost of their application, is quite high. And it has been assessed that the cost of any of those vaccines that are used when the transport and especially the cost of labor, because of their use with the health personnel, are included, those vaccines come to a very much higher figure—on the order of $5.00 per protected child. Now, this is a very high figure. If you remember, that in developing countries today, especially in Africa, which I know best, the cost of all health and hospital services for any of the African countries is on the order of not more than $2.00 per person per annum, well, this is going to be one of the considerable problems that we are facing.

The other point that I would like to stress is that most of those vaccines are usually given to the countries either by UNICEF or they are donated by a number of countries. Which means that, while the low cost of vaccine comes from the outside, the cost of its utilization in the field, the cost of its application, falls on the shoulders of the developing countries themselves, and this is one of the major problems in expanded immunization. And when it comes to malaria, the same sort of problem will come to our attention and will have to be solved in one or the other way.

General Discussion

PAIK: The presentation given by the previous speakers from four countries are very, very interesting. Also, Dr. Sharma gave us impressive data on immunization coverage in India. I will just mention the problems in a few countries in our region. In the Western Pacific region, you will see a great diversity among countries in terms of development. We have three very well-developed countries like Australia, Japan, and New Zealand. Also, we have now many advanced developing countries, such as China, also Malaysia, Philippines, Singapore, and the Republic of Korea. But we also have many countries newly emerging as independent countries where the social structure is still very, very less developed such as many small island countries, including Papua New Guinea. So, integration is good in theory, but to practice integration is not always an easy task. The other day, Professor Bruce-Chwatt cautioned about premature integration of the vertical malaria control program with horizontal general health care services, or primary health care system. Integration of two or three elements into one system requires skillful management. However, it is most discouraging to mention that malaria is still a major public health problem in many developing countries where the managerial capability of national health personnel is limited or unsatisfactory. For example, I'd like to mention a case that is a reality. The polio-saline oral vaccine is an appropriate technology as a result of sophisticated laboratory-based research. Yet, the coverage of immunization with this simple technology is far from the target in many, many developing countries, particularly in the small island countries in the Pacific. This is due to poor health infrastructure or to some logistic and managerial incapabilities. Sometimes this is also due to nonacceptance of immunization on the part of the population. So, this is one reality which really contrasts with what our respectful colleagues mentioned about the situations in their own countries. So, if we are talking about the integration of several elements, there will always

be some managerial problems. If malaria vaccines are potent and effective, particularly transmission-blocking vaccines available, which are also easy to be delivered, then maybe vector control activity can be decreased by vaccine as a major tool for control. However, such a possibility may be quite slim. Still, we have to think about using vaccine as a supplemental measure to the conventional method. There is another problem. If we combine too many methods, for example, indoor spraying, mosquito larva control, mass drug administration, and now vaccines, then some reasonable degree of perfection for each component cannot be maintained in some countries which would cause every component to become more or less haphazard. This, then, may be the danger. In this case, some operational criteria should be carefully set up on how to combine these different elements efficiently and efficaciously. Lastly, I am not totally pessimistic about integration. I have just mentioned the implications of inoperational integration in some less developed countries where malaria is still the major problem.

MEUWISSEN: I would like to make some additional comments on gamete vaccine. This is partly hypothetical and is highly technical, but it's not yet a gamete vaccine and there will be other vaccines. This is the situation where you have to rely on transmission-blocking vaccines. There should be one point added to that. At least in the *chicken* model, asexual parasitemia boosts the transmission-blocking capacity. If that would apply also to the human situation, then I could envision that we embark not on immunization of the total population but instead start with a gradual vaccination of the population just as you do in the normal immunization campaigns when you embark on the children and try to get, over a period of years, sufficient coverage by transmission-blocking herd immunities in the population. I think this would also make it possible to go gradually and to have it integrated in the normal immunization program without a special expanded program for immunization. I don't think that this question can be answered. It's just that I'd like for it to be considered as a possibility.

PERRIN: I wish to commend all the speakers this morning. I see the situation is very difficult to solve. However, it looks to me as though a lot will depend upon what kind of vaccine we will get and upon what kind of operational efficiency, what is the price, and so on. To discuss over and over again the applicability of vaccine, I think, is premature before we know what we are going to deal with.

MARTIN: Perhaps, as a preliminary to the use of vaccines in malarious areas in developing countries, it may be of interest to look at some of the populations who have moved from malarious areas into malaria-free regions and who, therefore, would have antibodies against malaria from

their prior experience but not be currently exposed to new malaria. Such a population may, in fact, be suitable to address this specific question as to whether the vaccine will boost levels of the natural immunity. That type of study could be relatively easier to perform since it could presumably be done in the major centers of the developing countries.

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C. STRATEGIES FOR FUTURE MALARIA VACCINE TRIALS

Panel Discussion
Future Malaria Vaccine Trials

BRUCE-CHWATT: It has been decided that because of a shortage of time only the representatives of countries involved in this conference will be able to express their point of view at this afternoon meeting. As you fully understand, this means representatives of the countries from:

People's Republic of China:	Dr. Shou-Pai Mao
India:	Dr. V.P. Sharma
Thailand:	Dr. Natth Bhamarapravati
Malaysia:	Dr. Ezaddin Mohamed
Indonesia:	Dr. Nyoman Kumara Rai
Philippines:	Dr. Crispin Echiverri
Pacific Basin:	Dr. Jacqueline Cattani
	Dr. I.S. Dulay
WHO Regional Office–New Delhi:	Dr. David Clyde
WHO Regional Office–Manila:	Dr. Yung-han Paik

It is fully understood that the persons that I name a moment ago are here in the capacity of representing their countries but also, in their personal capacity, to express their own views, their own plans, and their own proposals concerning the strategies for future malaria vaccine trials within this area.

It seems to me that they should use all their experience and all their wisdom to plan ahead and that they should not hesitate to indicate their personal views that are evolving and that evolved during this meeting. It seems to me that they may do this freely, even though they may be not in this stage allowed to express the opinion of their own government. They are not committing in any way their government at the present time, but they commit themselves and express their opinion. It also might be a very good idea that, in the course of these discussions we are going to have with the panel, some ideas may be given to the organizers of this meeting concerning the conclusions and recommendations which are going to be formulated and maybe discussed, approved, and

amended tomorrow. I understand that Dr. Siddiqui has received already a number of recommendations which may be amended, and it would be very useful, very valuable, if more of these recommendations could come as a result of this afternoon meeting.

MAO: I would like most for my colleagues to know that in the past I have been more interested in schistosomiasis than malaria. So, when I came here upon the invitation of Dr. Siddiqui, I found myself rather a student of malaria rather than a contributor. In the past few days, I learned a great deal. I think that vaccine would be a very useful tool to be used in a malaria control program. But I answer as several participants did this morning that each country has a definite aim or a definite target population for the malaria immunization. For instance, in China, as my colleague, Dr. Huang mentioned, malaria immunization may be used for troops in the frontier area, for travelers, and for laborers coming from non-immune regions to the hyperendemic areas. In this context, I would think that the vaccine to be used should respond to the need of the target population. For instance, if the vaccine is to be used for travelers or laborers who will go to an area with a drug-resistant strain of *Plasmodium falciparum*, then the vaccine should be prepared with the appropriate strain. Unfortunately, I don't know whether the Indochina strain used either by Dr. Collins or by Dr. Ristic is a drug-sensitive or drug-resistant strain. I am wondering whether the protective immune response would be the same to a drug-sensitive strain as that to a drug-resistant strain? I wonder whether there would be cross-response or different patterns of immune response? Another example is the point raised the other day by Dr. Nussenzweig, I think, about the immune state of the population. Now, if the vaccine is to be used not for travelers or for laborers or for immigrants but to prevent or to curtail another breakout of the epidemic in a region, then the strain used should be, then the vaccine to be used, we have to consider the immunostatus of the population. I think that so far in all the experiments, all the animals immunized were clean animals which were then challenged. But in the endemic areas this is not the situation. The population have existing or existed infection, so they have a different immune state as Dr. Nussenzweig mentioned. They may be immunosuppressed to a certain degree. So, I think, in order to have a potent vaccine, these two points should be taken into consideration.

SHARMA: It is ultimately the Government of India which is going to decide the use of future malaria vaccines. The Government of India has developed a bilateral agreement with the U.S. Government for the development, production, and use of vaccines against many microbial agents and malaria could perhaps be considered one of them. The country affords tremendous opportunities for the trial of vaccines against

malaria. There are areas exclusively under the influence of *An. culicifacies*, which is a rural vector, areas exclusively under the influence of *An. stephensi* and areas where both vectors exist. There are exophilic vectors in the northeast—*An. balabacensis* and foothill areas where there is stable malaria under the influence of *An. minimus*. I think the true value of vaccines would come out in the demonstration of control of malaria in stable areas.

Then, the country also has a great parasite distribution. We have areas highly dominated by *P. vivax* or areas highly dominated by *P. falciparum* and areas with each. *P. malariae* is also found in India. Therefore, when we introduce *P. falciparum* vaccine in a population, these species will be replaced by *P. vivax*. The public will get the feeling that malaria is still around in spite of the usage of vaccine. This point must be kept in mind. The third factor to consider is the genetic background of the people. We know there are a lot of genetic factors in the human population like G6PD, thalasemia, sickle cell anemia, or even the Duffy group antigen. I mean, we know fully well why *P. vivax* is not prevalent in Africa. These may modify the course of parasitemia in the populations and may ultimately result in our misinterpretation of results. The vaccines will definitely find a lot of acceptability in troops and travelers and would have a lot of value in nonimmune populations. We also have the problem of drug-resistant malaria, as I have already mentioned in both of my talks. The first preference for the trials would perhaps be given to areas where we have the problem of drug-resistant *P. falciparum* because if we can get rid of resistant malaria, I think the chemotherapy could be very much advanced the other way around. Instead of changing the drugs we could continue to use the same drugs. Then, no intensive epidemiological work has been on mixed infection. What they do is that they would examine the slide. The moment they find it positive, they mark it either *P. vivax* or *P. falciparum*. Still, there are 2–5% mixed infections. If a careful study is done, then you find a large number of mixed infections—maybe 5 or 7–8%. While we are controlling one type of infection by one vaccine, the other infection may be still very much around and we are back to square one that the patient is still having malaria although he was vaccinated. So, these are some of my thoughts on it. There is a possibility that the vaccines, because of the antibodies in endemic areas against the infection, may act as a booster.

NATTH: My observation here will be first on my personal feelings about vaccine development in general and, secondly, what I see as important in terms of malaria vaccine. I would like to say, also, that I'm not an expert in malaria. I'm a pathologist. But I feel that this problem of vaccine development is so important that I have given considerable thought on it in addition to my own experience in the research for the develop-

ment for dengue vaccine. I think that infectious disease control through vaccine development and deployment is crucial for a developing country like Thailand. We should try to facilitate the accelerated development as much as we can to contribute to the whole scene of vaccine development and employment. It's very important that in order to be able to facilitate this vaccine movement, we have to be able to mobilize the various expertise available in the country, as well as to also create some new structures to facilitate this development. As has been mentioned earlier, one aspect of the pathway of vaccine development is through clinical trials in your own population with the environmental and genetic nutritional interaction which is a very, very important thing to measure. In this respect, I have tried to think of what are our various expertise in our country. I would like to show for just a brief period a transparency. These are the expertise or the probabilities that are dealing with various aspects of malaria vaccine program in the country from the ministry and from the university standpoint. Of course Dr. Udom and Dr. Surin have discussed the ministry standpoint. But on the university standpoint, we have several groups working on the biomedical and epidemiology aspects plus health service research, clinical trial of drugs, and so on. There are two new structures that we have just added to our groups in the university. These are a vaccine science center in which we have facilities to back up the vaccine packaging system safety test, immunodiagnostics tissue culture monoclonal antibody production and all the like, and a vaccine clinical trial center where some 20 well-informed healthy male volunteers as well as females can be housed after receiving vaccine for at least 2 weeks of observation and very close group clinical supervision. These are the structures that are being added to the university as well as to the ministry. In addition to the ultimate goal of reduction of morbidity and mortality of malaria, my personal feeling is that the malaria vaccine trial which would lead ultimately to deployment is a good developmental model for a country like Thailand. We have to harness and mobilize science and technology toward the solution of our health problems. This would involve policy formulation, planning, research for development, technology transfer, manpower development, and even systems modeling for a pilot trial and an actual trial. We have heard during these past 4 or 5 days through this conference which has been excellently organized and implemented that the situation now is so dynamic and basic biomedical progress is being made so rapidly that I would not presume to clarify the various issues.

I think that in the situation where we could do something, I would like to create a malaria vaccine policy study group. This malaria vaccine policy study group should be, in my opinion, created jointly by the Ministry of Public Health and the university. The main function of the

malaria vaccine policy study group would be to act as an information, documentation, R&D, and policy planning and formulation group for the ministry and for the university. This policy study group would not be a permanent structure. It will be in a way a center without walls in which there would be a couple of people who are interested and are willing to dedicate the next 4 or 5 years or even 10 years of their careers to the study and to be facilitators for malaria vaccine policy formulation. It will be a coordinating group, where the various information and dealing with the various aspects of supporting systems for the whole of malaria vaccine planning and implementation could be focused. It would be a center or secretariat for a network of people who have expertise to deal with the various aspects of malaria vaccine and who identify key people who would come together in a network. The malaria vaccine policy study group should promote the idea and should try to create these concepts through conferences, consultative meetings, so on and so forth. I think that this will be for us a key strategy to pursue before we would be able to say what would be the specification or requirement for malaria vaccine policy and plan to pursue. Thank you very much.

EZADDIN: I would just like to explain a few procedures for any research or trials to be done in my country, Malaysia. Now the protocol for any research or clinical research in Malaysia will be screened by three national committees. First, by the National Medical Research Committee which will go through the necessity of having the research to find out any duplication or repetition of similar activities. Secondly, it must be cleared by the National Drug Control Authority which looks into the introduction of any new drug or vaccine into the country. All the drugs and biologicals to be introduced into the country must be registered by this authority. Third, of course, we have the National Ethical Committee which looks into the ethical aspects of all the research and studies. These committees, of course, will look into the type of the drug, type of research, and the form of the drug or vaccine, the mode of distribution, the safety and whether it will give a long term or short-term protection, the transportation problem or cold chain, the ease of administration—whether it is to be made available easily—the cost, of course, and the target groups. As far as malaria control program is concerned, of course, you identify certain target groups, such as the high-risk groups—mainly the mobile immigrant laborers and some areas where you have chloroquine resistant *P. falciparum* cases and certainly prophylaxis for people visiting highly malarious areas and maybe some of our own health workers in the malarious areas. But when I looked at the classification or the basis of clinical trial in Dr. Bruce-Chwatt's paper yeste - day, it is very difficult to actually pick and identify high-risk groups for clinical trials and fit a Phase I in the healthy human non-immune

group, preferably male. I think it is very difficult to identify in Malaysia at the moment, which is the area where you have the non-immune group because the malaria infection has been going on for so many years and because we have managed to reduce incidence, I think, in the last 10 years or so and adults, especially male adults, I'm sure, have already developed some degree of immunity against this infection. We can only think in terms of the children, and, actually, we are very reluctant to expose the children to these new experiences in clinical trials. With regard to Phase II, to the field population groups exposed to the natural challenge, I mentioned people just now like the immigrant laborers. But immigrant laborers are peculiar in Malaysia because they are not our Malaysian people, actually. They come from other countries and they don't stay too long in our country. They are very mobile, and I don't see how we can do the clinical trials in this group of people because they come and stay for about two months, then they go back to their country of origin. Similarly, with Phase III, once we can identify the target group of Phase II. Actually, it is very difficult to identify Phase III and, of course, Phase IV later on. Now, one big issue which our national Medical Research Committee and Ethical Committee will look into will be the real necessity of having this malaria vaccination program in addition to existing malaria control strategies and activities, because unless it can supersede or really give positive supplementation or complementation to the existing strategies, then they must really have a strong reason to introduce a new strategy. We have learned a lot from our own antilarval measures which were in existence in the country for over one hundred years and have given real positive and effective results. Thank you.

RAI: As far as Indonesia is concerned, I will express my own thoughts. I mentioned in my talk on the first day that the use of vaccine will not bring dramatic changes in the control strategy. However, if it is made available, we would like to use it in the first instance among the transmigrants and then, secondly, in infants under five. This is my personal thought. If the Government of Indonesia is willing to join the field trials, I would think of some factors that should be considered. Factor number one is the vaccine. What is the type of vaccine? Is it the sporozoite or merozoite type of vaccine? Then, what is the duration of immunity conferred? Third is the storage and fourth—is it the Phase I, Phase II, or Phase III, and, finally, what is the cost? The second factor would be the target group. Is it meant for all age groups or for a selective age group? And then, immunity level for the target group, and, finally, whether it is to be used in the urban or rural immunity. The third factor would be the implementation. Is it a multicenter or unicenter kind of field trial? And, also, we have to bear in our mind that this kind of trial is a multisectoral trial. We have to involve other sectors in the country.

Also, it is a central and peripheral health units factor. With regard to the factors of site, that is, to factor number four, we have also to ask the question—do we need virgin areas? By virgin areas I mean areas which are not covered by any measure in the malaria control. Or would we like to have hyper- or holo-endemic areas with high *P. falciparum* rate, and then we would have also to consider the distance from the central research teams and finally, the vectors. The fifth factor is the duration of the trial. I think this will be up to the duration of the immunity conferred by the vaccine itself. So, the duration of the trial will be mairly decided or depend upon the immunity conferred by the vaccine. These are five factors at the present moment I feel need to be considered. For the purpose of the trial, this is the map of Indonesia showing the endemicities, that is, direct areas denotes hyper- to holo-endemic areas. So, here, we can see that there are many places in Indonesia which are, I think, suitable for the sites of the trial. Also, for your information, I don't remember exactly, but I think it must have been in 1972, that we conducted a vaccine trial. But it was a smallpox vaccine in collaboration with Dr. Hector from Holland. It was, I think, a tissue culture smallpox vaccine. But it is quite different, I think, from the malaria vaccine because in that smallpox vaccine trial, we didn't challenge the population, we merely measured the take rate of the vaccine. This shows that we have experience of field trials with vaccines in Indonesia. I think that's all, Mr. Chairman. Thank you very much.

ECHIVERRI: If we consider and give weight to some remarks this morning that each country has varied factors in the epidemiology of malaria, then foremost I suggest that each country should be given the chance and be supported to conduct the malaria vaccine trials. Now, I also mention that these vaccines have their own or respective indications. As far as strategies for future malaria vaccine trials, that will depend on cohorts. If our cohorts are travelers or migrants, then the indication would be probably vaccine against sporozoites. If then, to enhance interruption of transmission, the vaccines against the gametes would be indicated, then if we try to reduce morbidity or mortality, then vaccines against these merozoites would be indicated. Well, in our country, the procedure is that the protocols are submitted to a research committee for ethical review and that our service, the malaria eradication service, has a covering paper indicating the justifications and recommendations. After the committee has reviewed the protocol, it passes to a management staff headed or chaired by our Minister of Health, who will approve this protocol. Then, these protocols will have to be implemented. That is all, Mr. Chairman, that I would like to say.

CATTANI: The Papua New Guinea Institute of Medical Research is a statutory body of the government of Papua New Guinea, and, there-

fore, it's certainly not our position to comment on policy or even make observations on policy of the government of Papua New Guinea. And I don't believe that that's why Dr. Michael Alpers was invited to this conference. I have, actually, a very elementary contribution to make. Over the past three years, as I indicated in the paper on the first day, we've set up a population. We've collected base line data and we've encountered a number of potential problems facing the malaria vaccine trials in terms of confounding. I don't think that we would pretend, at this point, that the Madang area is an ideal place for vaccine trials. It may or it may not be, but I believe that other areas will also have problems. They may be similar problems. They may be different problems. From our experience, I believe we should start collecting base line data now for a vaccine trial. I think that the countries that are interested in becoming involved in trials need to think about base line data as much, if not more, than the actual policy of conducting or procedures of conducting trials. There are implications for training local staff and being prepared to supervise the quality of data collected, both in terms of a base line and in terms of the vaccine trial itself. And also, small-scale pilot studies should be done before selecting a population in which to initiate a major trial. In these pilot studies, which would need many fewer resources than setting up an infrastructure to do a complete trial, I think a minimum of parameters should be investigated. Perhaps as minimum as parasite and spleen rates, the vector species that are present, the species' prevalence, the prevalence of other endemic diseases, the health infrastructure that already exists or that might be required to conduct a trial and the health status of the population in terms of nutritional parameters, drug sensitivity, and genetic diversity. These all can be done on a fairly small scale and should point out some problems that would be insuperable from the very beginning. From these pilot studies, one could choose a better candidate for a population and then begin to monitor that population more carefully. I think the last thing that I would like to mention is that any vaccine trial that's begun in any country, must be done with international involvement and approval. I don't think it can be a single country decision because of the very important implications of the success or failure of any trial. It's got to have the optimal chance of acceptance and, therefore, it must involve decisions and policy input from a variety of sources. Thank you.

DULAY: Of the three million population in Papua New Guinea, about 85% of the population continues to live in endemic areas. About 1/3 of the 85% of the population lives in hypo- to meso-endemic areas. Our experiences in malaria control which were initiated from a pilot project conducted in 1957 to our present problems have alerted the government to address itself to its policies for malaria control. The present policy for

malaria control is approved by the higher authorities with the modified objective of reduction or prevention of mortality, shortening of clinical episodes, and, if possible, to reduce morbidity or prevalence. In this direction, we realize that the use of a single measure alone will not achieve our desired results, what we expected earlier. For this reason, the objectives have been modified. The policy for malaria control is also to continually explore the possibility of alternate or supplementary measures that would be appropriate subject to local conditions. Bearing this in mind, if Papua New Guinea would be considered apart from those considerations that have been elaborated by my colleague here, we would certainly welcome the introduction of vaccine trial to participate in the definition of some features or many of those that have already been discussed to clear doubts or other problems that need to be addressed towards its operational usefulness. The present structure of the Health Department is such that the end results services towards primary health care. The ratio of health facility population now is about 1 to 1,000, which makes it possible if the vaccine perhaps could be integrated in the existing health care delivery system and the targeted groups. Of course, in spite of our limitations, we have an organization which does have the initial expertise that would assist in finding possible areas for trial. Before I came here, two provincial governments had endorsed their express willingness to participate in this venture. I think I cannot add further than that, but there was already an initial commitment. Thank you, Mr. Chairman.

CLYDE: A number of points have been made in relation to future malaria vaccine trials within the countries affected by malaria. I may comment and possibly enlarge on some of these. First, it has been noted that there is a need to repeat Phase II trials among volunteers in each country since indigenous populations may vary widely in reactivity owing to preimmune status, nutritional status, genetic variations and blood dyscrasias, and concomitant infections. This is particularly relevant in respect to erythrocyte stage vaccines, while the booster effect of naturally transmitted sporozoites on sporozoite-vaccinated subjects must also be considered. Secondly, the necessity has been stressed of determining and analyzing epidemiological situations as prerequisites to trials of Phase II and later, and not relying on old and obsolete data. The focal nature of such epidemiology is emphasized and the need to utilize the new techniques, such as the identification of sporozoite loads and species by IRMA and ELISA methods. Thirdly, it has been stressed that field trials need to be designed to eliminate a multiplicity of confusing variables, such as concomitant malaria control methods, particularly chemotherapy. Specific data need to be obtained initially from specific and pointed trials. However, it is the consensus, certainly

amongst the representatives of the Southeast Asia countries, that vaccination in practice will be an adjunct to, and not replace, other methods of malaria control amongst the indigenous populations. Therefore, late phase trials should be designed to include evaluation of the effects of other ongoing control operations, particularly chemotherapy. In respect to this point, the influence of natural transmission, acting as boosters in Phase III sporozoite vaccine trials in endemic areas, must be very carefully considered in the study design, as I mentioned a moment ago. Finally, it can hardly be emphasized enough that protocols and procedures for each phase of trial should be standardized to the extent possible, recognizing diversities between countries and populations. It is recognized that most countries would like to undertake Phase III and later trials of vaccines that pass through Phase I and II trials, and some countries with specialized facilities, such as has just been described by Professor Nath, might indeed embark on Phase II studies. The questions that must be raised in this respect, however, include the following: are most national facilities adequate in terms of funds, experience, and equipment to adhere to the necessary standardized protocols and not skip or condense essential parameters and procedures? Secondly, above all, are the national testing institutions and organizations powerful enough to resist political and military pressures to implement operational use of the vaccines prematurely, before the infrastructure is adequate? Similarly, are the commercial interests that will produce the vaccines willing to resist demands for large-scale demand for these products before they have been fully tested? Thank you, Mr. Moderator.

PAIK: I have spoken of the sad situation of malaria in the Western Pacific region, but this time I will speak about the more positive things because it is hoped that malaria vaccine may bring about a breakthrough in malaria control activities in the region in the future. Although I represent the WHO regional office in Manila, what I am going to say is purely my own personal view. In indicating, in a very crude manner at this stage, areas of malarious countries in the region for field trials on malaria vaccines, I am thinking to use existing problem areas of different kinds with different epidemiological characteristics for field testing of different types of malaria vaccines, not only for testing itself but also for eventual problem-solving if vaccines really work in these selected areas. Of course, this is very optimistic thinking on my part. The three major problem areas existing in the region are: (1) Indochinese countries where prevalence of drug-resistant *P. falciparum* infections is very high and management of severe malaria cases is the problem; (2) Sabah State of Malaysia where the main malaria vector is *An. balabacensis* and prevalence of *P. falciparum* drug-resistant infections is still very high, so transmission levels are also very high; and (3)

Papua New Guinea and the Solomon Islands, where exophilic effective *Anopheles farauti* is the main vector.

Three major types of stage-specific malaria vaccines are being developed and the Western Pacific region can offer unique opportunities for field trials for different types of vaccine because of the diversity of the epidemiological situation. The following indications for three different stage-specific vaccines can be postulated.

First, the sporozoite vaccine for causal prophylaxis on a personal basis will be tried out in international travelers. Non-immune travelers from Australia to Melanesian countries would be most suitable in view of Australia's proximity to highly malarious neighboring countries. Since a malaria surveillance system for imported malaria cases is operated quite efficiently in Australia due to the contributions made by Professor Robert Black of the Commonwealth Institute of Health, evaluation would be easier and a comparative study can be planned to check instances of imported cases in the two groups, one vaccinated, the other unvaccinated, who return from PNG, the Solomons, and Vanuatu.

Second, the merozoite vaccine for the prevention of severe clinical effects of malaria will be tried in the southern part of Vietnam, particularly in the vicinity of Ho Chi Minh City where severe malaria cases are quite rampant and, possibly, in the Sabah State of Malaysia where management of severe malaria due to *P. falciparum* is a problem because of drug-resistant strains.

The third vaccine which may block transmission will be tried as a public health vaccine on the northern coast of Guadalcanal (Solomon Islands), where the national program tried out all kinds of control measures. For example, after six monthly cycle of DDT spraying, they switched to another insecticide and then tried out ultra low-volume spraying together with mass drug administration but were unable to interrupt transmission. The addition of the current vaccine may give some input. We may also try this vaccine in some parts of Papua New Guinea, particularly in the northern coast, where one province called Olla has a very high exophilic tendency of vector and the housing conditions are not always suitable for spraying. Maybe this kind of area could be an indicative area to try out interruption transmission using malaria vaccine.

My general comment is that in the selection of countries and localities for field trials, some of the factors to be taken into account are the parameters for evaluation of different types of vaccine and the cycles of behavioral aspects of the population subjected to the trial. Some Melanesian people (particularly in the Solomon Islands), are fond of injection with needles because there was very successful Yaws control organized by WHO in 1960s where they gave insight into the modern medicine for the islanders. Because of the dramatic effect of penicillin

(one dose for Yaws), they all now believe that the syringe is better than tablets. Then we must consider the political will of the government of the country. Here again, Dr. Cattani has pointed out in her paper about over-enthusiasm of politicians or policy makers or the unrealistic expectations on the part of the population. This may again cause some problem if the vaccine will not work as we expect it. Then, of course, the speakers mentioned about field operational facilities, cold chain. Here again, an important consideration is the ethical one for the control group, or experimental group, because sometimes it is very difficult to get so-called informed consent in some countries in the region due to high illiteracy rate among adults. So a more cautious approach should be taken. In Papua New Guinea there has been a trial on pneumococcal vaccine. Maybe experience gained from this vaccine trial may be useful in planning for malaria vaccine. Lastly, the health systems as horizontal vs. vertical should be considered if future vaccine is to be combined, particularly with services to protect mothers and children. These comments reflect my own thinking about the future of vaccination. Thank you.

BRUCE-CHWATT: Well Dr. Paik mentioned a number of countries. Naturally, we did not intend specifically to name any countries at the present stage. On the other hand, it was unavoidable to mention some countries as an example of epidemiological or any other situation. Well, during those eight presentations, I've been trying to bring together as best I could the comments that were made. I have them here. They are not in proper order. I am quite sure our rapporteurs have done the same thing and far better than I could. But if you will allow me, I'll just read them higgledy-piggledy as they are right now. They may, perhaps, stimulate some ideas for specific or less specific recommendations.

1. Areas with highly endemic malaria subject to little migration, limited geographically and inaccessible, with at least two very distinct epidemiological stratifications. One part, the area of higher endemicity and the other, perhaps of lesser endemicity. Inhabited (and that's a sheer guess) by 10,000-15,000 people with a reasonably homogeneous ethnic population with a normal, or as close as normal, age group distribution of the population.

2. Good land communications, certainly accessible by air, and, if possible at all, close to academic or research institutions with a reasonably well-organized basic health service and a facility or existence of a basic laboratory and possibility of housing for additional technical staff.

3. Approval of national health authorities and of the relevant provincial administration. A complete assurance from the government and from the administrative powers of an understanding and cooperation of

the population involved. Absence of political or any other disturbing interference.

4. Commitment of an international body or an international institution or a bilateral body to technical and financial assistance for the duration of not less than three years, with the possibility of further extension. The extent of this assistance and its character to be specified at a later date.

5 and 6 together. Availability of fullest base line epidemiological data relevant to the prospective clinical trial and also, as point number 6, standardization of protocols and procedures and availability of technical facilities and also of a recording facility, because the data, as you remember, that are going to be collected when the quantity is so tremendous that modern recording methods will be necessary.

Well, here, I remember fully well what Dr. Cattani said, quite rightly in my view, that the collection of the fullest possible and necessary epidemiological data of any area will be impossible before the area is known. And yet, we cannot name the area at the present stage, so those epidemiological studies will have to be delayed until that area is selected. Well, this is a typical situation of the story that "if we had some fish, we could have some fish and chips, if we had some chips." Well, I don't know. How should we run the remainder of the meeting?

Well, this is open to general discussion, reminding you if I may, that no demands for any specific geographical area at this stage may be made.

REESE: I'd just like to make a comment relative to one of the points that Dr. Mao brought up. Some years ago, Dr. Trager and I did a study in which we partially addressed the question of how at least immunized monkeys would respond to parasites of different degrees of resistance to chloroquine and other drugs. And I would say that's where vaccine would provide another means of coping because it works off of an entirely different sort of system, and there's no reason to think certainly, from the available data on the blood in the blood-stage system. I'm sure it's going to be the same thing in the sporozoite or any other way. There's no reason to think from the available data that because an organism is drug-resistant, it will be any less susceptible to the immunization procedure or immunologic protection.

BUCK: During the past eight weeks I had the opportunity to attend five different meetings by different organizations in which the same subject was discussed. I think it really illustrates, in my book, that there is a case for coordination and orchestration of efforts. I think it is not that I ask for the creation of yet another bureaucracy to coordinate the other bureaucracy, but there have been examples like CADA, the Concerted

Action for the Development of Africa, in which it was possible with a minimum of bureaucracy to have interested parties meet from time to time to exchange their views and to pool their resources, which are usually scarce not only in money but particularly in manpower. I also believe that it is time now, as was suggested by the panel, to enter into epidemiological studies to lay the base line for whatever vaccine will come along. It is also time now to start the evaluation on the different ecological conditions of the new tools that have already become available and that will be used to understand the natural history including the protective immunity of man. Eventually, I think it will be possible to have an international network of centers of competence which would include other parts of Africa and of Latin America, in which one can make use and get clues from the differences that are observed in the endemic areas of *P. falciparum*, if a certain nucleus protocol were used, which could be enlarged and modified to meet local demands but should have one nucleus in which the differences would give us an insight into determinants of malaria as a disease. And I also would say that I agree with Dr. Bruce-Chwatt that there should be minimum requirements in the countries that would participate in Phase III trials. Namely, that they have referral systems, that they have an excellent laboratory "back-scrubbing," that they have national commitment for 3 or 5 years to support these studies, that they have sufficient manpower, that they have data equipment and data processing facilities and a logistic infrastructure that make it possible to carry out such investigations.

COLLINS: One thing that always appealed to me about selecting areas is to get isolated areas. A number of years ago, we looked at island populations in the New Hebrides. The Banks, for example, in which everybody had a positive response to *P. falciparum* antibodies. It seems if island populations were available with such good facilities then that would be an ideal site.

HUSSAIN: I'm a layman in parasitology. Actually, I have some acquaintance with viruses. The conference is on malaria, but it seems to me that everything has boiled down to only vaccination against malaria. Since there was some discussion on cost aspect of disease or negative or positive disease or vaccination, I am interested to know whether any real negative cost information exists with any organization or with anybody regarding the mortality and especially relative mortality in the present situation, and then morbidity and its duration, and then hospitalization rate with malaria and its duration. Because these are very necessary for finding out the negative costs and a country like ours (Bangladesh) where the government is very poor and people are very poor and cannot pay huge amounts of money as in developed countries. In fact, in our situation we think more about money than human lives. So, these are

more important to us than waiting for another 5 or 10 years to get an effective vaccination. Does this information exist?

BRUCE-CHWATT: Well, Sir, I am not sure that it exists. Wherever it exists, it's probably not fully reliable. It certainly is completely unreliable when it comes to any part of tropical Africa. In other parts of the world, like Sri Lanka, like India, like many other parts, I'm quite sure that it is very reliable. Perhaps not as perfectly as you might request, but it certainly is available. After all, this will be part and parcel of the epidemiological survey if the information is not fully available.

HUSSAIN: I think it is. In fact, the section of the people who are affected mostly is more important, whether the people affected are from the working group or people who are not working. So, this has one cost importance because if the working people are affected, we suffer economically more than if the people who are not working are affected. I also am interested to know, say, if we find an effective vaccine, will there still be some people who won't develop sufficient immunity with vaccination, which happens with every kind of vaccine? So, what are you going to do for those people who don't show any high immunic level even after sufficient doses of effective vaccines, if it is available?

BRUCE-CHWATT: This subject to me is a very difficult question and I am not prepared to answer it. There are other people here in the audience who might be able to answer it far better than I could. Perhaps they will cogitate a little on this almost philosophical question.

TRAGER: This will change the subject slightly. As I was listening to the emphasis on the proposed use of the vaccines, particularly in isolated regions with severe and resistant *P. falciparum*, I was wondering whether it might not be wise if a gamete vaccine should be available at roughly the same time as an asexual erythrocytic vaccine and that they be used together particularly in such regions because, certainly, an important objective must be to try to contain the transmission of chloroquine resistant falciparum malaria. This might be done by primaquine or some improvement on primaquine. But it might also be done by the gamete vaccine. It's theoretically possible, certainly, that the asexual vaccine would still permit development of gametocytes and would not be fully interferring with the transmission.

BRUCE-CHWATT: Dr. Perrin, in view of the fact that at the previous session, you wondered what we were talking about, perhaps you'd like to state your point of view once again, which would be very much appreciated.

PERRIN: I don't have any answer to Dr. Trager's questions or comments. However, I have two other questions. What is the moral issue?

For sure, you are going to modify what's going on at this place and you cannot just watch people like mice. If you find that there's a cause of transmission of malaria and you spend so many thousands of dollars to look at that, you should treat them at the same time. So, at the time you would do the vaccine trials the situation would not be the same as the time you begin to study the region in question. That's the first question I would like to ask you. The second question is that epidemiologic studies don't mean so much if you speak of 50 epidemiologic studies for basic level of immunity in a given region. You should be precise what you speak about. Do you seek to have the name of all the inhabitants, their age, the present parasitemia, the antibody titer against sporozoite, the antibody type against blood stage, and so on? Because you can get quite different base line data depending upon which parameter you choose. I think this is an issue which should be discussed by people who have experience in this field and is an issue which may be discussed along with the moral issue.

COPPEL: I'd like to extend the point of Luc Perrin and, essentially, that of yourself, Mr. Chairman, that at some stage, whether at this particular meeting or in some other forum, there's going to need to be set out a fairly specific set of criteria for selection of a Phase III trial. I think, perhaps, the place might be at World Health Organization or something like that. But, in order to aid the various governments in assessing whether they have an area in their country that is amenable, that would be useful for a Phase III testing, they're going to need a fairly specific set of guidelines.

MITCHELL: Yes, I think it was Dr. Clyde who raised the issue and asked the question of whether the commercial interest in this whole exercise would resist the pressure to make things available more widely on the basis of some preliminary titillating results, and I think that point is certainly well taken. It will be very difficult to exclude commercial interests in Phase III and subsequent Phase IV, etc. Dr. Clyde, can you perceive any special problems with respect to the testing of malaria vaccines with respect to these commercial interests? At the moment, commercial interests are just an abstract entity. Do you see any special problems which we'll have to deal with in this case?

CLYDE: It's difficult to go into this without naming companies and concerns and aligning it with chemotherapeutic practice. So, I don't think we want comments in a meeting like this on specifics of this problem. It's a philosophical and a possible one, though. I wouldn't like to say anything on that issue.

WERNSDORFER: My question, Mr. Chairman, regards pressure. That could be warded off as long as it is Phase III—preregistration. I see

more difficulties once the vaccine is registered for Phase IV. However, there might be pressure to hurry that very Phase III, and that is the pressure we should avoid. I agree with Dr. Buck when he said that we should come to a logical action criteria first and then develop agreed upon framework protocols to the specific requirements of a particular trial in keeping with its special objectives with the epidemiological setting and the social and economic conditions of the trial area. We could then do the best for this particular study and prepare for it by strengthening and building up staff resources and also physical resources and facilities which see these pre-vaccine trial studies through and pave the way for the vaccine studies in a kind of synoptic and continuous fashion. Thank you.

PAPPAIOANOU: I think that, epidemiologically, there is nothing more complex than trying to assess the efficacy of a vaccine in a field situation. I think that rates as number one in the highest level of difficulty. This would be the first time that a vaccine involving a parasite is aimed for use in a human population. But there have been, through some of the presentations here at this conference, some bacterial and viral vaccines that are now being used in the population. There have been extensive field trials that were carried out for those, and certainly, polio was one of the first and there were something like 750,000 people who were enrolled in that field trial. Certainly, we should really do everything we can to benefit from the experience of those field trials as we try to assess how we are going about carrying out this one.

BRUCE-CHWATT: I am very grateful to you, Dr. Pappaioanou, for pointing out that the difficulty that we seem to have at this meeting is due to the fact that, really, it is the first time in the history of tropical medicine that we are attempting to develop a vaccine against a parasite. With the exception of veterinary medicine with lungworm, there hasn't been anything of that kind, and that this is going to be several projects specifically designed for improving the health conditions in developing countries which lack the basic administrative and other services. No wonder that we are in a bit of difficulty. But I am quite sure that this discussion that we are having right now is going to clear up a lot of doubts in our minds and guide us in the right direction.

CLYDE: In fact, while this is the first artificial vaccine for protozoa, it's not the first vaccine. The Soviet Union extensively uses live continuous leishmaniasis vaccination all across the southern Soviet Union and most of the Soviet military forces involved nowadays in the southern Soviet Union and adjacent areas have live leishmaniasis vaccination. There are very extensive epidemiological studies done by those personnel and the experts from Martinovsky on the epidemiology and success of this

vaccine and, indeed, from the people I've spoken to, it does appear to be an individual's extremely successful vaccination. So, possibly, that literature could be looked into.

BRUCE-CHWATT: You are quite right, Dr. Clyde, and I am thankful to you for reminding us that this is not the first time. It's very nearly the first time. Well, it's like the story of the lady who was just a little bit pregnant, that's all. But, I'm not quite sure that it will be easy to get from the Soviet Union all their epidemiological data. They are notoriously cagey about that.

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Summarized Comments from Panel

1. **Dr. Shou-pai Mao,** *People's Republic of China*

Vaccine would be very useful to malaria control programs. Each country would have a definite target population in mind. For instance, in the People's Republic of China, there may be needs for use in military, travelers, and laborers moving from non-endemic to highly endemic areas. If this is a major need, then the vaccine should be prepared for their purpose, with attention to the appropriateness of the antigen for the specific parasite strains of the endemic area. A question was posed as to the possibility of strain differences (e.g., drug-resistant vs. drug-sensitive strains) which would affect the efficacy of the vaccine. There was concern that all early pre-clinical and clinical studies would utilize non-immune populations subjects. This would not be typical of the human populations utilizing the vaccines.

2. **Dr. V.P. Sharma,** *India*

Trials of vaccines could be planned for India, and the government would welcome such trials. The true value of the vaccines would be in areas where there are severe problems of drug and insecticide resistance. There is a question as to the acceptability of a vaccine which affects only one species of parasite in an area where multiple species exist. A number of human factors (e.g., genetic population variants) still need to be investigated. Troops, migrating labor, etc., would provide appropriate populations.

3. **Dr. Natth Bhamarapravati,** *Thailand*

It is felt that vaccine development is crucial for Thailand and that the country should do everything possible to facilitate this development. Expertise exists in several institutions in the country, such as the Malaria Division, the university, and the Ministry of Health. It is felt that a vaccine trial would provide a good development model for a

country such as Thailand. It would be advantageous to have established a malaria vaccine policy study group, drawing resources from a number of appropriate institutions. This would be a "center without walls," a coordinating group with a small permanent nucleus and a network of expertise and resources.

4. **Dr. Ezaddin Mohamed,** *Malaysia*

In Malaysia, initiation of vaccine trials would involve three national committees: a Medical Research Committee, the Drug Biological Authority, and the Ethics Committee. A number of pertinent factors relating to conduct of the trial would be considered by these committees. There are appropriate population groups in Malaysia for field trials, but characterization and final selection, the most appropriate areas, would be difficult. The one large issue in determining eventual vaccine introduction would be whether there is a real necessity for adding a vaccine to a current control program.

5. **Dr. Nyoman Kumara Rai,** *Indonesia*

The introduction of vaccines will not necessarily alter present field strategies of malaria control. Initial prime candidate groups would be transmigrants and infants and under-fives. The major questions which must be considered in testing a vaccine would include: (1) characteristics of the vaccine and its activity, (2) target groups proposed, (3) characteristics of the implementor institution, (4) characteristics of the site chosen for the trial, and (5) the duration of vaccine effect.

6. **Dr. Crispin Echiverri,** *Philippines*

Each country should have the opportunity to conduct vaccine trials, and support for such trials should be assured. Vaccine cohorts should be identified for each type of vaccine, according to the different objectives of vaccine administration. Protocols for testing vaccines must be reviewed carefully within the country where the trials are to be conducted.

7. **Dr. Jacqueline Cattani,** *Papua New Guinea*

In Papua New Guinea, careful studies have been done. The area studies may indeed not be the best area for testing vaccines because of problems which have been detected—but all areas well characterized will have a range of similar problems. Careful studies are essential for early characterization of prospective trial areas, but only a limited number of parameters should initially be examined. Initial small-scale studies can be followed by more intensive investigations if an area is eventually selected for vaccine trials. It is strongly felt that vaccine trials must be truly an international effort—not just a national or bilateral effort.

8. **Dr. I.S. Dulay,** *Papua New Guinea*

Government policy (PNG) is now toward a malaria central program, with continual exploration of alternative measures appropriate for local conditions. Operational vaccine would be integrated into the control program. The government would welcome a vaccine trial in PNG.

9. **Dr. David Clyde,** *WHO Regional Office in New Delhi*

It would seem important to conduct Phase II trials in *each* country where the vaccine will be used. There is still a need to develop and test new technology which will be essential to the field testing of vaccines. The accumulation of pre-trial data base is highly important, and some field trials need to be done to clarify issues. Late Phase III trials must take into consideration on-going control measures in the trials area. To the extent possible, protocols and procedures should be standardized. A number of important questions will emerge as the vaccine passes into and through the later trial phases. Are the national resources and facilities adequate? Are the testing institutions powerful enough to resist demands by national groups for premature expansion of vaccine use? Are commercial interests willing to postpone premature distribution of the product.

10. **Dr. Yung-han Paik,** *WHO Regional Office in Manila*

There are a number of appropriate and differing epidemiologic situations in WPR which would provide the potential for vaccine evaluation —including all three types of vaccine. The selection of countries and localities as trial sites would be governed by: (1) malaria characteristics; (2) population behavioral characteristics; (3) the will of the government to support the trial, avoiding over-enthusiasm and over-expectations; (4) logistical considerations; (5) ethical considerations; and (6) the existence of appropriate health delivery systems.

SESSION VI

GENERAL CONCLUSIONS AND RECOMMENDATIONS

- 039 -

General Conclusions and Recommendations Asia and Pacific Conference on Malaria

April 27, 1985

WASIM A. SIDDIQUI
President of the Conference
Chairman of the Organizing Committee

1. General Conclusions and Recommendations

It is both a great honor and heavy responsibility to stand here on behalf of the Organizing Committee of the "Asia and Pacific Conference on Malaria: Practical Considerations on Malaria Vaccines and Clinical Trials" to make a few concluding remarks.

Last Monday in my opening remarks, I mentioned that the uniqueness of this conference is that it has, for the first time, brought research scientists currently working on the development of malaria vaccines together with scientists and policy makers from the nations of Asia and the Pacific Basin for the purposes of developing a strategic plan that will be used in evaluating and field testing malaria vaccines when they become available for human testing. I also stated that in order to achieve the objective of the conference, it was necessary to evaluate the current status of the development of malaria vaccines and to identify those laboratory and field studies which are required to be completed before malaria vaccines are available for testing in human subjects.

For the last five days, we deliberated on relevant topics related to the objective of this conference. An update of information on the current status of malaria and malaria control in Asian and Pacific countries was provided by leading malariologists of these nations. Current status on the development of malaria vaccines was discussed by the leading workers in this field. Production of Plasmodia antigens by new biotechnology and applicability of information of modern vaccines for the production of malaria vaccines were elaborated and debated by leading molecular biologists. The topics of pre-clinical studies and technical aspects of clinical trials were discussed in detail by leading malaria epidemiologists. Strategies for future malaria vaccine trials in Asian and Pacific countries were deliberated and discussed by experts in this field including leading malariologists of these Asian and Pacific countries. Details of these deliberations comprise the 5-volume working docu-

ments which have already been distributed to the participants of this conference. Critical reviews prepared by the rapporteurs are important components of these documents. I am extremely grateful to each of the rapporteurs for their hard work and valuable contributions.

Despite nearly three decades of extensive control activities, malaria remains a major health problem in all endemic countries in the Asia and Pacific region. Three main factors are responsible for the current situation: (1) decreased effectiveness of residual DDT spraying which has long been the major weapon in malaria control; (2) widespread drug resistance to chloroquine and recent emergence of resistance to other antimalarials in some countries in the region; and (3) a number of administrative and logistical problems that have hampered implementation of existing control activities.

The use of DDT has not had a significant impact on exophilic vectors which have maintained high levels of transmission in areas such as Sabah, Malaysia, the Solomon Islands, Burma, Bangladesh, and other countries in the Pacific region. Research is desperately needed to find means of controlling the major exophilic vectors such as *A. b. balabacensis/A. dirus*. Other vectors, though not exophilic, have adopted change in behavior which allows them to avoid DDT sprayed surfaces.

Chloroquine has long played a major role in the control methods in every country in the Asia and Pacific region. At the current time, resistance to the drug is found in every country. In some countries, resistance remains confined to specific areas but in others it has spread throughout the country. In addition, resistance to other antimalarials such as Fansidar and even quinine have appeared, severely hampering any control efforts.

Administrative and logistical problems have long plagued control programs in many countries. The change to an integrated health care approach has been too rapid. In many instances, this has compounded existing difficulties. This has also left some programs in a state of limbo allowing a resurgence of malaria cases and an erosion of previous accomplishments toward controlling the disease.

Other administrative problems concern the decreased financial support for malaria control programs in many countries and the departure of trained personnel.

Unless new methods of control are introduced and increased funding is provided, the projection of existing data graphs indicate that little further reduction of malaria will occur by the year 2000.

Thus, in view of this most unsatisfactory and dangerous situation, USAID has planned and implemented research aimed at the development of new and innovative tools which could act as supplements to the existing antimalaria armamentation.

The urgent need for a vaccine and a long-term readiness of receptive

countries has been well established during the deliberation of this conference. This is indeed a milestone where the validity for the development of vaccine is no longer debated. In contrast, we look forward toward the implementation of laboratory findings in field situations in the years to come.

2. Current Status on the Development of Malaria Vaccines

How far along are the immunologists and molecular biologists in preparing a vaccine that is suitably engineered for clinical trials? Perhaps a time estimate is revealed when asking the question, have strong antigen candidates been defined for each developmental stage?

For the gametes, we heard impressive progress reviewed by Dr. Meuwissen. The evidence that 48kd/45kd and 25kd gametocyte surface antigens are the direct targets of transmission-blocking antibodies is now on solid ground. Moreover, there is no evidence of strain diversity. Because neutralizing and non-neutralizing monoclonal antibodies exist, the possibility of defining the protective epitope is good. Having identified the protective antigens, the molecular biologists must now clone the gene, and we can all look forward to rapid progress in this area.

For a long time now, the sporozoite vaccine has been leading the field. The current status has not changed. Not only is there a single defined candidate antigen, but now the Nussenzweigs have defined a single epitope, asparagine-aspartic acid-asparagine-proline (NANP). These four amino acids repeated three times comprise the minimal CSP epitope. The NANP3 repeat is found in strains from all areas of the world. Monoclonal antibody which binds the NANP3 repeat neutralizes infectivity. We are all eagerly awaiting good news of trials of the synthetic peptide in conjunction with an appropriate adjuvant.

How do we answer the question for the blood-stage antigens? Is there a single defined strong candidate antigen? Our Pacific Basin and Southeast Asian colleagues must feel that we are hopelessly lost on this question. On Tuesday morning, there appeared to be a hopeless array of bands and molecular weight polypeptides from which to choose. Unexpected direct evidence was revealed for the first time in the afternoon session.

A large number of candidate asexual blood-stage antigens were listed in the morning session. These were grouped into five categories: (1) merozoite/schizont membrane associated; (2) high molecular weight rhoptry; (3) low molecular weight rhoptry; (4) antigens on membranes of infected erythrocytes; and (5) antigens associated with the parasitophorous vacuoles.

Out of these five categories, the Perrin 41kd antigen in the rhoptry now looms as the single most impressive asexual blood-stage candidate

on the basis of both indirect and direct evidence. The monoclonal antibody, at microgram quantities, is capable of *in-vitro* inhibition. More impressively, immunization with the native 41kd results in a delayed patent period and a parasitemia of no more than 0.2%, whereas squirrel monkeys immunized with candidate antigens in other groups had parasitemias as high as 2 to 19%. Thus, to the question of whether there is a single asexual blood-stage candidate antigen that stands above the others, there must be a growing inclination to answer in the affirmative that, yes, it is the low molecular weight rhoptry polypeptides.

Dr. Pereira da Silva asked the question, "Can the ideal malaria vaccine be developed without being polyvalent?" Graham Mitchell might answer, "No, we should develop a polyvalent vaccine containing RESA, rhoptry-1, rhoptry-2, pf190, GBP, Sequestration antigen, FIRA," and so on. Although only a pilot experiment, our direct evidence in the *Aotus* monkey hints that we might not need so many. We were able to achieve what appeared to be a sterile immunity using a polyvalent antigen combination of a group I (merozoite/schizont membrane-associated antigen) and a group II (high molecular weight rhoptry antigen) with three injections of Freund's complete adjuvant. When challenged, no a single parasite was detected in thick blood films, whereas the control developed the expected parasitemia in *Aotus* of the karyotype used.

Thus, we may be further along in defining candidate antigens for all three stages than we think.

3. Practical Considerations of Malaria Vaccines

When the protective antigens are ultimately defined and cloned, how should we engineer the polyvalent vaccine? If the weight of history were to favor any vaccine for malaria in the developing countries, it theoretically would be polyvalent recombinant proteins delivered in the vaccinia virus. However, Dr. Moss informed us that the CSP gene introduced into the vaccinia genome was not expressed at the virus surface, and Dr. Pereira da Silva found that antibodies produced in squirrel monkeys to a recombinant protein did not bind to the native protein.

We are then left with perhaps the most powerful approach. That being to take the gene sequences that the molecular biologists give us and to then define relevant B-cell and T-cell epitopes, deleting, as Graham Mitchell had suggested, irrelevant, non-productive, or suppressive epitopes. With the development of the powerful synthetic adjuvants which stimulate both cell-mediated and humoral immunity reported by Drs. Kotani and Chedid, namely B30-MDP and murabutide, respectively, it becomes possible to chemically synthesize judiciously selected epitopes in tandem, flanked with synthetic adjuvants in a single synthetic molecule. This has great promise. Thus, there is ground for opti-

mism that a polyvalent vaccine comprised of sporozoite, asexual blood stage, and gametocyte vaccines may be ready for clinical trials in the future.

4. Pre-Clinical Studies

At present, then, it appears that the putative candidate malaria vaccine will be prepared by recombinant DNA technology and/or chemical synthesis once a vaccine candidate has been identified by experimental work and suitable methods have been developed to produce the antigen in reasonable quantity.

Pertinent pre-clinical studies on candidate vaccine were enumerated and discussion focussed on requirements of FDA or similar regulatory agencies before a vaccine is approved for clinical trials.

The scientific session on Thursday related to pre-clinical studies and evoked the most extensive and most rigorous general discussion in the conference. It is appropriate to highlight some of the points stressed in the session: (1) the need to obtain definitive data on specific antibodies and levels that in fact reflect functional protection; (2) the extent of unanswered questions on adjuvants, carriers, multivalence, and how relevant data on those questions can best be obtained for rational decisions about which preparations should be brought forward for clinical study; (3) the need to take into account not just the United States Food and Drug Administration regulations, but also the regulatory agencies of other nations; (4) the significance of ethical concerns and issues that directly influence what specific types of clinical studies can or cannot be conducted, notably in particular relation to the evaluation of merozoite vaccine preparations that may not afford complete protection but may modify the course of malaria and evaluation of transmission-blocking agents; (5) the importance of concerted efforts to coordinate plans for clinical trials and field studies to maximize compatibility of design and of interpretation; (6) the appropriation of confined studies in animals during or before clinical trials to evaluate the possibility that selective vaccine pressures may result in expression of new and different epitopes as the parasites attempt to circumvent or escape from the vaccines' impact; and (7) the immense additional need for conclusive, basic pre-clinical data in animal models, including *Aotus* monkeys and, possibly, chimpanzees to resolve fundamental question prior to the initiation of clinical studies.

5. Clinical Trials

General objectives: the overall objectives of malaria vaccine trials are the assessments of the safety and efficacy of the vaccine in individuals of different ages, ethnic or geographical origins, and malaria experiences and

the determination of optimum conditions for the induction and maintenance of functional immunity. Furthermore, the epidemiological impact of immunization against malaria will have to be determined and include the assessment of the acceptability of and compliance with immunization procedures in communities of population groups exposed to malaria risk.

Specific objectives: Detailed protocols for Phases I, II, and III clinical trials were thoroughly discussed and form the most important component of the conference document. However, I wish to highlight a few points which have special relevance to some of the general recommendations which will be proposed this morning:

5.1 Declaration of Helsinki with later appendices stipulating the ethical condition under which all clinical trials are to be performed.

5.2 Phase I trial should first be conducted in adult volunteers in nonendemic areas. After evaluation of results, Phase I trials should be extended to endemic areas. The Phase I malaria vaccine trial center in an endemic area should meet standards of a vaccine trial center and should be located in a country with a strong motivation for the eventual use of the specific type of vaccine in its malaria control program. Establishment of a Malaria Vaccine Trial Center requires the creation of a viable infrastructure.

5.3 Phase II trials will constitute the bridge between Phase I and the commencement of Phase III field trials. The centers in which they will be carried out will be expected to lie closely with the institutions involved in Phase I investigations and play a major role in the eventual planning and conduct of Phase III operations.

Before I make a few remarks on Phase III trials, I would like to highlight some of the points raised in the Thursday session, namely, (1) differing viewpoints about the potential and need for Phase II clinical trials on merozoite preparations that may not be completely protective and on gamete-blocking agents, one core issue being the potential with reference to ethical and other grounds for conducting such studies in partially immune subjects having experimentally-induced infection; (2) the apparent value of *in-vitro* means to assess the effect of transmission-blocking agents; (3) the role of field study personnel and of field studies in diagnosis and treatment of malaria not directly related to study populations in study areas; and (4) the unsettled question of chemotherapy at the onset of or prior to field studies of malaria vaccine efficacy.

6. Phase III Trials

There is no doubt that the field studies or Phase III trials will be difficult to carry out. However, assuming that sporozoite, asexual blood stage, and transmission-blocking vaccines will be available someday, the later

alternative might not require Phase III testing except perhaps in combination with one or two other antigen(s). All expertise available should be utilized including parasitologic, immunologic, epidemiologic, statistical, and administrative knowledge. Every effort should be made to plan the studies so thoroughly that no question of the scientific value will remain after the exhaustive endeavours in the field.

7. Criteria for Selection of Sites for Phase III Clinical Trials

7.1 The site should be situated in a country which has displayed a strong motivation and desire for the eventual use of malaria vaccine(s) in its malaria control program.

7.2 The site should have strong local and national commitments as well as have potential international support for Phase III and eventual Phase IV trials.

7.3 The site should involve a high level of malaria endemicity, a low level of migration, ethnic homogenicity, and a total population of approximately 10,000 consisting of the usual age distribution.

7.4 The site should be affiliated with an established national institution which has basic laboratory support and its own field team or the site should be in a position to work with outside experienced teams.

7.5 The site should be reasonably accessible by land/air and should possess a reasonable level of basic health services.

7.6 The site should be within easy access to malaria treatment facilities.

7.7 The site should offer adequate facilities for the efficient collection and analysis of data.

7.8. The site should possess a long-term knowledge of the local epidemiology of malaria or possess the potential to collect requisite epidemiological and other baseline data.

8. Recommendations

8.1 The Asia and Pacific Conference on Malaria, having met in Honolulu, Hawaii, U.S.A., on April 21–27, 1985, is aware of the fact that malaria continues to be one of the major human diseases afflicting some 1–2 billion people throughout the world, particularly in the developing countries and the tropics, and *recommends* that the international institutions and national governments increase their technical and financial assistance to foster the improvement of methods and means for the continued fight against this disease which retards the human welfare and prevents the economic development of the Third World.

8.2 The Asia and Pacific Conference on Malaria recognizes with admiration the foresight and sponsorship of the United States Agency for International Development and applauds the leadership of the University of Hawaii for convening this conference and initiating a milestone in the development of a strategic plan to implement significant laboratory findings into field clinical trials.

8.3 The Asia and Pacific Conference on Malaria recognizes the worldwide need for closer collaboration of scientists working towards the development of malaria vaccines and people to be responsible for evaluating these vaccines in clinical trials in institutions and in the field. It is, therefore, *recommended* that the national and international sponsoring agencies with interests in all aspects of malaria vaccines devise ways and means for fostering international collaboration.

8.4 The Asia and Pacific Conference on Malaria applauds the proposed plan of the U.S. Agency for International Development in sponsoring similar conferences for America and Africa in the near future. This will foster closer collaboration among malaria workers on a world-wide basis.

8.5 The Asia and Pacific Conference on Malaria recognizes the pivotal leadership role of Hawaii for the forthcoming implementation of laboratory findings into field clinical trials in countries of Asia and the Pacific Basin. It is *recommended* that appropriate facilities be established at the university to coordinate the logistical and technical details in the proposed field clinical trials in Asian and Pacific countries.

8.6 The Asia and Pacific Conference on Malaria recognizes that malaria vaccines, when available, will only be a supplement to the existing control measures. Despite encouraging progress being made towards the development of malaria vaccines, it should be stressed that it will be many years before any such product becomes widely available. It is, therefore, *recommended* that governments, national and international agencies currently involved in malaria control programs should not allow any decrease in the scale of current or proposed programs. On the contrary, control programs using conventional and currently available means should be maintained and even expanded.

8.7 Using the Asia and Pacific Conference on Malaria as a springboard, it is *recommended* that a scientific technical committee with expertise in epidemiology, general and clinical malariology, immunology, statistics, and clinical field trials be established. This committee will then have the specific task of:

a. Developing specific criteria for the selection of areas appropriate for Phase III clinical trials,

b. Developing specific protocols for field epidemiological investigations, and

c. Insuring all clinical trials meet the ethical requirements of internationally accepted standards and the stipulations of the Helsinki Declaration.

It is also urged that malaria experts from the nations of Asia and the Pacific Basin should be an integral part of this committee and this committee should also serve as a forum to facilitate exchange, review, and disseminate information regarding scientific and field clinical trials developments as they arise.

8.8 Based on the scientific deliberations of this conference, it was evident that a malaria vaccine will be available in the not too distant future. It is, therefore, *strongly recommended* that sites for future malaria vaccine trials be selected in the immediate future and rapid measures be taken to initiate epidemiological investigations as soon as possible so as to obtain baseline data in endemic areas. Special attention should be given to this very important recommendation. Regarding the selection of a site or sites in an endemic area, it is gratifying to note that the countries of Asia and the Pacific Basin which are represented at this conference are very receptive to the idea of incorporating malaria vaccines in malaria control measures in the future.

8.9 The Asia and Pacific Conference on Malaria is aware of the fact that the effective implementation of pertinent laboratory findings into the field will only be possible if there is a strong and long-range commitment by the national and international political and technical agencies to achieve this goal. Therefore, it is *recommended* that these agencies should adopt a policy of long-range support in developing expertise, in training manpower, in providing facilities with necessary equipments, reagents, and other logistic support. *Special attention* should be given to provisions for training and careers in field malariology. This should especially be directed towards young people in the countries where malaria is prevalent. The coming Phase II and Phase III trials of malaria vaccines will require knowledgeable field malariologists and they are in short supply.

8.10 The Asia and Pacific Conference on Malaria recognizes the rapid and significant progress achieved in the development of sporozoite vaccine and the availability of two distinct sporozoite candidate vaccines for Phase I trial. Hence, it is recommended that these two distinct candidate vaccines should be evaluated and compared for their immunogenicity and tolerance using similar protocols.

8.11 From lessons of history, a strong case can be made for the notion that the time when a major new control procedure is to be intro-

duced is precisely the time to accelerate work on second generation control measures. Sporozoite CS protein vaccines will be tested in Phase I and Phase II trials in volunteers before asexual blood-stage vaccines. Regardless of any promising results obtained in these trials, the necessity for maintaining impetus on research and development of non-sporozoite vaccines is emphasized and *strongly recommended*.

8.12 Since it is quite likely that a sporozoite vaccine may not give complete protection, it is essential to accelerate the development of asexual blood-stage vaccines. For this, *Aotus* and *Saimiri* monkeys are of special value. Accordingly, it is *recommended* that the first priority for the use of susceptible karyotypes of *Aotus* monkeys be given to tests of asexual blood-stage vaccines.

8.13 It is also recognized that only two or three of the numerous asexual blood stage antigens are potential vaccine candidates. It is therefore *recommended* that *Aotus* monkeys should be set aside for carefully controlled immunization tests in the not too distant future. If immunization tests are promising, it is *further recommended* that no time be lost in preparing these antigens in a form appropriate for Phase I and II trials in human volunteers and that such trials be carried out.

8.14 The Asia and Pacific Conference on Malaria recognizes the evidence that adjuvants in conjunction with a malaria antigen is required in inducing functional immunity. At present there is no safe and effective adjuvant available for use in humans. Hence, it is *strongly recommended* that the development of a safe and effective adjuvant should be given high priority.

8.15 Because there remain many questions relating to vaccine efficacy, such as persistence of functional immunity, relationship of activity with existing homologous and heterologous malaria infections, variables existing in the immune status in the hosts, optimal routes and schedules for immunization, adjuvant selection, relationship to chemotherapeutic intervention, etc., which would be most difficult to investigate adequately in new world monkey models for the human malaria parasite or in Phase I and Phase II human trials, it is *recommended* that simian malaria models be further explored and utilized to provide this needed information.

8.16 It is recognized that the assessment of efficacy of malaria vaccine trials in Phase III and Phase IV will require the use of sensitive, simple, and reliable tests applicable in field conditions and therefore it is *recommended* that accelerated research efforts should be made to develop tests to meet this need.

8.17 Since the assessment of a malaria vaccine efficacy in Phase III and Phase IV clinical trials will require careful statistical analyses of

many factors, it is *recommended* that the methods of statistical analysis to be used for evaluating vaccine efficacy be identified *prior* to the collection of baseline data in the pre-trial design phase.

Discussion

TRAGER: Thank you very much, Dr. Siddiqui, for the excellent synopsis of the proceedings of the meeting and for presenting the summary recommendations. Now the floor is open for a discussion of these recommendations and for additional comments and recommendations.

BRUCE-CHWATT: I would like to congratulate Dr. Siddiqui on his remarkable summary of the discussions that took place in this conference and on an excellent series of recommendations. I wonder if, to start from the general and proceeding to the particular, a general recommendation specifying what is happening today throughout the world in the field of malaria and how mankind as a whole, if you will allow me to use this lofty and very often mortal phrase, should respond to the challenge. And I propose, perhaps, that the following general recommendation may precede or follow the number of recommendations that Dr. Siddiqui so ably presented to us. I propose the following text that can be amended. I have no copyright on it and no proprietary authorship.

"The Asia and Pacific Conference on Malaria, having met in Honolulu, Hawaii, U.S.A., on April 21–27, 1985, is aware of the fact that malaria continues to be one of the major human diseases afflicting some 1–2 billion people throughout the world, particularly in the developing countries and the tropics, and recommends that the international institutions and national governments increase their technical and financial assistance to foster the improvement of methods and means for the continued fight against this disease which prevents or slows down the economic development of the Third World." Thank you, sir.

TRAGER: Thank you Dr. Bruce-Chwatt. I think there is no need to discuss this as I think we are all in unanimous support of this recommendation. Am I right, Dr. Siddiqui?

SIDDIQUI: Absolutely right. I wholeheartedly agree with the recommendations proposed by Professor Bruce-Chwatt.

TRAGER: Are there any other questions?

JEFFERY: I have a comment on a couple of the recommendations which Dr. Siddiqui has offered. I think it is obviously admirable that the Asia and Pacific Conference has taken a position of leadership in bringing people together in the area of vaccine development and the later stages

of vaccine development. I am a little disappointed that the resolution does not recognize the world-wide need for coordinating and collaborating bodies but only speaks to a consortium of nations in the Asia-Pacific area. I do think that region can take the leadership presently; it should carry some responsibility to include some mention of the need for taking in the rest of the world in this effort. Thank you sir.

TRAGER: That is an excellent comment. Dr. Siddiqui, would you like to reply to that?

SIDDIQUI: Yes, I completely agree with this comment. Incorporation of Professor Bruce-Chwatt's recommendations will cover the comment just made by Dr. Jeffery.

TRAGER: I would like to suggest that with the inclusion of Dr. Bruce-Chwatt's introductory recommendation and perhaps some slight expansion of that, we could in fact cover this very important point.

MITCHELL: Just following on Dr. Jeffery's point. I think I read his comments—maybe I am taking liberties here—but another recommendation would be the Asia and Pacific Conference on Malaria recognizes the need for international involvement and collaboration on any approach toward the development of a malaria vaccine, which of course brings into many other international agencies, most of whom are represented here. Is that the feeling I perceive, or not? A more specific recommendation would be that we really do recognize the need for closer collaboration between all those with an interest in the development of malaria vaccines at the international level.

TRAGER: Yes. I think that essentially what you have said should be written down and could well follow Dr. Bruce-Chwatt's statement. I think we all recognize that this is a beginning and it seems likely that similar conferences may well be held with regard to other malarious regions.

Would anyone else care to comment? Dr. Siddiqui, will there be some provision so that as people think of things that they feel are very important to be included, they can write to you?

SIDDIQUI: Yes, and they are very welcome to do so. If anyone thinks of any other recommendations, either general or specific, please do send them to me, even if this occurs after the conclusion of the conference.

SAVANAT: As you are all aware, the World Health Organization also has this interest in the development of malaria vaccine. The content of this kind should somehow try to complement each other in order to reach a common goal and not to compete in any way about the activities of these two important organizations.

JEFFERY: I have a question for the chair. Are rather specific recommendations now being asked for or welcomed, or should all recommendations be in a fairly general sense? I think there may be several rather specific points which some would like to include in the recommendations, if this is acceptable.

SIDDIQUI: As I stated earlier, specific recommendations are also welcome.

JEFFERY: May I offer one?

SIDDIQUI: Please. But do also submit it in writing.

JEFFERY: It is in my writing. I will try to summarize it. This is, as I say, a rather specific recommendation and may not be within the scope of the meeting. Because there remain many questions relating to vaccine efficacy, such as persistence of effect, interrelationship of activity with existing homologous and heterologous malaria infections, variables existing in the immune status in the hosts, optimal routes and schedules for immunization, adjuvant selection, relationship to chemotherapeutic intervention, etc., which would be most difficult to investigate adequately in new world monkey models for the human malaria, or in Phase I or Phase II human trials, it is recommended that simian malarial models be further explored and utilized to provide this needed information. Efforts should be made to develop and provide antigens for selected simian parasite species, which simulate the major human species, to allow for significant studies in the more easily obtainable *Rhesus* monkey. Thank you.

COLLINS: I have been given a three-page recommendation to hand in from our particular branch. The concern primarily is with the prior to Phase III and Phase IV to try and identify all the variables and then have the statisticians prepare the proper, shall we say, "what if" statistical analyses in advance, if at all possible, so that the appropriate data measuring selected essential variables will be collected in standardized fashions and, etc., so that there is no chance of missing out on any of the variables and their effect on the effect of the vaccine. The malaria branch has enumerated every variable and every statistical analyses they can think of. We would like to submit this for your consideration.

MARTIN: I think we have all been very impressed by the secretarial support that Dr. Siddiqui has provided to this conference in the rapid production of workshop summaries and summaries of the discussions. One of the activities of a scientific or technical committee that might be established might well be the rapid dissemination of informal results and information so that we will maintain the momentum of interest in

what is going on in the various pre-clinical and, hopefully, clinical studies on the various vaccines.

SIDDIQUI: Dr. Martin's suggestion is well-taken. USAID and the University of Hawaii will try to implement this specific recommendation. For the sake of information, I may mention that the proceedings of this conference will be published in the form of a book. These books will be distributed free to key countries and laboratories.

Closing Remarks

TRAGER: I think that the University of Hawaii has certainly outdone itself in its efforts to provide a wonderful ambiance for the meeting and for the publication of the proceedings. The idea of rapid dissemination at this point is a very good one which I feel should be considered in regard to how it could be implemented.

BRUCE-CHWATT: With regard to what Dr. Trager said, I personally am so tremendously impressed by the effort deployed by the Secretariat here that I would like to see a reflection of our gratitude as a collective thanks extended to Dr. Siddiqui, to the organizers of the conference, and in particular to the Secretariat staff. I am tremendously impressed and most grateful. Thank you.

TRAGER: I am very grateful to Leonard for saying that because I was going to say that among my very closing remarks. There is no question of the great effort of the Secretariat. Well, now I think it is time to close. We have only one more pleasant duty and that is to again express the great appreciation of all of us to Dr. Siddiqui, and to his faithful colleagues, for the wonderful organization of this conference. Thank you all.

SIDDIQUI: Dr. Trager, I thank you for your very generous compliment. The University of Hawaii, with the assistance of USAID and other sponsoring agencies, will take the initiative to disseminate the knowledge gathered during and after the conference.

BRUCE-CHWATT: I was inspired Thursday evening by the beauty of the spectacle that we saw, by the beauty of the island, and by the marvelous food and congenial atmosphere. That night, not being able to sleep a great deal, I said to myself that it may be a good idea to present to the meeting this morning a Japanese haiku for which this island is well known. Now, as you know, a haiku is a brief poem of not more than three lines. The first line should be composed of five syllables, the second of seven syllables, and the third of five syllables again. So, I prepared this sort of haiku:

"Palm-fringed island
Group of learned wise men
Fighting old disease

Yashi no shima
Kenja no tsudoi
Okori utsu

Malaria was called OKORI in old Japan.
The calligrapher is *Mrs. Masumi Nakabayashi.*

柳子の島

賢者はつづい

疫討つ

一九八五年四月

中林ますみ書

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The Ballad of the Plasmodium

LEONARD J. BRUCE-CHWATT

Plasmodium has a lot in store
And works in stages by the score
Anopheles that probes your skin
Pumps many sporozoites in
They lose no time, move into liver
And settle down before you shiver
Some hypnozoites go to sleep
A late relapse intending keep
But others grow, divide like mad
And move from liver into blood
In red cells do their very worst
Expanding to make each cell burst
As merozoites they are vexed
By being greatly undersexed
Their life is tedious, rather stale
Without a female and a male
This truth they soon will realize
And change assuming larger size
So now, when a mosquito bites
It must suck up gametocytes
And microgametes being sucked
Can now perform the amorous act
When in the stomach of the gnat
They find a macrogamete fat
The love's great feat is now complete
As female forms ookinete
Then losing all her self-control
She goes through insect's stomach wall
And there encysted feels more able
To raise a new plasmodial stable

When thousand sporozoites fit
Will prove that she has done her bit
To salivary glands they wend
And start again; there is no end.
The moral of this age-old story
Is that we can't aspire to glory
Until our principal objective
Will make control much more effective
We try to fight Anopheles
With our weapons in a mess
Since DDT that was the norm
Is now refusing to perform
And most of new insecticides
Have some unwelcome costly sides
By now *P. falciparum*
Is causing very great malarium
Although these protozoan vultures
Can now be grown *in vitro* cultures
Thus faced with chloroquine resistance
We must depend on more assistance
And find a new medicament
To solve our great predicament
Or to develop other means
To fight those vicious little fiends
Which no one who is sound of mind
Can be defending from Mankind

Program

SUNDAY EVENING
Opening Ceremonies and Dinner

MONDAY
Session I
Current Status of Malaria and Malaria Control Programs in Countries of Asia and the Pacific Basin

TUESDAY
Session II
Current Status on the Development of Malaria Vaccines
A. Candidate Antigens
B. Evidence of Protection

WEDNESDAY
Session III
Practical Considerations of Malaria Vaccines
A. Production of Plasmodia Antigens by New Biotechnology
B. Applicability of Information on Modern Vaccines for the Production of Malaria Vaccines

THURSDAY
Session IV
Strategies for Future Malaria Vaccine Trials I
A. Experiences from Clinical and Field Trials of Antibacterial and Antiviral Vaccines
B. Phase I, II, and III Clinical Trials: General Considerations
C. Technical Aspects of Phases I, II, and III Clinical Trials

FRIDAY
Session V
Strategies for Future Malaria Vaccine Trials II
A. Methods for Evaluation of the Impact of Vaccination on Control of Malaria
B. Integration of Vaccine with Other Malaria Control Programs
C. Strategies for Future Malaria Vaccine Trials

SATURDAY
Session VI
General Conclusions and Recommendations
A. General Conclusions and Recommendations
B. Closing Remarks

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