VACCINES AND PUBLIC HEALTH
ASSESSING TECHNOLOGIES AND POLICIES
FOR THE CHILDREN'S VACCINE INITIATIVE

Antigen Processing of Oligosaccharide Protein Conjugate Vaccine

B Cell

Immunoglobulin

Proteolytic Enzyme
Digestion of Carrier

Class II MHC

Peptide Association with Class II MHC

T Cell

T Cell
Receptor

Peptide Presentation

Class II MHC

National Institute of Allergy and Infectious Diseases
Fogarty International Center
National Vaccine Program

November 5 and 6, 1992
Pooks Hill Marriott
Bethesda, Maryland
Thursday, November 5

8:30  Continental Breakfast

9:00  Introduction to the Conference
      John R. La Montagne

9:10  The Genesis of the Children's Vaccine Initiative (CVI)
      D.A. Henderson

9:20  The CVI in a Changing World
      Richard Bissell

PANEL I: THE CHILDREN'S VACCINE INITIATIVE: THE CHALLENGE
      Chair: D.A. Henderson

9:30  Introduction: D.A. Henderson

      Vaccine Technologies and Public Health: Why a Critical Review Now?
      Anthony Robbins and Phyllis Freeman

      The Promise of New Technologies
      John R. La Montagne and Regina Rabinovich

      Desired Field Performance Characteristics of New Improved Vaccines
      for the Developing World
      Ciro de Quadros, Peter Carrasco and Jean-Marc Olive

      Desirable Immunologic Characteristics of Vaccines for the CVI
      Gordon Ada
11:30 Lunch

PANEL II: IMMUNOLOGIC ISSUES IN VACCINOLOGY
Chair: Richard M. Krause

12:30 Introduction: Richard M. Krause

Modern Concepts in Immune Recognition and Lymphocyte Activation
Ronald N. Germain

Development and Maturation of the Immune System
Alexander R. Lawton

Development of Effective Mucosal Immunity
Jerry R. McGhee and Hiroshi Kiyono

Adjuvants and Immune Enhancement
Anthony C. Allison

Glycoconjugate Vaccines
Kathryn E. Stein

3:00 Coffee Break

PANEL III: MEASURING THE IMPACT OF VACCINE RESEARCH AND DEVELOPMENT
Chair: Scott Halstead

3:30 Introduction: Scott Halstead

The Role of Vaccine R&D in Scientific Development of Newly Industrializing Countries
Adolfo Martinez-Palomo, Malaquias Lopez Cervantes, and Phyllis Freeman

Do Vaccines and Immunization Programs Stimulate Economic Development?
Terrel Hill and David Parker

The Effectiveness of Means of Controlling Communicable Diseases
Kenneth S. Warren
Friday, November 6

8:00  Continental breakfast and coffee

8:30  Introduction: Kenneth Bart

PANEL IV: PERSPECTIVES IN PUBLIC HEALTH POLICY
Chair: Isao Arita

8:40  Introduction: Isao Arita

Public Investment in Science for Public Health

Transfer of Vaccine Technology to Developing Countries: The Latin American Experience
Robert Knouss and Akira Homma

PANEL V: VACCINE TECHNOLOGIES FOR THE CVI
Chair: John R. La Montagne

9:30  Introduction: John R. La Montagne

Combination Vaccines
Ronald W. Ellis and R. Gordon Douglas

Live Attenuated Vaccine Vectors
John J. Mekalanos

Synthetic Peptides and Purified Antigens as Vaccines
Fred Brown

11:00  Coffee

11:30  Novel Approaches to Controlled Release Antigen Delivery
Smadar Cohen, Maria J. Alonso, and Robert Langer

Thermostability of Vaccines: Technologies for Improving the Thermal Stability of Oral Poliovirus Vaccine
Stanley M. Lemon and Julie B. Milstein

Maternal Immunization
Richard Insel, Marvin Amstey, Kathleen Woodin, and Michael Pichichero

1:00  Lunch
PANEL VI:  CHALLENGES IN VACCINE MANUFACTURE AND QUALITY CONTROL

Chair: Philip Russell

2:00 Introduction: Philip Russell

Global Capacity for Manufacturing Vaccines: Prospects for
Competition and Collaboration Among Producers in the Next Decade
Anthony Robbins and Isao Arita

New Challenges in Quality Control and Licensure:
Technologies
Carolyn Hardegree

New Challenges in Quality Control and Licensure: Regulation
R. Gordon Douglas and Kenneth R. Brown

2:30 A New Technological Synthesis
S. Ramachandran and Philip K. Russell
Dr. Gordon Ada, John Curtin School of Medical Research, P.O. Box 334, Canberra, A.C.T. 2601, Australia. Tel: (06) 249-2596; Fax: (06) 249-2595.

Dr. Anthony C. Allison, Syntex Research, 3401 Hillside Ave., P.O. Box 10850, Palo Alto, CA 94303. Tel: (415) 855-5096; Fax: (415) 354-7554.

* Dr. Isao Arita, Agency for Cooperation in International Health, ACIH, 4-11-1 Higashimachi, Kumamoto City 862, Japan. Tel: 81-96-367-8899; Fax: 81-96-367-9001.

* Dr. Kenneth J. Bart, National Vaccine Program, Parklawn Bldg., Rm. 13A-53, 5600 Fisher's Lane, Rockville, MD 20857. Tel: (301) 443-0715; Fax: (301) 443-3386.

Dr. Fred Brown, Plum Island Animal Disease Center, P.O. Box 848, Greenport, N.Y 11944-0848. Tel: (516) 323-2500; Fax: (516) 323-2507.

Dr. Smadar Cohen, Ben Gurion University, Dept. of Chemical Engineering, Beer Sheba 84105, Israel. Tel: (011) 972-57-461-798; Fax: (011) 972-57-36-446.

Dr. Ciro de Quadros, Regional Advisor, Expanded Program on Immunization, Pan American Health Organization, 525 23rd Street, NW, Washington, DC 20037. Tel: (202) 861-3247; Fax: (202) 861-6089.

Dr. R. Gordon Douglas, Jr, Merck Vaccine Division, P.O. Box 2000, Rahway, NJ 07065-0900. Tel: (908) 594-3234; Fax: (908) 594-5009.

Dr. Phyllis Freeman, University of Massachusetts Law Center, Boston, MA 02125. (617) 287-7372; Fax: (617) 287-7379.

Dr. Ronald Germain, NIAID, National Institutes of Health, Bldg. 10, Rm. 11D18, Bethesda, MD 20892. Tel. (301) 496-1904; Fax: (301) 496-0222.

* Dr. Scott Halstead, Department of Health Sciences, The Rockefeller Foundation, 1133 Avenue of the Americas, New York, New York 10036. Tel: (212) 869-8500; Fax: (212) 764-3468.

Dr. Carolyn Hardegree, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892. Tel: (301) 496-1014; Fax: (301) 402-0763.

* Dr. Donald A. Henderson, Associate Director for Life Sciences, Office of Science and Technology Policy, Old Executive Building, 17th & Pennsylvania Avenue NW, Washington DC 90024-1521. Tel: (202) 456 2892; Fax: (202) 395-1571.
Dr. Terrel Hill, United Nations Children's Fund, Child Survival Unit, HBF, Three United Nations Plaza, New York, NY 10017. Tel: (212) 326-7328; Fax: (212) 326-7336.

Dr. Richard A. Insel, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642. Tel: (716) 275-0414; Fax: (716) 442-7222.

Dr. Robert Knouss, Pan American Health Organization, 525 23rd St., N.W., Washington, D.C. 20037. Tel: (202) 861-3178; Fax: (202) 223-5971.

* Dr. Richard Krause, Fogarty International Center, Building 31, Room B2C39, 9000 Rockville Pike, Bethesda, MD 20892. Tel: (301) 496-7611; Fax: (301) 496-2173.

* Dr. John R. La Montagne, Director, DMID, NIAID, National Institutes of Health, Solar Bldg., Rm. 3A18, Bethesda, MD 20892. Tel: (301) 496-1884; Fax: (301) 480-4528.

Dr. Robert Langer, Department of Chemical Engineering, Massachusetts Institute of Technology, Bldg. F25, Rm. 342, Cambridge, MA 02139. Tel: (617) 253-3107; Fax: (617) 258-8827.

Dr. Alexander R. Lawton, Vanderbilt University School of Medicine, D-3237 Medical Center North, Nashville, TN 37232-4397; Fax: (615) 343-6249.

Dr. Stanley M. Lemon, University of North Carolina, CB# 7030, 547 Burnett-Womack, Chapel Hill, NC 27699-7030. Tel: (919) 966-2536; Fax: (919) 966-6714.

Dr. Adolfo Martinez-Palomo, Centro de Investigaciones y de Estudios Avancados, Apartado Postal 14740, Mexico 14 D.F. Tel: 525-754-5116; Fax: 525-754-5116.

Dr. Jerry R. McGhee, Department of Microbiology, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294. Tel: (205) 934-5045; Fax: (205) 934-3894.

Dr. John J. Mekalanos, Department of Microbiology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115. Tel: (617) 432-1000; Fax: (617) 738-7664.

Dr. Anthony Robbins, Boston University School of Public Health, 80 East Concord St., T3C, Boston, MA 02118-2394. Tel: (617) 638-4620; Fax: (617) 638-4857.

* Dr. Philip Russell, The Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe St., Baltimore, MD 21205. Tel: (410) 955-1624; Fax: (410) 550-6896.

Dr. Kathryn E. Stein, Laboratory of Molecular and Developmental Immunology, FDA, Bethesda, MD 20892. Tel: (301) 496-6916; Fax: (301) 496-0222.

Dr. Kenneth S. Warren, Maxwell Communications Corp., MacMillan, Inc., 866 Third Ave., New York, NY 10022. Tel: (212) 702-9515; Fax: (212) 605-9395.
Vaccine Technologies and Public Health: Why a Critical Review Now?

Anthony Robbins, M.D.* & Phyllis Freeman, J.D.

*Profesor Investigador Visitante
INSTITUTO NACIONAL DE SALUD PUBLICA
AV. UNIVERSIDAD 655
COL. STA. MARIA AHUACATITLAN
C.P. 62508 CUERNAVACA, MORELOS
MEXICO

Institute Switchboard 52 73 11 01 11 ext. 2759
Telephone & Telefax 52 73 17 55 29
Vaccine Technologies and Public Health: Why a Critical Review Now?

Researchers, public health professionals, and a growing cadre of others concerned about the health and future of the developing world have scrambled to assemble the Children's Vaccine Initiative. The aggregation of creative energy has been impressive. However, today is the first time that colleagues from the world of global public health policy and from the world of laboratory science (including a few who reside in both camps) have convened to take a critical look at the policies that guide vaccine and immunization programs world-wide and the technologies that are available to advance the Children's Vaccine Initiative. In this brief introduction to the conference we suggest why it is important to assess both policies and technologies--now and together.

Since Jenner's time there have always been enthusiasts and skeptics about the role of vaccines in disease prevention. Positions have often been argued with more ardor than rigor. Both this conference and the special issue of the *International Journal of Technology Assessment in Health Care* in which the finished papers will appear are meant to push all of us to review that history and to analyze the prospects for the future. How much do infectious diseases affect the health of the world's people? What role have vaccines played in limiting the damage caused by communicable diseases? What lessons have we learned about designing, developing, producing, and delivering vaccines? For the future, can these lessons be applied to improving strategies for research, development and delivery of vaccines? If we do our work well, both the impact of vaccines and the audience for our analysis can be broadened. This meeting is predicated on that thesis.

There is little debate that until this century, infectious diseases were a, if not *the* major cause of early death and disability everywhere in the world. Historians have credited vaccines and medicine for little of the improvement in health. In Europe, North America and other countries that industrialized first, major infectious diseases waned first. In fact, we can almost define development by the measures that have reduced the burden of infectious diseases. Improved nutrition was a product of new agricultural technology. Protection of water supplies and food from contamination by human wastes were followed by better personal hygiene, pasteurization, refrigeration, and sanitary preparation of food, and all contributed to better health. For industrial nations, reduced crowding at
home and work, control of rats and other carriers of disease, and safer workplaces played a far larger role than vaccines. And only after World War II could much improved health be attributed to medical practice—diagnosis and treatment.

Yet even the most demanding of analysts usually credit immunization as having had a significant role in reducing the impact of smallpox, diphtheria, tetanus, polio and measles. For other diseases, knowledgeable skeptics argue that vaccines may have played a minor role in industrial nations, but the incidence and prevalence of most infectious diseases was already waning in response to what we call development.

In industrial countries of the 21st Century vaccines can become increasingly important as the most cost-effective means to prevent a group of diseases that have not yielded to development, public health measures, or treatment. Today, even in those countries with the highest standard of living, the lowest infant mortality rates and the longest life span, certain infections have eluded prevention and treatment. Typically these are viral diseases, spread from person to person—by inhaled droplets or sexual contact.

Just as the so called "industrial nations" range from those with little poverty to those where substantial populations live without the benefit of adequate nutrition and effective environmental, public health, and medical services, so too the "developing nations" are anything but homogeneous in standard of living and patterns of disease. The distinction between "industrial" and "developing" countries has blurred further, as millions of people in Eastern Europe have lost the benefit of disease prevention and medical services they once enjoyed. Furthermore, on top of poverty—one way we define developing countries—many nations in the southern hemisphere confront the additional challenge of "tropical diseases" (which Dr. Warren will discuss). Backsliding also occurs in nations at war—civil or international, declared or undeclared.

In less developed nations, in war zones and in areas of severe economic crisis, infectious diseases plague the population. Where clean water supplies are unavailable or unpredictable, where sewage is not treated consistently, where disease carrying insects and rodents persist in human communities, where housing and working conditions are crowded and dirty, infectious diseases continue to thrive and play a far larger role in the health landscape.
Analyses drawn from industrial world experience may not prophesize the role of vaccines in developing countries and in development. Vaccines and their delivery have proven to be inexpensive compared to other population-based approaches to disease prevention and control, and to medical diagnosis and treatment. Thus, in the future, vaccines may be the vanguard of development, rather than a technology and practice introduced to mop up the diseases that did not disappear with improved living conditions. Can vaccines be used to improve child survival and health so that economic development can proceed more easily? (A topic to be discussed by Parker and Hill.)

In the early 1970s as the smallpox eradication campaign neared its goal, public health workers saw a new and expanded role for vaccines—-not just one vaccine against one disease, but a battery of vaccines to prevent the deadly and disabling childhood infections. WHO created the Expanded Program on Immunization, setting the goal of universal childhood immunization—vaccinating every child in the world against six diseases for which vaccines were available: diphtheria, pertussis, tetanus, tuberculosis, polio, and measles. After a decade of false starts, non-productive competition, and duplication of effort, WHO and UNICEF resolved to work together. They rallied country after country to close in on their 1990 goal of full immunization for all children.

Success might have been defined in disease control terms, but lacking adequate surveillance and confronting real obstacles that made 100% coverage an impossible goal, the international organizations redefined universal childhood immunization downwards. No one claims that we more than brushed the 80% target in 1990, the year in which UNICEF and WHO proclaimed victory. The international community had begun to talk and worry about "sustainability"—a realistic and necessary but uninspiring goal. How much longer would the world keep investment in immunization a top priority without a new sense of excitement?

New excitement emerged slowly and from an entirely new quarter, the biomedical research community. Molecular biology took a leap forward with recombinant DNA technology and these National Institutes of Health provided the bulk of support for the new work. Tools crafted for studies of genetics in microorganisms became essential to all biomedical research. In the late 1970s, researchers at the National Institute of Allergy and Infectious Diseases recognized the power of the new biology for the development and production of new and improved vaccines.
Unfortunately the National Institute of Allergy and Infectious Diseases' initiative for accelerated development of new vaccines surfaced just as the Reagan Administration was threatening the first major cut in the history of NIH extramural grants. True to its constituency of current grantees, the Director of NIH stopped funding for the vaccine initiative before it could come to fruition. NIAID never did receive the support it requested for accelerated vaccine development, but the vision has been elaborated, refined (and captured in the annual Jordan report\(^1\) ). It continues to inspire and excite.

In 1983, NIAID asked the Institute of Medicine, then headed by Frederick Robbins, to convene a panel of experts to ratify and refine the conclusion that biotechnology portended a revolution in vaccines. The IOM concluded on the basis of existing scientific knowledge that there were 14 vaccines for the United States and another 19 of special interest to the developing world for which there was a better than even chance they could be licensed and available within a decade\(^2\).

Even before the ink was dry on the IOM reports, we predicted that the IOM estimates based on the science alone were far too optimistic. The market for vaccines was not driving improvement of old vaccines or development of new vaccines as rapidly as the science would allow. Past experience, starting with the "liability crisis" surrounding swine flu and then pertussis provided unimpeachable evidence that the process of vaccine development, production and distribution could easily get stuck because of deficient investment, reflecting an unacceptable lack of urgency. The history of vaccine development for countries of the third world was more dismal, for they lacked both hard currency to buy vaccines and the elements of a system to develop their own.

Encouraged by scientists and public health officials who saw the need to avoid delays in vaccine development, production, and use, the United States Congress perceived the need for integrated planning and management of the vaccine development and immunization. Each step from laboratory research through product development, field testing, licensure, production, distribution, use, and surveillance had to proceed rapidly from the previous one if vaccines were to be used optimally. With paltry public health

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\(^1\)Starting in 1979, Dr. William Jordan authored an annual report on the prospects for accelerated development of vaccines. The National Institute of Allergy and Infectious Diseases continues to issue the report, that bears Dr. Jordan's name.
spending on immunization and outstanding profits available from investments in pharmaceuticals and diagnostics, it was unlikely that the market for vaccines would drive the process to the best results. In 1986, over the objections of the Reagan Administration, Congress enacted the National Vaccine Program, the world's first integrated planning program for vaccines.

The world community, on reaching the goal of universal childhood immunization--80% of the children born in 1990 were fully immunized--but with no clear course charted for the future, made a diagnosis not unlike the Congress' and resorted to a similar strategy. In planning for the World Summit on Children (September 1990 in New York), harnessing new science to overcome old obstacles to new and improved vaccines for the Expanded Program on Immunization became one key to future disease prevention. The Children's Vaccine Initiative emerged as the strategic program of product development to assure rapid availability of vaccines suited to the diseases and conditions in the developing world. The Children's Vaccine Initiative would seek improved efficiency and efficacy from immunization programs, defined in practical terms well understood by public health personnel in the field.

The first goal was to reduce the number of contacts required to immunize a child fully. UNICEF estimates that today it takes over 500,000,000 separate contacts annually to achieve current levels of immunization against six diseases(1). A second goal was to reduce dependence on refrigeration. The cold chain costs fully as much as the vaccine themselves. A third goal was to reduce the use of needles, syringes and sterilization equipment which are expensive and pose a threat from hepatitis B and HIV. Fourth, the protection offered by vaccines had to be achieved at an earlier age and last longer. Fifth, new vaccines should permit prevention of at least another dozen diseases that persist as major hazards to children. And throughout, these advances and improvements were to be affordable to developing countries and contributing donors.

Because these ambitious goals required new vaccine products and delivery technologies, The Children's Vaccine Initiative is principally a product development program, but it is intimately linked to planning and investment that will assure production of the new products, the means to deliver them, and the resources to acquire them where needed. The primary goal is to assure that scientific advances that are applicable to vaccine development, production and administration are exploited promptly for the benefit of public health.
The objectives of this conference emerge naturally from the purpose and program of the Children's Vaccine Initiative. For the benefit of researchers and developers who seek to respond to The Children's Vaccine Initiative we must clarify what public health demands of vaccines in the future. If the technology were available, what are the characteristics of vaccines that we would prescribe and how would they be used? For the benefit of the public health professionals who have enlisted practitioners of research and development to advance the cause of immunization, we must assure an understanding of the technologies that are at hand or on the horizon. What immunization problems can be conquered by the application of science? What does the future hold? For example, must new vaccine technologies always be more expensive or is it the profit motive that has promoted the development of expensive vaccines for industrial world markets?

We will succeed if we stimulate active interchange between the denizens of the laboratory and the policy makers and public health practitioners who are far more comfortable at a desk or in a remote village. To succeed, we must present more than conventional wisdom. (However, be reassured, we are not asking that your critical assessments topple your beliefs of last year, last month, or yesterday--simply that you present your analysis so we will all understand your critical assessment of the science, the technologies, and the policies, and your conclusions for today.)

We are particularly grateful to the National Institute of Allergy and Infectious Diseases, the Fogarty International Center, and the National Vaccine Program for sponsoring this conference. Strong backing from the editors and publishers of the International Journal of Technology Assessment in Health Care encouraged us to join public health policy and molecular biology in a single conference for a special issue of the journal. And now a commitment from the United Nations Development Program to buy and distribute the results of everyone's work means that we can reach the global audience that will benefit from these new insights about and for the Children's Vaccine Initiative.
REFERENCES

Meeting on "Vaccines and Public Health: Assessing Technologies and Global Policies for the Children's Vaccine Initiative"
Bethesda, Md. 5-6 November, 1992

Section III: Vaccine Technologies


Ciro A. de Quadros, M.D., M.P.H.
Peter Carrasco, M.B.A.
Jean-Marc Olive, M.D., M.P.H.

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6. Management and Logistics

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I. INTRODUCTION

This chapter examines the field experience of some of the vaccines that have been or are currently being used by immunization programs to combat disease. It illustrates the current problems and obstacles that field workers face when using the currently available vaccines. Using field experiences with the different vaccines which have been introduced on a wide scale, the chapter compares and discusses the advantages of an ideal vaccine and some of the benefits that could be obtained should such new or improved vaccines become available.

While the vaccination initiatives undertaken over the last decade are the cornerstones for protecting the world's children and helping assure their development and survival, the current vaccine technologies being employed may become obstacles for further disease reduction or eradication. The reason being that the characteristics of the vaccine technologies presently in use in routine vaccination programs complicate or increase the burden of health care infrastructures to maintain or expand vaccine delivery systems.

Eradication of Smallpox

The first systematic effort to use a vaccine technology to prevent disease was Edward Jenner's in 1796 who organized the first initiative to vaccinate the public against smallpox. The first global initiative to apply the modern principles of immunization and a vaccine technology to eradicate a vaccine preventable disease was the successful W.H.O.'s Smallpox Eradication Program. The successful eradication of smallpox in 1977 has become the gold standard by which all immunization initiatives and disease eradication/elimination efforts will be measured by.

As new vaccines became available during the decades of 1940s, 1950s and 1960s they were integrated into the different vaccination programs in each country. One of the most celebrated vaccines to be made available was the inactivated poliomyelitis vaccine (IPV) that was immediately used to combat the large polio outbreaks which occurred during the early 1950's in Europe and the U.S.A. and in the 1960's in Latin America, and elsewhere. With the availability in 1961 of the oral poliomyelitis vaccine (OPV) which was much
easier to administer many countries began mass campaigns to vaccinate their children against poliomyelitis.

Despite the availability of new vaccines between 1950 and 1970 the use of them in consistent and sustained programmatic fashion did not occur in all countries. Generally, it can be said that it was only in those countries, with a strong health care infrastructure, that successfully integrated these new vaccines into their health care services. Thus countries like the U.S.A., Sweden, and other European countries were the first to control and or eliminate poliomyelitis, diphtheria, and measles. During this period many other countries in the world introduced these new vaccines into their public health care system and undertook concerted efforts to control disease outbreaks such as poliomyelitis but were unable to sustain and or provide the commitment to these vaccine technologies to assure their integration into their routine health services.

Even the global effort to eradicate smallpox undertaken in 1959 by W.H.O. stumbled during its first seven years because of lack of sufficient funds that were necessary to organize and set up the management and logistics required for such an audacious undertaking (2). However, once the Smallpox Eradication Program received special funds for an intensified program in 1967, W.H.O. was able to augment the health infrastructure or even temporarily create some for purposes of eradication in the different countries reporting cases of smallpox. What made this possible was the type of vaccine that was being employed and the mode of administration.

Smallpox vaccine was highly heat stable, did not require refrigeration in the field and required only one dose to confer protection. Its administration with a bifurcated needle that later need only to properly sterilized was relatively easy to handle by health workers with relatively little training. These characteristics of smallpox vaccine were fully exploited by W.H.O. and country program management and greatly contributed to the eventual success of the smallpox eradication program in 1977.

Expanding Immunizations

In 1974, W.H.O. launched the Expanded Program on Immunization which at that time had the twin goals of providing immunization
services to all children by 1990 and reducing the mortality and morbidity of some vaccine preventable disease. To achieve these goals W.H.O., UNICEF and other donor agencies in conjunction with the countries agreed that the self reliance in the delivery of immunization services would be best achieved by integrating immunizations into the comprehensive health services of each country. This initiative became a global crusade that enlisted all governments and international and national donor agencies to support the commitment that had to be made if the governments around the world were to achieve and sustain the goal of universal childhood immunization.

The commitment required to reach the goal was dictated by the available vaccine technology, the number of antigens, doses, and scheduling of doses required to fully immunize a child against the target diseases. Unlike the eradication of smallpox, the expansion of immunizations require commitment for a rather large and sustained investment to provide and augment the capacity of the health infrastructure to deliver the initially targeted vaccines DPT, BCG, OPV, Measles vaccine and TT).

As countries committed themselves to attain high levels of coverage, the idea of eradication or elimination of some of the target diseases (e.g., poliomyelitis and neonatal tetanus) crystallized as feasible goals which could be further used to strengthen the global commitment towards disease control.

Eradication of Poliomyelitis

Led by PAHO, and supported by USAID, UNICEF, the Inter American Development Bank, the Canadian Public Health Association and Rotary International, the Region of the Americas was the first in the world that achieved a dramatic decline in confirmed cases of polio between 1985 and 1992. Since August, 1992 when wild poliovirus was recovered from a paralytic two year old child in Peru no paralytic case of poliomyelitis due to wild poliovirus was detected anywhere in the entire Western Hemisphere. This spectacular success in the Americas not only led the way for the global initiative to eradicate the wild polio virus by the year 2000, but also paved the way for the English Speaking-Caribbean countries to launch an initiative aiming at the elimination of measles from the subregion by 1995 (4). Cuba, Brazil, Chile and the Central American Countries
have launched similar efforts.

The available polio and measles vaccination technologies proved that they were capable of helping governments reach very high levels of vaccine coverage, eradicating polio, at least in the Americas, and supporting the elimination of measles in the English Caribbean. But the support required to sustain these technologies may prove to be too much of a burden on the capacities of the health structures, and the national health care budgets in other countries of the world.

Because most presently available vaccines require multiple doses or must be scheduled within the first twelve months life, the health structure in each country must provide for recurrent costs in order to sustain their capacity to deliver immunization services routinely. Because the vaccines also require refrigeration at all levels, the cold chain infrastructure must be maintained and or expanded.

A new set of improved vaccines or a vaccine that contains all the childhood antigens or a large subset of antigens, that requires only one dose or two doses at the most, that can be given earlier in life, that are more heat stable and therefore do not require refrigeration in the field, that are effective against a wide variety of diseases not currently targeted by immunization but which take a heavy toll of needless deaths and that are affordable, would not only help assure the control and or eradication of certain diseases but would also reduce the cost burden on health care system and parents and would encourage the community to demand the vaccination of their children.

II. OBSTACLES AND OPERATIONAL NEEDS

1. Vaccination Schedules

One of the most important obstacles that the currently used childhood vaccines present are the complicated vaccination schedules that must be followed in order to properly confer immunization and the logistical system that must be put in place or maintained in order to routinely deliver and conserve the necessary doses for each person or child.
The current W.H.O. immunization schedule call for a child to receive a single dose of BCG and OPV at birth followed by three doses of DPT and OPV at 6, 10, and 14 weeks. Measles vaccine is provided at 9 months of age. This schedule has shown to cause the following field problems in many countries of the world:

a) High drop-out rates: parents do not return to the vaccination clinic to immunize their children with the second or third doses of the required vaccine. The drop-out rates can be due to: lack of parental information or the necessity for additional doses, inappropriate clinic hours for the parents to take their children to get vaccinated, refusal of clinic staff to open a vial of vaccine at the end of the day because of the high wastage rate. In addition, the interval between the third OPV/DPT dose and measles is so long that the parent may forget or does not have the time to return at the appropriate time because of many other factors. While it is not well documented, field experiences in some Latin American countries suggest that due to rising fuel and transportation costs, as well as, increased medical costs, families will delay or default on their follow-up visit to the health services. Therefore, families may postpone their visit for vaccinating their children with the second or third doses of an antigen because of economic reasons.

b) Missed opportunities: this refers to the failure of the health establishment to actively check the immunization status of each child that visits the health establishment regardless of the reason for child visit and vaccinate the child if necessary. While there are many methods to capture children in order to review their immunization status, it is obvious that an antigen or set of combined antigens that require one or two doses will not only result in less missed opportunities but would dramatically reduce the drop-out rates, as well as, help reduce the economic costs to the parents in vaccinating their children. Cost savings to health care system would also be realized because of the reduced paperwork and follow-up systems that are now currently in place in order to track each child for the multiple dose vaccines.

2. Heat Stability

Because the currently available vaccine technologies in general require that two or three doses of vaccine be administered to
confer immunity and the important fact that all the current vaccines are not heat stable, requiring refrigeration, health systems in all countries must enhance, extend, and or create an infrastructure capable of assuring proper storage and timely delivery of vaccines. Furthermore, each health establishment has to be provided with the necessary refrigeration equipment to assure that vaccines are transported at proper vaccine temperatures. A heat stable vaccine will eliminate the use of kerosene refrigerators, which have a very poor history of temperature performance because of the polluted or very poor quality kerosene available in most developing countries.

Perhaps the greatest operational need that health care workers would like to see would be more heat stable vaccine that could be carried practically in their pockets. This would free health workers to more easily plan their vaccination rounds. A more heat stable set of vaccines would offer the following tactical advantages: a) a greatly reduced number of vaccine failures because the potency of the vaccines would no longer be dependent on proper temperature storage. b) free health care workers to reach areas of difficult access without worrying about the cold chain.

3. Adverse Events

Besides the necessary visits that parents must make to assure that their child is fully immunized according to the immunization schedule, another important problem that creates further obstacles for the demand of immunization are the adverse events associated with some of the vaccines, and their route of administration. Except for OPV, all other vaccines are administered with injection technology. If the injection procedure is not followed correctly then there is a higher risk that the recipient may experience not only unnecessary pain but can also develop swelling or an abscess. Other very rare severe complication may also occur. When there is an untoward high rate of abscesses in a community, the parents may be very hesitant to continue with their child's immunization schedule. The injection procedure can be affected by improper sterilization, unhygienic practices - that can occur in field situations if the health workers are not well trained, unsharpened needles are used, and poor application or improper introduction of the needle into the tissue of the arm, thigh, or buttocks.
Complicating the adverse reactions due to improper injection techniques is the reactogenicity of the vaccine themselves. Adverse vaccination events also creates a burden on the health care system by: 1.) increasing the cost of immunization by providing the treatment costs for the affected individuals. and 2.) maintaining an enhanced surveillance system for the monitoring of these adverse events, especially for those vaccines that require multiple doses. Because of the current sets of antigens require multiple doses the health care system must plan for health education activities, as well as, social mobilization campaigns in order to mobilize all segments of society and draw upon the untapped additional resources needed for assuring that the highest vaccine coverage are obtained. A multiple doses vaccine will require that multiple messages to the community are delivered to assure compliance with each dose. Because generally compliance with the third dose is the most difficult to obtain much effort and resources are directed towards the community to get parents to bring in their children for this last dose.

4. Vaccine Failures

Another major problem the a vaccination program can have is vaccine failure. The community is the first to suffer and with this the credibility of the health services. Vaccine failure can arise from improper storage of vaccine, i.e. cold chain failure, improper techniques of administration including wrong dosage, wrong age of administration, and host immune response. When vaccine failures do occur they must be properly documented and explained to the community otherwise this will create an obstacle that will hamper future efforts to protect the health of individuals and maybe eradicate or eliminate a disease.

5. Recurrent Costs

While in principle these considerations are not necessarily obstacles to the delivery of immunization, they represent management obstacles and create a recurrent cost burden. The magnitude of this recurrent cost burden will vary from country to country. In countries which carry heavy debit burdens, or, small health budgets, these recurrent costs are a major obstacle to maintaining, enhancing, and extending the delivery of immunization services to all the population.
Since many of vaccines currently available require a syringe for their effective administration, the health care system must either develop or augment waste management procedures for the proper disposing of needles and syringes, set up and develop an infrastructure to train, equip and resupply all health establishments with the sterilization equipment and fuel. The recurrent costs associated with injection technology and logistical supply may be financial and management obstacles and hence impede and disrupt the vaccination program, thereby delaying the achievement of targets.

Surveys carried out in Latin America showed that recurrent cost associated with enhancements, or extensions to the cold chain can account for as much as 50% of total costs of the immunization programs. Of the total recurrent cost associated with the cold chain in the different countries surveyed a good proportion (range 22% - 42%) is dedicated to supporting activities related to the distribution of vaccine and per diem and transport costs associated with rural vaccination costs. If new vaccines are made available, especially a single antigen vaccine that combines several antigens the annual cost savings that can be accrued if immunity is conferred in one single dose can be substantial. These recurrent costs (associated with the currently used childhood vaccines) that would be saved range from fuel and transportation costs associated with the multiple delivery of vaccines and syringes, combustible fuels associated with the storage of vaccines; spare parts and energy costs for the refrigeration equipment used; per diem and transportation costs associated with repair and maintenance of the cold chain.

In the early days of yellow fever campaigns, for example, the vaccinator was hampered because of rather bulky field kit that was required to be assembled and transported to wherever he was scheduled to vaccinate. More than fourteen pieces of equipment were necessary for administering the vaccine. By comparison the current administration of yellow fever vaccine is quite simple requiring a syringe, diluent, and the freeze dried vaccine plus a cold box to transport the vaccine.

6. Management and Logistics

There are other management obstacles that may surface because
of the currently available vaccine technology. One obstacle resulting from a vaccine requiring multiple doses is that supervision of the vaccination program is process oriented to assure that the proper data is collected and that the logistical operations are in fact in place and effectively producing the expected outputs to assure that each dose of vaccine handled by the health establishments is recorded and remains potent.

A new set of vaccines that do not require multiple doses or do a parenteral route of administration would be a major advance in vaccine technology. For example, if a new generation of more immunogenic vaccines that can confer the proper immunity after a single, oral dose, became available, the need for syringes and needles would be eliminated. Costs with supervision would also be reduced in the process. Such new vaccines would give health workers the ultimate tool to protect children and eradicate or eliminate a disease.

Some of these characteristics were presented by the smallpox vaccine, particularly heat stability and ease of administration. Vaccination rounds were quickly and easily executed as personnel did not have to worry about ice making or having the appropriate number of vaccine carriers and or cold boxes. Bulky syringes did not have to be transported and or properly disposed. The technological advantage offered by the smallpox vaccine was clearly illustrated during control activities that had to be quickly taken in the face of a suspected outbreak. The local smallpox team located in an interior town in Bangladesh, for example, could in a matter of hours mobilize itself and be up a river with a containment team because the smallpox vaccine did not have to be refrigerated and therefore one did not have to worry about finding ice or carrying extra quantities of ice to maintain the cold chain. The smallpox team therefore traveled very quickly, sometimes by the moonlight to gain time and rapidly implement containment vaccination. Because only one dose of smallpox vaccine was necessary to confer protection and more importantly, stop disease transmission, a second or third round of vaccination did not have to be planned for. In a large outbreak area community volunteers could be easily trained as vaccinators.

The polio eradication efforts in the Amazon basin in Peru offers a contrasting picture. Because of a limited infrastructure
a scout team had to spend several days exploring the rivers and mapping out the area to set properly a logistical system that could effectively support the transportation and safe storage of the very heat labile OPV vaccine. Fuel depots had to be established at certain logistically important points in order that the vaccine and ice could move along to the desired points. Rather large cold boxes had to be used to transport large quantities of ice that were necessary for assuring that sufficient ice was available for the smaller vaccine carriers. Because of the remoteness of the area other antigens were offered, therefore syringes also had to be planned for. The only advantage that OPV afforded was that is was orally administered and because of this community volunteers could be quickly trained to assist in vaccination activities. Only after this careful and time-consuming planning, where a rather costly infrastructure had to be put into place and maintained, could the health team return and execute the planned vaccination program in two rounds.

While PAHO has shown that polio could be eradicated with the existing OPV vaccine there are doubts that the same can be achieved in other parts of the world were the health care infrastructures are not as well developed as in the Americas. Therefore, the logistical barriers that the current polio vaccines have, require a sustained commitment and good management system to be in place, which for some countries maybe too costly.

If a heat stable polio vaccine was developed with similar characteristics as the smallpox vaccine, the rapid eradication of the wild polio virus could be more easily envisioned in other parts of the world, mainly because of the tactical edge that would be provided and the greatly reduced infrastructure that would be needed to be put into place with lower recurrent costs.

Should a new set of vaccines become available as has been already described, then the health care system and parents can immediately benefit from the following: 1.) reduced visits to the medical provider and hence reduced cost; 2.) reductions in adverse events and therefore better compliance with the immunization schedule of the individual. In fact, if the ideal vaccine is made available, i.e., single dose to confer immunity, orally administered, and has little or no side effects, then the demand for immunizations should be explosive. 3.) reduced costs to the
health system because of savings realized in less monitoring of adverse events and social mobilization activities. 4.) reduced vaccines failures and therefore enhancing the acceptability of preventive health behavior.

This new technology would likely lead to greater community participation because parents would realize that with one or at most two visits to the health facility they could completely protect their children from vaccine preventable diseases and perhaps help eradicate some diseases forever.

III. Conclusion

Despite the advancement and introduction of modern medical technology into the hands of health care workers, vaccines still are the most effective and powerful tools that we have to prevent disease. Vaccines are the simplest and cost effective medical intervention that the health care system has at its disposal. The eradication or elimination of a disease or pathogen using a vaccine is the ultimate expression of preventive medicine. The development and or improvement of vaccines to permit that the eradication of a pathogen or disease must always remain high on the list of technological advancements in bio-technology.

This chapter has presented the field viewpoint of current problems that can be encountered with the available vaccines. Yet, the world has never enjoyed such high vaccine coverage rates. Health care services have the tools before them to reduce or even eradicate some of the vaccine preventable diseases. The PAHO experience with the eradication of poliomyelitis clearly demonstrated that the correct combination of strategies, coupled with the necessary political will, can accomplish the goal it set out. If new and improved multi-antigen vaccines become available, removing the present barriers for field delivery, several other diseases may be conquered in the foreseeable future. In fact, if the different disease eradication/elimination efforts are to reach their goals the obstacles mentioned above have to be removed.

It remains to be seen if the CVI will fulfill these promises.
Desirable Immunological Characteristics of Ideal Vaccines for the Childrens' Vaccine Initiative.

Gordon Ada,
Division of Cell Biology,
John Curtin School for Medical Research,
Australian National University,
PO Box 334, Canberra, ACT, 2601,
Australia.

Phone 61 6 2492560
FAX 61 6 2492595.

Running Title. Immune responses to ideal vaccines.
Abstract.

The efficacy of vaccines for prophylactic use is a function of the immune response by activated lymphocytes. Based on the current understanding of these responses, their induction and the most effective ways to obtain long-lived immunity, a novel protocol for the vaccination of children against seven childhood diseases, involving only two visits for vaccine administration, is proposed.
Introduction.

There are two sets of parameters which a vaccine, to be considered 'ideal', should fulfill (2). The first set has two components - safety and efficacy. The former aspect will not be discussed except to say that, more than ever previously, a vaccine must now pass stringent safety standards in order to be registered or licensed for public use. The efficacy of a vaccine depends almost entirely on the immune response induced by the vaccine, and that is the major topic for discussion in this paper. The second set of properties are those that make a vaccine practical to deliver, particularly in third world countries, and they include:

1. Providing long-lasting immunity, including appropriate B and T cell responses, after one or two doses;
2. Be heat stable, no cold-chain being required;
3. Be easy to administer, preferably orally;
4. Be multivalent, protecting against several diseases,
5. Be affordable by the country;
6. Be acceptable to the public and to the health authorities in a country.

The nature of the antigens in the vaccine, their formulation and the immune responses generated are directly implicated in items 1-5, so those aspects will be discussed.

The Childrens Vaccine Initiative aims to provide improved vaccines which will fulfill to the maximum extent possible these requirements. This paper documents the nature and importance of the different immune responses to infections. The aim is review briefly current knowledge in this area, and then to ask - can this
knowledge be used to propose vaccine formulations and an administration schedule which approximates closely to the "ideal EPI vaccine?"

This paper also serves as an introduction to some of the later presentations at this meeting. Several aspects mentioned very briefly here are discussed in more detail by later contributors.

**Vaccines in the WHO Expanded Programme of Immunization.**

Three types of vaccines are used - live attenuated viruses (measles, polio) and bacteria (BCG), inactivated whole bacteria (Pertussis, P) and two toxoids (tetanus, T and diphtheria, D). DPT is administered as a tri-valent vaccine. The recommended schedule for administration is shown in Table 1. Other vaccines may well be added to this list, the first being hepatitis B vaccine. Some oligosaccharide based vaccines have also been mentioned. Thus, these vaccines comprise the three major types of current preparations - live attenuated, inactivated and subunits, although only an antibody response is required to neutralize the bacterial toxins.

**Vaccine efficacy.**

Most vaccines are designed to be used for prophylaxis. When used in endemic regions, a situation which certainly applies to many countries where WHO/EPI vaccines are used, some vaccine recipients may well have been already exposed to the wild-type agent.

Normally, a person is vaccinated at a given time, from birth onwards, and at some time following vaccination - weeks, months or years, the immunized person may be exposed to the wild-type agent. Fundamentally, the success of a vaccine so administered depends on
generating immunological memory. The only component of the immune system which displays both specificity and memory is the lymphocyte, both T and B cells.

**Lymphocyte responses.**

The essential immune responses of lymphocytes are:

(i). The production of antibody by mature, differentiated B cells. This antibody has several roles. The most important is its function as the first line of specific defense - to neutralize the infectivity of the infectious agent, be it a virus, bacterium or parasite. Such antibodies recognize specific epitopes, which may be continuous or discontinuous; that is, the conformation may be secondary (a linear sequence), tertiary (separate but adjacent sequences from different folds of a protein) or quaternary (adjacent molecules contributing short sequences to an epitope). A second role is to facilitate the lysis of infected cells which display the foreign antigens on their surface. Specific antibody recognizing these antigens may lyse the cells by one or the other of two mechanisms - antibody-dependent cellular cytotoxicity (ADCC) and/or antibody plus complement. A third role is to facilitate the phagocytosis of cellular debris resulting from the infection.

(ii) The production of cells with cytotoxic activity - cytotoxic T lymphocytes (CTLs). As primary cells, i.e., as they occur in the infected host, these are almost if not exclusively class I MHC restricted, CD8+ T cells. They have two main properties. They can lyse infected cells in vitro shortly after infection by, say, a virus (28) and some time before the cell would produce infectious progeny. It has been difficult to demonstrate this activity directly in vivo, though all
findings are consistent with such an activity. They also secrete a specific pattern of lymphokines, including in particular interferon gamma (IFN-γ). There is now evidence (47) that in the absence of specific CTLs, production of this cytokine by other cells may result in clearance of a viral infection.

(iii) The production of cells with helper activity. These are usually and in vivo possibly only class II MHC restricted, CD4+ cells. They are involved in the switch of B cells to different Ig isotypes, such as IgG, IgA and IgE and in the formation of B memory cells. The extent to which they help in the generation of CTLs is uncertain as mice lacking CD4+ cells can still generate CTL activity (42). T cell-independent antigens do not induce the switch to the IgG, IgA or IgE isotypes, and do not generate B cell memory (27).

Delayed-type hypersensitivity (DTH) activity is also usually mediated by CD4+ cells, although CD8+ T cells may also mediate this activity (32).

The reason why CD4+ and CD8+ cells may sometimes show similar activities is the finding that there are at least two subsets of CD4+ cells, TH1 and TH2 (Table 2) (39). These are distinguished mainly by the pattern of lymphokines produced. It is thought that there may be a precursor population, TH0, which produces a broader spectrum of lymphokines. However, their existence is not certain as, at least in vitro, some lymphokines, such as IL-10, can inhibit the production of others, such as IFN-γ (40).

Non-adaptive responses.

There are a number of responses to an infection, which are called non-adaptive or non-specific, because they do not demonstrate
memory and their action, though it may have some selectivity, is not highly specific. The three main types are:

(i). The formation of cytokines, mainly alpha and beta interferons which activate certain cell types and have an anti-viral effect;
(ii). Cell types, such as NK cells, eosinophils and polymorphs;
(iii) Complement which can lyse directly some enveloped viruses.

The time-dependent fashion of the immune responses to an acute infection.

This is illustrated by the curves in Figure 1 (1) which indicates the CTL and antibody response in murine lungs following an intranasal inoculation of influenza virus. Virus (curve A) replicates in the lungs, reaches maximum titres by day 3-6, and then decreases so that infectious virus is no longer detectable after day 9-12. (Viral RNA can also not be detected by the PCR reaction after 14 days, 15). Curve B represents CTL activity which peaks and then disappears a few days later. There is no evidence that this disappearance is due to suppressor activity, but rather reflects the short half life (days) of these effector cells. By about 2 weeks, memory T cells are detected, in numbers between 20-100 fold higher than their precursors (naive cells) and they persist for a long time. In contrast, antibody-secreting cells (ASCs) are detected for a very long period, reaching maximum numbers about 3 months after the initial infection. Memory B cell numbers also peak about this time, varying from 100-1000 fold more in frequency compared to naive B cell numbers (29). Though not shown in the figure, T helper cell activity is the first lymphocyte activity detected, between 2-3 days after the infection begins.
Memory B cells, their formation, persistence and recruitment to form ASCs.

ASCs have a short half life, of the order of days or a week (37). The constant presence of ASCs (Figure 1) for more than 80 weeks after the infection is due to the persistence of antigen, in the form of antigen:antibody complexes attached via Fc or C′ receptors to the surface of follicular dendritic cells (FDCs) in lymphoid tissue. This localization of antigen in primary follicles was first observed in 1963 (44). In the ensuing nearly 30 years, it has been established that:

(i). The antigen may persist for very long periods (> 1 year) and in an undegraded form (35);
(ii). B cells during maturation follow one of two paths (eg., 34, 48). After stimulation, one group matures and secretes IgM antibodies and other Ig isotypes in a primary response; the other group migrates to the follicles and with T cell help, undergoes clonal expansion to become memory cells expressing Ig isotypes other than IgM. Over time, somatic mutation leads to affinity maturation of the cell’s Ig receptors;
(iii). Memory cell formation does not occur in the absence of localized antigen (32);
(iv). Memory cells need to live for long periods, and this also seems, possibly only initially, to require the presence of antigen (21);
(v). Antigen:antibody complexes bud off the FDCs to form iccososomes. These are taken up and processed within the B memory cells which, with further T cell help, differentiate into mature ASCs (60, 61).
(vi). As time passes and in the absence of a second infection or vaccination, decreasing amounts of antigen on the FDCs progressively select the memory cells with receptors of increasing affinity, thus providing an explanation for one of the most important features of immune responses - the production of antibody of increasingly higher affinity. It must also be realized that some of this antibody recognizes discontinuous epitopes, i.e., epitopes formed by adjacent peptide sequences in a protein molecule, or by sequences contributed by closely adjacent protein molecules (trimers or quadrimers) (43, 51). It therefore follows that such complexes must also persist on FDCs.

(vii). It is probable that idiotypic/anti-idiotypic reactions contribute to this effect but evidence that this occurs over a long time period is lacking.

(viii). The presence of antigen has also been reported as being required for persistence of CTL memory cells (22). As, in vivo, infectious virus is required for the formation of CTLs, this claim is inconsistent with the finding that viral RNA has been shown not to persist (15).

Nevertheless, the long term persistence of antigen in its native conformation is a critical feature of a long-lived high affinity antibody response. It helps to explain why some viral infections, e.g., measles (9) can give 40-50 years of protection without re-exposure to the agent during that time. It is likely that some of the attenuated live viruses are highly successful as vaccines for the same reason.

The dichotomy of the immune system: the requirements for T cell activation.
In complete contrast, T cells recognize peptides, the degradation products of protein antigens. This processing of antigen to form peptides is an essential property of antigen-presenting cells (APCs). Such cells also produce interleukins which help to initiate the activation of the T cell. The peptides resulting from antigen processing in the APC interact with MHC antigens to form a complex which is transported to the cell surface where it may be recognized specifically by a T cell receptor. Generally, non-infectious antigens are endocytosed and digested in lysosomes. Peptides of c.15 amino acids in size (range, 12-24) associate with class II MHC antigens (52). In the case of an infectious agent such as a virus, some of the newly synthesized viral proteins in the cytosol (as well as peptides from self proteins) are degraded. In this case, nonapeptides associate with class I MHC antigens (18) and are transported to the cell surface. The peptide fits into a groove at the tip of the MHC molecule. The points of contact between MHC molecule and the peptide determinant have been accurately mapped in a number of cases, so it is now known that some amino acid residues in the peptide for a given MHC allele are highly specific while others can vary considerably. There are thus two requirements if a peptide is to be 'successful': it must fit properly into the MHC groove, and secondly, the resulting epitope, formed by the peptide determinant and the MHC groove, has to be recognized by a T cell receptor. There are thus two levels at which the genetic make-up of the host can substantially influence the T cell response and thence, the overall immune response.

This means that while an antigen, particularly one recognized by neutralizing antibody, remains outside a cell, it should retain its
native conformation. Once inside an antigen presenting cell, it should be susceptible to proteolytic degradation to form peptides. Exposure to pH 5 in lyzosomes would be sufficient to denature many proteins, making them susceptible to proteases and thus a source of Th cell determinants.

In contrast, the internal antigens of enveloped viruses are protected from environmental proteases. Some may therefore be susceptible to breakdown during synthesis in the infected cell and thus a prime source of CTL determinants, e.g., the nucleoprotein of influenza virus (38). On the other hand, the non-structural, cytosolic proteins of flavivirus infections are also a rich source of these determinants (26). One could in fact speculate that this a raison d'être for the production of such antigens.

**Immunological requirements for a highly successful vaccine.**

To achieve high efficacy, a vaccine should ideally fulfill four requirements (modified from 1). These are:

(i). Activation of the APC, synthesis and secretion of cytokines and processing of antigens entering the cell by either route to form appropriate peptides. Dendritic cells are particularly important in this regard;

(ii). Activation and differentiation of specific T and B cells resulting in a high yield of memory cells;

(iii). Generation of TH and CTLs to several peptide determinants preferably from conserved regions of several antigens, and also to minimise the restrictive effects of MHC polymorphism in an outbred population.
(iv). Long-term persistence of antigen in its native conformation on FDCs to allow continuing formation and recruitment of memory B cells to form ASCs, thus ensuring the steady production of antibody.

Though successful live attenuated vaccines, by extrapolation from animal studies, probably fulfill all these requirements, many other vaccines do not. Non-infectious preparations generally do not induce CTL responses. The latter assume particular importance where there is great antigenic variation in 'neutralizing' B cell epitopes, as a strong CTL response then acts as a safety net, facilitating a more rapid recovery from what might otherwise be a lethal infection. Such vaccines depend rather upon inducing the continuing presence of neutralizing antibody, but to achieve this may involve multiple administrations. Even a vaccine which only induced good memory responses could tip the balance in favor of survival by allowing a more rapid response after the challenge infection.

Immunopotentiation and delivery of vaccines.

Adjuvants

Generally, soluble proteins and oligopeptides are poorly immunogenic and must be administered with preparations which potentiate the immune response. Alum, first used more than 50 years ago, is still the only preparation registered for general medical use. The 'gold standard' is Freund's complete adjuvant consisting of an oil/water emulsion and a Mycobacterium. With so much interest in subunit and peptide-based candidate vaccines, many new preparations are now being investigated, varying from active fractions of Mycobacteria, to pluronic block polymers and to preparations which activate complement. Some favor the formation
of particular Ig isomers, some induce CTL responses, and some are less toxic than others. Within the next few years, several are likely to be registered for general use (3,5).

Adjuvant formulations have at least two functions - to provide a depot for antigen and to activate APCs to produce inter alia different cytokines. The pattern of cytokine interactions with cells is extremely complex. Among other approaches, the use of 'doctored' mice which lack particular genes is demonstrating a considerable redundancy such that one cytokine can sometimes simulate the functions of another. The day has almost arrived when specific cytokines such as IFN-γ (or their genes - see later) will be included as a component of an adjuvant preparation.

Delivery systems.

There have also been advances in the 'packaging' of antigens to emulate some of the advantages of live agents, such as the targeting to particular cells, constructing a mosaic pattern or specific aggregates of protein molecules, and so on. Three will be discussed briefly - liposomes, iscoms and biodegradable microspheres/capsules.

Liposomes as vectors of antigen have been used for many years but with variable results. They are avidly taken up by cells such as macrophages. In a recent modification, liposomes containing monophosphoryl lipid A (as adjuvant) were adsorbed to alum (to achieve a depot effect) and a repeat region of the Plasmodium circumsporozoite protein incorporated as the antigen. This gave much higher titers of antibody compared to the use of alum alone (20). It
was also shown (20,49) that such preparations can induce CTL formation as well as the other T and B cell responses.

In immunostimulating complexes (iscoms), multimers of antigen are held in a cage-like structure, about 40nm in diameter, together with quil A, a strong adjuvant. Two administrations have resulted in high levels of antibody and either memory or effector CTLs (30,57).

A third approach is the use of biodegradable microspheres/capsules containing the antigen, with or without adjuvant. Varying the ratio of \( p \)-lactide and glycolide controls the rate of solubility of the polymer and release of the antigen. Thus constant and pulsed release patterns have been obtained. This approach has the potential not only of achieving long-lasting immunity after a single administration (55) but also of inducing both mucosal and systemic immunity if given orally (16).

Each of these formulations shows considerable promise as a means of obtaining improved immune responses.

**Portals of entry of pathogens.**

There are three main natural routes of infection - via a mucosal surface, by an abrasion in the skin, or via a biting vector. Many infections occur via a mucosal route, the main sites being the respiratory tract, the gut, the eye, the urogenital tract and the rectum. Despite this, most vaccines currently are administered parenterally. There is increasing interest in developing oral administration as a more general route of vaccination for vaccines to combat enteric organisms. In addition, cells sensitized by antigen at one mucosal site can circulate to other mucosal sites; because of this
common mucosal system (36), immunity can be gained at mucosal sites other than the one where sensitization originally occurred. In some cases, it has also been an effective way to induce systemic immunity.

Certain immune parameters are characteristic of the mucosa. The first is secretory IgA. As polymeric (dimeric) IgA passes through cells lining the mucosal lumen, it complexes with the secretory component (sc), thus allowing passage into the lumen. Though a correlation between s.IgA levels and protection from infection has been demonstrated in different systems, direct proof that s.IgA can specifically and completely prevent infection at a mucosal surface was shown only in 1991 (50). Being a dimer, s.IgA not only has increased avidity but because of the complexing with sc, is also resistant to proteolytic attack, a very useful property especially in the gut. Nevertheless, some bacteria produce proteases which cleave s.IgA.

The second characteristic is the presence of intra-epithelial T lymphocytes which have gamma/delta T cell receptor chains. Though CD8+, these cells are not restricted by the major MHC antigens (63). One group of cells has a specificity for heat shock proteins, whereas another group has a wider spectrum of specificities, including some heat shock proteins. Their specific role in different infections at a mucosal surface has not been well documented so far, but it is likely that they have a role similar to their more common counterparts with alpha/beta T cell receptor chains.

The potential of the new technologies for vaccine development.
Three new general approaches to vaccine development are being examined intensively for their potential. They are the development of synthetic vaccines composed of oligopeptides, the use of anti-idiotypic antibodies to mimic antigens, and several approaches using recombinant DNA technology (R.DNA). Despite the theoretical attraction of anti-idiotypic antibodies as a surrogate antigen in that they could mimic epitopes of a tertiary or quaternary conformation, the data available on their use is not sufficiently encouraging to devote space to this field in this short review.

**Synthetic polypeptides as candidate vaccines.**

Two general approaches have been used. Until recently, the main approach was to conjugate short peptides representing B cell epitopes (ie., haptens) to proteins which thereby acted as carriers, and as a source of T cell determinants. Bacterial toxoids have been used extensively in this way, the rationale being that many people would already possess memory TH cells because of an earlier vaccination. Thus, a 37 amino acid sequence of the B subunit of human chorionic gonadotrophin hormone has been linked to diphtheria toxoid (54) as a candidate vaccine to control human fertility. It has successfully undergone phase I trials in infertile women (31) and is about to undergo phase II trials in fertile women (23). Other proteins such as hepatitis B virus core antigen (12) are rich source of the TH determinants. Even though this molecule is substituted with peptides, protein:peptide conjugates assemble as a virus-like particle, and this may result in a greatly increased immune response to the hapten.
A second recent approach has been to devise entirely synthetic vaccines composed of linked peptides. In one case for example, a B cell epitope, TH cell and CTL determinants and a fusion sequence were linked in a linear fashion. When used to immunize rabbits, T helper and CTL activity in addition to the antibody response were obtained (25). Inclusion of the fusion sequence was to ensure that some molecules entered into the cytoplasm of the cell, thus allowing the CTL response. Generally, such constructs are poorly immunogenic but immunogenicity can be considerably enhanced if tandem arrays of epitopes are made, or even better still, if a multiple antigenic peptide (MAP) is made, in which multiple copies of the epitope are synthesized onto a 'dendritic matrix' of lysine residues (58).

Synthetic vaccines constructed of oligopeptides have several advantages but also have some disadvantages (reviewed in 3). It is too early to say whether this approach could be useful in a future EPI but it should not be dismissed as a possibility.

Application of R.DNA technology.

There are three approaches which offer prospects for developing vaccines for global use. They are:

(i). Transfection of cells with DNA coding for the disease antigen(s) of interest;

(ii). Construction of chimeric live vectors, either viruses or bacteria, which will express the DNA coding for the foreign antigens; and

(iii). Use of site-directed mutagenesis to vary the amino acid sequences in important epitopes/determinants.
The first two only will be discussed here as they currently demonstrate the greatest potential for application to the CVI Programme.

**Transfection of cells with R.DNA.** Three types of cells are used as substrates - prokaryotic (bacteria), lower eukaryotic (yeast and insect) and higher eukaryotic (mammalian). The first genetically-engineered vaccine to be registered for medical use is the hepatitis B surface antigen, HBsAg, produced by transfected yeast cells. It appears to be equally as effective as the earlier product isolated from the blood of infected people (62) but up to 17% of recipients are either poor or non-responders because of their genetic background (14). The use of bacteria as the substrate for the production of this antigen proved to be unsatisfactory (17), as it was also for the synthesis of influenza virus hemagglutinin (13). In contrast, a number of vaccines for veterinary use are composed of bacterial proteins made in this way. The picture emerging from studies like these is that bacteria, though satisfactory for the production of other bacterial proteins, may not be generally suitable for the production of proteins normally produced by the synthetic machinery of mammalian cells. The baculovirus/insect cell system has been shown to give a high yield of the foreign proteins (46). The intensive research at present underway to develop an HIV vaccine has allowed a comparison between mammalian, yeast and insect cells as substrates for the production of the highly glycosylated envelope protein, gp120. Though each preparation induces the formation of antibodies, the product produced in mammalian cells was most effective at inducing neutralizing antibodies, particularly
against conformational determinants (24,7). A similar finding has been made with herpes viral antigens (10).

**Chimeric live vectors.**

**Viruses.** A number of viruses and bacteria has been used as vectors for foreign DNA, but currently, work on four is either more advanced or seem to hold particular promise - two families of virus, pox and adeno, and two bacterial preparations, BCG and Salmonella. Two of these, poxviruses and BCG are normally administered intradermally whereas adenovirus and Salmonella are given orally, and are therefore of interest as a way to induce mucosal immunity.

The use and potential of pox viruses as vectors has recently been extensively reviewed (8). For many reasons, vaccinia virus has been used in this regard far more than other vectors. For example, DNA coding for antigens of more than a dozen other viruses has been expressed in cells infected by the recombinant virus; in many cases, protection has been achieved in animal models against a challenge by the donor of the DNA, and most convincingly in the case of a vaccinia virus constructs with a rabies viral glycoprotein (45) and antigens from rinderpest virus (64). In higher primates, the results have not been quite so impressive (41), but vaccinia has generally been considered to have many advantages as a vector. There has also been much concern about the prevalence of side reactions experienced during the smallpox eradication campaign (19). Inclusion of genes coding for some lymphokines such as IFN-γ has conferred a degree of attenuation (47), but recently, further significant advances have been made in two respects. A remarkable degree of attenuation has been achieved by the selective deletion of 18 open reading frames
without diminishing the potential of the virus as a vector for a number of foreign genes in animal tests (59). Equally remarkable have been the results obtained when canarypoxvirus was used as a vector. Avipox viruses undergo only abortive infection in man so that the canarypox virus preparation, ALVAC, should be regarded as a very safe vector. Despite this lack of productive replication, the construct proved as effective a vector as the highly attenuated vaccinia virus in animal experiments (11). Equally encouraging is a report that an ALVAC construct carrying the gene for the rabies glycoprotein, after a single administration, yielded substantial antibody titers in humans in phase I clinical trials (6). There will be great interest in seeing this approach repeated with a range of other antigens. It is notable that in successful trials of the vaccinia virus:rabies glycoprotein construct, the vaccine was administered in bait to the wild fox population, opening the possibility that poxvirus constructs might also be effective in man if taken orally.

In contrast to the above studies, work with adenovirus as a vector is less well advanced. Genes coding for proteins from several other viruses have been inserted into the adenovirus and have been expressed correctly in infected cells (53).

Bacteria. A considerable amount of study has been devoted to establishing Salmonella bacteria as a vector of foreign antigens for oral administration. As Dr. Curtiss will report on this later in the program, it will not be discussed here except to say that there are now several reports that these constructs induce good levels of CMI responses, including CTL formation, a not-unexpected finding. However, the antibody levels generated have been variable.
The use of BCG as a vector is more recent and there are only a few reports (eg.,4, 56), but these are of great interest because of the long record of BCG as a safe and reasonably effective vaccine when it is administered at or near birth. In these preliminary findings, significant CMI responses (as with Salmonella) have been obtained as well as good antibody responses to a foreign bacterial protein. In both reports, antibody responses to a viral glycoprotein, the env protein of HIV, were disappointing.

Table 2 summarizes the potential of recombinant live viral and bacterial vectors for inducing the immune responses to the expressed foreign gene(s).

Prospects for improved vaccines and delivery systems for the Children's Vaccine Initiative.

The current immunization schedule for the WHO/EPI vaccines involves at least five visits from a vaccination team. There is often a window of time between cessation of breast feeding and immunization with the measles vaccine when many infants are susceptible to a natural infection, and up to 2 million die each year. Consequently, there is great interest in vaccinating against measles at a very early stage with a preparation which would be effective in the presence of maternal antibody.

How can more effective immunization with fewer administrations be achieved, based on the knowledge we now have of the different microorganisms and the nature of the most effective immune responses? If hepatitis B is included in the schedule, then a scenario like that outlined in Table 3 might be appropriate and can
form a basis for discussion. It would have several advantages, such as:

(i) The tetanus and diphtheria toxins, engineered by site-directed mutagenesis to remove toxicity without the loss of immunogenicity, and the relevant protein antigens of pertussis would be produced by chimeric BCG. As BCG persists in the body, this should provide a continuing stimulus which could, if necessary, be boosted by the later injection, at about 10 weeks of age, of DPT in biodegradable microcapsules/spheres. The P in this DPT could either be the whole inactivated *B. pertussis*, or the individual proteins present in current acellular preparations. If the former, the immunity resulting from the individual pertussis proteins given at birth via BCG would help to protect against any subsequent side effects if the whole pertussis bacteria was administered at the later time. The use of BCG in this way has the additional advantage that this organism has strong adjuvant activity;

(ii) The protective viral antigens of measles, hepatitis and polio would also be provided *in their correct conformation* at birth via the safe canarypox virus or the highly attenuated vaccinia virus vector. The measles and hepatitis antigens could also be administered a second time in biodegradable microspheres at 6-10 weeks, together with oral polio. Administering an initial dose of poliovirus subunits followed later by oral polio should protect against those rare cases of paralysis due to reversion to virulence of type 3 virus following the initial use of live virus;

(iii). Additional doses of oral polio could be administered at later times if found to be necessary.
Other scenarios could be outlined which made more use of the oral route of vaccine administration, such as the use of adenovirus and Salmonella. As pointed out earlier, chimeric vaccinia virus has been administered orally to foxes, so it might be possible to adopt this route with humans. Both vaccinia virus and BCG have been given orally to humans on previous occasions.

This immunization protocol goes a long way towards fulfilling at least the first five items in the second set of properties for children's vaccines outlined in the introduction. Immunological considerations must be an important component of any scenario proposed for the CVI. The proposal in Table 3 may perhaps be regarded as wishful thinking but if the main goal of the CVI is to become more than a dream, the validity and practicality of this or other comparable scenarios should be the subject of active discussion and planning now.

References.


53. Burke, R. L. Contemporary approaches to vaccination against herpes simplex virus. *Current Topics in Microbiology and Immunology*. 1992, 179, 137-158.


Figure 1. Time sequence of the infectious process and two components of the subsequent immune response.

Ordinate: Increase in infectivity/effector cell numbers.

Abscissa: Time after initiation of infection.

Curve A. Increase of, followed by disappearance of, infectious virus
Curve B. Appearance and disappearance of effector CTLs.
Curve C. Appearance and continuing presence of antibody-secreting cells.

The arrows point to the time when maximum levels of memory lymphocytes occur.

Reproduced from (1), with permission.
Table 1. The currently recommended EPI schedule with special reference to developing countries.

<table>
<thead>
<tr>
<th>Age</th>
<th>Vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth</td>
<td>BCG and OPV</td>
</tr>
<tr>
<td>6 weeks</td>
<td>DPT and OPV</td>
</tr>
<tr>
<td>10 weeks</td>
<td>DPT and OPV</td>
</tr>
<tr>
<td>14 weeks</td>
<td>DPT and OPV</td>
</tr>
<tr>
<td>9 months</td>
<td>Measles</td>
</tr>
</tbody>
</table>
Table 2. The potential of recombinant live vectors for inducing different immune responses to the expressed foreign gene(s).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Antibody Epitopes</th>
<th>CMI</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contin.</td>
<td>Discontin.</td>
<td>Cd4+</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccinia</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Avipox</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Adeno</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella</td>
<td>++</td>
<td>?</td>
<td>+++</td>
</tr>
<tr>
<td>BCG</td>
<td>++</td>
<td>?</td>
<td>+++</td>
</tr>
</tbody>
</table>
Table 3. An immunologist's concept of future developments towards a Children's Vaccine.

Schedule.

1. Vaccines administered at a single visit at or near birth.
Recombinant BCG containing genes for
   (i) 1-3 *B. pertussis* antigens;
   (ii) 'detoxified' diphtheria and tetanus toxins.
   plus
Recombinant avipox vector, ALVAC, or vaccinia vector, NYVAC, containing genes coding for
   (i) HA and F proteins of measles virus,
   (ii) HBsAg, and
   (iii) Poliovirus VP1 (3 serotypes);

2. Vaccines delivered at a single visit at 6-10 weeks of age.
DPT, HA and F proteins of measles virus and HBsAg with adjuvant in biodegradable microspheres
   plus
oral polio virus.
MODERN CONCEPTS IN IMMUNE RECOGNITION
AND LYMPHOCYTE ACTIVATION:
RELEVANCE FOR DEVELOPMENT OF USEFUL VACCINES

Ronald N. Germain, M.D., Ph.D.

Lymphocyte Biology Section, Laboratory of Immunology, NIAID, NIH, Bethesda, MD 20892

Running head: Antigenicity, Immunogenicity, and Vaccines

Dr. Ronald N. Germain
Laboratory of Immunology
Bldg. 10, Rm. 11N311
NIH
Bethesda, MD 20892
Phone: 301-496-1904
FAX: 301-496-0222
**Abstract**

Adaptive immunity requires both specific recognition of antigen and its translation into appropriate lymphocyte responses. The striking differences in B and T lymphocyte antigen recognition are reviewed, the pathways for conversion of protein antigens into peptide-major histocompatibility complex molecule ligands for T cell receptors are detailed, and the roles of co-stimulatory signals in lymphocyte activation are summarized. This information is used to suggest new approaches for rational vaccine design.
Acknowledgements

I wish to thank the members of the Lymphocyte Biology Section for their role in generating and discussing the data on which much of this overview is based. I also thank Bob Chanock for many valuable discussions about protective immunity, especially for being sure I always remember the importance of antibodies, and Jay Berzofsky for emphasizing the issue of improving on nature in developing vaccines for infections that do not typically lead to protective immunity.

**Introduction**

The practice of active prophylactic vaccination arose from observations that individuals having survived a first infection were frequently partially or fully protected from disease upon re-exposure to the same organism (94). The development of qualitatively or quantitatively enhanced immune responses upon re-challenge with a specific antigen is defined as adaptive immunity, a type of pathogen resistance that is distinguished from other forms of host defense that do not show this special phenomenon of antigen-specific recall. All successful modern vaccines are based on elicitation of protective adaptive immunity using altered or inactivated forms of the pathogen, or individual chemical constituents of the organism.

Despite knowledge of this general principle of immunization and the success of many vaccines against a broad range of infectious agents, the rational design of new vaccines from first principles of immunology is an uncertain task. The difficulties lie in three areas: 1) gaps in our knowledge of basic mechanisms of immunity; 2) lack of definitive understanding of the effector modalities that actually mediate protective immunity to a given pathogen; and 3) difficulties in creating and administering vaccines that promote the specific types of immune responses necessary for protection.

It is not only the absence of vital pieces of information that hamper vaccine development. The problem is made worse by the reality that scientists tend to specialize in one of these three areas and are often only incompletely aware of both the advances and the obstacles being faced in the other two. Thus, individuals studying how lymphocytes function are largely unconcerned with whether the activation patterns they analyze are directly relevant to any specific disease process; those investigating whether T cells or antibodies protect against a given organism often do not know the latest findings about how a critical isotype of antibody or subset of T cells can be stimulated; the vaccine designer may not know the most recent findings on how the balance of activation between specific T cell subsets contributes to protective vs. pathologic immune responses.
In the past, integration of knowledge at these three levels was not essential to the development of effective vaccines. For diseases in which protective natural immunity was known to exist, it was often sufficient to manipulate the pathogen by trial and error to rid it of disease-inducing potential, and to then empirically determine an effective immunization scheme that led to protection. In some cases, this remains perhaps the most efficient strategy. However, the emergence (HIV), re-emergence (TB), or persistence (malaria) of infectious diseases that do not typically engender fully protective immunity in infected hosts has made imperative the development of vaccines that improve upon natural immunity (7). Accomplishing this goal, within the limits of the effector activities available to our immune systems, requires the careful and complete integration of research on all three fronts. Those studying protective immunity must make the basic scientist aware of which effector activities need more intensive study, to improve our ability to predictably evoke such a response. Those studying basic mechanisms and protective modalities must make the vaccine designer aware of advances in their areas, so that the proper formulation and delivery methods can be used to produce the necessary responses, while avoiding stimulation of counter-regulatory activities that would interfere with such immunity or lead to disease exacerbation (e.g., enhancing antibodies). Those involved in vaccine design must make the other two groups aware of limitations in practical vehicles for vaccine delivery and schedules of immunization, so that optimization can be achieved based on focused studies on lymphocyte activation and the kinetic and quantitative development of protective responses.

It is not possible in the brief space available here to even superficially summarize the extensive recent advances in areas such as lymphocyte homing, cell adhesion receptors, region and organ specific immunity, the myriad activities of the increasing number of cloned and characterized cytokines, or intracellular signalling pathways, to name just a few. Instead, I will focus on two closely related subjects with special importance to vaccine development - immune recognition (antigenicity) and of how such recognition is translated into development
of effector function (immunogenicity), using these overviews as examples to illustrate how such new knowledge can guide rational vaccine design.

**Basic Parameters of B and T Lymphocyte Immune Recognition**

**B Cells vs. T Cells**

Adaptive immunity in vertebrates depends on the activity of two major subsets of lymphocytes, B and T cells. The specificity of adaptive immunity lies in the interaction of clonally distributed receptors present on these lymphocytes with individual chemical compounds produced by the pathogen. Therefore, stimulation of a selective lymphocyte response minimally requires adequate binding of a component of the infectious organism to one of these receptor structures. The ability of a molecule to interact with suitable affinity with an clonotypic immune receptor is termed antigenicity. The structural and chemical requirements for such interactions with the receptors of B cells and T cells are very different and vaccine design must take these marked differences into account.

A surface form of serum antibody is the antigen-specific receptor on B lymphocytes (50). The recognition characteristics of such antibody molecules are well known, and the rules of ligand binding appear to be the same as for the soluble form of this receptor later secreted as antibody by the progeny of these B cells. In particular, the diversity of chemical structures recognized by such immunoglobulins is very wide and not limited to any particular class of chemical compound. Furthermore, for protein antigens, conformation plays an important role in the recognition process (8). Finally, direct binding of intact antigen in solution is adequate for these receptors, although multivalent surface display of ligand may have a special role in eliciting certain B cell reactions.

These characteristics of B cell immunoglobulin receptors and the direct relationship of these receptors to effector antibodies make clear that antigenicity is not a limiting feature of
the B cell limb of the immune system. It is correspondingly easy to evoke antibodies against pathogen-derived material that will not protect against a toxic effect nor aid in removal of the organism itself. It is therefore essential to ensure that of the many possible target structures against which antibodies can be generated, vaccination elicits those that mediate protection against the pathogen or its products. Thus, antibodies to denatured proteins not found associated with live organisms nor possessing toxic potential are undesirable, as are antibodies that upon binding not only fail to enhance the pathogen’s destruction, but possibly contribute to its improved survival or replication (enhancing antibodies). The critical antigenicity issue for B cell responses therefore relates to focusing the response on the relevant target molecules, not in achieving the potential for immune recognition per se.

The antigenicity issues for T cells are very different. Three major characteristics of materials able to elicit T cell responses were well recognized by 1980 (8). First, only proteins or chemically modified proteins seemed to be effective. Second, direct interaction of these proteins with the T cell’s recognition system did not seem to occur - the antigen had to be associated with the membrane of another cell and metabolic activity of this latter cell was required for effective antigen display. Third, T cell responses to protein antigens were controlled by the allelic forms of class I and class II major histocompatibility complex (MHC) molecules expressed by the antigen presenting cell (APC). These MHC molecules are the very polymorphic membrane glycoproteins responsible for vigorous tissue graft rejection, and exist in two major forms: class I molecules with broad tissue distribution and class II molecules found mainly on B cells, dendritic cells, macrophages, or γ-IFN treated cells (43).

The structural basis for these features of T cell antigenicity are now well understood. T cells possess clonally distributed receptors related to but distinct from immunoglobulin, either the αβ receptors of both CD4+ and CD8+ T cells, or the γδ receptors of a less prevalent group of T cells (16). The αβ receptors are specific for complexes of small peptides derived from protein antigens and specifically bound to class I or class II MHC molecules
(4;19;90;105). The reason for the existence of two distinct classes of MHC molecules, both
of which possess peptide binding activity and are recognized by αβ TCR, appears to relate to
the different function of CD4+ and CD8+ T cells. CD4+ cells interact productively with B cells,
macrophages, and dendritic cells that primarily acquire a multiplicity of antigenic proteins from
the external milieu. These proteins do not constitute markers of the APC themselves. In
contrast, CD8+ cells are specialized for destruction of cells that have been infected or
undergone malignant transformation, where the presence of a particular protein marks the cell
for death before mature virus release or further tissue invasion occurs. It is thus necessary to
access peptides derived from extracellular proteins entering the endocytic pathway in the
former case and peptides derived from cytoplasmic proteins in the latter. This has been
accomplished by evolving structural features of class I and class II molecules to favor peptide
binding at distinct sites, the endoplasmic reticulum for class I and acidic vesicles for class II
(11;29;108). Additional proteins, some but not all of them also encoded in the MHC,
contribute to peptide generation and either peptide or MHC molecule transport to appropriate
interaction sites.

**Antigen Presentation by MHC Class I Molecules**

A great deal is now understood about peptide generation, transport, binding, and
display by class I MHC molecules. Degradation of proteins in the cytosol is apparently
mediated by enzymes forming part of a large multifunctional protease (proteasome). Some of
the enzymes are common to all cells, but others are encoded in the LMP genes of the class II
region of the MHC (13;33;48;63), and these may serve to produce peptides of the right length
or chemical nature for transport to and binding by class I molecules. The peptides produced in
this manner move into the lumen of the rough endoplasmic reticulum (ER) via a channel or
transporter (TAP = Transporter of Antigenic Peptides) consisting of a heterodimer of proteins
with homology to the ABC family of permeases that includes the multi-drug resistance
The genes encoding the TAP lie adjacent to those encoding the proteasome-associated LMP subunits in the class II region of the MHC. In the ER, the peptides interact with class I heavy chains or heavy chain-β2 microglobulin dimers (104) that are arrested in the ER and prevented from moving to the Golgi by the p88 chaperone calnexin (17;18). The peptides may already be processed to an optimal length (42) or they may be trimmed in the ER (23). The next step involves a structural change in the class I molecule that stabilizes β2 microglobulin (β2m) association with the class I heavy chain (21;73) and contributes to a transport competent state. This permits the trimeric class I heavy chain-β2m-peptide complex to move through the default secretory pathway (69) to the cell surface for recognition by CD8+ T cells. Because peptide is necessary for stabilizing class I-β2m association and for release from the p88 chaperone, this strongly biases class I molecules towards peptide binding at this site. Molecules that don't acquire peptide here are unlikely to move to the cell membrane, and those that have done so cannot acquire peptide elsewhere until the first peptide dissociates. For most class I molecules, this dissociation is a slow event, taking place on average at longer times than the natural turnover rate of the class I protein itself (14;21).

As a result of direct peptide elution and microsequencing (24;44;107), as well as crystallographic analysis class I-peptide complexes (9;27;28;62;64;91), the biochemistry of peptide-class I interactions and the characteristics of peptides that bind to class I molecules are now well understood. The peptide binding groove of these MHC molecules favors peptides of 8-10 residues in length (24;44;62;107), which can be accommodated in the binding site with the N and C termini in fixed positions where they make numerous H bonds to conserved residues in the class I molecule (27;62;64). Binding subsites or pockets formed by the polymorphic residues that differ among alleles of class I (27;28;64;91) bind to special anchor or motif residues of the peptide and control the allele specificity of peptide presentation. A subset of class I molecules is specialized for binding peptides that have the
N-formyl methionine group characteristic of many prokaryotic proteins (75), adding an additional feature to self-non-self discrimination. Other residues of the peptide extend out and above the binding site for contact with the TCR, giving specificity to the recognition of the peptide-class I complex (27;62;64). The source proteins for the class I associated peptides are as expected from the processing scheme just discussed, coming from abundant cytoplasmic or nuclear proteins (44;47).

Because of the existence of optimal lengths and critical motif residues, it is now possible to scan the sequence of proteins from a given pathogen and predict with good accuracy the peptides from those proteins that will be presented by given allelic forms of class I. This will be a key element on the future design of subunit vaccines.

**Antigen Presentation by MHC Class II Molecules**

Class II molecules are assembled as relatively stable αβ dimers in the ER, a process that in contrast to the situation with class I molecules does not require peptide (G. Otten and R. Germain, unpublished observations). However, emergence from the ER is dependent on association of these dimers with another, non-MHC encoded chain termed the invariant chain (Ii) (3;56). This association also prevents peptide binding to the class II dimer in the ER, reserving the class II binding sites for peptides at another location (30;83;84;103). These "empty" class II-Ii complexes move to the trans-Golgi, where a signal in the cytoplasmic tail of Ii deviates the complex from the normal secretory route and targets them to early endosomes (6;54;60;86). In early endosomes, excess free Ii can decrease transport rates and cause endosomal enlargement, possibly increasing the mixing of class II with incoming antigen present in low concentrations (86). Optimal antigen uptake occurs when receptor-mediated endocytic mechanisms are active, such as via surface immunoglobulin of B cells (55;85) or immune complex binding to Fc receptors (2). The class II-Ii complexes and antigen materials move to late endosomes/ or prelysosomes (76;86), where most of the Ii is
degraded and removed from class II (10). Simultaneously, the antigen is degraded into shorter fragments. These fragments bind to the exposed class II binding groove in the acidic environment of this late endocytic compartment (30;37;38;54;68;69). Once free of residual Ii, the peptide-class II complexes leave this site and by an uncharacterized route, moves to the cell surface for recognition by the receptors of CD4+ T cells. Like in the class I pathway, there is “editing” of unoccupied class II molecules. This seems to take place in the endocytic loading compartment, and involve the aggregation and destruction of Ii-free class II that fails to capture peptide (31). A conformational change (31;89) akin to that mediated by peptide binding to class I molecules prevents this aggregation and allows the useful, loaded class II molecules to reach the cell surface.

The characteristics of peptides bound to class II differs from those associated with class I. Rather than a narrow length range, class II-associated peptides are both longer and more diverse in size (12-24 residues) (15;45;70;87;88). A single core determinant may be flanked by staggered ends at the N and / or C termini. Nevertheless, motif residues can be identified for class II-associated peptides that presumably bind to polymorphic binding pockets, just as with class I-binding peptides (15;45;88). However, the conserved regions that anchor the N and C termini of peptides to the class I binding site are absent from class II molecules (12;62), and so the detailed characteristics of the peptide-MHC molecule association may differ significantly. The source proteins for naturally processed and bound peptides is as expected, coming from either membrane proteins that enter endosomes or highly abundant serum proteins that undergo extensive endocytic / pinocytic uptake (15;45;70;87;88).

**Immunogenicity and Co-Stimulation in Lymphocyte Responses**

Although antigenicity (receptor binding) is essential for the initiation of specific immune responses, mere possession of this property is not by itself adequate for
development of most effector activities. A second characteristic that is called immunogenicity is required for the B and T lymphocyte differentiation events we associate with a full immune response. This property appears to involve the delivery of additional signals to the lymphocytes beyond those transduced through the antigen-specific clonotypic receptors. The antigen itself might possess the capacity to evoke such additional signals, or associated but chemically distinct entities (naturally produced during infection or exogenously added during vaccination) might carry out this function; such materials are would fall in the category of immunological adjuvants.

**Inflammatory (Th1) vs. Helper (Th2) T Cell Subsets**

Two divergent, mutually exclusive patterns of mature CD4+ T cell effector activity have been described (66). One involves production of a set of lymphokines termed "inflammatory", including IL-2, γ-IFN, and TNF-β, whereas the other entails production of IL-4 and IL-5, without release of the inflammatory cytokine set. Cytokine production of IL-2, γ-IFN, and possibly TNFβ by the Th1 type of CD4+ cells appears to require non-TCR mediated signals, the absence of which leads to T cell unresponsiveness (92). This co-stimulation cannot be provided by any of the known soluble cytokines tested to date (IL-1 through 7, γ-IFN, TNF, and others) and cell to cell contact appears to be essential (67). Although integrin-ligand pairs contribute to T cell activation, both by providing intracellular signals and enhancing cell adhesion (51), these do not seem to be the source of the critical co-stimulatory signals. CD28 on the T cell and B7 on the APC is the major ligand-receptor pair whose interaction has a dominant role in regulating the activity of Th1-type T cells (32;39;53;59;82).

B7 expression is highly regulated and its expression varies with both the specific cell type and the state of differentiation of that cell (25;26;80). B cell activation can upregulate B7 expression, and some evidence suggests that aggregation of MHC class II molecules
contributes to increasing B7 expression (53). If so, then T cell receptor recognition of peptide-class II complexes on B cells after antigen uptake and intracellular processing may stimulate the B cell to express B7 and in turn, contribute to full T cell activation in the inflammatory pathway. Therefore, the immunogenicity of a peptide may directly relate to its antigenic properties. Some peptide-MHC complexes might be suitable for delivering certain signals to the T cell, but lack the capacity to efficiently evoke reciprocal co-stimulator molecule expression by the APC (22;79). These antigens would lack adequate immunogenicity in the Th1 pathway, and they could tolerize such cells or deviate them to the Th2 pathway.

Th2 cells producing IL-4 and IL-5 (cytokines that play central roles in B cell clonal expansion and isotype switching) (58), do not appear to require B7 / CD28 signals for cytokine production. IL-1 is a co-factor in the clonal growth of these cells in vitro (46), and may help regulate their activity in vivo. The conversion of resting unstimulated T cells to Th2 type cells is poorly understood, but an emerging theme is that IL-4 itself promotes differentiation towards the Th2 phenotype (i.e., there is a positive feedback loop established between IL-4 and IL-4 production) (57;101). Thus, in certain parasitic diseases, the outcome of infection depends on whether the response is predominated by Th1 or Th2 CD4+ cells, and such phenotypic dominance results from the positive feedback effects of the early cytokine responses to the organism (40;41;93). This is clearly a key site for vaccine-based intervention, with the possibility of locking the immune response in the protective inflammatory Th1 mode as opposed to unprotective Th2 mode. This balance also dictates the isotype of antibody produced, with Th1 cytokines favoring IgG2a in mice and IL-4 favoring IgG1 and IgE production (95).

**B Cells and CD8 Effector T Cells**

Co-stimulation is also important for development of cytotoxic CD8+ T cells responses
and for B cells proliferation and antibody secretion. Similar co-stimulatory signals to those observed to control CD4+ Th1 production of IL-2 and γ-IFN, possibly delivered via B7 and CD28, are involved in production of these cytokines by CD8+ cells (74). However, the cytotoxic activity of CD8+ cells does not seem to be equally dependent on such signals after initial differentiation, although this may not be true for the virgin cytotoxic cell precursors. Effector CTL differentiation from resting T cells usually requires CD4+ T cell activity, and CD8+ T cell receptor signalling in the absence of suitable CD4+ help leads to a state of tolerance in the CD8+ compartment (35;81). Thus, it is necessary to maintain CD4+ T cell immunogenicity in subunit vaccines aimed at evoking CD8+ T cells or to provide adequate elicitation of co-stimulation for CD8+ cells to provide their own Th1-like helper function, to avoid tolerizing the very effector population one seeks to stimulate.

In the same vein, CD4+ help is essential for most B cells responses and high affinity antibody production. Because most (though not all) protective antibodies are directed at conformational determinants on intact proteins, these must be preserved in the design of vaccines. B cell uptake of antigen via surface Ig is a critical mechanism for achieving suitable antigen concentrations for efficient loading of class II with peptides (55;85), and competition for such uptake by large amounts of serum antibody directed to non-neutralizing sites on a target can markedly compromise appropriate B cell responses by competing for antigen binding with surface receptors on the useful B cells. In addition, the antigen must possess suitable determinants for class II presentation to CD4+ T cells. A lack of interaction with CD4+ Th and with the cytokines they produce renders an antigen non-immunogenic, and may contribute to tolerization of the B cells if the antigen is presented in polymeric form (34).

B cell activation and differentiation is now known to require membrane contact with activated T cells in addition to T cell derived cytokines (72). One candidate for a critical ligand-receptor pair is the CD40 molecule on the B cells and the CD40 ligand on the T cell (71). The latter is rapidly and transiently upregulated after initial T cell stimulation. The
CD40L-CD40 interaction serves to allow optimal movement of the B cell into cell cycle and differentiation for antibody production. A topic of future concern will clearly be to understand the rules for induction of CD40L on T cells and the delivery of this necessary signal to co-operating B cells that are the source of protective antibody.

**Closing Remarks**

For both CD4+ and CD8+ T cells, only proteins or peptides have the potential for antigenicity. To stimulate the CD4+ T cells that respond to peptides presented by class II MHC molecules, the protein must be delivered efficiently to the endosomal processing compartment, give rise to peptides containing the motifs necessary for capture by the available allelic forms of class II in the individual, and produce adequate levels of a peptide-class II MHC complex that is structurally distinct from self-peptide class II complexes that have already acted to tolerize the T cell compartment. For CD8+ T cells, the protein antigens must gain access to the cytoplasm, produce peptides suitable for transport into the ER that can bind to available allelic forms of class I and again, avoid self-peptide class I MHC mimicry. For B cells, suitable conformation is necessary, as is attachment to a protein yielding a peptide meeting the CD4+ antigenicity requirements to stimulate T cell help.

Immunogenicity for CD4+ cells requires delivery of co-stimulatory signals that are distinct for the two major CD4+ T cell phenotypes. Th1 cells depend on B7-induced CD28 signalling events for production of inflammatory cytokines, whereas IL-1 may contribute to development of Th2 responses. In both cases, antigenicity may affect immunogenicity by virtue of T cell receptor-dependent induction of co-stimulatory molecule upregulation in the APC. CD8+ T cells depend on either these same signals and/or an undefined component of CD4+ effector products, possibly IL-2. B cells require contact to promote CD40 ligand-CD40 interactions. The response pattern to antigen stimulation and co-stimulatory signalling is
further modified by the impact of the cytokines produced by the reactive T cells. These cytokines can both modulate B cell antibody quality and quantity, and also mediate feedback regulation of the differentiation state of the T cell itself.

Optimal synthetic vaccine design therefore requires a variety of conditions to be met. First, it is critical to identify peptides or proteins containing peptides able to form antigenic complexes with the prevalent MHC alleles expressed by individuals in the target population. The definition of sequence motifs controlling peptide-MHC class I or class II interaction will permit the identification of candidate antigenic peptides within proteins of a pathogen, as the sequences of such proteins become known. Alternatively, direct sequence analysis of peptides eluted from infected cells will permit identification of peptides that have been naturally processed and bound to MHC molecules and that can be incorporated into synthetic vaccine formulations. The identification of preferred anchor residues can also allow modification of naturally occurring antigens to optimize effective MHC molecule presentation for initial induction of immunity. Even though the natural material may have a less favorable residue at a key position, it is substantially easier to boost a primed response than to elicit a primary response (1). It is also possible to identify key residues controlling T cell specificity (epitopic residues) rather than MHC molecule binding and to produce vaccine material with multiple substitutions at such positions to preclude escape from immune destruction due to pathogen sequence variation at these sites (102). Second, in the cases where peptides are not used to bypass processing requirements, the delivery of the parent proteins to the proper processing compartment of APC is essential. The different routes involved in class I and class II-dependent presentation make it clear that improved delivery vehicles are needed to ensure a proper balance of entry into the two different pathways. pH- and enzyme-sensitive liposomes (36), antigen:anti-surface receptor conjugates (96), and live vectors able to produce intracellular proteins (61) are now being used to direct antigen efficiently into the class I and class II pathways. In both cases the presenting cell to which antigen is delivered
must include be capable of initiating primary responses; such initiating cells appear to be
different from the B cells that will produce the desired antibody (100), and a strategy for
appropriate targeting of these specialized accessory cells is important in maximizing
immunogenicity.

Third, there needs to be development of improved adjuvants that regulate
co-stimulatory signalling in an appropriate fashion. A balance between provision of adequate
levels of such co-stimulation and excessive co-stimulation that might override controls on
self-reactivity and engender autoimmunity must be achieved. The identification of key
molecules involved in co-stimulation raises the possibility of incorporating inducers of these
molecules directly in vaccine preparations, and of coupling the inducers to the protein antigens
to ensure delivery to the same presenting cell.

It should be apparent from this very brief summary of progress in only a narrow area of
basic immunology that the knowledge explosion in the field makes it difficult for the most
recent advances to rapidly move into investigations of protective immunity and vaccine
design. I have tried to summarize here some of the opportunities that basic studies of
antigenicity and immunogenicity have provided for improvements in the development of
effective vaccination strategies. Hopefully, new mechanisms for information exchange among
the investigators in the three fields relevant to vaccine produc tion will emerge, so that the
goal of effective vaccines for many diseases still plaguing the world population, and especially
children, will be met in the shortest time.
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DEVELOPMENT AND MATURATION
OF THE IMMUNE SYSTEM
Alexander R. Lawton, M.D.
Departments of Pediatrics and Microbiology and Immunology
Vanderbilt University School of Medicine
D-3237 Medical Center North
Nashville, TN 37232 (615)322-4397
Running Head: Immunology of the Neonate
Abstract:

Generation of diversity of T and B cells begins early in gestation. Selective restrictions in expression of V genes occur in fetal life, but insufficient clonal diversity is not likely to limit newborn immune capabilities. Functional immaturity of neonatal T and B cells is beginning to be defined. Virgin T cells lack the capacity to produce diverse lymphokines, while neonatal B cells are less responsive to lymphokines promoting terminal differentiation to plasma cells.
Immunology of the Neonate

Knowledge of the neonatal immune system stems from description of serologic and histologic responses to infections or intentional immunizations, much of which is now ancient history, and on recently obtained detailed information on molecular mechanisms involved in differentiation and function of T cells, B cells, and antigen-presenting cells. If our collective goal is to design vaccines which will effectively immunize in utero or in the neonatal period, we need information on processes of the immune response which have been recognized for some time but are only recently amenable to study. Maturation is a reasonable term for changes in immune capacity which involve post-thymic or bursa-equivalent cells and may not entirely depend on antigen-specific stimulation.

Ontogeny of T and B Cell Development

Although earlier precursors have been defined for both B and T-cell lineages, (3;4;18) the most generally useful markers are the clonally distributed antigen-specific receptors: immunoglobulin molecules for B cells and the related γ-δ and α-β T cell receptor (TCR) molecules for T cells. The genetic mechanisms by which these receptors are generated have been recently reviewed (1;27;29;38). In brief, both types of receptors are assembled by splicing of several genes segments mediated by a unique recombinase enzyme. Immunoglobulin heavy chains, and TCR β and γ chains, are produced from libraries of V
(variable), D (diversity), J (joining), and C (constant) genes, while Ig κ and light chains and TCR α and δ chains utilize only V and J and C segments. Each of these chains is encoded in a separate locus with its own set of VDJ or VJ genes. Numbers of germ line V genes vary from less than 10 to perhaps as many as 1000. There are fewer D genes (<50) and J genes (<10).

Recombination of these tandemly arranged gene segments contributes to receptor diversity in 3 ways: First, joining of each V gene with different D and J segments creates a different configuration of the antigen-binding site. This occurs because the VDJ junction contributes about 1/3 of the amino acids forming the site. Second, the precise locations of DJ, VD, or VJ joints vary. Third, additional nucleotides are added at splice junctions by an enzyme, terminal deoxynucleotidyl transferase, which does not require a template. The latter characteristics of the splicing mechanism may delete, change, or add amino acids in the binding site. Shifts in the transcriptional reading frame or introduction of stop codons producing many abortive rearrangements are inherent in this process.

From a teleological perspective, this mechanism seems designed to generate maximum diversity from a minimum number of inherited genes by introducing random alterations into a highly restricted site of the assembled receptor molecule. Potential clonal diversity of \( >10^{10} \) specificities may be generated from approximately \( 10^3 \) genes. For B, but not T cells, diversity is
further increased by somatic mutations within the binding site during antigen driven differentiation. The evolutionary strategy is to ensure sufficient diversity to recognize any and all antigens rather than to conserve particular specificities. The cellular requirements are an intrinsically high rate of division among precursor cells, allowing for the high frequency of abortive rearrangements, and mechanisms for eliminating or controlling the activities of self-reactive clones. These requirements are met in the thymus and in the fetal liver/bone marrow, where production of T and B cells is many-fold greater than the numbers exported to peripheral lymphoid tissues. In the thymus, developing T cells are subjected to both positive and negative selection, with the latter process eliminating highly autoreactive cells. (22;37) Immature B cells similarly pass through a phase shortly after their emergence during which contact with antigen results in clonal energy or deletion (16;26).

T and B cell development begins within the thymus and fetal liver respectively at 7 to 8 weeks gestation. It has recently been demonstrated that the omentum is also a site of B cell generation (32). Immunoglobulin heavy chain gene rearrangements precede those of light chains so that pre B cells can be identified as a population containing intracytoplasmic \( \mu \) chains but no light chains. Successful production of light chain is rapidly followed by export of intact monomeric IgM molecules to
the cell surface where they become functional receptors. By 10-
12 weeks gestation the fetal liver contains roughly equal
populations of pre-B and receptor-bearing B lymphocytes. The B
lymphocyte population expands rapidly, so that by 15 weeks the
proportions of B cells in spleen and blood are similar to those
in post-natal life (15).

Precursor T cells contain intracytoplasmic CD3 prior to
eexpression of TCR B chains; such cells are present in fetal liver
as well as thymus. Expression of cytoplasmic TCR B chain
precedes that of α chains. T cells with a membrane-associated
TCRB-CD3 complex are present in fetal thymus at 12 weeks or
earlier and comprise approximately 1/4 of thymocytes throughout
the remainder of gestation. Receptor-bearing T cells are absent
from fetal liver prior to 10 weeks, but found with increasing
frequency thereafter. Only a small minority of T cells in thymus
express the alternative TCRγ-δ (4).

**Ontogeny of Repertoire Expression**

There is experimental evidence in inbred mice strongly
implying ordered expression of particular antibody specificities
(5;31) Antibodies to carbohydrate antigens are frequently
dominated by one or a very few clonotypes. Such readily
identifiable clonotypes are expressed at a predictable
developmental time (31). In mice and humans certain VH genes are
preferentially expressed in fetal life (28;30). Members of VH families mapping closest to the D genes comprise most of the expressed fetal repertoire in mice, an observation which suggested a location-dependent mechanism for VH readout. However, a high proportion of the human fetal repertoire is not D-proximal, the positional hypothesis now seems less likely (discussed in 1).

The murine fetal antibody repertoire is dominated by clonotypes secreting polyspecific antibodies and exhibiting connections through idiotypic interactions (10;35;36). Idiotypes associated with polyspecific autoantibodies are prominent in human fetal spleen (23). B cells bearing CD5 are associated with production of polyreactive autoantibodies (6); and are present in relatively higher proportions early in ontogeny in mice and humans (3;10). Recent studies have demonstrated that the fetal omentum is a preferred site of origin of pre B cells committed to the CD5 lineage (32). It is tempting to link fetal VH restrictions, a uniquely "connected" repertoire and the ontogenetic prominence of the CD5 subset. However, conventional as well as CD5+ fetal B cells express restricted, although different, VH genes (21).

Is expression of a restricted human fetal B cell repertoire an important consideration for vaccine development? This question addresses two issues. First, intentional immunization
might alter repertoire development by modulating idiotypic networks, as has been demonstrated in mice (37). Second, restricted VH usage might constrain diversity so that not all epitopes are recognized.

The developmental period during which the murine immune system exhibits uniquely restricted diversity begins at about day 13 of gestation and ends about 3 weeks later, 2 weeks postnatally. Even very young mice express considerable clonal diversity (5;24). Based on histologic appearance of lymphoid tissues and relative numbers of peripheral B and T cells, the newborn mouse is similar to the human fetus at about 12 weeks gestation. The additional period of human gestation almost certainly assures a more diverse immune system at birth. A recent study comparing VH usage and frequency of polyspecific antibodies between neonatal and adult Epstein-Barr virus transformed clones failed to find a difference (17). The fetal T cell Vβ repertoire early in the second trimester is indistinguishable from that of adults (11). Constraints on the neonatal immune response are much more likely related to activation of T and B cells than to generation of clonal diversity.

Maturation of Immune Capabilities

B lymphocytes bearing IgG or IgA on their surface are
present in adult frequencies in fetal blood and spleen by 15-16 weeks gestation and at higher than adult frequencies in neonatal blood (15). While adult IgG or IgA-bearing cells express only a single isotype, neonatal cells characteristically also express IgM and IgD (14). Multiple isotypes expressed on B lymphocytes are apparently generated by RNA processing rather than switch recombination of VDJ from Clμ to a downstream isotype. IgA-bearing cells from IgA-deficient patients, (9), and IgG-bearing cells from patients with panhypoglobulinemia (13) share this immature phenotype. In all three instances there is a striking lack of association between expression of diverse isotypes on B cell membranes and the capacity to generate plasma cells secreting IgG or IgA, in vivo or when stimulated by polyclonal activators. Early studies implicated both T and B cells in the impaired neonatal response. In addition to poor helper function, neonatal T cells were active suppressors (20). These studies were technically flawed because the polyclonal activators used stimulated only a small fraction of B cells recently pre-activated in vivo. However, recent investigations using a very efficient culture system in which B cell differentiation is initiated by cognate interaction with anti-CD3 activated T cells has confirmed the earlier results (33).

Neonatal lymphocytes secreted little or no immunoglobulin of any isotype when stimulated by immobilized anti-CD3, although both lineages were induced to proliferate and T cells secreted
nearly as much IL-2 as adult T cells. Substitution of adult for neonatal T cells, or supplementation with supernatants from activated adult T cells (TF) increased the IgM response but had little effect on IgG or IgA production. Neonatal T cells were competent to activate adult B cells to proliferate, but relatively little immunoglobulin was produced unless cultures were supplemental with TF.

The functional deficits of neonatal cells can be overcome by addition of sufficient quantities of IL-2 and either IL-4 or IL-6. The effect of IL-2 is critical, since antibodies to the IL-2 receptor abolish responses to IL-2, IL-4, or IL-6. IL-4 has the independent effects of increasing production of IL-2 by neonatal T cells and enhancing helper activity by an IL-2 independent mechanism. Neonatal cells supplemented with IL-2 and IL-4 or IL-6 were capable of secreting all isotypes, with no cytokine-specific effects (34). In summary, these investigations demonstrate that neonatal B cells require substantially higher concentrations of IL-2 and IL-4 or IL-6 for optimal differentiation than do adult B cells. Neonatal T cells, while producing IL-2 sufficient to drive proliferation, do not provide additional cytokines necessary for terminal differentiation of B cells.

Differential expression of isoforms of the leukocyte common antigen CD45 distinguish adult CD4+ cells with helper activity
for B cell differentiation from those with suppressor-inducer function (CD45RA⁺). CD45RO cells appear to constitute a memory population derived following antigen stimulation of naive CD45RA⁺ cells (7).

Neonatal CD4⁺ T cells are 90% CD45RA⁺ where 50% of adult T cells bear this marker. The small fraction of neonatal CD4⁺CD45RO⁻ T cells has helper activity comparable to the same population of adult cells whether stimulated by pokeweed mitogen or anti-CD3 antibodies. The reciprocal CD45RA⁺ neonatal T cell population lacks helper activity and actively suppresses helper function of CD45RO cells. This radiation-sensitive suppressor function is absent from the corresponding adult CD45RA⁺ cells. Neonatal CD45RA⁺ T cells initially stimulated with phytohemagglutinin and then cultured for long periods with IL-2 become CD45RA₀, lose suppressor activity and acquire helper activity comparable to freshly isolated CD45RA₀ cells (8). However, irradiated neonatal T cells activated by immobilized anti-CD3 and supplemented by cytokines appear capable of helper function even though retaining CD45RA (34).

Direct comparison of adult and neonatal T cells has confirmed important differences in their capacity to produce cytokines. Similar amounts of IL-2, tumor necrosis factor and lymphotoxin are produced, but neonatal T cells produce little or no IFN-γ, IL-3, IL-4, IL-5, IL-6, or GM-CSF (12;25;38). The
differences in cytokine production reflect reduced numbers of neonatal cells producing the cytokine. For example, approximately 40% of adult T cells, but only 3% of neonatal T cells, were positive for IFN-γ mRNA by in situ hybridization (25). Synthesis of diverse cytokines is linked to prior activation of T cells. CD4⁺ CD45RO⁺ adult T cells, for example, have higher frequencies of cells producing IFN-γ and IL-4 than unfractionated CD4⁺ cells while the reciprocal CD45RA⁺ population has very few (38). Neonatal T cells activated by anti-CD3 and cultured with IL-2 for 7 to 10 days produced and adult pattern of lymphokine mRNAs on restimulation. This maturation event was not blocked inhibition of DNA synthesis. Whether such cells lose CD45RA was not determined (12).

Changes in CD45 isoform expression from RA to RO thus appears to be a very useful marker for T cell maturation. The proportion of RO⁺ cells rises gradually with increasing age, reaching a plateau at puberty (21). Immunization can accelerate this process. For example, transfused infants have higher proportions of CD45RO cells than controls of the same age (19).

Conclusions

Generation of clonal diversity of T and B cells begins early in gestation and proceeds at a rapid rate. The lack of precision of the VDJ recombinase enzyme in making coding joints of Ig and TCR genes stands in striking contrast to the fidelity of other
enzymes involved in DNA replication and repair, emphasizing the evolutionary importance of extensive diversity in the immune system. Although the fetal B cell repertoire is distinctly different from that of the adult, it is unlikely that lack of sufficient clonal diversity constrains the neonatal immune response.

A great number of studies have documented that immune responses of neonates are less vigorous than those of older children or adults. All lineages involved in host defense, T cells, B cells, antigen presenting cells, and professional phagocytes, appear in some ways less competent than their adult counterparts.

Immaturity of neonatal lymphocytes, and particularly T cells, is beginning to achieve molecular definition. Naive helper T cell populations lack the capacity to produce a variety of cytokines, particularly IL-4, IL-5, IL-6, and IFN-γ, and are suppressors of B cell differentiation.

Stimulated naive T cells lose suppressor activity, and a fraction acquire the ability to synthesize one or more lymphokines. Distinct patterns of lymphokine secretion characterize functional T cell subsets which play a major role in many chronic infections (2). Better understanding of post-thymic T cell differentiation related to lymphokine expression is an
exciting future prospect.

The immaturity of neonatal B cells with regard to T-dependent triggering appears to be quantitative rather than qualitative, since it can be overcome with optimal quantities of cytokines. The relative inability of newborns and young children to respond to carbohydrate antigens, as well as the restrictions of anticarbohydrate antibody with respect to isotype, $V_H$ and $V_L$ usage remain to be explained.
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EFFECTIVE MUCOSAL IMMUNITY: CURRENT CONCEPTS FOR VACCINE DELIVERY AND IMMUNE RESPONSE ANALYSIS

Dr. Jerry R. McGhee
Professor of Microbiology
The University of Alabama at Birmingham
UAB Station; BHSB 392
Birmingham, Alabama 35294-0005
Tel: (205) 934-5045

Running Title: DEVELOPMENT OF EFFECTIVE MUCOSAL IMMUNITY
EFFECTIVE MUCOSAL IMMUNITY: CURRENT CONCEPTS FOR VACCINE DELIVERY AND IMMUNE RESPONSE ANALYSIS

By

Jerry R. McGhee and Hiroshi Kiyono

From

The Mucosal Immunization Research Group, The Immunobiology Vaccine Center, Departments of Microbiology and Oral Biology, The University of Alabama at Birmingham, Medical Center, Birmingham, AL 35294
ACKNOWLEDGMENTS

Work summarized in this review was supported by U.S. Public Health Service Contract AI 15128. HK is the recipient of NIH Research Career Development Award DE 00237. We thank Dr. Masafumi Yamamoto, Mr. Masahiko Amano and Ms. Sheila Weatherspoon for their help in the preparation of computer graphics and of this review, respectively.
INTRODUCTION

It is now established that the mucosal immune system is separate and is regulated in a different fashion than occurs in peripheral lymphoid tissues (the systemic immune system). For example, the mucosal immune system can be divided into discrete inductive sites where vaccines / antigens are encountered and are taken up, processed and presented to B and T cells, and separate areas where immune cells actually function (mucosal effector tissues) (21, 31). B cell commitment to IgA occurs in these inductive sites. Further, through the help provided by Th cells and cytokines, these B cells respond to antigen and undergo expansion including memory cell formation and following their emigration to mucosal effector tissues, the B cells rapidly develop into IgA plasma cells, and the latter represents a major characteristic of mucosal tissues. It also appears that antigen-specific Th cells, and perhaps even CD8+ cytotoxic T lymphocytes (CTLs) can make this circular journey (along with B cells) from inductive to mucosal effector sites, and mucosal immunologists have termed this the Common Mucosal Immune System (CMIS). In this brief review, we have selected five areas which we feel are of major importance in our goals for development of mucosal immunity in the Children's Vaccine Initiative (Table 1).

1. WHY DO WE NEED MUCOSAL IMMUNITY?

The mucosal immune system seems daunting to vaccinologists due to its enormous size and seeming complexity. It has been estimated that the mucosal immune system spans a surface area of > 400 m² or greater than 250 times larger than the skin surface! Further, vaccines must be given in an appropriate inductive site, e.g., gut-associated lymphoreticular tissues (GALT) and, depending upon the antigen, the mucosal IgA response is often measured in a completely different site, e.g., the oral cavity or the mammary gland. Thus, all vaccines except one are today administered by systemic routes, and as will be discussed below, this results in effective cell-mediated
and antibody responses in systemic tissues, but is essentially ineffective for induction of mucosal immunity in humans who have not previously suffered a mucosal infection by the causative organism. Furthermore, attempts to induce mucosal immunity by vaccination through routes thought to be appropriate, e.g., orally, are sometimes successful but more often are not. It would therefore be useful to attempt to simplify our approaches to mucosal immunization and place emphasis on relevant lymphoid cells and molecules involved in mucosal immunity and memory (Table 1).

Among immunoglobulin (Ig) isotypes, IgA predominates in humans, and represents greater than 60% of all antibody isotypes produced (13, 34). As indicated above, most IgA produced in mammals is derived from plasma cells in mucosal effector sites, for example the lamina propria (LP) regions of the gastrointestinal (GI) and upper respiratory tracts (URT) and in ascinar regions of glands (21, 31). Approximately 80% of mucosal (S-IgA) antibodies are derived from the GI tract (Fig. 1) and of course this is required since the gut is by far our largest organ. This clearly suggests that a major commitment has been made by the host for T and B cell immunity in the intestine and indeed the gut could be considered to be the largest lymphoid organ in the immune system. Thus, many studies in humans and especially in mice have focused on the GI tract, and much of our understanding of the mucosal immune system has been derived from these studies.

If we focus only on IgA and the two subclasses in humans (IgA1 and IgA2), remarkable differences are noted and this again reinforces our need for a separate mucosal immunity (14, 17, 35). For example, we know that approximately 30 percent of human serum Ig is IgA, where 90-95% is IgA1 and only 5-10% is IgA2 (17, 35, 36). Furthermore, most human serum IgA is monomeric (85-90%) and thus could not reach mucosal tissues, since only polymeric IgA (pIgA, usually dimeric) is transported across epithelial cells by the polymeric Ig receptor, secretory component (SC) (reviewed in 36). This raises a second point, e.g., most vaccines are tested in mice, rats and rabbits,
and in these species a large proportion of serum IgA is pIgA and is selectively transported by liver hepatocytes into bile by SC (hepatobiliary transport; 36). Hepatobiliary transport does not occur to any extent in humans since our hepatocytes do not produce SC. Thus, serum IgA responses are of little relevance for mucosal protection in humans, and in experimental animals transport of serum IgA into the upper GI tract via the hepatobiliary route could obscure measurements of local S-IgA responses there. In this regard, it is always important to assess the numbers and isotype of antigen-specific plasma cells which reside in mucosal effector tissues. Elucidation of antigen-specific IgA producing cells at the single cell level is essential for proper evaluation of mucosal vaccines in experimental animal models.

2. **IS ORAL IMMUNIZATION ALONE SUFFICIENT FOR INDUCTION OF MUCOSAL IMMUNITY?**

The distribution of IgA1 and IgA2 plasma cells in various human mucosal effector sites provides major evidence that separate mucosal inductive sites, e.g., nasal-associated versus gut-associated lymphoreticular tissues (NALT vs GALT) may differ in their roles for provision of the necessary B cell precursors for S-IgA antibody responses. For example, the nasal mucosa, lacrimal glands and LP of the URT contain mainly IgA1 plasma cells; IgA2* cells are infrequent (Fig. 1) (9, 20). In contrast, higher numbers of IgA2 plasma cells are seen in the LP of the lower intestinal tract (Fig. 1) (9, 20). It has recently been suggested that the tonsils (or NALT) are a major inductive site in humans, and indeed may represent a site for IgA precursor B cells destined for the URT and perhaps other mucosal areas. For example, pokeweed mitogen triggered B cell cultures derived from tonsils produce all major Ig isotypes, including IgA (8). Interestingly, greater than 95% of the IgA produced is of the IgA1 subclass, supporting the notion that tonsils are indeed a possible site for precursor IgA1* B cells (15). Likewise, human appendix, which may be representative of GALT, contains both IgA1*
and IgA2+ B cells; however IL-6 induces much higher IgA2 responses in appendix B cell cultures (15). This would suggest that GALT is a major source of IgA precursor B cells which populate the effector regions of the small and large intestine with some preference for the IgA2 subclass.

It was in former years assumed that bronchus-associated lymphoreticular tissues (BALT) are the normal counterpart for GALT, e.g., Peyer's patches (PP) in the GI tract. BALT was first described in rabbits and several other species where organized lymphoid tissue was present at the branch points of the airways, most frequently between the bronchus and the artery (4, 5, 44). The BALT in rabbits and rats are covered by a stratified columnar epithelium which contains specialized epithelial cells which resemble microfold, (M) cells, and these cells most likely function in antigen uptake in a manner analogous to the dome region of the PP (see below) (45). In fact, it can be stated that the most extensive studies of BALT have been done in rabbits and rats and this has been reviewed in detail (46). Recent work (43) have failed to demonstrate organized BALT structures in normal human lungs. It should be emphasized that these studies were done on lung tissue of patients without evident respiratory diseases, and on subjects of various ages who would normally encounter the microbial flora of the upper respiratory tract. A recent viewpoint (42) was taken that the human lungs contain lymphoid cells in other compartments, e.g., the intravascular pool, the interstitium and bronchoalveolar spaces, and may represent the cells which respond to antigenic insults in the lung (42). Thus, this might suggest that a classical BALT is more involved in the systemic immune system in the lungs instead of the mucosal immune system.

Thus, it would be appropriate to re-evaluate our past suppositions that BALT is a universal tissue in mammals and is the GALT counterpart in the lungs. As we indicated above, the major organized human lymphoid tissue in the upper respiratory tract are the follicles present in the nasal cavity. These large, anatomically distinct masses are
termed Waldeyer's ring. The most prominent follicles are the palatine and pharyngeal
tonsils. These tonsils are covered by a stratified squamous epithelium, but certain
regions have columnar epithelial cells and some of these bear characteristics of
microfold ( $M$ ) cells. The possible roles for this nasopharyngeal lymphoid tissue in
mucosal immunity has been recently reviewed (23). Of more importance, a number of
studies have shown that high frequencies of B cells expressing surface IgA occur in
human tonsils. The majority of these surface IgA+ ( sIgA+ ) B cells express IgA1 and
mitogen stimulated tonsillar cultures produce mainly IgA1 antibodies (8). Further, it
was recently shown that IL-6 also induces activated tonsillar B cells to become IgA1
secreting cells (15). It is tempting to speculate that these are the B cell precursors for
nasal mucosa and LP regions of the upper respiratory tract. If indeed the tonsils or
NALT supply precursor B cells to the URT, then the central question becomes- what is
the relative importance of tonsils in mucosal immunity? In other words, does the NALT
compare, for example, with the PP ( GALT ) in terms of serving as major mucosal
inductive sites? If they are of importance, then vaccine strategies should begin to
consider antigen presentation and uptake in human tonsillar tissues. Finally, it would
be appropriate to now refer to human tonsils as NALT and not BALT.

Recent studies in SCID mice given human periperhal blood lymphocytes suggest
that intestinal LP regions are selectively repopulated with human IgA plasma blasts
(25). This should now provide a model to test the hypothesis that tonsillar tissues
(NALT) preferentially contain more IgA1 precursors destined for mucosal tissues,
especially in the URT. Likewise, the appendix may contain greater numbers of mucosa­
seeking IgA2 precursors. Experiments along these lines are currently underway in our
group to determine if SCID mice reconstituted with representative tissues of human
NALT and GALT exhibit preferences for IgA subclass responses and possibly result in
repopulation of SCID mouse mucosal effector tissues in patterns which mimic the
natural human host.
In summary, we should return to the question of whether oral immunization will be suitable for induction of mucosal immunity in sites distant from the GI tract by the CMIS. The answer at this point would be that it may be an over-simplification to assume that GI tract (oral) immunization will provide the protection required for adequate immunity in the lungs and the female reproductive tract. In addition, it is also important to consider other routes of mucosal immunization (e.g., vaccine targeting to NALT) in addition to oral immunization for the induction of mucosal immunity in the URT and the oral cavity. Although most investigators now agree that the concept of the CMIS is still important for the induction of antigen-specific immune responses in different mucosa-associated tissues (33), we now must also realize that a better understanding of how the CMIS is compartmentalized will be essential in order for us to use the mucosal immune system for the development of effective vaccines (Table 2).

3. ANTIGEN UPTAKE DIFFERENCES IN MUCOSAL INDUCTIVE VERSUS EFFECTOR TISSUES

Studies in a number of laboratories have shown that lumenal antigens/vaccines may be taken up by the host through both mucosal inductive and effector sites. In this regard, mucosal IgA inductive sites are equipped with specialized antigen uptake cells, termed microfold (M) cells (7, 41). The current view is that microorganisms and complex antigens are sampled and passed intact through endocytotic vesicles and delivered to underlying lymphoid cells for further antigen processing and presentation (Fig. 2). It has been suggested that M cells may express class II MHC and thus potentially serve as antigen presenting cells (APCs). However, a recent immunochemical study of human PP suggested that epithelial cells in the dome region express class II MHC, while M cells do not (10). Thus, it is safe to conclude that M cells can transport complex antigens (vaccines) into IgA inductive sites and may also act as APCs. These functions are not mutually exclusive; for example certain viruses
(reovirus types 1 and 3 and rotavirus) or bacteria (E coli-RDEC) bind to receptors on M cells and may pass through in nondegradative endosomal/lysosomal pathways (18, 50). Other macromolecules may enter endosomes which fuse with class II+ secondary lysosomes with subsequent processing of peptides. APCs provide CD4+ Th cells with the triggering signal via peptide-MHC-class II and result in the initial activation steps and formation of memory cells. B cells in germinal centers also receive triggering signals by antigen and develop into IgA precursor stages and into memory cells. Immediate homing to mucosal effector sites precedes the second signals required for actual immune responses.

The results of studies which show that antigen-specific B and Th cells repopulate mucosal effector sites via the CMIS have provided the rationale for development of oral vaccines and unique antigen delivery systems which attempt to optimize antigen uptake by M cells (Table 2). A number of oral vaccine delivery systems are currently being tested (Table 3). Perhaps the best studied system involves the co-administration (or direct coupling) of cholera toxin (CT) or CT-B subunit with vaccine immunogens, since CT induces good mucosal S-IgA responses and redirects mucosal and serum antibody responses to proteins which often induce oral tolerance when given separately. Other major delivery systems include microspheres which encapsulate (and thus protect) vaccines and ISCOMS which facilitate antigen-delivery into and induction of B cell responses in germinal centers (see Mucosal Memory in Vaccine Development section below). Both microspheres and ISCOMS are taken up by M cells rather inefficiently, and current studies involve methods of enhancing their uptake in the GI tract by changing their surfaces. Recombinant viruses (Adeno- and Poliovirus) and bacteria (e.g., avirulent Vibrio cholerae, Salmonella typhimurium or rBCG in mice and avirulent V. cholerae, S. typhi and rBCG in humans) are also promising approaches to delivery into the PP for disseminated S-IgA responses in distant mucosal effector sites (Table 3).
In mucosal effector tissues, antigen uptake and presentation also occurs; however, important differences are noted. For example, vaccine antigen may be endocytosed by epithelial cells, and in certain situations the epithelial cells themselves express class II MHC (30) and can process the antigen with subsequent association of immunogenic peptides with MHC class II (Fig. 2). It is tempting to suggest that this type of presentation leads to anergy, and this may represent a major function of epithelial cells in response to food antigens. It is known that some responses occur; however, this may be diminished by the presence of anergic T cells (Th2 cells?) which cannot provide help for what could become an exaggerated local S-IgA response. The induction of anergic Th2 cells could result from inappropriate signal two delivery by epithelial cells, which prevent appropriate delivery by 'normal' mucosal APCs. In other situations, intact proteins can traverse tight junctions, and in this instance intact vaccine antigen could trigger B and T cell responses. For example, sIgA+ B cells may bind antigen and through endocytic pathways process and present peptides, together with MHC class II, to Th cells. Macrophages in LP regions also could serve this function for more complex antigens. The simplest scenario would be presentation by class II+ sIgA+ B cells to Th2 cells, and this would complete signal two allowing full activation of Th2 cells with IL-4, IL-5, IL-6 and IL-10 cytokine release (21, 31) (see below). sIgA+ B cells would receive signal two from activated Th2 cells and derived cytokines (IL-4, -5 and -6), for subsequent B cell proliferation and differentiation into IgA producing plasma cells with specificity for the vaccine antigen (21, 31) (Fig. 2).

4. CONSIDERATION OF MUCOSAL MEMORY IN VACCINE DEVELOPMENT

The induction of memory in the mucosal immune system has been incompletely studied; however some have concluded that no memory exists for mucosal IgA responses. Thus, many studies have shown that IgA responses are sometimes short-
lived, and boosting may not always induce IgA responses faster, to higher levels or of increased avidity, all of which are properties of memory T and B cells involved in anamnestic responses. This has tended to confuse those who work with vaccines, since it is sometimes assumed that continual restimulation of mucosal surfaces will be required for protective S-IgA responses. This clearly may not be the case, since there is good evidence now for mucosal memory to certain proteins. However, the nature of the vaccine antigen, e.g., protein versus polysaccharide is a major determinant of memory B cell induction for anamnestic mucosal IgA responses. In this regard, we have placed emphasis on the often forgotten fact that IgA responses can be induced to so-called T cell independent antigens, including bacterial polysaccharides and lipopolysaccharides of importance in vaccines. Clearly, these IgA responses lack memory and point to the need for use of polysaccharide-protein (conjugate) based vaccines for potential induction of B cell memory for S-IgA anti-carbohydrate antibody responses (Table 2).

The first encounter with an antigen leads to clonal expansion of antigen-specific B cells which develop into a germinal center. It is thought that memory B cells also arise in these germinal centers, including those present in the PP B cell zones. These germinal center B cells include memory cells and acquire unique properties including loss of sIgD and enhanced expression of Peanut Agglutinin (PNA) receptors (sIgM+, sIgD−, PNAHI). It is usually assumed that a single B cell can give rise to the progeny for a germinal center and hybridoma analyses have shown that an entire secondary antibody response is generated by a small number of clones (~20) (6). It is probably safe to conclude that this would also apply to anamnestic IgA responses in mucosal sites; however this assumption has not been experimentally addressed. Elegant recent studies have directly shown that somatic mutations occur during germinal center responses, a time when the antigen-specific B cells generate Ig receptors with higher affinity (3, 19, 24). During germinal center responses and memory B cell generation,
switches to other isotypes occur and for IgA high frequencies of sIgA+ B cells occur in PP germinal centers (12). Further, it is plausible to suggest that these memory B cells exit the germinal centers and pass directly into T cell areas enriched in CD4+ Th cells (22). It is known that memory cells do not begin to recirculate until 2-3 weeks after antigen priming (26) and memory B cells (and T cells) may therefore remain in IgA inductive sites such as the PP for long periods.

In this discussion of memory, we normally assume that the first encounter with a systemic foreign protein (vaccine) results in antibody responses characterized by IgM and later IgG isotypes. This is also accompanied by induction of memory B and T cells, which respond to a second vaccination with higher affinity IgG antibodies in higher titers. This secondary response is due to the generation of B cell clones with surface Ig receptors of higher affinity, which arise through somatic mutations in the germinal centers. We postulate that a similar series of events likely occurs following oral immunization with protein vaccines; however in these instances the primary and boosted response are both of the IgA isotype. The prediction from this would be that the first oral vaccine encounter induces low affinity S-IgA antibodies and following clonal expansion and somatic mutations, memory IgA B cells could respond to a second oral vaccine encounter with heightened, S-IgA antibodies of much higher affinity.

Other investigators have clearly shown that oral immunization with cholera toxin (CT) induces memory in the mucosal immune system (1, 27-29). Mice orally primed with CT maintained memory for a two year period, and secondary type IgA anti-CT antibody producing cells were seen in lamina propria when these mice were orally challenged with CT (27, 29). Further, in vitro stimulation of lymphoid cells from these aged mice gave enhanced IgA and IgG anti-CT antibody responses, and these were most pronounced in PP B cell cultures (27). Additional studies showed that adoptive transfer of B cells from mesenteric lymph nodes of mice orally primed one year earlier with CT, supported secondary-type IgA anti-CT responses in LP of recipient
mice (1). Finally, these investigators have shown that human PBMC of volunteers receiving oral whole cell-CT-B cholera vaccine could be triggered with antigen in vitro (28), presumably due to memory B (and T) cells in the blood circulation. Numerous other studies have also provided evidence for memory in the mucosal immune system, and in all cases these responses were induced to complex antigens (vaccines) containing proteins. Nevertheless, additional studies should be done with conventional protein-based vaccines, e.g., tetanus or diphtheria toxoid (TT or DT) together with CT to study in more detail memory B and T cell responses in IgA inductive and effector sites. Studies along these lines are underway in the Mucosal Immunization Research Group at UAB.

Studies in a number of laboratories have shown that IgA antibodies can be induced to polysaccharides and two examples should suffice to show that IgA antibody is readily induced to polysaccharides. Oral administration of pneumococcal polysaccharide vaccines result in IgA responses in external secretions and in serum (38). Although few studies have been done in athymic, nude mice, evidence has been provided that these animals make IgA to T-independent antigens following immunization by systemic or oral routes (2). The most definitive studies on the role of S-IgA anti-polysaccharide responses and protection have been carried out in mice orally immunized with live cholera Vibrios (49). The PP B cells were used to generate IgA hybridomas with specificity for V. cholerae LPS (0 polysaccharide side chains) (49). When an IgA myeloma of this specificity was grown in the upper backs (piggy back model), it was shown that pIgA anti-LPS was transported into GI tract secretions, and more importantly the S-IgA antibodies were protective (49). This work has more recently been applied to IgA anti-Salmonella LPS mAbs, and protection against an oral Salmonella challenge was shown (37). It should be noted that many studies have shown that IgA anti-LPS responses are induced to gram negative bacteria, and high levels of S-IgA (usually S-IgA2) occur in human external secretions (11). One must
conclude that IgA anti-bacterial polysaccharide responses can be (and often are) induced in the mucosal immune system. Nevertheless, it is equally clear that IgA anti-polysaccharide responses lack memory and effective immunity will require continual restimulation (Table 2).

**Possible Applications of Conjugate Vaccines for Induction of Memory IgA Responses**

Recent advances in vaccine development have included the coupling, by chemical means, of complex bacterial carbohydrate capsules (Hemophilus influenzae type b; Hib) to well defined protein vaccines, e.g., TT, DT and bacterial outer membrane proteins (OMP). This 'converts' the vaccine to a T cell dependent form and thus carries with it several advantages. For example, conjugate vaccines (Hib) induce human IgG subclass responses to the polysaccharide, whereas the carbohydrate alone induces mainly IgM and IgG2 antibody responses (16). Thus, the Th cell dependent response supports IgG1 and IgG3 anti-polysaccharide antibodies which serve more effectively for opsonization, C fixation and phagocytosis. Further, conjugate vaccines have been given to infants at earlier times, with resultant anti-carbohydrate responses, whereas immunization with carbohydrate alone at these early times generally fails to elicit antibody responses.

The use of conjugate vaccines is clearly a major advance; however several important questions remain in terms of the nature of the immune response induced. It is often assumed that the protein conjugate in the moiety induces CD4+ Th cells which help B cell responses on the one hand to the protein and on the other to the polysaccharide. However, no studies to date have shown that human CD4+ Th cells enhance antibodies to the carrier protein as well as to the polysaccharide. In fact, few studies have simultaneously measured the isotype, including IgG and IgA subclasses, and titer of antibodies to the carrier protein and compared these with antibodies to the
polysaccharide. If Th cells are providing cognate help for polysaccharide-specific B cell clones in germinal centers, then it is possible that affinity maturation, switching and memory B cell formation occurs. If this is true, then conjugate vaccines could potentially overcome the problem of lack of memory to carbohydrate vaccines. Thus, efficient priming could result in memory B (to carbohydrate) and T (to carrier) cell formation. What would be the significance of this for the mucosal IgA response? The obvious answer would be that conjugate vaccines could prime mucosal sites for heightened S-IgA anti-polysaccharide responses. These S-IgA antibodies would appear faster (and thus prevent colonization) and would have higher affinity for the bacterial capsules.

5. T Helper Cell Subset Regulation of the IgA Response

Antigen-specific T helper (Th) cells use the α/β T cell receptor (TCR) and normally express CD4+, thus signifying that these Th cells are activated through TCR binding to foreign peptide presented by MHC class II. These Th cells can often be further subdivided into at least two subsets, Th1 and Th2, based upon unique profiles of cytokines produced and major functions in host immune responses (39, 40, 47). For example, Th1 cells secrete IL-2, interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β) and Th1 cells function in cell mediated immunity (CMI) for protection against intracellular bacteria such as *Mycobacterium tuberculosis* and *Salmonella typhi*. Th1 cells may provide help for B cell responses and the IFN-γ produced supports IgG2a responses in mice (47). The Th2 cells preferentially secrete IL-4, IL-5, IL-6 and IL-10 and provide effective help for B cell responses, most notably for IgG1 and IgG2b subclasses, and for IgE and IgA (47). Other studies have also shown that T cells and certain cytokines, e.g., IL-5 and IL-6 are of particular importance for induction of committed sIgA+ B cells to differentiate into IgA producing plasma cells (reviewed in 21). One would predict from this that higher frequencies of Th2 cells may occur in
mucosal effector sites, and indeed this has been shown by our recent studies (32, 48). Nevertheless, these studies have involved measurements of total Th2 cell populations and IL-5 and IL-6 induced IgA responses were done in polyclonal B cell populations. More relevant work should assess the role of Th2 cells and secreted cytokines in antigen-specific systems in response to mucosal vaccination.

In vivo studies of the frequency of Th1- and Th2- type cells would be greatly facilitated by methods which would allow the quantitative measurement of individual T cells which produce cytokines. Thus, we have now developed ELISPOT assays which allow detection of T cells producing IL-2 and IFN-γ (for Th1-type cells) and IL-4, IL-5 and IL-6 (for Th2-type cells). We have used these assays to show that murine Th2-type cells predominate in mucosal effector sites such as the lamina propria of the GI tract (48) and the salivary glands (32). However, significant numbers of Th1-type cells are also found in mucosal effector tissues (32, 48). Therefore, measurement of total numbers (polyclonal) of CD4+ Th1- and Th2- type cells provide only limited information regarding the relative importance of these two subsets in regulation of antigen-specific mucosal IgA responses; nevertheless these studies do show that activated Th2-type cells are the major subset in mucosal effector sites.

In order to focus on antigen-specific Th cell responses, we have enumerated numbers of antigen-specific Th1- and Th2- type cells present following antigen-specific priming of mucosal inductive sites by oral immunization. Our initial studies revealed the surprising finding that oral immunization with a T cell-dependent antigen, sheep erythrocytes (SRBC) preferentially induced SRBC-specific Th2 cells in PP, while systemic immunization (intraperitoneal; i.p.) resulted principally in Th1 cell responses in spleen (51). The results of this study, if generally applicable to protein vaccines could have significant implications for design and delivery of oral vaccines. For example, oral vaccines which induce effective levels of mucosal S-IgA antibodies do so by induction of Th2 cells in GALT (Table 2). Likewise, vaccines which induce
significant Th2 cell responses in PP will result in significant S-IgA responses in mucosal effector tissues, while vaccines which preferentially induce Th1 cell responses will not be as effective for provision of help for B cells undergoing IgA responses. Therefore to test these assumptions, we have now used more relevant vaccine antigens, e.g., CT, first as an oral immunogen and second as an adjuvant with TT to assess whether oral immunization induces Th2 cells that directly correlate with S-IgA responses in the GI tract of mice (52, 53). Controls for these studies have included systemic immunization of mice with CT only or with CT plus TT by the intravenous (i.v.) route.

In these studies, one group was given CT by gastric intubation and was boosted orally two weeks later (53). A different time course was followed for mice receiving CT plus TT, where it was shown that optimal oral immunization was obtained by three occasions at one week intervals (52). For systemic immunization, mice were given CT only or CT plus TT by the i.v. route. Both antigen-specific T and B cell responses were examined either 3 days (CT) or 7 days (CT + TT) after the last immunization (52, 53). The numbers and isotype of antigen-specific, antibody producing cells (spot forming cells; SFC) were determined in lymphoid cell suspensions of spleen (SP), PP and LPLs by the ELISPOT method. The levels and isotype of anti-CT and anti-TT antibodies were determined in fecal extracts and sera of mice orally immunized with CT alone, or CT plus TT, respectively by ELISA. As one might expect, oral administration of CT induced high CT-specific IgA responses in fecal samples of immunized mice. Further, mononuclear cells isolated from LP of these orally-immunized mice contained increased numbers of CT-B specific IgA SFC (52, 53). In contrast, antigen-specific IgA responses were not induced in the LP of mice systematically-immunized with CT. When mice were orally immunized with TT in the presence of CT as a mucosal adjuvant, a dramatic TT-specific IgA response was induced in mucosal effector sites (52). In this regard, high antigen-specific IgA antibody levels in fecal samples and elevated numbers of TT-specific IgA SFC in LPLs were evident in these orally immunized mice.
In terms of antigen-specific Th1 and Th2 responses, PP CD4+ Th cells from mice orally immunized with CT and stimulated \textit{in vitro} with CT-B resulted in significant Th2 cell responses as manifested by IL-4 and IL-5 SFC (53). Significantly higher numbers of Th2-type cells occurred in these cultures at all time intervals when compared with Th1-type cells producing IFN-\(\gamma\) and IL-2. Further, SP CD4+ Th cell cultures from mice orally immunized with CT also showed higher frequencies of Th2-type cells. On the other hand, SP CD4+ Th cell cultures from mice given CT by the i.v. route exhibited increased numbers of both Th1 and Th2 cells. These results suggest that CT, unlike other antigens such as SRBC, induce Th2 (and Th1) cell responses when given by the i.v. route and may explain why significant IgG anti-CT responses occur following systemic immunization with a single dose of this antigen (Table 4).

It was of importance to determine if CT could enhance Th2 cell responses to other protein vaccines when given by the oral route. When PP CD4+ Th cells from these orally-immunized mice with TT and CT were examined for frequencies of TT-specific Th1 and Th2 cell responses, clearly, higher numbers of TT-specific IL-5 SFC were induced in PP CD4+ Th cell cultures when compared with IFN-\(\gamma\)SFC (52). Our results have shown that both CT and TT when given orally to mice, preferentially induce Th2 cell responses (Table 4).

The studies summarized above show that oral immunization results in enhanced Th2 cell responses in IgA inductive sites, e.g., PP (Table 4). Furthermore, this immunization regimen also resulted in significant IgA anti-CT and anti-TT SFC responses in of the GI tract, a major IgA effector site (Table 4). The responses in LPLs were entirely of the IgA isotype, while splenic SFC responses to CT and TT were largely of IgG and IgA isotypes (Table 4).
SUMMARY AND FUTURE DIRECTIONS

Oral immunization preferentially induces Th2 cell responses which directly correlate with antigen-specific IgA responses in mucosal effector sites. It is tempting to suggest that activated, antigen-specific Th2 cells which are induced in PP are continuously supplied to mucosal effector sites for regulation of IgA responses. It is likely that Th2 cells producing IL-5 and IL-6 direct antigen-specific sIgA+ B cells to become IgA producing plasma cells. Nevertheless, additional studies will be required to establish that IgA responses to T cell dependent antigens depend upon Th2 cell-derived help.

What are the implications of these studies for current oral vaccines, including novel antigen delivery systems? The most obvious would be that vaccines should now be optimized for induction of Th2 cell responses in IgA inductive sites such as GALT (Table 2). It is now clear that induction of Th2 type responses in both IgA inductive and effector sites are essential for oral vaccines to induce S-IgA responses. However, antigens delivered by live vectors such as S. typhimurium in the murine system and S. typhi in humans must consider T cell responses induced against live vector in addition to the inserted recombinant antigen. In this regard, it has been shown that these microorganisms induce CMI responses which largely results from Th1 type cells. Thus, one can envision that oral immunization with live vector vaccines may induce elevated Th1 (and possibly Th2) responses against the vector and the recombinant antigen, respectively. Thus, the concept of Th2 type cell induction for effective antigen-specific IgA responses by purified antigen may not apply to this live vector delivery system. In this situation, one must consider an appropriate balance between Th1 and Th2 cells for the induction of antigen-specific IgA responses. In addition, it is also important to consider the kinetics of Th1 and Th2 type responses in orally-vaccinated subjects, especially for vaccines to virus-associated diseases. For virus vaccines, an ideal situation would be virus-specific Th2 type cells in IgA effector sites, in order to generate
neutralizing virus-specific S-IgA responses. Subsequently, CD8+ cytotoxic T lymphocytes (CTLs) and Th1 type cells need to be induced later in IgA effector sites as well as in systemic sites for elimination (clearing) of virus-infected host cells. These issues need to be addressed in order to initiate effective oral vaccines for different infectious diseases. The MIRG at UAB is currently carrying out studies for induction of Th1 and Th2 cells, and CD8+ CTLs by orally administered vaccines, including live vectors, purified proteins and mucosal adjuvants, (CT) in delivery systems such as microspheres. In conclusion, several issues which are listed in Tables 1 and 2 should always be kept in mind during the planning for development of effective mucosal vaccines.
REFERENCES


### Table 1. Major Issues For Development of Mucosal Immunity In The Children's Vaccine Initiative.

1) Why is the mucosal immune system of importance in vaccine development?
2) Will oral immunization alone be sufficient for induction of mucosal immunity?
3) What is the role of antigen-uptake in induction of mucosal immunity?
4) What are the requirements for induction of memory in the mucosal immune system?
5) Can Th cells and cytokines directly regulate IgA responses to mucosal vaccines?
<table>
<thead>
<tr>
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<th>Important Considerations For Effective Mucosal Vaccines In Order To Induce Optimal IgA Responses</th>
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<tbody>
<tr>
<td>1.</td>
<td>Induction of Th2 type responses</td>
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<tr>
<td>2.</td>
<td>Generation of memory IgA B cells</td>
</tr>
<tr>
<td>3.</td>
<td>Enhanced vaccine uptake by M cells</td>
</tr>
<tr>
<td>4.</td>
<td>Manipulation of IgA1 and IgA2 subclass responses</td>
</tr>
<tr>
<td>5.</td>
<td>Realization that the CMIS is compartmentalized</td>
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</table>
Table 3. Antigen Delivery Systems For Inducing S-IgA Responses by Oral Immunization

<table>
<thead>
<tr>
<th>1. Nonliving Carriers</th>
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<tbody>
<tr>
<td>- Cholera Toxin (CT) and CT-B Subunit</td>
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<tr>
<td>- Microspheres</td>
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<tr>
<td>Lactide - Glycolide co-polymers</td>
</tr>
<tr>
<td>Phosphazenes/Alginates/Starch/Others</td>
</tr>
<tr>
<td>- ISCOMS</td>
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<tr>
<td>- Liposomes</td>
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<tr>
<td>2. Living Carriers</td>
</tr>
<tr>
<td>- rSalmonella</td>
</tr>
<tr>
<td>- rV. cholerae</td>
</tr>
<tr>
<td>- rBCG</td>
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<tr>
<td>- rLactobacillus</td>
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<td>- Adeno and Poliovirus</td>
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29
Table 4. Oral Immunization Induces Th2 Cells Which Mediate IgA Responses: Implications For Vaccines

<table>
<thead>
<tr>
<th>Antigen and Mode of Delivery</th>
<th>Th Cell Subsets</th>
<th>B Cell Responses (Isotype)</th>
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<tbody>
<tr>
<td></td>
<td>GALT</td>
<td>Spleen</td>
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<tr>
<td>Oral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRBC</td>
<td>Th2 &gt;&gt; Th1</td>
<td>Th2 &gt; Th1</td>
</tr>
<tr>
<td>CT</td>
<td>Th2 &gt;&gt; Th1</td>
<td>Th2 &gt; Th1</td>
</tr>
<tr>
<td>CT + TT</td>
<td>Th2 &gt;&gt; Th1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRBC</td>
<td>0</td>
<td>Th1&gt;&gt;Th2</td>
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<tr>
<td>CT</td>
<td>0</td>
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<td>Th1=Th2</td>
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ND = Not done.
Figure 1. Disproportionate distribution of IgA1- and IgA2- producing plasma cells in mucosal effector sites. Note that upper body sites, e.g., nasal mucosa and lacrimal glands are major IgA1 producing areas while the lower GI tract are major IgA2 plasma cell areas. This figure was adapted from a chapter by Dr. Per Brandtzaeg (9).
Antigen Uptake And Presentation For The Th2 Type Cell Responses In The Mucosal Immune System

Figure 2. Major pathways for vaccine antigen uptake in mucosal inductive sites. The M- cells in the epithelium endocytose/phagocytose intact antigens/vaccines and deliver them to underlying T and B cell areas where dendritic cells (DC) and class II+ B cells may serve as major APC cell types for induction of Th2-type cells. Antigens/vaccines are also taken up in mucosal effector sites and may be processed and presented by class II+ epithelial cells. Intact antigen may pass tight junctions and following processing by APCs [including macrophages (MO), dendritic cells (DC) and class II+ B cells] may induce activation of Th2 cells for regulation of IgA responses.
Table 3. Antigen Delivery Systems For Inducing S-IgA Responses by Oral Immunization

1. **Nonliving Carriers**
   - Cholera Toxin (CT) and CT-B Subunit
   - Microspheres
     - Lactide - Glycolide co-polymers
     - Phosphazenes/Alginates/Starch/Others
   - ISCOMS
   - Liposomes

2. **Living Carriers**
   - *rSalmonella*
   - *rV. cholerae*
   - rBCG
   - r*Lactobacillus*
   - Adeno and Poliovirus
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<td></td>
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<tr>
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<td>Th2 &gt;&gt; Th1</td>
<td>Th2 &gt; Th1</td>
</tr>
<tr>
<td>CT</td>
<td>Th2 &gt;&gt; Th1</td>
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<td>CT + TT</td>
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<td>Th2 &gt; Th1</td>
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<tr>
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<td>0</td>
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<tr>
<td>CT</td>
<td>0</td>
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ND = Not done.
IgA1 AND IgA2 PRODUCING CELLS IN HUMAN MUCOSAL EFFECTOR TISSUES

<table>
<thead>
<tr>
<th>Mucosal Effector Tissues</th>
<th>IgA1</th>
<th>IgA2</th>
</tr>
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<tbody>
<tr>
<td>Nasal Mucosa</td>
<td>93%</td>
<td>7%</td>
</tr>
<tr>
<td>Lacrimal Glands</td>
<td>80%</td>
<td>20%</td>
</tr>
<tr>
<td>Salivary Glands</td>
<td>64%</td>
<td>36%</td>
</tr>
<tr>
<td>Mammary Glands</td>
<td>60%</td>
<td>40%</td>
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</table>

Mucosal Surfaces > 400 m²: 80% are in the GI tract.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>IgA1</th>
<th>IgA2</th>
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<tbody>
<tr>
<td>Duodenum</td>
<td>77%</td>
<td>23%</td>
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<tr>
<td>Jejunum</td>
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<tr>
<td>Ileum</td>
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<td></td>
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<tr>
<td>Colon</td>
<td>36%</td>
<td>64%</td>
</tr>
<tr>
<td>Rectum</td>
<td>43%</td>
<td>57%</td>
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</tbody>
</table>

Figure 1. Disproportionate distribution of IgA1- and IgA2- producing plasma cells in mucosal effector sites. Note that upper body sites, e.g., nasal mucosa and lacrimal glands are major IgA1 producing areas while the lower GI tract are major IgA2 plasma cell areas. This figure was adapted from a chapter by Dr. Per Brandtzaeg (9).
Antigen Uptake And Presentation For The Th2 Type Cell Responses In The Mucosal Immune System

**Inductive Sites**

- Vaccine
- Intact or Processed Vaccine?

**Effector Sites**

- Tight Junction
- Vaccine
- Endocytosis
- Intact Vaccine
- Processed Vaccine

**Induction of Th2 Cells: Mechanisms**

- DC
- Th2 > Th1
- B cells
- Th2 > Th1
- class II+ (IgA)?

**Figure 2.** Major pathways for vaccine antigen uptake in mucosal inductive sites. The M- cells in the epithelium endocytose/phagocytose intact antigens/vaccines and deliver them to underlying T and B cell areas where dendritic cells (DC) and class II+ B cells may serve as major APC cell types for induction of Th2-type cells. Antigens/vaccines are also taken up in mucosal effector sites and may be processed and presented by class II+ epithelial cells. Intact antigen may pass tight junctions and following processing by APCs [including macrophages (MØ), dendritic cells (DC) and class II+ B cells] may induce activation of Th2 cells for regulation of IgA responses.
ADJUVANTS AND IMMUNE ENHANCEMENT

Anthony C. Allison

Syntex Research, Palo Alto, CA, U.S.A.

Running title: Adjuvants and immune enhancement

Correspondence: Dr. A.C. Allison, S3-5, 3401 Hillview Avenue, Palo Alto, CA 94304, U.S.A.
Introduction

Improved and novel vaccines are urgently needed. They could prevent the spread of human immunodeficiency virus, protect against infections susceptible groups of humans, including young children and the elderly, and eliminate some cancers, including primary hepatocellular carcinoma and nasopharyngeal carcinoma. New methods for producing antigens have been developed. These include recombinant DNA technology, exemplified by hepatitis B virus surface antigen and site-specifically mutated pertussis toxin, and glycoconjugates, exemplified by *Haemophilus influenzae* vaccine. Moreover, our understanding of the immune system has been greatly advanced in the last few decades. The challenge is how to use this information to produce a new generation of safe and efficacious vaccines.

It is generally agreed that subunit antigens efficiently elicit cell-mediated and humoral immune responses only when they are administered with adjuvant formulations. These can be defined as formulations that augment cell-mediated and humoral immune responses to antigens. The formulations frequently have two components, an adjuvant (e.g. a muramyl dipeptide or lipopolysaccharide analog) and a vehicle (e.g. a squalane emulsion or liposome preparation). The term vehicle is used to make the distinction from a carrier, which is an immunogenic molecule bound to a molecule of low immunogenicity and able to augment immune responses to the latter, e.g. a protein bound to a bacterial capsular polysaccharide.

Two adjuvant formulations have a long history. One is based on mineral oil emulsions, with or without mycobacteria, and the second on adsorption of antigens to aluminum salts. In recent years three adjuvant formulations have been developed: liposomes, immune-stimulating complexes.
(ISCOMs) and squalene or squalane emulsions. Although several types of surface-active agents have adjuvant activity, most attention has been focused on saponin-like Quil A molecules in ISCOMs and Pluronic® block co-polymers which are used to make stable squalene or squalane emulsions. Analogs of muramyl dipeptide (MDP) and lipopolysaccharide (LPS) have been produced with the objective of preserving adjuvant activity while minimizing side effects. In experimental animals promising results have been obtained with the new adjuvant formulations, and trials of their efficacy in humans have been initiated.

To allow selection of the most appropriate adjuvant for use in a particular vaccine, it is necessary to establish what adjuvants are required to do and the side effects to be avoided. A clear understanding of the latter may facilitate regulatory approval. Fortunately there are signs that the hitherto inflexible opposition of regulatory authorities to the use of adjuvants other than aluminum salts in human vaccines is changing.

**Affinities and Isotypes of Antibodies**

Traditionally the efficacy of adjuvants has been judged by the levels of antibodies elicited, using a convenient test such as ELISA or hemagglutination. While these assays have provided useful information, they should now be supplemented by other measures of the quantity and quality of antibodies elicited. Preferably, antibody levels should be quantified by tests relevant to function, such as neutralization of bacterial toxins or viruses. Because of potential problems with solid-phase assays, at least some measurements of antibody levels using fluid-phase assays should be made. In addition to the quantities of antibodies elicited by a vaccine, two properties of the antibodies are likely to be important for protection: their affinity for antigen and their isotype. To neutralize a virus or bacterial
toxin, antibodies should bind them with sufficiently high affinity. If the complexes are not removed by phagocytic cells, antibodies must bind to a virus or toxin with an affinity at least of the same order as the natural receptor. We have developed methods for measurement of the quantities and affinities of antibodies in the fluid phase (32). These should be useful in studying the efficacy of vaccines.

Another important property of antibodies is their isotype. Antibodies of the IgG class pass from the vascular to the extravascular compartment more easily than those of the IgM class, and only the former are transferred across the placenta or by milk to fetuses and newborn animals. Antibodies of some isotypes efficiently activate complement, bind to high-affinity receptors on monocytes, and act synergistically with antibody-dependent effector cells to produce cytotoxicity. Examples are IgG2a antibodies in mice and IgG1 antibodies in humans, both of which bind to high affinity Fcγ receptors (60). Studies with isotype-switch variants of murine monoclonal antibodies (which have the same Fab regions, so binding to antigen is comparable) show that IgG2a antibodies confer better protection against tumors than those of other isotypes (30). Antibodies of the IgG2a isotype are also involved in protection against at least some infectious agents (64). Studies with "reshaped" human antibodies, genetically constructed so as to have antigen-binding hypervariable regions like those of rodent monoclonals, confirm the superiority of the human IgG1 isotype in ADCC-mediated lysis (52). The desirability of developing an adjuvant formulation that preferentially elicits high-affinity antibodies of the IgG2a isotype in mice and IgG1 in humans is therefore apparent.

Antibodies elicited should be directed to determinants exposed in native antigens: modern adjuvants augment the formation of such antibodies whereas Freund's adjuvant can denature antigens and elicit antibodies against internal determinants (31).
Cell-mediated Immunity

Helper T-lymphocytes are required for the formation of antibodies against most antigens. In addition, cytotoxic T-lymphocytes can lyse infected cells or produce mediators, such as IFN-γ, following interaction with antigen in a genetically restricted situation (44). A role for IFN-γ and TNF in protection against viruses has been demonstrated by the use of vaccinia vectors (51). Cytotoxic T-lymphocytes able to lyse autologous cells expressing several antigens of human immunodeficiency virus (HIV) are demonstrable in infected persons (62), although it is not yet known whether they have a protective role.

It is therefore likely that for optimal protection against some infectious agents, e.g., herpesviruses and possibly HIV, the elicitation of cell-mediated immunity (CMI) is desirable. Tests for CMI should include not only delayed hypersensitivity but also proliferative responses to the antigen and the release of IL-2 (10) and IFN-γ. Cytotoxicity for autologous or syngeneic infected target cells should also be studied. If mice or rats are used, syngeneic target cells are readily available. With outbred species, such as humans and subhuman primates, B-cells transformed by Epstein-Barr virus, transfected with a vaccinia virus vector expressing the antigen under consideration (e.g., HIV antigens, reference 8), can provide autologous target cells for studies of genetically-restricted cell-mediated cytotoxicity.

According to traditional wisdom replicating viruses are required to elicit cytotoxic T-lymphocyte responses. That is not the case: recombinant envelope glycoprotein of HIV in ISCOMs elicits CD8+ MHC-class I-restricted cytotoxicity in mice (58). In our hands, recombinant gpD of HSV-2 in SAF was found to elicit CD4+, class II-restricted T-lymphocytes in guinea pigs. Hence subunit vaccines in an efficacious adjuvant can elicit cytotoxic T-cell responses.
Undesirable Effects of Adjuvants

The first complication of the use of an adjuvant is acute tissue damage at the injection site, or a later granulomatous reaction. Many surface-active adjuvants produce tissue damage at injection sites. A convenient test is measurement of creatine phosphokinase in the circulation after intramuscular injection (10). Limits of circulating creatine phosphokinase acceptable to regulatory authorities for injections of drugs and vitamins have been defined. Granulomas can be assessed by histological examination at various times following vaccine injection, or by measurement of leukocyte enzymes following intramuscular injection (17). Even alum produces an appreciable granulomatous response at the injection site, much greater than is elicited by MDP-A. We have never observed lesions at either the primary or secondary injection sites in many experiments in which guinea pigs were vaccinated subcutaneously with repeated doses of antigen and the threonyl analog of MDP discussed below. In fact, at necropsy, the injection sites were difficult to locate. This is in contrast to the necrotic lesions at injection sites described by Nagao and Tanaka (47) using [αl-]MDP.

A second undesirable effect of adjuvants is pyrogenicity. A regulatory requirement of biological products introduced into humans is that they should not be pyrogenic. Naturally occurring adjuvants, such as MDP and LPS, are pyrogenic. However, some synthetic analogues of MDP, including murabutide (15) and N-acetylmuramyl-L-threonyl-D-isoglutamine, [Thr]-MDP (10), are potent adjuvants with greatly reduced pyrogenicity compared with naturally occurring MDP. The monophosphoryl derivative of lipid A (MPL) retains adjuvant activity with reduced pyrogenicity (26,53).
Human infections with gram-negative bacteria sometimes produce Reiter's syndrome, a complex including anterior uveitis, arthritis and urethritis. Although the full-blown Reiter's syndrome is relatively rare, components of it, including an influenza-like syndrome, muscle cramps and generalized joint discomfort, are common. Certain individuals, particularly those of the HLA-B27 haplotype, are genetically predisposed to develop one or more components of the Reiter's complex (22). This can happen when they are exposed to small amounts of bacterial products or even when they are given certain drugs such as levamisole. Some Reiter's symptoms (an influenza-like syndrome and generalized joint discomfort) can be produced by injection of 1 mg or more of MDP analogs into humans (46). If an adjuvant is to be used in millions of people, some genetically predisposed, it might produce or exacerbate Reiter's symptoms, of which the most serious is anterior uveitis. Hence separation of adjuvant activity from capacity to induce anterior uveitis is a major safety requirement.

The rabbit and cynomolgus monkey provide appropriate experimental models. Small doses of LPS or MDP injected intravenously in the rabbit increase vascular permeability in the eye, as shown by passage of fluoresceinated macromolecules into the anterior chamber (63). Histological examination shows leukocyte emigration into the uveal tract that can lead to irreversible changes. Synthetic analogs of MDP were compared for their capacity to produce uveitis and to function as adjuvants (63). Nor-MDP, which was used in a clinical trial with BHCG in Australia (29), was among the analogs with the highest capacity to produce uveitis in rabbits (63). Repeated administration of nor-MDP produced in cynomolgus monkeys granulomatous inflammation in the eye and blindness. The threonyl analog of MDP was found to be a potent adjuvant with low pyrogenicity and low capacity to produce uveitis (29). MDP, murabutide and lipophilic derivatives such as muramyldipeptide-phosphatidylethanolamine (MTP-PE) activate macrophages to produce nonspecific resistance to infection (46); [Thr$^3$]-MDP lacks this activity (20). Production of mediators, including prostaglandins, by activated
macrophages and endothelial cells is likely to contribute to the pathogenesis of uveitis (20). The lipophilic MDP analogs tested were potent in the induction of uveitis (20), and administering them in liposomes aggravated this activity rather than preventing it. While the use of the lipophilic MTP-PE in cancer patients (46) is ethically justifiable, the safety of this formulation in widely used adjuvants is questionable.

MDP and some derivatives can produce adjuvant-type arthritis in the rat (48). The rat can serve as a model for the arthritic component of Reiter's syndrome. It is essential that adjuvants for human use do not have this activity. [Thr]-MDP does not produce adjuvant arthritis in the rat (10).

Mineral Oil Emulsions

The study of adjuvants was initiated in 1916 when Le Moignac and Pinay found that suspending Salmonella typhimurium in mineral oil increased antibody formation. Oil-based adjuvants have since been used to increase humoral responses of farm animals to many inactivated bacterial and viral vaccines (38). Freund's incomplete adjuvant (FIA) is a water-in-mineral oil emulsion stabilized with the detergent Arlacel A; Freund's complete adjuvant (FCA) contains also killed mycobacteria (21). In general, protein antigens in FIA elicit antibody formation but not DTH; protein antigens in FCA elicit also DTH. Freund's adjuvants have been widely used in laboratory animals to elicit high levels of antibodies, cell-mediated immunity and protection against challenge with viable microorganisms. FCA has not been approved by regulatory authorities for use in human or veterinary vaccines because it elicits tuberculin hypersensitivity and granulomatous reactions at injection sites. The use of FCA in laboratory animals is now being discouraged, and an efficacious and safe alternative is urgently needed.
Aluminum Salts

Glenny and his colleagues (24) precipitated diphtheria toxoid by potassium alum and found that the precipitate elicited the formation of anti-toxin much more effectively than did the unprecipitated toxoid. This was the first of many observations showing that aluminum salts, especially aluminum hydroxide and aluminum phosphate, are adjuvants. Aluminum hydroxide (alhydrogel) is the only adjuvant currently authorized for human use by the Food and Drug Administration. The efficacy of aluminum hydroxide in increasing antibody responses to diphtheria and tetanus toxoids is well established. Hepatitis B virus surface antigen, both serum derived and recombinant, is adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. Not all antigens are adsorbed to alhydrogel. For example it is ineffective for influenza vaccination (49) and inconsistently elicits cell-mediated immunity (9). The antibodies elicited by alum-adjuvanted antigens are mainly of the IgG1 isotype in the mouse (9), which may not be optimal for protection. Adsorption to alum can denature proteins (9). Alum can increase the formation of IgE antibodies in rabbits and rodents (61); antibodies of this isotype can produce hypersensitivity.

Saponin and ISCOMs

Saponins are surface-active agents widely distributed in plants. A group of triterpene glycosides extracted from the South American tree Quillaia saponaria, termed Quil A, has adjuvant activity (16). Saponin is a potent adjuvant for strong antigens but is relatively ineffective for weak antigens (8). Saponins have been used in several veterinary vaccines, including that for foot-and-mouth disease virus (16). Saponin is also an effective adjuvant for vaccines against parasites, including Trypanosoma cruzi and Plasmodium yoelii (50).
Nevertheless, saponin has a number of undesirable side-effects: it is irritating, pro-inflammatory, binds to cholesterol and lyses red blood cells (8). Hence ways to retain the adjuvant activity but diminish the side effects have been sought. One approach has been the production of "immune-stimulating complexes" (ISCOMs) which were originally described by Morein et al. (42) as particles consisting of Quil A and membrane proteins. The preparation of ISCOMs allowed reduction in the Quil A concentration required for adjuvant effects. Later studies showed that lipids are also essential for the formation of the regular cage-like structures characteristic of ISCOMs, whereas preparations lacking lipids tend to form aggregates or micelles (42).

ISCOM preparations have been reported to increase antibody responses to a number of viral membrane proteins, compared to responses obtained with aqueous or liposome preparations of the antigens. Proteins from bovine herpesvirus type 1, cytomegalovirus, hepatitis B virus, Epstein-Barr virus and canine distemper virus are among those which have been tested with ISCOMs (41). ISCOMs have also been used to improve an equine influenza vaccine, and a commercial ISCOM-influenza vaccine is now licensed for use in Sweden. ISCOMs containing the recombinant envelope protein of human immunodeficiency virus (gp 160) elicited CD8* cytotoxic T-lymphocytes in mice (58).

All these studies demonstrate that ISCOMs are potent adjuvants for many antigens. However several questions remain unanswered. ISCOMs were developed for viral envelope glycoproteins and may not be suitable for some other antigens. Quil A is heterogeneous, and the chemical entities in it are only partially defined. The glycoside required to make ISCOMs is different from that increasing immune responses (K. Dalsgaard, personal communication). Under the circumstances it will be difficult to assemble a full toxicology profile, including tests for carcinogenicity that are likely to be requested by regulatory authorities for general use. Although large-scale production of ISCOM vaccines is feasible,
it is easier to mix antigens with preformed emulsions. Monkeys immunized with SIV glycoproteins in ISCOMs (34) were sensitized so that when challenged with virus they showed acute hypersensitivity; animals immunized with the antigens in MDP-A showed protection but were not sensitized. Presumably ISCOMs, like saponin, can elicit IgE antibodies. Whether this would be a problem in practice is unknown.

**Muramyl Dipeptide Analog Formulation**

The mycobacteria in FCA have several disadvantages. They contain tuberculin and other proteins, so that after a single injection animals become sensitized. A second injection of FCA produces a massive delayed-type hypersensitivity response. It was a considerable advance when Ellouz et al. (18) showed that the minimal adjuvant-active component of the mycobacterial cell wall is N-acetylmuramyl-L-alanine-D-isoglutamine, also known as muramyl dipeptide (MDP). When protein antigens are administered to guinea pigs in FIA + MDP, delayed-type hypersensitivity and antibodies of the \( \gamma_2 \) isotype are elicited.

As discussed above, MDP has undesirable effects, including pyrogenicity and capacity to induce anterior uveitis and arthritis. Over 130 MDP analogs were synthesized at Syntex Research in an attempt to separate adjuvant activity from side effects. The threonyl analog of MDP (N-acetylmuramyl-L-threonyl-D-isoglutamine), shows the greatest separation of adjuvant activity from side effects so far obtained (10,63). This analog was therefore selected as an acceptable counterpart of mycobacteria in an adjuvant formulation (10). Our next challenge was to develop an alternative to the mineral oil emulsion of Freund's adjuvant that would be suitable for human use.
After much experimentation with liposomes, several oil preparations and various surface-active agents, we found that squalene or squalane emulsions, prepared with the Pluronic® block-copolymer L-121 and stabilized with a small amount of Tween 80, provided a versatile vehicle for antigens (10). Hunter et al. (27) had used L-121 and related molecules with mineral oil as adjuvants. In L-121 a central block of polyoxypropylene is hydrophobic while two flanking blocks of polyoxyethylene are hydrophilic because of hydrogen bonding with water. Since it is surface-active, L-121 associates with membranes, but it does not penetrate into membranes and disrupt their structure, unlike saponins which bind cholesterol and are cytolytic. Squalane is saturated and stable in formulation, unlike squalene, which is unsaturated and becomes oxidized. Our microfluidized squalane-L-121 emulsion is remarkably stable, even when frozen. It does not produce reactions at injection sites in humans, unlike squalane emulsions prepared with higher concentrations of membrane-active detergents.

Our interpretation of the effectiveness of the squalane L-121 emulsion as a vehicle for antigens is as follows: electron micrographs show that labelled protein antigens are concentrated on the surface of the squalane microspheres (fig. 2). Antigens are retained there partly because they are amphipathic and partly by hydrogen bonding to L-121, as shown in fig. 3. The squalane L-121 emulsion system is therefore more versatile than squalane emulsions lacking the block copolymer. It is also more versatile than liposomes, the structure of which has to be optimized for each antigen. The squalane L-121 microsphere particles also activate complement and migrate from injection sites to lymph nodes of the drainage chain. The C3b on the surface of the microspheres should target them to follicular dendritic cells, major antigen-presenting cells. A depot of antigen on follicular dendritic cells is more important for immunogenicity, as well as better for the patient, than a depot at the injection site.
Thus the function of the squalane-L-121 emulsion is to target antigens to antigen-presenting cells. The function of the MDP analog is to induce expression of cytokines and increase expression of major histocompatibility genes and intracellular adhesion molecules, which are required to trigger cell-mediated immune responses (see below). The combination of the threonyl analog of MDP with the squalane-L121 emulsion is termed the MDP adjuvant formulation (MDP-A), also known as Syntex Adjuvant Formulation (SAF).

**Lipopolysaccharide and Monophosphoryl Lipid A**

Gram-negative bacteria such as *Escherichia*, *Salmonella* and *Pseudomonas* have endotoxins which induce fever, changes in leukocyte count, hypotension, shock and uveitis. The endotoxins are lipopolysaccharides (LPS), consisting of a hydrophilic polysaccharide covalently linked to the hydrophobic lipid A component. LPS has potent immunological adjuvant activity, as well as inducing macrophage activation and non-specific stimulation of immune responses. The structures of lipid A from *Escherichia* and *Salmonella* have been elucidated (54) and are quite similar. Observations with synthetic lipid A and analogs confirm that the biological activity of LPS resides in the lipid A portion of the molecule. Chemical modifications of the lipid A structure have been made in attempts to decrease the toxicity without reducing the adjuvant activity of the molecule. Synthetic Monophosphoryl lipid A (MPL) of *E. coli* has been synthesized by Imoto *et al.* (38), while Ribi *et al.* (53) have chemically removed the phosphate moiety from the C-1 position of the toxic diphosphoryl lipid A of *Salmonella*. MPL is less toxic than the diphosphoryl molecule, but retains much of the adjuvant and mitogenic activity of LPS. MPL also induces non-specific protection against bacterial infections (53). MPL, formulated in a squalene emulsion like that we devised for MDP analogs, or in liposomes, has useful adjuvant activity (26).
Liposomes

Allison and Gregoriadis (1) showed that liposomes increase immune responses to bacterial toxoid. Liposomes are versatile vehicles since their size, composition, surface charge and structure (multilamellar or unilamellar) can be varied. Antigens can be entrapped within liposomes or bound to their surface, and adjuvants such as MPL or lipophilic MDP analogs can be used with liposomes. Gregoriadis (25) has reviewed many applications of liposomes for this purpose.

Targeting Vaccines to Antigen-presenting Cells

The traditional view was that adjuvants such as mineral oil emulsions or aluminum hydroxide form at the injection site depots from which antigen is slowly released. However, excision of the injection site after three days was found to have little effect on immune responses. We performed the first systematic study of the cells responding to antigens and adjuvants using cell transfers (56,59). These showed that adjuvants such as LPS or Bordetella pertussis initially interact with antigen-presenting cells (APC) and not lymphocytes. However, adjuvants could not by-pass the requirement for helper T-lymphocytes (2). More recently, APC have been better defined. While cells of the monocyte/macrophage lineage can function as APC when activated, for example during infections, three other cell types function as APC under physiological conditions, for example in a human or other animal responding to vaccine antigens.

Langerhans Cells: Cells of the Langerhans cell lineage originate in the bone marrow, migrate through the blood to the skin where they remain for about a week and then migrate through afferent lymphatics to the T-dependent areas of lymph nodes, where they are termed interdigitating cells (5).
Dendritic cells isolated from the spleen (39) have similar properties and may be of the same lineage. Because of possible confusion with follicular dendritic cells, which have a different location and properties, the term Langerhans cells is used in this presentation. Cells of this lineage efficiently present antigens associated with their surfaces, for example contact-sensitizing chemicals and myelin basic protein, to elicit T-lymphocyte-dependent immune responses (36).

**Follicular Dendritic Cells (FDC):** As their name implies, these cells are found in lymphoid follicles in lymph nodes, spleen and other sites, where their branching cytoplasmic extensions are closely associated with B-lymphocytes. FDC express CD4 and high-affinity C3b receptors (CRI). Immune complexes activating complement injected into mice became localized on FDC, and this process appears to be required for the generation of B-lymphocyte memory, in other words proliferation of clones of B-lymphocytes responding to antigen with consequent priming for a secondary response (33). Immune complexes binding FDC become associated with beaded cell membrane extensions which are readily taken up by follicular B-lymphocytes expressing class II major histocompatibility antigens (57). The antigen can be demonstrated for at least one week by immunocytochemistry in endocytic vacuoles within B-cells; in such a compartment they may be partially digested for presentation to T-lymphocytes.

**B-lymphocytes:** Evidence has accumulated that B-lymphocytes efficiently present antigens to T-lymphocytes (55). In fact, depletion of B-cells by repeated injections of antibody against the μ-chain of immunoglobulin markedly decreases responses to antigens of T-lymphocytes in peripheral lymphoid tissues. A major role of surface membrane immunoglobulin receptors for antigens on B-cells may be to bind the antigen for subsequent presentation to T-cells. Targeting of antigens to FDC may be a crucial factor in the efficient presentation to B-lymphocytes and, through them, to
T-lymphocytes. In secondary immune responses this occurs through the formation of complement-activating immune complexes. Adjuvant formulations can facilitate such localization by themselves activating complement. This is true of LPS and MDP-A; liposomes of compositions that activate complement are better adjuvants than those that do not. Such complement activation should be moderated so as to have enough C3b on the antigen-bearing micelles, emulsions or liposomes to allow targeting of associated antigens, but not sufficient complement activation at injection sites to elicit inflammatory lesions. The importance of targeting antigens to dendritic cells is illustrated by experiments in which avidin conjugated to a monoclonal antibody recognizing these cells elicits high levels of IgG antibodies whereas other conjugates (e.g. targeting antigen to macrophages) were ineffective (14).

Selection by Adjuvants for the Production of Antibodies of High Affinity and Protective Isotypes

For reasons discussed above it is frequently desirable to elicit isotype antibodies of high affinities and protective isotypes, e.g. IgG2a in the mouse. It has long been known that the use of particular adjuvants can influence the isotypes of antibodies. An example is the use of low doses of antigen with alum, Bordetella pertussis or saponin to produce IgE antibodies in the mouse. Antigens administered to guinea pigs in FIA elicit mainly antibodies of the γ1 isotype whereas with the complete adjuvant γ2 antibodies are formed (65). We have compared antibodies elicited by human serum albumin and recombinant human interleukin-1α administered to mice in different adjuvants by the intraperitoneal and subcutaneous routes (31). Considerable differences were observed. FCA elicited high levels of antibodies, but these were not of high affinity; many were directed to epitopes not exposed on the native molecule. MDP-A (SAF) elicited the highest proportion of antibodies of the
IgG2a isotype. The antibodies against IL-1 were potent in neutralizing the biological activity of the molecule, and cells from the mice were used for production of monoclonal antibodies. Aluminum hydroxide and Quil A elicited antibodies largely of the IgG1 isotype (see also 9).

Thus adjuvants can select for the isotype of the antibodies formed. Moreover, production of hybridomas does not require the barbarous traditional procedure of immunizing intraperitoneally with FCA. Subcutaneous or intramuscular immunization with MDP-A or Quil A, depending on the desired isotype, is equally effective (31). The use of FCA for laboratory animal immunization is already restricted in several large research centres and, since more humane adjuvants are equally effective, they should be used everywhere.

Role of Cytokines in Isotype Selection

Until recently the mechanisms by which the formation of antibodies of particular isotypes are favoured were unknown. Now evidence is accumulating that cytokines play a role in isotype selection in the mouse, together with indications that the same is true in cultured human cells (19). IFN-γ augments the production of IgG2a antibodies in mice, whereas IL-4 augments IgG1 and IgE antibodies. These findings explain why adjuvants that are designed to increase cell-mediated immunity, such as FCA and MDP-A, concurrently select for antibodies of the IgG2a isotype. Potent T-cell-mediated responses to antigenic stimulation release IFN-γ (which can occur from both the helper and cytotoxic subset of T-cells), and IFN-γ augments the formation of IgG2a antibodies. Adjuvants which less consistently stimulate T-cell responses, e.g. aluminum hydroxide and Quil A, favour the production of IgG1 and IgE antibodies, presumably by stimulating the release of more IL-4 than IFN-γ.
Syntex Adjuvant Formulation (including the threonyl analog of MDP) augments early production of IFN-γ (but not IL-4 or IL-5) in lymph nodes draining sites of antigen injection (K. Merrill, unpublished). Early production of IFN-γ is also elicited by monophosphoryl lipid A (26).

Use of Adjuvants in Vaccines

Examples of the use of modern adjuvants in vaccines include SAF in inactivated virus vaccines for feline leukemia virus and simian AIDS virus (37) as well as simian immunodeficiency virus (45), and in a recombinant HIV-1 vaccine in chimpanzees (23). In the absence of an efficacious adjuvant little or no protection is observed. The same is true with subunit vaccines of herpesviruses. Recombinant gpD of HSV-2 in FCA elicits strong protection in guinea pigs against genital challenge with the virus; protection is not observed with other adjuvants (6). GpD in SAF is also highly protective in this model (3). The major surface glycoprotein of Epstein-Barr virus (gp340) in SAF protects cottontop tamarins against a 100% lymphomagenic dose of the virus (43). In this model alum and vaccinia constructs are less effective or toxic. SAF is also efficient at eliciting anti-idiotypic antibodies and protecting against B-lymphomas in mice (13).

Two examples will suffice to illustrate that adjuvants can help to overcome the effects of age on immune responses. In general, infants and persons over the age of 65 show lower responses to vaccines than do older children and young adults. Indeed it is estimated that less of one third of old recipients of influenza hemagglutinin (HA) show antibody responses (4). Alum is not an effective adjuvant for HA (49). In mice SAF augments responses to HA, especially in very young and in old animals (11). If this holds true in humans, the efficacy of influenza vaccines will be improved in populations where they are most needed.
Where hepatitis B virus is prevalent, e.g. South-East Asia and Africa, immunization of infants can prevent chronic infection, which is associated with hepatitis and hepatocellular carcinoma. Mice of some genetic constitutions are low responders to HBsAg (40); SAF improves responses to HBsAg in young mice and overcomes inherited low responsiveness (12). A potent adjuvant can thus improve immune responses when they are deficient because of age or genetic constitution.

Prospects

The mode of action of adjuvants is being defined in terms of contemporary immunology. Several efficacious adjuvant formulations have been developed, and they do not have limiting toxicity in experimental animals or humans. Studies of their efficacy in humans are in progress. The new adjuvants could improve vaccines already in use, e.g. influenza and hepatitis B, and make possible the development of others, e.g. against herpesviruses, RSV and HIV. Immunization against EBV and cytomegalovirus before organ transplantation is desirable. In South-East Asian and African countries immunization of infants against HBV and EBV could eliminate two common cancers, primary hepatocellular carcinoma and nasopharyngeal cancer. In this way promise of recombinant and peptide antigens could be fulfilled.
References


GLYCOCONJUGATE VACCINES: WHAT NEXT?

Kathryn E. Stein, Ph.D.

Laboratory of Molecular and Developmental Immunology, CBER, FDA

Running head: Glycoconjugate Vaccines

Dr. Kathryn E. Stein
Laboratory of Molecular and Developmental Immunology
Center for Biologics Evaluation and Research, Food and Drug Administration
8800 Rockville Pike
Bethesda, Maryland 20892-0029
Phone: (301) 496-6916
FAX: (301) 402-5943
or 402-2776

1
Abstract

The principle that infants can be protected from invasive diseases due to encapsulated organisms has been proven with the introduction of Haemophilus type b conjugate vaccines. The use of glycoconjugates to implement the goals of the CVI requires clear definitions of the chemical and immunologic requirements for optimal vaccines.
Acknowledgements

I wish to thank my colleagues in the Division of Bacterial Products for many valuable discussions related to conjugate vaccines during the last twelve years. Special thanks to Drs. Bascom Anthony, Carl Frasch, William Habig, Carolyn Hardegree, and Willie Vann. I owe a great deal of gratitude to those with whom I’ve worked directly on glycoconjugates, especially Martha Braun, Harold Jennings, Carole Miller, William Paul, Leonard Rubinstein, and David Zopf.
Avery and Goebel described carbohydrate-protein conjugates in 1929 (4), yet more than 60 years elapsed between their report and the approval at the end of 1990 of conjugate vaccines for the prevention of invasive diseases caused by *Haemophilus influenzae* type b. This event marked the first introduction of a new vaccine for routine use in infants in 28 years and more importantly, demonstrated that glycoconjugate vaccines could prevent invasive disease caused by encapsulated bacteria in infants (5,16). The challenges of the future regarding the application of conjugate technology toward the development of vaccines for the prevention of diseases caused by other encapsulated bacteria and regarding specific goals of the Children’s Vaccine Initiative (CVI) are formidable. Now that the efficacy of glycoconjugate vaccines has been established, it is time to move to a new generation of conjugates, based on a firm understanding of the requirements for optimal immunization. In addition to the issues related to combinations of vaccines, major research in the chemistry and the basic immunology of conjugates will be required in order to optimize the use of these vaccines. Combinations of vaccines containing haemophilus, meningococcal and pneumococcal conjugates and a number of carrier proteins and other antigens, will necessitate determining the optimal size and extent of coupling of the saccharide and the optimal choice of carrier proteins before they can be successfully formulated into a single vaccine preparation that provides adequate immunogenicity of all components. Moreover, the basic immune mechanisms by
which these vaccines work in infants are poorly understood and represent an area that must be studied, in depth, if we are to make real progress in this field. Along with this research, it will be necessary to determine how to manufacture these vaccines reproducibly and to develop appropriate models for the prediction of immunogenicity in human infants. In this paper, some of the basic areas of investigation regarding the chemistry and immunology of glycoconjugates will be discussed within a framework of what is currently known. It is not my intention to provide a comprehensive review of the literature of conjugate vaccines, but rather to highlight certain areas that warrant consideration with respect to the goals of the CVI. A more detailed discussion of some of the issues raised here have been published by several groups in a book on conjugate vaccine (1).
Glycoconjugate Formation--an Infinite Number of Choices

The virtual disappearance of invasive diseases caused by *Haemophilus influenzae* type b through the introduction of routine vaccination with *Haemophilus b* conjugate vaccines has been an extremely exciting and rewarding event to witness and be a part of. This does not mean, however, that work on the development of glycoconjugate technology is over. Rather, it is just beginning. It is now time to do the basic research on minimum requirements for conjugate immunogenicity as well as to optimize currently licensed conjugates, a process that was less pressing while thousands of cases of haemophilus b disease were occurring annually. This could lead to the development of principles that can be applied to any polysaccharide whose structure is known. Figure I illustrates the virtually infinite choices that exist in developing a glycoconjugate vaccine. In this section I will attempt to address some of the issues regarding these choices.

The most obvious variable is the size of the saccharide to use in the conjugate. This question has not been satisfactorily or systematically studied. In theory, the size of the saccharide need be no larger than the size of the antibody combining site. This was the premise that we used a number of years ago when we developed a model system (15) using the B512 dextran that had been previously studied by Kabat (reviewed in (8)) who determined using oligosaccharide inhibition that the upper limit of the anti-dextran antibody combining site was six to seven sugars, although he also
described a population of antibodies with a smaller combining site. Conjugates using six sugar oligosaccharides, (isomaltohexaosyl, IM6) coupled to an immunogenic carrier, keyhole limpet hemocyanin (IM6-KLH) were compared to conjugates using a smaller three-sugar hapten (isomaltotriosyl, IM3) coupled to KLH. We found that in a variety of mouse strains, IM6-KLH induced antibodies that were completely cross-reactive with the native polysaccharide. Using the smaller hapten conjugate, IM3-KLH, the ability to make anti-dextran antibodies was dependent on the allotype of the mouse strain, probably related to the linked immunoglobulin variable region genes. We concluded that a site filling hapten was sufficient to induce anti-polysaccharide antibodies. Both conjugates were derivatized with approximately 45 oligosaccharides per 100 KD of KLH. Conjugates made with 5-10 oligosaccharide chains per 100 KD of KLH were far less effective (Stein, K. E. and Zopf, D. A., unpublished). Earlier studies from the Lindberg laboratory compared 2, 8 and 12 sugar oligosaccharides made from Salmonella O-antigen 4, which has a 4 sugar repeat unit and found that comparable antibodies, measured by ELISA or functional assays, were elicited by both conjugates and that they were as effective as heat killed bacteria (7). The literature has conflicting reports as to what length of sugar is optimal. Anderson (3) studied a number of different conjugates, however, chain length was not the only variable. Seppälä and Mäkelä (12) compared small oligosaccharides (1000 to 4000 Daltons) to a polysaccharide and found that the oligosaccharides produced conjugates of greater immunogenicity than the polysaccharide. Jörbeck found that conjugates made from small oligosaccharides of Salmonella typhimurium O-antigen 4 were highly
immunogenic in stimulating functional antibody (7). Paoletti et al. (13) found that intermediate size oligosaccharide-protein conjugates were most immunogenic. In contrast, Szu et al. compared high and low molecular weight polysaccharides of Vi antigen and found that a conjugate of the high molecular weight polysaccharide was more immunogenic than the lower molecular weight polysaccharide conjugate (20) and Peeters et al. found conjugates of pneumococcal type 4 polysaccharide more immunogenic than oligosaccharide conjugates (14). Based on my own studies and others mentioned above, I believe that an oligosaccharide that provides at least one intact repeat unit and is site filling will be the minimum size and may, in some instances, be ideal, but this should be determined in a systematic fashion.

The size issue needs to be addressed in the context of the other issues raised below. In addition, the answer, in part, lies in what type of conjugate is desired from an immunologic standpoint. As will be discussed below, if the goal is to produce a thymus-dependent conjugate, then the saccharide should probably be a haptenic oligosaccharide. There are two caveats. One, is that there must be a high enough oligosaccharide density on the protein and two, that the size of oligosaccharide required has been determined. The size that will be site-filling will depend on the length of the repeat unit and the conformation. Conjugates made of short, site-filling oligosaccharides, coupled at one end via the reducing end of the saccharide (In one study, Seppälä and Mäkelä (12) found that oligosaccharides coupled via the reducing end were more immunogenic than if the were multiply attached.) should provide
conjugates with properties that conform to hapten-carrier immunology, about which a
great deal is known.

A second issue regarding the saccharide portion of glycoconjugates is whether
to derivatize it. This will depend on the structure of the polysaccharide and whether
there are available groups that can be easily derivatized without destroying the
antigenicity of the molecule. In this regard, coupling through the reducing end of the
oligosaccharide chain leaving at least one repeat unit intact, should preserve the
antigenicity of the oligosaccharide. For larger oligosaccharides, coupling to groups
on the repeat unit, such as hydroxyl groups, runs the risk of over-derivatization as
well as loss of some of the available repeats. Another consideration is whether the
coupling is random or defined. This will impact on the issue of immunogenicity as
well as manufacturing consistency.

It is essential to test the effects of chain length and the extent of coupling in a
systematic way for each polysaccharide of interest (see Figures 2 and 3). This should
be done using conjugates of different oligosaccharide chain lengths within a specified
range, where all have the same number of chains per molecule. Once the size is
determined, then the density of oligosaccharides can be varied. When using a
haptenic oligosaccharide, the density of the hapten on the carrier is critical for
stimulating a good antibody response.

The coupling chemistry presents another set of choices. First is whether to
use a linker or not. Regardless of the decision, there are a number of choices that can be made and these will be dictated largely by the primary structure of the polysaccharide. Coupling by reductive amination has been widely used (3) but more novel methods also have been developed (11).

The choices of the carrier are theoretically as numerous as the number of available proteins. The distinction between "relevant" and "irrelevant" relates to whether the protein is immunologically useful. A relevant protein could either be a protein that is a constituent of an existing vaccine for which the immune system is already or will be simultaneously primed, such as an antigen present in diphtheria-tetanus-pertussis (DTP) vaccine. Another type of "relevant "carrier would be a protein present on the organism against which the glycoconjugate is intended to immunize. For example, for Haemophilus b conjugate vaccines, an outer membrane protein from H. influenzae would be considered relevant inasmuch as exposure to the organism would be expected to boost the response to the carrier protein in the vaccine.

The question of whether or not to derivatize the protein, is one that must be considered in the context of the desired immune response, as well as the chemistry. If one wants to take advantage of carrier-specific immunity stimulated by other vaccines such as DTP, one would like to know that the epitopes present on the protein in DTP are present on the glycoconjugate. Extensive derivatization could mask
important epitopes. Similarly, if the carrier is heavily derivatized, it may stimulate immunity specific for the derivatized protein that will react poorly with the native molecule. If the native molecule is a toxin then the molecule would need to be toxoided as well as derivatized. The alternatives to derivatized proteins are to use native proteins which have reactive amino groups that could be used in the coupling. This will not be possible for carrier proteins which are toxins, however, cross-reacting mutants (CRM) that are non-toxic can and have been used successfully. If one is to mix a number of different glycoconjugates for use in a single vaccine, then the carrier used on each glycoconjugate must be carefully considered. It may be desirable to mix glycoconjugates with saccharides from different bacterial capsules each coupled to the same carrier protein, or it may be more desirable to use several carrier proteins. Again, this is an issue that should be addressed in a systematic way.

The Immunology of Glycoconjugates

The aim of using a using a glycoconjugate vaccine to stimulate polysaccharide immunity, as compared to the polysaccharide, itself, is to convert the response from a thymus-independent (TI) response to a thymus-dependent (TD) response. I have recently reviewed the differences between TI and TD responses to PS antigens and I will not cover all of the same material again (18). The most important issue to reiterate regarding the CVI is to note that immunity to polysaccharides develops late in ontogeny as compared to immunity to protein antigens. Thus, the main purpose in
converting the response to a TD response is to be able to stimulate protective immunity to polysaccharides in infants and this will be dependent on efficient stimulation of carrier-specific T helper cells. The basic mechanisms underlying the delay in maturation of immunity to polysaccharides and the means by which conjugate vaccines overcome this ontogenic delay, however, are poorly understood. Much work needs to be done on the basic immunology of glycoconjugate vaccines if we are to be able to utilize them most effectively in fulfilling the aims of the CVI. Below I will discuss some aspects of the basic immunology which are understood and others that need to be addressed in future studies. T cell immunity and antigen processing, particularly with regard to vaccine development, has been discussed by Germain elsewhere in this volume.

Our studies of the response to IM6-KLH in mice were conducted with the aim of comparing the anti-dextran response to the TI polysaccharide with that to the TD form of dextran, IM6-KLH, in mice immunized at different ages (19). We found that whereas the response to dextran did not mature until mice were 12 weeks of age, the anti-dextran response to IM6-KLH matured in mice immunized at 3-4 weeks of age. Thus, the age of peak response was shifted to a much younger age when the TD antigen was used, nonetheless, there was a developmental regulation of the response to both antigens. The same type of developmental increase in antibody responses to two Haemophilus b conjugate vaccines has recently been demonstrated in human infants (2,10). The reason for this developmental regulation is not clear, however, in
our mouse studies, we showed that the ability to respond to polysaccharide
determinants, whether stimulated by TI od TD immunogens, correlates with a late-
developing subset of B lymphocytes (19). As these mouse studies were, indeed,
predictive of the response of humans infants to glycoconjugate immunization, one
might speculate that there is a similar, late-developing, subset of B cells in humans
that is required for anti-polysaccharide antibody formation. This remains an area for
active investigation.

Despite the clear developmental regulation of the response to Haemophilus b
conjugate vaccines with both products licensed for use in infants, the slopes of the
curves and the response to a secondary immunization are different for these two
vaccines. While the response to HibTITER™ (PRP-CRM₁₉₇) increased dramatically
with increasing age at initial immunization, it was typical of responses to TD antigens
in that it showed a very good booster (nearly 10-fold) upon secondary immunization.
In young infants, the primary response was relatively weak (10). In contrast, the
response to PedvaxHIB™ (PRP-OMP) stimulated a higher antibody response in
younger infants than PRP-CRM₁₉₇ but it was not typical of TD responses in that the
booster was only 2-4 fold upon secondary immunization (2). The response to PRP-
OMP was reminiscent of responses to TI type 1 antigens. Subsequently, we showed
that, using murine lymphocytes, PRP-OMP did have an in vitro property of TI type
1 antigens (18), polyclonal B cell activation, and this has been confirmed (9).
Although the TI type 1 properties of PRP-OMP are compatible with its behavior if
one were immunizing mice with it, namely a good antibody response in young mice but a weak booster, one can only speculate that these properties might be responsible for its different behavior in human infants than observed with PRP-CRM\textsuperscript{197}. Again, there is a need for further studies on the basic immunology of the responses to conjugate vaccines, particularly if one is to consider the use of such vaccines in very young infants as part of the CVI as well as to better understand the current vaccines.

Another poorly understood aspect of glycoconjugate immunity is how these antigens are processed and presented. As exogenous antigens, they will be processed and presented to T cells on MHC class II antigens. Although much is known about antigen presentation in general (see (17)), very little is known about the effects of glycosylation on antigen presentation. One study in which the effects of sugars on T cell recognition of known peptide epitopes was examined was recently published (6). They found that, depending on where the peptide was glycosylated, there was little or no effect on T cell recognition or it was completely ablated. The implication for glycoconjugate vaccines is illustrated in Figures 5 and 6 and is dependent on whether the glycoconjugate is an oligosaccharide or polysaccharide conjugate. In either case, many peptide epitopes not directly involved in the conjugation, will be processed in lysosomes normally and will be presented to T cells on the surface of a B cell or antigen presenting cell in the context of MHC class II antigens. Epitopes in close proximity to the sugars, however, might be dealt with differently depending on the
size of the carbohydrate moiety. In the case of oligosaccharides, an oligosaccharide tail on a peptide might not interfere with effective presentation of the peptide to the T cell. In contrast, polysaccharides, for which there are no or few degradative enzymes in lysosomes, may remain undigested and present steric barriers to effective epitope presentation to T cells. Thus, the chemistry of the conjugate will be very important in determining which epitopes of a given protein result in carrier-specific T cell immunity. This may be an important factor in consideration of vaccines for genetically isolated or homogeneous populations. As mentioned above, the protective efficacy of glycoconjugate vaccines for \textit{H.influenzae} type b in infants has been demonstrated (5,16). The molecular events involving antigen processing and other aspects of the immune response to glycoconjugates, however, are not understood. If we are to optimize the manufacture and use of glycoconjugate vaccines basic immunology research must be expanded in this area.

Finally, we may need to alter our thinking on the effects of mixing vaccines on the outcome of an immunization. If a (glycoconjugate) vaccine as a single component stimulates 5X the protective level of antibody as a single vaccine but as a component of a mixture it only stimulates 2X the protective level, is that unacceptable? Classical thinking would say that the mixture shows interference, however, in both cases, more than protective levels of antibody were stimulated. In order to evaluate this issue, we must know what levels of antibody are protective. Moreover, in the context of TD immunogens where immunologic memory is
stimulated, we need to understand what the requirements for protective levels of antibody are. In other words, does one need a protective level of antibody or will adequate priming of the immune system suffice?

Conclusions

First generation glycoconjugate vaccines have proven to be effective in stimulating protective immunity to the capsular polysaccharides of encapsuled bacteria that cause invasive diseases in infants. Much remains to be done, however, to optimize the synthesis and to understand and optimize the immunology of such vaccines. Only then will the complex problems of satisfying the goals of the CVI and providing effective mixtures of glycoconjugates to protect against a number of different bacteria be overcome. A full understanding of the chemistry will permit the reproducible manufacture of such vaccines. In addition, it is essential not only to know how to make these vaccines reproducibly, but also what the in vitro correlates are for satisfactory immunogenicity in human infants. A thorough understanding of the immunology of glycoconjugates will permit better design and selection of vaccines and will be required in order to determine if immunization at birth or polysaccharide antigens will be possible.
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VIRTUALLY INFINITE NUMBER OF GLYCOCONJUGATES FOR A GIVEN POLYSACCHARIDE:

\[ 24^{n}m^{o} \] CHOICES*

24 COMBINATIONS

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* \( n \) = number of crosslinker types

* \( m \) = number of different carrier proteins

* \( o \) = 3 (native, mutant or toxoided carrier)
THE ROLE OF VACCINE R&D IN SCIENTIFIC DEVELOPMENT OF NEWLY INDUSTRIALIZING COUNTRIES

AIXOLO MARTINEZ-PAIOMO
MALAQUIAS LOPEZ-CERVANTES
PHYLIS FREEMAN

1992
1. Science for Development

As a new global strategy for improving public health, the Children's Vaccine Initiative [UNICEF, 1992] implicitly raises, once again, the question of the role of science in developing countries. If there are to be new and improved vaccines, better delivery systems and simplified immunization schedules, there will be substantial analysis, laboratory activity and fieldwork before the new products can be ready for effective use. Who will participate in the different phases of that work? What role will scientists and institutions in developing countries play? And what could such participation contribute towards health and development within the developing world? If scientific institutions from developing regions are not engaged from the level of basic science through all phases of the process, what are likely to be the consequences for health and development?

This is not a new debate, but the issues are more subtle and complex than often understood. Developing countries need more vigorous research in order to raise their levels of education and to improve the utilization of the achievements of applied science and technological innovations. No matter how efficient technology is, it becomes sterile without the support of science. Better science is necessary in order to fully comprehend, analyze, select, and adapt concepts and methods developed elsewhere. Poor countries must constantly row against the prevailing ignorance which, in turn, fosters intolerance to change. [Martínez-Palomo, 1987]

The differences between the more and the less developed nations of the world are partly explained by the lack of utilization of scientific knowledge and technology in the latter, and, by the virtual nonexistence of scientific capacity to address local problems and produce the knowledge and technology necessary to solve these problems. In turn, the meager condition of science is one of the causes that maintains the underdevelopment of the poor countries of the world. For these reasons it is important to ask how science for
health and development within developing regions and the Children's Vaccine initiative will interact.

2. Universal Science or "Another Type of Science" for Poor Nations?

Could it be true that the poor countries require another type of science, distinct from that carried out in the industrialized nations? In view of the urgent requirements for adequate food and education, decent housing, health care, and more, the question "Why not rely upon innovations from abroad?" must be addressed.

At some point the World Bank concluded that, in view of the limited scientific and technological resources of the Third World, and the many problems that are difficult to resolve, the only solution is to combine the scientific and technological potentials of the industrialized world with the local knowledge of scientists and professionals in developing countries, who would then have the responsibility only of applying the new technology developed and donated or sold by the First World. [Evans, et al, 1981]

Several questionable implications reside in this proposed solution. One is that technologies and products invented abroad will actually be useful and effective in the diversity of nations which comprise the developing world--without local scientific support or intervention. Or, that enthusiastic application of imported technologies or products will not result in creation of new and even more difficult challenges to health and development. Another is that the useful goods and services will be offered on a basis which supports healthy development. Still another faulty implication is that scientists and industrialists abroad will invent a sufficient range of technologies and products for optimal social and economic development. While there is no doubt that some imported innovations have helped improve the conditions of life in the developing world by reducing infant mortality, controlling certain infectious diseases, and increasing the mean life expectancy of the population, they have also contributed to forces which sustain underdevelopment.
As a complement to the production of hard knowledge of universal worthiness, the most important social function of science in poor countries, and least appreciated, is the fostering of a rational and optimistic attitude on the part of scientist and public alike, enabling them to analyze the present and influence the future. Access to universal thought forms, sophisticated levels of analysis, and objective methods of problem solving are necessary to combat the frustration and dependence that stifle so many countries.

These crucial elements for development depend on continuing, systematic and institutional nurturance from early childhood education through professional placement and advancement everywhere in the world. Global initiatives in disease prevention, or in any other field which depends on analysis and planning, should be attentive to enhancing the environment for science, not on evading such basic investments.

3. The Challenge: Present Socio-Economic, Health and Scientific Conditions

The Developing World is composed of more than 120 poor countries; the annual mean income is 40 times less than in the developed nations. The imbalance is such than three-fourths of the inhabitants of the planet have to subsist with only a fifth of the available resources. [Martínez-Palomo, 87]

Typically, in these countries fertility was very high and mortality began to drop dramatically in the second half of the present century. [Frenk et al, 89] Improved agricultural techniques have contributed to extending life spans. As population growth rates increased neither the per capita incomes, nor employment rates, have kept pace. Some countries successfully implemented family planning programs which help to diminish socio-economic demands
while industrialization is taking place. Unfortunately, most underdeveloped nations have worsened their socioeconomic condition under these pressures and that of a huge economic debt. [ICHRI, 90]

In developing countries, life expectancy at birth is, on the average, 51 years, in contrast to the 74 years that correspond to a developed country. Perinatal and infant mortality are approximately 10-20 times greater in some poor nations, the main causes being infectious diseases such as diarrhea, pneumonia and other respiratory infections, tetanus, diphtheria, measles, and whooping cough. [King, 1983; Gomez de Leon & Frenk, 1991]

Malnutrition is often associated with infectious morbidity and mortality among small children. Once a child has reached the age of 5, his/her life expectancy is only 8 or 9 years less than their peers in the wealthy nations. Also, for them, the causes of death will be similar: cancer, stroke, myocardial infarction, diabetes and injuries. Nevertheless, the developing countries are also plagued by endemic diseases such as tuberculosis, malaria, schistosomiasis, trypanosomiasis, onchocerciasis, and leprosy, among others. [Martinez-Palomo, 1987 and 1989] Many of these ailments are effectively and economically controlled in developed countries but not in the developing world. [Evans et al, 1981]

Currently, developing countries bear about 2 percent of the total cost of science and technology. The other 98 percent is contributed by the developed countries. Expressed as a percentage from the gross national product, the investment in science in developing countries is, broadly speaking, 10 times less than in the rich nations. [Wade, 75; ICHRI, 90] The result is that developing countries produce a feeble kind of science, of doubtful quality, that has not succeeded in playing a decisive role in development—so far.

In 1990 the International Commission on Health Research found that 9.3% of the world’s burden of preventable mortality falls
on those residing in developing countries, yet only 2.5% or $835 million of the annual investment in health research is spent on research conducted in developing countries. Of that, the largest portion, 82%, is provided by the developing countries. In Mexico, less than 1% of health research now deals with public health problems. [TFHDRD 1991]

The combined investments of local governments and international organizations are insufficient qualitatively and quantitatively. Research salaries often fall below a decent living wage. At the beginning of the 1980's the Latin American region contained 2.4% of the world's scientists and engineers and produced only 1.3% of the world's scientific publications. [Sagasti and Cook, 1985] The percentages of gross national product dedicated to research and development was less than 0.7% in many countries and the percentage of health budgets devoted to research varies markedly. From 1980-1984, health received only 5.2% and reached 22.5% in Venezuela. Investments and productivity in the region increased markedly in the early 1980's with the economic boom only to slow again with the deepening recession. Brazil may be the only country which continued to build its scientific and technological infrastructure as a strategy to surmount the economic crisis. [Martinez-Palomo and Sepulveda 1989]

Scientists work in isolation from their peers. Career paths are virtually non-existent and biomedical research institutions often compete badly with private practice or other private sector activities for scientific talent. [Martinez-Palomo 1987; TFHDRD 1991]

Nevertheless, science continues to hold a promise for cultural and socio-economic development, and this is one of the few hopes that can still offer access to more decent living conditions for underdeveloped countries.

4. Exceptional Examples Inspire and Reveal the Limitations of Science Without Systematic Support
Even with the meagre investments in biomedical research, local scientists in Latin America have made contributions which legitimize the hope and reveal the defects of the current situation: Science in Latin America still relies on exceptional individuals rather than having become an integral part of the society. Around the turn of this century local researchers discovered some of the most important tropical diseases prevalent in the region including Chagas' disease and onchocerciasis [Martinez-Palomo and Sepúlveda 1989]. Carlos Chagas of Brazil discovered the parasite which transmits the parasitic disease that now bears his name [Chagas 1991]. Rodolfo Robles of Guatemala defined the principal characteristics of human onchocerciasis, including blindness, when it was regarded as a dermatological diseases of little medical interest by industrial world scientists [Figueroa Marroquin 1963 and Robles 1917].

There are both older and new landmarks as well. Among the early works two stand out: Daniel Carrion's experiment in Peru elucidated bartonellosis (and took his life) [Schultz 1968] and Carlos Finlay's discovery in Cuba of the role of the mosquito in transmitting yellow fever [Rodriguez Esposito 1951]. More recently the malaria vaccine devised by Patarroyo's group in Colombia and the immunotherapy for leprosy and leishmaniasis described by Convit in Venezuela demonstrate the continuation of the enduring tradition [Patarroyo et al 1988; Convit et al 1987].

Advances toward solving health problems which are virtually non-existent in industrial nations derive special benefit from the attention of researchers in affected areas, as in the example of onchocerciasis. A more recent illustration has been provided in the field of amebiasis. This experience also demonstrates the importance of international communication and collaboration.

Rough estimates have indicated that approximately 1/10 of the world's population harbor the protozoan parasite Entamoeba histolytica in the large intestine. The infection is particularly common in rural and suburban settings where sanitation is inadequate and
water sources are not safe. Out of every 10 people infected with the parasite 1 or 2 will develop a clinical condition that may ultimately produce death, i.e., dysentery or liver abscess.

Many more people than necessary are subjected to prolonged and expensive treatments with antiamebic drugs that have unpleasant and occasionally harmful side effects. These unnecessary treatments come at a considerable cost to the society. While the ultimate solution lies in the improvement of sanitary conditions and the provision of safe water, the health systems of those countries most affected by the disease will benefit from improved guidelines to make a more efficient use of specific measures to cope with amebiasis.

Research has provided new alternatives. In 1925 the father of French parasitology, Emile Brumpt, hypothesized that amebiasis comprises not one, but two infections: one produced by a nonpathogenic parasite, affecting 90% of those infected, and a second infection, produced by a similarly looking protozoan, but endowed with virulent properties that may cause a fatal outcome. [Brumpt 1925]

His theory lay untested for almost 30 years until one of us (A.M.P) found in 1973 a laboratory demonstration of differences between pathogenic and nonpathogenic amebas. [AMP et al 1973] This finding was extensively confirmed in many developing countries with a reliable biochemical technique called isoenzyme electrophoresis, and more recently, by the application of the most modern tools of biotechnology: monoclonal antibodies and recombinant DNA probes.

As a result of this research activity, spanning over more than half a century, we now know that 90% of people with amebas in their intestines do not need treatment, and that control measures should aim at those inhabitants of regions where invasive intestinal or hepatic amebiasis is prevalent. Short of development sufficient to
ensure access to ample supplies of clean water and an environment free of human wastes, useful approaches to those at high risk of the virulent form of the disease include treatment of symptomatic cases, development and use of an effective vaccine, and/or finding a harmless chemical that would block the production of the resistance phase of the parasite, the cyst, responsible for the transmission of the infection.

The strategy for addressing this world wide prevalent infection has changed fundamentally as a result of the application of biomedical and epidemiological research findings. Not only the control can be now envisaged under more optimistic terms, but also, tens of millions of people will be spared from unnecessary costs and unpleasant side effects of drug treatment with antiamoebic drugs. Without interdisciplinary collaboration between scientists in the North and South our knowledge of this important disease would be no different than the one we had at the beginning of this century.

6. Transfers of Technology Without Sufficient Local Scientific Expertise

The impact of transfers of technology are best evaluated in the broad context of the role of science for development. There are many examples from which to choose in illustrating limitations of the prescriptions offered by the World Bank and others.

A substantial number and diversity of products and technologies have been made available to developing countries from abroad. Some have been of great use. Others have been neither very useful nor effective, or have introduced a whole new series of burdens. To demonstrate the recurring lesson of the importance of local scientific capacity to successful transfers of appropriate technology we offer two examples. The first is the use of pesticides. The advantages of their importation include improved yield and production in agriculture and better control of mosquito populations and other disease vectors. The disadvantages are dramatic. These
pesticides have polluted water and soil extensively causing a range of adverse health effects. Yet many of these products are still being imported into developing countries despite their having been banned in industrial countries.

Were the culture of science more supportive of making selective judgments about which technologies to import and which to ban, and if the competence to support such technical judgments were readily at hand, dangerous pesticides might not be so widely used in poor countries today. Export decisions are often purely commercial and the ability of the importing nation to analyze the merits of sales pitch far too limited.

Similarly, the importer may be choosing from an altogether unsuitable range of technologies. Perhaps selection of different crops chosen for nutritional value and lesser susceptibility to pests would be a better use of limited resources. Only with a substantial scientific base can such judgments be made effectively. And only if the judgments are effective will political decision makers be likely to increase reliance on science as a criterion for allocation of scarce resources. If the problems imported with the technology are severe they may set back development, not nurture it.

Another example is the use of antibiotics and many other types of drugs. These products have been used indiscriminately, without sufficient understanding of their adverse effects. We include in our definition of adverse effects not only the development of untoward reactions or the creation of resistant germ strains, but also the waste of economic resources. When every dollar is critically needed at other levels of the health care system, the substitution of newer and more expensive drugs to treat conditions that are equally amenable to less costly ones fosters deprivation. Thus, the transfer of technology has been and can be useful in solving some problems but, in the absence of local capacity to evaluate, regulate and adapt imported technology to local conditions and needs, the process often backfires creating new and unexpected problems.
Up to now, it seems fair to say that science has not been instrumental in modifying the condition of underdevelopment, although its absence has had adverse effects. Isolated examples show how the applications of scientific findings can improve agricultural production, nutritional status and the general health level in certain impoverished populations. There is no doubt that scientific resources in developing countries are scanty and that the problems that we confront are very difficult to solve. Nevertheless both the positive and negative examples serve to demonstrate that science has already become a decisive factor in development and that local capacity to discover, invent, produce, apply and regulate technologies and products is crucial to the betterment of the quality of life in these nations.

In 1990 the International Commission on Health Research for Development addressed these issues and concluded that because research can play a key role in improving the health of the people it is essential for each developing country to establish and strengthen an appropriate health research base. This Commission also urged developing country governments and international development agencies to invest in health research.[ICHRD, 90]

7. Vaccine Research and Development as a Cornerstone of Science for Health

Vaccine research and development offers a possible cornerstone for the reinforcement of science and health in developing countries. The range of activities needed to achieve optimal results potentially link the whole spectrum of health sciences to technological advances intended to resolve significant public health problems which now deter development. What is urgently needed in developing countries is 1) integration of the different types of health research into a system capable of breaking from dependence which stifles development; 2) technological innovation suited to addressing socio-economic, cultural, health challenges described in Section 3; 3) greater commitment of governments to
Public health priorities and to investing in science, its applications and evaluation to assure continuity, effective and cost beneficial interventions; and, 4) International cooperation and collaboration at all levels to foster long term productivity towards healthful development. This last item includes international support for sound, scientifically based regulatory judgments by developing nations about which available technologies to embrace and adapt and which to avoid or ban.

The elements of health research which need to be integrated into a coherent system include biomedical, clinical, epidemiological, health systems and social research. Similarly, mechanisms for industrial scale production, must be in place and governmental dedication to immunization and evaluation must not falter. Together with regulatory systems designed to assure safe use and wise allocation of scarce resources these elements can consolidate the role of science as a key contributor for socio-economic development. A systematic, enduring and integrated approach to resolving major societal problems can enhance credibility of the efforts being made in developing countries, internally and internationally.

It is terribly important that the promotion of science as a key to development proceed openly. Local populations need greater exposure to science, the opportunity to feel pride when their neighbors make significant contributions and eagerness to participate themselves. At best, expectations of positive results can infuse the culture with a more optimistic vision. (We are not arguing for technocratic solutions where human cultures and experiences are subjugated anew.)

Several factors combine to make the opportunity to enter the field of vaccine R&D attractive, especially for middle income, populous countries such as Brazil, India, Mexico, or Thailand. First, countries such as these have already established traditions of biomedical research, so there are individuals and institutions capable of making significant contributions. Although there has been scant regional collaboration to date and national governments must bear primary responsibility for new investments, the larger countries
with the stronger capacities could become important links to advancement for their lesser developed neighbors under favorable political circumstances.

Second, despite the ascendance of chronic diseases in mortality statistics as a major feature of epidemiologic transition, it is clear that infectious diseases will continue to be a major threat to health in the developing world for years to come, especially for children. [Frank et al., 1989] Intestinal and respiratory infections still outrank all other causes of death for children ages 1-4 throughout Latin America and remain among the top 5 causes of mortality in 10 of 15 Latin American countries for all ages [PAHO, 1986]. Over the last ten years, resurgent malaria, dengue, choiera and even yellow fever have coincided with the appearance of AIDS and other viral infections such as epidemic conjunctivitis. [Martinez-Palomo and Sepulveda, 1980] To date, prevention strategies for problems such as infectious and parasitic diarrhea and acute respiratory infections have been unsatisfactory. However, recent advances in cell and molecular biology offer to break the traditional barriers to design and production of effective vaccines.

Third, national and international investments in vaccine R&D, especially for high priority diseases, are likely to increase because confidence is growing among policy makers that this area has potential for such positive impact on development. [World Bank Development report planning process or some other source?] In Mexico, less than 10% of health research now deals with public health problems. [THIRD, 1991] Coupled with the opportunity to collaborate on the frontiers of molecular biology with laboratories around the world, new investments create a strong incentive for recruiting able scientists to prevention of important infections.

Similarly, expanding vaccine development may serve to draw indigenous talent to epidemiology, an increasingly sophisticated discipline whose concepts and methods can focus biomedical science on critical health needs in every country and allow for evaluation of the usefulness of immunization strategies. Recent experience in Latin America features the difficulties of bringing a vaccine from the
bench to fruition without adequate integration of basic and applied laboratory science with field studies. Optimism about an effective malaria vaccine was reawakened when Patarroyo and his group in Colombia reported very promising results from initial laboratory and clinical tests. Unfortunately, international enthusiasm waned as the design and execution of further field studies proved inadequate.

Fourth, the original EPI strategy, which consisted of buying vaccines after the R&D investments had already been recouped by industrial nation purchases, will not stimulate economic investments sufficient to meet many developing country public health needs. [Robbins and Freeman 1988]. Even a modest agenda of operational research to improve field characteristics of existing vaccines was stalled until the advent of the Children's Vaccine Initiative by dim prospects for profitability and inadequate public attention globally. Elaboration and accomplishment of a research and product development agenda which can add the most recalcitrant of tropical diseases to those which ultimately become preventable by vaccines on an affordable, cost effective basis is likely to require and benefit from the most intense involvement of scientists from all regions, especially those most affected.

Fifth, the Children's Vaccine Initiative itself serves as an impulse. This project presents a significant opportunity for developing countries to become partners for the long haul in design of appropriate, high quality health products. To date, international partnerships have had limited success. According to the findings of the International Commission on Health Research, most frequently researchers in developing countries become involved in short term, externally driven projects which generally address problems of interest to donor countries, which are less important for recipient countries. [ICHR 1990] That sort of joint venture may result in co-authorship of a scientific paper, but in little strengthening of local capacity institutionally or progress towards new products selected for their specific contribution to effective public health practice.
Finally, another attraction for developing countries lies in the knowledge that the Expanded Program on Immunization has relied more heavily on local production of vaccines than has been generally appreciated. [Reference to P. Evans/UNICEF data—up to 60% of some EPI vaccines are produced locally] Since the United Nations appears to have neither available funding nor the inclination to replace local production, and if new, combination vaccines are to replace earlier generation formulations, it is the larger developing countries which have the added inducement of economies of scale to produce vaccines as well as to use large quantities. Continuing success of local production depends on sufficiently attentive and powerful regulators who establish and insist on adherence to rigorous standards in field testing before licensure, on quality assurance in every industrial manufacturing facility and on independent quality control testing before releasing any batch of vaccine for use. High and consistent quality of these functions can only be assured in a dynamic scientific environment.

8. Towards an Integrated Strategy for Vaccine Disease Prevention in the Developing World

One attractive alternative, described in detail elsewhere in this volume, is the possibility of establishing research centers and public enterprises in developing nations, which would focus on the development of new vaccines, the improvement of those already available as well as practical field issues (such as simplifying the immunization schedule or reducing reliance on injection). A regional approach to vaccine research, development and field testing with continuing support of industrial country collaborators, was presented to a group of scientists at a meeting in Rio de Janeiro under the sponsorship of PAHO and the Rockefeller Foundation as early as 1988. The group reviewed the perspectives for the development of new vaccines in Latin America during the 1990's. In spite of the economic uncertainty and restrictions that prevailed in this region during past decade, these scientists supported the establishment of a regional vaccine R&D network.
The basis for this recommendation was the firm belief that such an enterprise—without precedent in the region—would allow scientific research to make an important contribution to the improvement of the health of the people. This vote of confidence predated the birth of the Children's Vaccine Initiative at the 1990 Children’s Summit. Now it is important to find a way for the two approaches to vaccine development to become operationally compatible and interdependent.

Most frequently when the role for developing countries and their scientists is discussed in international efforts for vaccine development, it is at the stage of large scale clinical testing. Often transfer of the technology essential to further innovation in design, formulation, production and quality assurance of vaccines is not included in the package. Least discussed are longer term benefits of and strategies for encouraging basic research in developing nations, even for complex tropical diseases which are endemic only in these countries.

Even when transfer of production technologies is contemplated on a non commercial basis, as in the creation of the Dutch-Nordic Consortium [add ref], there is, to date, little consideration of the range of scientific expertise needed for maintaining international standards for productivity and quality, or for incorporating technological advances. In planning the Children's Vaccine Initiative it is important to keep in mind that the field of vaccines offers simultaneously examples of successes [Homma and Knouss, 1992] and problems in completing technology transfers from developed to developing countries. The latter illustrate the urgency for strengthening research capacity as an integral element of stimulating vaccine development and use of new public health products.

Because Mexico had a tradition of vaccine production, and even before the revolving fund was established to reduce the price of bulk vaccine purchases as part of the Expanded Program on Immunization, Mexico acquired the technology to produce a measles vaccine. After some years of producing and applying the vaccine,
W.H.O. issued new quality standards for measles vaccines. These new conditions prompted Mexican health authorities to study the effectiveness of the vaccine. Epidemiologists found the effectiveness of the vaccine to be low, and further laboratory studies betrayed a rapid loss of vaccine potency due to the use of a bad stabilizer.

Because the formulation of vaccine stabilizers has become a trade secret, no help to solve this problem was available from firms with relevant experience, except on a commercial basis, which was unaffordable. Mexico halted production of the vaccine and local researchers confronted an unanticipated challenge. They had no choice but to try to turn the failure of the original transfer of technology into a success. A costly and lengthy laboratory process eventually yielded an adequate stabilizer.

At present Mexico is one of very few countries with capacity to produce any measles vaccine. The availability of the Edmonston Zagreb strain in Mexico facilitated epidemiological research which has demonstrated advantages in immunizing younger children. This possibility has influenced vaccination research and policies at the national and the international level as well. [Markowitz et al, 1990] Indeed, a measles vaccine which is safe and effective closer to birth has become one of the priorities of the Children's Vaccine Initiative. Although neither the work on the stabilizer nor the field studies on immunizing younger children against measles were originally part of a global public health or scientific development master plan, both demonstrate the integral role of local scientific capacity.

A partial transfer is only one of the problems that can interfere, and eventually prevent, a successful appropriation of a technology. In the absence of local research capacity countries are saddled with aging technology and products, which are only partial solutions for their needs. The Mexican response to the cholera epidemic which began in 1991 provides a case in point. Public health officials considered a variety of alternatives to interrupt the spread of the disease. Once again, reliable sanitation and water systems are the ideal—and currently unattainable resolutions of this and many other disease problems. In their absence, vaccination was
evaluated along with chlorination of drinking water, digging of latrines and health education about food and water safety and waste disposal.

Only if a vaccine were certain to be effective and less costly than the other available measures for the isolated, rural populations most at risk would investment in its purchase and delivery be preferable to intensifying health education and interim sanitation programs. The benefits of increasing reliance on a cholera vaccine also have to be weighed against the advantages of even modest improvements in hygiene and sanitation which inhibit other infections as well as cholera. The same, limited resources will be allocated, whatever the final judgment of the most effective strategy to stop the epidemic in Mexico.

The inactivated oral cholera vaccine which was tested in Bangladesh in the 1980's appeared to be the best prospect. Overall, this vaccine gave promising results, but the response was not satisfactory among blood type O subjects and efficacy was lower for infection by the vibrio biotype El Tor. Due to the high proportion of blood types O in Latin American populations, and because cholera El Tor has been identified as the cause of this epidemic, further studies were deemed necessary to demonstrate potential efficacy of the vaccine. If the vaccine proves sufficiently cost effective in the studies now in process, the public health authorities will combine it with other elements of a strategy to ensure most effective use of the resources at hand in the vulnerable communities.

These examples further illustrate the value of combining application of existing products and transfer of technologies with strengthening research capacity. Too often, the problems that interfere with successful technology transfers are not of equal urgency to health in the donor countries and collaboration for problem solving does not occupy the same favored status as the original transfer. Other times the export is cloaked in secrecy to protect industrialists and their Investors without comparable bargaining power or protection for the long term interests of populations at risk in developing countries.
Even the most sophisticated of multinational vaccine firms call in scientific talent from a vast array of disciplines to solve manufacturing problems. It has become common for these firms to train, recruit or contract for special expertise in vaccine design. Developing countries need to encourage evolution resulting in the same sort of capacity for reasons that become increasingly obvious and urgent. As the multinational vaccine industry consolidates with mergers and buyouts, competition diminishes. It seems unlikely that the prices for finished vaccine products will be affordable to the consumers who most need reliable supplies if a cartel or monopoly accountable primarily to private investors results from industry concentration— as the current trend portends. [Freeman and Robbins 1991]

The pricing of AIDS drugs offers cold comfort. This recent and particularly relevant history demonstrates the willingness of multinational pharmaceutical manufacturers to charge prices which exceed the capacity of all but the most privileged to benefit from new products, even when profits exceed industry estimates, even in the most profitable of industries and even when substantial research investments were contributed by public institutes, as in the case of AZT. [Arno and Teiden, 1992] If in the United States many HIV positive individuals have no access to life prolonging medications despite the best efforts of activist consumer groups which have focussed a media spotlight on drug pricing with unprecedented intensity, can one really assume new vaccines will be affordable for Third World populations?

When one looks twenty years down the road instead of two, this open question is even more troubling. If the CVI relies too heavily on such commercial manufacturers with little competition anywhere in the world, price inflation may astound even those with greatest faith in the market. Granted governments need to invest more in public health. But even so, what assurance do we have that multinational firms will continue investment in vaccines for relatively poor nations if they can command higher profits manufacturing therapeutics and diagnostics for more prosperous
buyers? From one perspective under this cloud of uncertainty it seems wise to assure alternative schemes for developing components for children's vaccines and for assuring competition.

9. Linking National, Regional and Global Initiatives into an Effective Strategy

Perhaps the greatest challenge for the long run is posed by the many problems of developing countries which are still not being addressed at all. International perceptions often generalize to the exclusion of local problems: commercial priorities lie elsewhere; and local scientists are few and many of the best are too busy working on topics without relevance to public health. How might we direct this heightened international attention to vaccine development toward a model for identifying and filling such gaps? In addition to current efforts to 1) reinvigorate EPI in the decades following the campaign to reach the 1990 goal of universal coverage; 2) mount a systematic and integrated regional approach for vaccine R&D for vaccines of high regional priority (SIREVA), and, 3) initiate a global vaccine development strategy (CV!)- there is a newly institutionalized and potentially complementary agenda for shaping health research at the national level.

Following the publication of the final report of the International Commission on Health Research, several countries are working intently on plans for what has come to be called essential national health research (ENHR). This strategic approach to influencing research priorities and health outcomes is meant to attract scientists to concentrate in areas which promise significant health benefits, and to attract greater support for projects so demonstrably related to national well being. A characteristic of the Mexican model (called COMISA) is the interdisciplinary and coherent manner in which each working group is expected to address a given health problem area. Once the appointed working group has identified a series of priority problems within an area, more specific research lines are to be stated, indicating the role of each one of the six types of research relevant to health outcomes in providing
elements for prevention and/or control, i.e. biomedical, biotechnology, clinical, epidemiological, health systems and social research. [COMISA ref]

This national effort shares a strategic vision for improving health with the Expanded Program on Immunization, with the regional system for vaccine R&D and with the Children's Vaccine Initiative. National experiences with setting and implementing agendas for essential national health research may provide valuable experience for stimulating more productive local health research activities and for increasing commitment of scientists and governments in developing countries to innovations to improve effectiveness and efficiency of immunization programs.

In closing we remind ourselves and our readers that even the most enthusiastic proponents for marrying scientific development to public health and socio-economic development objectives must reflect soberly on the considerable barriers to fulfilling these aspirations. It will take unprecedented creativity and persistence to manage these simultaneous processes (EPI, SIRIVA, CVI and ENHR) so that they succeed in mutual reinforcement of health and development.

The current lack of integration of health R&D programs into the national socio-economic development programs threatens to reinforce the extremely low resource allocation for health research unless greater political will and international solidarity inspires a revolution in political behavior. The full realization that health is an economic issue is a relatively recent phenomenon in the whole world. Hence there is a vicious cycle by which low levels of health undermine development efforts, and the latter do not explicitly incorporate plans to correct the former.

Always threatening the success of local participation is the limited capacity of scientists in developing countries to replicate themselves. The weakness of Institutions, which expresses itself as
Financial instability, low productivity and lack of scientific tradition, leads to fragmented and disorganized research efforts. The commitment to social priorities and productivity of scientists in the Third World is further aggravated by the nearly absent information systems and rather deficient communication networks, including bad libraries, incomplete and untimely health surveillance networks and lousy telephone and mail services. Even well intentioned scientists are tempted to retreat to topics of purely personal interests when reinforcing communication is so deficient.

For good reasons and for bad, international organizations always accede, in some way, to mounting pressure to spend more of their budgets in the countries whose populations are intended to be the ultimate beneficiaries of their programs. These customary expenditures can become more significant investments if EPI, SIREVA, CVI and ENHR can become integral parts of adhering to a long term strategy. Lessons from such an effort might inform attempts to develop better schemes for the prevention and control of chronic diseases or drug additions.

Despite these earnest words of caution, fifteen years of mounting international enthusiasm for the Expanded Program on Immunization, the propitious start of both SIREVA and CVI can combine with emerging national health and research priorities while reinforcing science.

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HOW HAS IMMUNIZATION CONTRIBUTED TO ECONOMIC DEVELOPMENT?

Paper prepared for the Conference on ---
Bethesda, Maryland, 4-5 November 1992

INTRODUCTION

This paper examines the contribution of immunization to different aspects of development, focusing particularly on the experience of the past decade. We use the concept of "development" is used in a broad sense, with at least three main dimensions: (1) the direct contributions made by health gains and deaths averted due to the EPI; (2) the effects of EPI on institutional development within the health care system; and (3) impacts on political and social processes of the concentration on health and children's well-being. This perspective goes beyond traditional definitions of development in terms of economic growth, using as its basis the concept of "human development", in which development is seen as the improvement in the capacity of people, and in the ability of societies to improve the people's situation (UNDP, 1991). Health forms one of the critical sectors in which investment in people takes place, and in fact the EPI has contributed considerably to the understanding of this process. Review of experience enables the identification of specific factors affecting the degree to which immunization has had impacts in these different respects.

Section 1 presents an overview of the EPI programme, how it was developed and has been implemented; and its various extensions. Section 2 reviews the processes and evidence regarding immunization and development in these different domains. Section 3 summarizes the principal factors affecting the success of immunization efforts, and concludes with several observations on future directions.

1. THE IMMUNIZATION EFFORT


The development of a wide range of vaccines and the demonstration of their efficacy has led to the increasing use of immunization during the past two centuries, especially in the industrialized countries. The adoption of immunization in developing countries has proceeded relatively more slowly, in part due to the limited extension of public health infrastructure, and in part to the difficulties of storing and transporting vaccines under proper conditions. However, for a
combination of economic and humanitarian reasons, vaccination was introduced in the developing world from the late 1800s onwards.

The major advance in the spread of immunization occurred in the campaign to eradicate smallpox, in which a relatively heat-stable vaccine proved successful, finally in Africa in 1972, in eliminating this disease. This achievement, based on concerted international action and the subject of worldwide recognition, led to a commitment to control other diseases, including tuberculosis, diphtheria, pertussis, tetanus, measles and polio.

The Expanded Programme on Immunization (EPI) was designed to take advantage of the investment that had been made in smallpox eradication, and sought to build on the success of the eradication of that disease. The EPI was launched by the World Health Organization in 1974, with the aim to provide coverage with a set of vaccines, especially to populations in developing countries. The programme was defined initially in relatively technical terms, with the aims to improve vaccination technology, to improve the skills of health personnel for vaccination, and to strengthen management systems for vaccine distribution and the collection and use of information.

Efforts were pursued to strengthen national immunization efforts along these lines during the late 1970s, through a combination of special programmes and, to a lesser extent, through incorporation into regular public health services.

Immunization was identified as one of the eight essential elements of primary health care in the Alma Ata Declaration of 1978. Along with other PHC interventions, however, immunization was subject to limited health sector funding, and attention was given to methods of identifying and promoting actions with the most significant health impacts. Reviews were made of the comparative costs and effectiveness, in terms of lives saved, of different health interventions within the framework of "selective" PHC, and these showed immunization, particularly against measles, to be an important area of health investment. Yet funding levels for EPI remained low during the 1970s and early 1980s, and coverage in most developing countries did not exceed 20%.

B. Acceleration and Going to Scale (1981-1990)

The survival of children was given a strong focus in the early and mid-1980s as a basis for mobilizing attention and resources for health and related actions in developing countries. Immunization was increasingly recognized as a key element of this effort, in view of its potential impact. Immunization was identified by UNICEF as one of four (later seven) interventions with low costs and being most critical for the survival of children. (Other "GOBI" components were growth monitoring, oral rehydration therapy for diarrhoea, breast-feeding, and family planning, food supplementation and female literacy.) The key to this strategy was a focus on feasible interventions for which support could be mobilized in within governments,
national populations and the international community. Immunization had the important features of being preventive in nature, well known and of demonstrable value, and with a basic infrastructure in place for implementation.

In a similar way, immunization and the control of diarrhoeal diseases were identified by USAID in 1984 as the "twin engines" of child survival within its assistance programmes. This increasing interest was organized, and additional support was developed, through a process of formal and informal consultations between WHO, UNICEF and other international organizations, bilateral technical agencies, and prominent individuals in the public health field. Within the framework of the interagency Task Force for Child Survival, Robert McNamara and other world leaders recognized the possibility to galvanize support around a single, common goal. Among the different candidate interventions, immunization was selected as the focal action for children.

Support was provided from many sources, including bilateral donors (principally the governments of Belgium, Canada, France, Italy, Norway, Sweden, the United Kingdom and the United States); from non-governmental organizations, such as the Rotary Foundation which took polio as a specific focus, and the Rockefeller Foundation; and from international agencies. The EPI was substantially strengthened during the mid-1980s. In 1986 the goal of Universal Child Immunization was adopted, with the aim of achieving at least 80% immunization coverage worldwide.¹

The working definition of EPI that evolved had several key elements that both consolidated and went beyond the features of the earlier approach, under the overall principles of PHC. First, there was a focus on population coverage, which led to increased attention to catchment areas, the identification of underserved population groups, and the establishment of routine surveillance systems. The early EPI had introduced an orientation to coverage monitoring, and methods were developed for immunization coverage surveys, but these had not been fully implemented and were only put in place on a wide scale in 1984-85.

A second feature was a strengthened attention to vaccination technology. Advances in vaccine development were applied to improve existing vaccines, such as new formulations of polio and measles vaccines; and to develop new vaccines to be added to the EPI, including hepatitis B. Efforts were made to improve the temperature stability of vaccines. However, as the cold chain remained the most vulnerable element in the vaccination process, particular focus in the early 1980s was given to new developments such as the solar power for refrigeration and, by 1984, the expanded use of cold boxes for transporting and storing vaccines in the periphery.

¹ The target of 75% was set in Africa, as an average for all the EPI antigens.
A third feature of the strategy was a greatly expanded approach to mobilization for vaccination, through a range of complementary strategies. Within the health system, this was done through the such means as the use of immunization coverage as an indicator of health system performance; through training to ensure that staff had the proper skills for EPI; and increasing the number of vaccination sessions in order to maximize contacts with mothers and children. However, in view of the limitations of the fixed facility system in many countries to reach the population, new approaches were taken to the use of outreach sessions and mobile vaccination teams. Beyond the health system, considerable effort was made to mobilize the political and social systems to recognize the importance of immunization and to facilitate the expansion of EPI coverage. With the involvement of other sectors besides health, and of social, religious and other non-governmental organizations, extensive publicity was given to EPI, often through nationwide or regional vaccination campaigns.

The EPI was intensively implemented in the 1987-1990 period. An important milestone on the way to the goal of universal immunization was reached in 1990, as 80% of all infants worldwide had been vaccinated against the six key diseases. The target of Universal Child Immunization (UCI) was achieved in 1990, and certified as such in November 1991 by WHO and UNICEF.

C. Sustainability and Disease Reduction (1990-2000)

Since the achievement of UCI, efforts have been redoubled to maintain and increase the high levels of coverage that were attained. For the 1990s, going beyond 80% coverage and achieving disease control goals will be more difficult than reaching 80%, because the population groups, which are often those at highest risk, will be hard to reach (often literally "unreachable") through the current infrastructure, geographically as well as socially. Even among those populations currently reached, substantial differences in coverage rates are often seen, between and within countries.

Four additional immunization goals for the 1990s were endorsed by the World Health Assembly in 1985 and 1988. (1) elimination of neonatal tetanus by 1995 - a disease for which coverage rates are low, only about one-third of all pregnant women having received the tetanus vaccine in 1990; (2) reduction by 95% in measles deaths and reduction by 90% of measles cases compared to pre-immunization levels by 1995, an important step in the global eradication of mumps; (3) eradication of poliomyelitis by the year 2000; and (4) to achieve and maintain at least 90% immunization coverage by the year 2000.

With the success of universal immunization as will be described below through strategies that have been adopted in some, the use of immunization to the extension of coverage of other health interventions. In a number of Asian countries, for example, "USI Flex" has been developed as a model for adding other specific actions for children and women to the health outreach structures.
New vaccines are also being added to the EPI. WHO already recommends that vaccination against hepatitis B be included in national immunization programmes, as a major cause of liver cancer and liver cirrhosis. The vaccine has existed for a number of years, but it has become available in large quantities only recently. The problem is that it is presently expensive, calling for prioritization among health actions.

2. THE CONTRIBUTION OF IMMUNIZATION TO DEVELOPMENT

A. Health Gains and Development

The most direct contribution of immunization lies in the reductions of illness, deaths and disability attributable to immunization. At the present time, it is estimated by WHO that some 3.2 million children's lives are being saved each year from vaccine-preventable diseases, and another 450,000 children who might have been crippled by polio are not being disabled.

The most basic indication of progress is seen in immunization coverage performance. Coverage in developing countries increased by nearly four times between 1981, from a base of about 20% to reach the 1991 goal of 90% coverage. As shown in Figure 1, at the end of 1990, coverage with BCG had reached 89%, and coverage with three doses of DPT had reached 81%, and with three doses of poliomyelitis vaccine, 83%. Measles coverage had reached 78%, in part because reporting is done only for vaccinations given to infants between 9 and 12 months of age. Surveys have indicated higher coverage with measles vaccine in many children up to 18 months of age (UNICEF 1991b).

Considering the potential mortality impacts of tetanus and measles immunization for the Matlab area of Bangladesh, Koenig (1992) suggests that under-5 mortality would decline by some 32%, from 208 per 1000 to about 140 per 1000 live births. In this scenario, infant mortality rates would decline from 116 to 91 per 1000 live births, due chiefly to tetanus immunization. Mortality of children aged 1-4 years is estimated to decline from 90 per 1000 to 50 per 1000 live births, principally as a result of measles immunization. Although there are limitations to the degree to which these figures can be generalized to other settings, they provide a clear indication of the order of magnitude of the numbers of lives that can be saved.

Worldwide, it is estimated that 2,000 thousand deaths due to measles are averted by immunization each year (compared to nearly 1,000 thousand deaths occurring); some 700 thousand deaths from neonatal tetanus are prevented (compared to about 600 thousand deaths occurring); and some 600 thousand deaths due to pertussis are prevented (compared with about 400 thousand deaths occurring) (Kim-Farley et al., 1992). These results are shown in Figure 2.
In interpreting these estimates, one important issue in considering the impacts of immunization concerns the extent of replacement morbidity and mortality. Under this hypothesis, if a child is at nutritional or other risk of a number of diseases, the prevention of a given disease by vaccination may merely result in sickness or deaths from other causes. However, a growing body of empirical evidence is demonstrating the long-term impacts of immunization on the reduction of child mortality. For example, a study by Koenig et al. (1990) examined the mortality experience of over 8,000 children vaccinated against measles, compared to a matched set of nonvaccinated controls. The results showed substantial impacts on child survival, with the mortality rates for vaccinated children being nearly one-half (46%) less than those for unvaccinated children, indicating that children saved from measles deaths are not dying significantly from competing causes of mortality. Figure 3 presents cumulative mortality by measles vaccination status from Matlab, showing the extent of this difference over time. Similar findings have been reported from the Kasongo Study in Zaire, and other studies from Matlab (Koenig, 1992).

A second issue affecting the quantification of immunization impacts concerns the synergy between morbidity and other factors in the determination of total mortality. The links between infection and nutrition have been well known for a number of years, as has the importance of clean water and other aspects of development in the mortality profile. Recent reviews of this literature have confirmed the broad pattern that the combination of morbidity from diseases such as measles and poor nutrition increases the risk of dying, and that these effects may be multiplicative, rather than merely additive (Pelletier, 1991). In other words, the effect of immunization in averting illness and deaths may be greater in groups which are already more vulnerable, i.e., those who are poor and otherwise at greatest risk.

The cases that are averted have had a direct contribution to economic welfare. First, the costs of illness and death to households and communities have been lessened. It is estimated that the out-of-pocket costs of illness may be as much as 8-10% or more of household income in developing countries, with one or more illness episodes per household member per year. Frequently, assets such as farm animals must be sold at a loss, or loans must be taken out at high rates of interest. Reductions of the disease burden thus have a tangible effect on household financial resources. However, these direct costs represent only a portion of the total household costs for receiving health care and caring for sick children. Women, especially, must spend substantial time in attending health facilities when children are ill, and often have to forego working time in order to care for children at home. There are also significant financial costs for funerals in most cultures, in addition to the emotional costs, incurred on the death of a child (Andersson et al., 1991).
The broader aim is control or, where possible, eradication, and hence the focus is on universal coverage. Smallpox has been recognized as offering the greatest potential for eradication, as a specific and achievable target, as expressed in the global target for polio eradication worldwide by the year 2000. To date, it appears that the transmission of wild poliovirus has been completely interrupted in the Americas region. This experience has led to the rapid development of plans of action for polio eradication at the global, regional, and country levels (Kimmelman et al., 1992).

Recent comparative reviews of the cost-effectiveness of different disease interventions have consistently shown the efficiency of expenditures for vaccination, and most consistently the high level of cost-effectiveness of measles immunization.

A major concern throughout the child survival movement has been that EPI and other interventions, by saving lives without taking any direct actions to reduce fertility rates, will lead to increased family size and populations. It has been suggested, with varying degrees of systematicity, that this process leads in many poor countries to increased poverty and declines in welfare, and to continuing burdens on the environment. However, there is increasing evidence that mortality reductions due to EPI and other child survival actions in fact contributes as well to future declines in birth rates, and promote the demographic transition to smaller families. Review of recent trends demonstrates the predictive value of declining infant mortality rates for the estimation of future birth rates (Foege, 1990).

This process occurs through several means. First, the survival of infants increases the period of breast-feeding, with its contraceptive benefits. Second, fewer infant deaths increases the interval between births, with a lowered need for replacement. Third, although in the short term a larger number of surviving children increases the dependency burden, over time there is a shift to fewer numbers of births as replacement or insurance against those who would have died. Finally, mothers' confidence in family planning and health services is increased with increased child survival. Review of the infant mortality-fertility relationship indicates strongly the synergistic relationship between child survival and family planning actions in reducing both infant mortality and fertility, and the importance of the promotion of breast-feeding and other health actions, particularly through increased female education (UNICEF, 1991).

B. Immunization and Health System Development

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2 The target of 80% was used in smallpox, where the concept of 'herd immunity' was adequate to stop transmission of the disease. In other cases this is not relevant, and higher rates must be achieved.

3 This summary will be expanded based on the results of Jamison (forthcoming), which is currently undergoing revision (10/92) in preparation for the 1993 World Development Report.
Immunization has made several specific contributions to the capability of the health system to meet the health needs of the population. These include a strategic focus on population coverage and the goal of universality; increased priority and resource availability for EPI and related maternal and child health services, including expansion of health care infrastructure and coverage; and technical improvements in health services.

Immunization holds universal coverage as a strategic and practical objective. To achieve this, enumeration of children by name in many countries has focused attention on the community members and meeting the needs of the entire catchment area population. Immunization often represented the first time that local populations were counted, and these listings of communities and households were used as a management tool. In Guinea and Benin, for example, the information systems developed with impetus for the EPI are now being used for a wide range of basic health service management purposes, including monitoring of health worker performance and follow-up of families (Knippenberg et al., 1990). Using immunization rates as a proxy measure for health service coverage has enabled attention to be focused on coverage attainments in different geographical areas, at the local as well as national levels.

A focus on universality also moves attention beyond those who visit health facilities, to attention to the needs of the poor and other underserved groups, and the problems of equity of access to services. The attention of health professionals as well as of communities has been directed to those groups not using the health services; to the reduction of dropout rates; and to eliminating missed opportunities for vaccination. It is estimated that, worldwide, immunization provides approximately 500 million separate contacts between health services and children, which provide opportunities for greatly improved services.

The linkage with other services, particularly efforts to promote behavioral change, are also needed for the achievement of disease reduction. In the case of tetanus, for example, in instances where the vaccine may fail, reliance on simple measures such as hand washing, cleaning of the cord-cutting tool, and reduction of other risk factors such as multiple cord ties is necessary to effectively reduce the risk of disease. This approach to analysis has led to increased measures for quality control, for example, in Bangladesh (Hlady et al., 1992).

Second, there has been increased budgetary attention to immunization and preventive health services. The focus on immunization in many countries was recognized as requiring a specific and strengthened allocation of resources for immunization. In some countries, at the height of the UCI drive, there was an annual review of the health budget with respect to the needs to achieve Immunization goals. In the situation of austerity faced in many countries, with health budgets were being cut, there was a stable or increased allocation to preventive health care. Line items were introduced into many health budgets for immunization, with a reordering of priorities. To some extent this entailed a shift from curative care and especially tertiary care serving only a small minority of the
population, usually among the better-off and in urban areas. While this had been a general objective of primary health care, relocation had in most instances not been successful. In countries such as Indonesia, Madagascar, Mexico and India, there is evidence that the drive to UCI increased the priority and resources for preventive and promotive activities.

Increased priority and resources for immunization also affected donor countries, in their effort for and approach to health sector aid. Assistance to the health has traditionally been weak, and carried out through a wide range of projects which have often focused on politically visible tertiary care hospitals, and on un-coordinated health service support. The UCI drive increased the volume of aid to health overall, well beyond the earlier levels [Data to be obtained from Harvard study for WDR 1993]. A number of governments, including Canada, Italy and the United States, developed specific initiatives for child survival which received widespread popular support. In the non-governmental sector, the Rotary Clubs launched the PolioPlus program, which raised over $ [x] million for immunization.

The focus on immunization coverage, combined with increased resources and availability overall, has led in many countries to improvements in the infrastructure for health at the peripheral level, and in immunization skills on the part of health providers. It also led to different approaches to increasing the coverage of health services, particularly where the coverage of fixed facilities was limited. In Indonesia, the number of posyandus was greatly increased and their functioning was enhanced, as President Suharto, in the effort to achieve UCI, accelerated expenditures on posyandus using Presidential funds, to place a post in every hamlet in the country during the late 1980s.

In many countries, the investment in vehicles and cold chain was applied directly to outreach strategies, using existing but poorly functioning dispensaries and health posts. In Bangladesh, for example, the system of outreach posts, including sites set up for family planning, was strengthened to provide immunizations and other essential services to populations not reached by health centers, thus increasing the coverage of a number of different activities. Mobile teams were used in countries, such as in Sahelian Africa and the Sudan, where teams had traditionally been a principal means of immunizing difficult-to-reach populations, in areas with widely dispersed groups. Through these various means, services have been brought directly to the people, and demand has been created to promote their continuing availability.

In such contexts, it has been widely recognized that immunization in itself is not to promote child health, but that the availability of five contacts with children, as well as antenatal contact with mothers, offers critical opportunities for the provision of further MCH interventions. As noted earlier, the concept of "UCI-Plus" was developed in countries where the EPI was particularly effective, but where the formal health system does not have extensive coverage. In India, for example, the activities developed through the Universal Immunization Programme are being
expanded within the framework of the 8th Five Year Plan to incorporate oral rehydration therapy, control of acute respiratory infections, prophylaxis against nutritional anaemia and against vitamin A deficiency, and a range of services for early childhood development and maternal support (Government of India, 1991).

In other countries, the EPI investment was translated directly into the strengthening of existing health centers and the opening of new ones. Approaches integrating EPI with other maternal and child health services were especially appropriate where the health infrastructure was potentially able to reach the majority of the population, but where additional resources and motivation were needed to make this coverage effective. In Tanzania, the EPI provided crucial additional resources that enabled the health system to link more effectively with communities. In Benin and Guinea, the system of fixed facilities was strengthened with an increasing range of health activities, and was coupled with the emerging strategy of community responsibility that was later developed as the Bamako Initiative (Knippenberg et al., 1990).

However, the resource use picture is mixed. The infusion of vehicles and equipment in some cases led to inefficient use, as in many cases which have been reported where vehicles were assigned only for the EPI and not used for other health activities, or where health workers devoted attention to EPI to the exclusion of other functions. Thus, while in countries where the health system was simply not functioning well, the immunization efforts represented a positive net gain. In other settings, the focus on EPI has been judged to detract from other actions. In a detailed study, Goodfield (1991), for example, observed that the acceleration of EPI in Uganda, with substantial social and political involvement, entailed a predominant focus on EPI rather than other services. Other evaluations have indicated that particularly the more vertical, donor-driven approaches have not always left as much behind as was hoped. However, recent reviews of EPI implementation -- and notably in some in which there was a strong mobilization for EPI -- have suggested that immunization is being established in a more regular role within the overall provision of maternal and child care, as a precondition for sustainability (Noto et al., 1991).

Third, immunization has contributed in a number of ways to the technical strengthening of the health system. Training has been a strong focus of the EPI, under the leadership of WHO. The management training materials developed by WHO for the EPI pioneered the use of modular training systems in other health programmes. Some areas of management development, such as in cold chain maintenance, have been principally relevant for the EPI alone. However, other aspects, notably in the areas of logistics, quality control and operational planning, are relevant to the overall operation of health services. EPI experience has contributed substantially, for example, to the development of logistic management techniques for the purchase and distribution of essential drugs, medical equipment and vehicles in primary health care (WHO, 1992).
The second major instance has been in the area of technology development. Where immunization is provided by workers with limited training in sterilization techniques, and with increasing awareness of the risks of contamination, particularly with the rise of AIDS, there was significant experimentation with single-use syringes for EPI during the late 1980s, especially with support by USAID and WHO. Single-use syringes are now in use in a number of countries. However, in most countries, for economic as well as operational reasons, multiple-use syringes are still the norm. Thus, there was widespread strengthening of sterilization equipment and training in sterilization methods as a part of EPI support. This has had important implications for sterilization of other medical equipment, especially at the peripheral level. Kerosene refrigerators have been improved to account for the lower quality fuel available in many countries. Similarly, the development of solar power mechanisms for running the cold chain, e.g., in Zaire and other countries particularly in Africa, has improved the capacity for refrigeration of antibiotics and other health supplies. Special temperature indicators have also been developed and used to monitor the status of the cold chain at all levels.

Along with the development of technology by industrialized countries, there has been increasing indigenization of vaccine production and other supplies. Currently some [xx]% of vaccines are estimated to be produced in developing countries themselves. For example, India and China each produce over 80% of their own needs for several vaccines. A number of other countries have increased their capacity to produce specific vaccines, although in many instances quality control concerns still remain.

C. The EPI and Social and Political Development

The achievement of UCI was characterized by a high level of social involvement and the active support by political leadership, to a much greater degree, in combination, than has been the case in other health sector initiatives. The result has been a major change in the approach to health issues in many countries.

It had often been perceived up through the early 1980s that immunization was one of the few public health interventions that did not require a high degree of social involvement to be implemented -- that the problems, including low coverage, could be addressed by technical solutions. However, experience in Burkina Faso, Colombia, Nigeria, Turkey and other countries contributed to a growing recognition that accelerated strategies involving widespread social support, serving to "transform health programmes into social movements", held the potential to achieve significant advances in EPI progress (Joseph, 1985).

* In Zaire, the sale of excess solar power has represented a major opportunity for income generation for the health care and other community activities (WHO, 1990).
Political leadership was mobilized, as many heads of state and government - many launched campaigns and gave personal support. Starting in 1984 with President Betancourt of Colombia, more than 30 heads of state have lent their personal prestige and political support to immunization, in roles ranging from publicly launching an EPI campaign and appearing on television or posters, to involvement in national coverage monitoring processes. The spouses of heads of state have in many countries also been active, as, for example, in Indonesia where the wives of provincial and local officials also played continuing roles in EPI promotion. Leaders have changed over time, but in most instances the political attention to EPI has been adopted by new officials. It has been generally felt that this level of involvement helped to mobilize sectors beyond those traditionally concerned with health. It also often brought additional resources into specific immunization campaigns.

The political support for EPI went considerably beyond relevance only in stable situations. Fighting in civil and military conflicts was suspended in a number of instances, some of which were widely publicized. These included, for example, the "Day of Tranquility" in El Salvador.

A similar approach was seen in the mobilization that took place at the local levels for EPI activities. Political leadership often played a key role in initiating activities. Social mobilization processes themselves have been widely varied. In some cases, national mass campaigns were carried out with the full involvement of religious leaders, teachers and other community leaders. In others, national immunization days were held with intensive media support. In some instances, early immunization was mandated in order for children to qualify for school or other services. Large-scale campaigns as were conducted during the mid-1980s, relying upon hundreds of temporary immunization points and thousands of non-health ministry workers to provide immunization services. These proved useful to initiate the acceleration of EPI, although some countries employing this strategy experienced declines in coverage until the transition was made to routine delivery of services.

This process has had several implications. A first is the precedent which has been established (or the practice which was greatly strengthened) for the use of public and private resources for health purposes. These have included the provision of free radio and TV time, the use of teachers and other workers in EPI campaigns, and the availability of vehicles and fuel from the public and private sectors, all of which represent resources which can be mobilized for other health actions.

A second implication has been the emergence of accountability of the health system to decision-makers outside of the health ministry -- making health not only a medical but a social responsibility. Awareness of national political attention, but more importantly, of attention and oversight by local government and by community organizations has in many countries improved the efficiency with which health workers approach their jobs. It has both had a positive effect on morale,
and has increased the orientation to performance within the health system. This has often spilled over into ongoing community structures for monitoring health system coverage and effectiveness.

In summary, the EPI strategy has been seen as involving a redefinition of the principle of participation, expanding from the earlier and traditional focus on involvement at the grassroots level, to a broader view of the role of society in health actions. The immunization drive has been characterized by mobilization processes at all levels of society, linking action at the national level to that at the local level (Knutsson, 1985).

At a more general level, we have seen the important role of child health goals as a motivating force for social action. The UCI drive was responsible in large measure for the momentum leading to the World Summit for Children, attended by over 70 heads of state and government in New York in September 1990. It is in turn full by some of the tiny notoriety that the Summit would not have occurred if not for the success of the UCI drive, hath because it brought actions for children into the political limelight, and because it fostered a "can-do" approach at the country level as well as within the international community. The global goals which emerged from the World Summit for Children include the overarching objective of, "between 1990 and the year 2000, reduction of infant and under-5 child mortality rate by one third or to 50 and 70 per 1,000 live births respectively, whichever is less." These goals are now being translated into national goals for children for the year 2000, and strategies are being developed to achieve these within the framework of National Programmes of Action (NPAs) in all countries (UNICEF, 1990). At present, more than 50 NPAs have been prepared by developing countries.

3. CONCLUSION

A. Critical Factors In EPI Effectiveness

The EPI experience has demonstrated the interlinkages between the health system and the social and political systems in achieving impacts on "development," including its economic dimensions. In this process, several factors may be seen as contributing to successful outcomes -- and, correspondingly, in whose absence progress and sustainability have been more difficult.

First, an orientation to clear and measurable objectives has served to focus attention and enable monitoring of progress and results. The clarity of policies and the discreteness of immunization as an activity is virtually unique in public health. The vaccine-preventable diseases are asserted by one discrete action at one time, or in a brief series of contacts, and require no recurrent action. Under the leadership of WHO, the principal parameters of the EPI, including vaccine selection, immunization schedules and other issues, have been accepted throughout the medical profession and among the public. The development of coverage survey
techniques and reporting systems and their application in nearly all countries has led to the continuous measurement of progress, and an orientation to common goals.

Second, the presence of political will and social mobilization helped to organize support and energize local actions to achieve the immunization goals. Very few countries were able to attain high levels of coverage through routine services without some form of social mobilization. In nearly all countries, the multi-sectoral approach with the active involvement of teachers, religious leaders, social organizations and other non-health sectors, together with radio, television and traditional media, provided the critical momentum for the EPI.

Third, extension of the health infrastructure and management effectiveness provided the basis for achieving high coverage rates. Despite extensive social mobilization and strong political support, there is the underlying need to provide adequate access to health services to the population, either through fixed facilities or through outreach strategies. Planning for accelerated activities also included extensive investment in staff training as well as the cold chain to meet national access targets. The immunization drive in many countries was responsible for extending the health infrastructure and improving overall health system performance, with support from media promotion and person-in-person communication.

Fourth, there is the operational need for reliable and affordable vaccine supplies. The UNICEF supply system has been effective in procuring vaccines at low cost, both for donation to programmes and on behalf of governments (and Rotary International). A total of some 4.400 million doses, at a cost of US$177 million, have been procured between 1982 and 1990. WHO has elaborated technical and biological standards for vaccines, and designated vaccine manufacturers which meet these standards. With the continued rise in demand for UNICEF procured vaccines, as coverage increases and disease eradication programmes accelerate, a number of measures are being taken to enable countries to purchase vaccines more efficiently, including with local currency.

Fifth, the EPI drive focused strongly on improved cold chain, sterilization and injection equipment, to protect vaccines from heat during the journey from the manufacturer to the most remote Immunization points in developing countries. WHO and UNICEF have collaborated effectively with the private sector in developing improved equipment to store and transport vaccines, as well as to provide for safe injections. WHO has provided guidance and technical specifications for EPI equipment, enhancing standardization and enabling procurement at low cost. A range of technical developments, including solar powered equipment, kerosene refrigerators, temperature indicators and steam sterilization have been critical to EPI implementation.

Finally, the success of the UCI drive has been dependent upon extensive international cooperation and resource mobilization. There has been a remarkable
level of cooperation between developing country governments and the international donor community. Large amounts of aid, now exceeding US$ 100 million annually, have flowed both through bilateral and multilateral channels. During the 1980s nearly US$ 500 million was contributed to immunization programmes through UNICEF. This assistance has in turn enabled the mobilization of substantial amounts of national resources for immunization. The effort has been supported by ongoing attention and mobilization in the international technical and political communities.

B. Future Directions

Although it is difficult to define precisely the contribution of immunization to "development", it has been that the EPI, particularly during the past decade, has made a wide range of specific contributions whose collective impact is substantial. These include direct impacts on mortality reduction, along with contributions to disease reduction and lowering of fertility rates; effects on the development of health system performance, infrastructure, and management capacity; and impacts on the relationship between health and social and political processes, leading to a much broadening of the base of support for health actions. In looking to the coming period of sustaining high coverage rates and reducing the disease burden, several trends may briefly be noted.

The scope of the EPI is being expanded. Technical developments, coupled with strategic support through efforts such as the Children's Vaccine Initiative, are leading to the improvement of existing vaccines and the development of new vaccines. The global EPI is increasing the range of suggested immunizations, while countries are entering into a process of diversifying their immunization schedules according specific needs and resource availability. These changes will require continued attention, technically, in terms of immunization policy, and at the level of health programme management.

The experience of the 1980s has begun a process of greatly expanding access to immunization and other health services, through strategies of infrastructure expansion as well as outreach. Management capacity has been strengthened to better enable adequate distribution of vaccines and the monitoring of population coverage and impacts. Additional efforts are needed to improve quality control and the efficient use of vaccines. In all of these areas, increased focus is needed in the planning process to ensure that adequate resources are available to meet coverage and disease reduction goals for the 1990s; management effort is needed to ensure cost-effectiveness; and monitoring systems must be further strengthened at the local and national levels.

Finally, the immunization is increasingly being used as a vehicle for increasing the range of health actions. EPI strategies themselves have evolved; in some countries there is greater integration of other interventions, such as micronutrients and CDD control, within structures developed for the EPI structures.
In ethoro tho EPI is itself being routinized within the services provided by fixed centers. The longer-term implications of this process include at least the need to consider immunization holistically within national health development processes, with supportive strategies and actions from the international community.
REFERENCES


FIGURES FOR THE PAPER

FIGURE 1: TAKE FIGURE 1 FROM UNICEF EXECUTIVE BOARD DOCUMENT E/ICEF/1991/L.8/ADD.1 (ADDENDUM), FIGURE ON PAGE 5.

FIGURE 2: TAKE FIGURE 3 FROM KIM-FARLEY ET AL. ARTICLE ON "GLOBAL IMMUNIZATION", ANNUAL REVIEW OF PUBLIC HEALTH (13) 1992 -- TERRELL HAS COPY -- FIGURE IS ON PAGE 226.

FIGURE 3: TAKE FIGURE 2 FROM KOENIG ET AL., BULLETIN OF THE WORLD HEALTH ORGANIZATION 68(4), 1990, PAGE 443 (IN LIBRARY). RERDRAW THIS IN HAKVARD GRAPHICS?
II. PROGRESS BY REGION

A. Asia

5. The goal of 80 per cent for each antigen was exceeded for the region as a whole, although 10 countries did not achieve this level. In 1990, the weighted average for all Asian countries was 94 per cent for BCG, 89 per cent for DPT3, 90 per cent for three doses of poliomyelitis vaccine and 86 per cent for measles. This was a dramatic increase over the 44 per cent for DPT3 and measles recorded in 1985.

East Asia and the Pacific

6. East Asia and the Pacific leads all other regions in coverage, with rates of greater than 90 per cent for all of the vaccines administered to children. This reflects a programme that is reaching 33 million of 36 million infants five times during their first year of life. Figure 2 below shows the coverage for DPT3 and measles for each of the countries, ranked by DPT3.
new and improved vaccines, delivery strategies, immunization schedules, and monitoring and evaluation methodologies (11) provide a technical basis for advancing program policies and strategies;

6. Emphasis on training in technical skills, planning, and management develop the needed human resources at senior, middle, and peripheral levels; and

7. Some diseases can be eradicated from the face of the earth; the cost of such an effort ultimately saves money by halting the need to immunize against the diseases or treat its victims.

The progress and lessons learned provide optimism that the new challenges set by the WHA for global immunization programs in the 1990s will be met.

GOALS

In May 1989, the Forty-Second World Health Assembly set the 1990s agenda for the EPI in resolution WHA42.32 (16). Six major challenges to be addressed during the decade were cited:

1. Achieving and sustaining in all countries full immunization coverage with all the vaccines used by the EPI;
2. Controlling the target diseases, including reduction of measles by 90% compared with pre-immunization levels by 1995, elimination of neonatal...
Table 2. Results of life-table analysis of mortality risks according to measles vaccination status; Matlab intervention area, 1982-85

<table>
<thead>
<tr>
<th>Time from vaccination at beginning of interval (months)</th>
<th>Non-vaccinated</th>
<th>Vaccinated</th>
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<td>0</td>
<td>8135</td>
<td>47</td>
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<tr>
<td>14</td>
<td>870</td>
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</tbody>
</table>

n = number of observations at beginning of interval; d = number of deaths during interval; w = number of withdrawals during interval; a = effective number of observations, and q = conditional probability of dying during interval.

The large number of withdrawals arose mainly because many children in the study were only followed for 12 months. For example, a child vaccinated in August 1984 could be followed for a maximum of 3 months, until October 1985, the end of the study.

Fig. 2. Cumulative risk of death by vaccination status, Matlab intervention area, 1982-85.

Potentially highly significant (Gehan-Wilcoxon test: \( \chi^2 = 4.18, 1 \) degree of freedom, \( P < 0.0001 \)). Although the overall mortality rate declined with increasing age, statistically significant lower mortality risks were still observed for children vaccinated both at 12-23 months and at 24-35 months of age (\( \chi^2 = 17.18, P < 0.0001 \), and \( \chi^2 = 11.16, P = 0.001 \), respectively). While a reduction in mortality risks was also observed for children vaccinated at 36 months of age or older, this was not statistically significant (\( \chi^2 = 2.57 \), not significant), probably because of the relatively few deaths among such children.

Potential selection bias in the study

Although vaccinees and nonvaccinees were randomly matched for age and survival status up to the time of vaccination, the possibility of selectivity among vaccinated children remains a potential source of bias. Children who received measles vaccine in blocks A and B could constitute a special group who may have had higher chances of survival, even if they had not been vaccinated. This possibility was considered from several different perspectives.

THE EFFECTIVENESS OF MEANS OF CONTROLLING COMMUNICABLE DISEASES

Kenneth S Warren*
Consulting Editor
Charles Scribner's Sons

Running head: Controlling communicable diseases.

* Mailing address:
125 Southlawn Ave
Dobbs Ferry, N.Y., 10522
U.S.A.
914 693 0350
Abstract

Most communicable diseases are caused by infectious agents which are not visible to the naked eye, leading to early beliefs in miasmas and control by quarantine. While microscopes revealed the agents in the 18th century, they weren't associated with disease syndromes until the late 19th century. Today, vaccines are the most cost/effective means of control.
Communicable Diseases

Communicable diseases are caused by infectious agents which are all clearly parasites, defined as organisms which live within or on the body of other organisms from which they take their food. Rudolph Hoepli's (6) classical study of parasitic infections in early medicine concluded, "Human parasites like human diseases are as old as the human race and petrifacts of very ancient, long extinct animals show that they also had parasites. Pre-historic man had in all likelihood a certain, although very limited, knowledge of a few ectoparasites and intestinal worms. This knowledge probably resembled that of certain primitive races of today." Most infectious agents including bacteria, viruses and protozoa are not visible to the naked eye. Even the helminths, the adult stages of which are all visible, were not associated with disease syndromes. The development of the microscope in the eighteenth century enabled the visualization of protozoa and bacteria, but they were not associated with disease until about 1880, Pasteur and Koch revealing the bacterial etiology of anthrax, cholera and tuberculosis, and Loesch and Laveran the protozoan etiology of amebiasis and malaria. The development of fine porcelain filters at the turn of the century enabled the discovery of viruses, but the field didn't burgeon until the 1930s.
At the beginning of the 20th century bacteria were called vegetable parasites, and protozoa and helminths animal parasites, not in relation to their hosts, but themselves. Recently, James Watson has described the viruses as molecular parasites. In the early part of this century there was an unfortunate division of the parasitic infectious agents into two different biological and medical fields, microbiology which included bacteria and viruses, and parasitology comprising protozoa and helminths. In a study of the emergence and early development of parasitology, Worboys (24) claimed that it "provided the zoological underpinning for tropical medicine. Its subject matter was defined on the one hand by the tropical medicine curriculum and on the other by the botany-zoology division." At the inauguration of the London School of Tropical Medicine in 1899 Sir Patrick Manson (8) stated, "Today the protozoan and the helminth, as regards tropical pathology, are in the ascendent. In this school, although the bacterium will not be neglected, necessarily a large share of your time will be occupied with animal parasites, a subject which I fear has not been sufficiently studied hitherto in our medical schools."

This dichotomy between microbiology and parasitology has had broad and unfortunate consequences. The bacteria and viruses of microbiology were accorded particular importance in the developed world of the North, while the protozoa and helminths of parasitology appeared to be the major problems in the developing world of the South. Microbiology was sited in the medical schools
where, in the United States, infectious diseases evolved as a major clinical subspecialty, while parasitology was located in the schools of public health in the United States, and in schools and institutes of tropical medicine in Europe. Microbiology spawned the basic science disciplines of immunology and molecular biology and powerful means of treatment (antibiotics) and prevention (vaccines), while the major accomplishments of parasitology were insecticides and molluscicides (17).

An important new development has been the redefinition of infectious diseases by Anderson and May (2) into those caused by microparasites and macroparasites:

"Microparasites [bacteria, viruses and protozoa] may be thought of as those parasites which have direct reproduction - usually at very high rates - within the host. They tend to be characterized by small size and a short generation time. Hosts that recover from infection usually acquire immunity against reinfection ... and the duration of infection is typically short relative to the expected life span of the host."

"Macroparasites [helminths and arthropods] may be thought of as those having no direct reproduction within the definitive host. They are typically larger and have much
longer generation times than microparasites, with the
generation time often being an appreciable fraction of
the host life span. ... Thus macroparasitic infections
are typically of a persistent nature, with hosts being
continually reinfected."

This is of particular importance because the fact that the
macroparasitic helminths do not replicate directly within their
human definitive hosts not only has remarkable clinical
consequences, but results in very different strategies for both
treatment and control. In an editorial entitled "The guerrilla
worm" (16) it was noted that helminths "follow the precepts of
guerrilla warfare as outlined by Chairman Mao, repeatedly
infiltrating host defenses as individuals or in small groups and
gradually building into large forces; warfare is usually by
attrition and tends to be prolonged." Clinically, "Infection is
not synonymous with disease. Many patients invaded by
schistosomes, trichinellae, ascarides, filariae and hookworms never
had and never will have overt signs or symptoms of disease. The
respective manifestations caused by these worms - liver fibrosis,
myositis, intestinal obstruction, elephantiasis, and anemia - occur
only when there is an unusually heavy attacking force or when large
numbers of parasites have accumulated." With respect to treatment
it is not necessary to eradicate the invaders since the few
organisms remaining after therapy can only be increased by further
infiltration which tends to occur slowly. Furthermore, there is an
overdispersed distribution of the worms within their hosts with only about 10% of human populations having heavy, disease producing worm burdens. Thus, while vaccines are clearly the method of choice for controlling rapidly reproducing microparasites, chemoprophylaxis with the new single-dose, oral, broad spectrum, non-toxic anthelmintics is a most acceptable alternative for helminth infections (21). 

The overweening association of parasitic diseases with the tropics has also had a negative effect with respect to the proper allocation of resources to deal with infectious diseases in the South. Hookworm was targeted by the Rockefeller Foundation at its inception in 1913 as the first major disease to be eradicated from the face of the Earth. The campaign was carried out in "52 countries, 6 continents and 29 islands of the seas" and involved sanitation, shoes, and treatment with drugs such as carbon tetrachloride. Much was learned from this complex campaign, but it failed to achieve its goal; there are still 900,000,000 cases of hookworm in the world today. After the second World War, with the development of highly effective anti-malarial drugs such as chloroquine and insecticides such as DDT, the World Health Organization (WHO) embarked on a campaign to eradicate malaria; it lasted from 1955 to 1970 when WHO "threw in the towel" because of the development of resistance to both the drugs and insecticides. Global eradication of a major communicable disease was finally achieved in 1980 using a simple measure discovered in 1780. The
disease was smallpox, it was caused by a virus, and it was eradicated by a vaccine.

It is of particular interest, therefore, that it was not until 1979, when the audacious smallpox eradication campaign was approaching its successful conclusion, that bacterial and viral diseases were revealed to be of overwhelmingly greater importance in the tropics than protozoan and helminth infections (Table 1) (15). Using data from an update published in 1988 (14) the 10 principal causes of mortality in the developing world was compared to that of the 10 principal parasitic infections; the former rate totalled 32 million per year of which about 60% was due to bacterial and viral agents; in contrast protozoa and helminths were responsible for a total of 2.4 million deaths per year (20).

Tropical Medicine/Tropical Health

At the height of the colonial era in the late 18th century the field of tropical medicine was invented by an Englishman, Sir Patrick Manson. As mentioned above Manson played the major role in the parasitization of tropical medicine, but he also put an emphasis on science as the primary approach to dealing with the immense problems of health in the tropics. "I now firmly believe in the possibility of tropical colonization by the white races. Heat and moisture are not in themselves the direct causes of any important tropical disease. The direct cause of 99% of these
diseases are germs. To kill them is simply a matter of knowledge (7)." Ronald Ross, the Nobel laureate who discovered the mosquito transmission of malaria, was initially a protege of Manson. His emphasis, however, was on public health approaches to disease control - "Do not think that when you have made your discovery, great or small, you have finished the matter. Medical research is not a mere academic amusement consisting in the publication of elegant articles adorned with colored plates. The discovery is only half-way up the mountain, and beyond it extends the arduous summit of the practical application (11)." Ross, therefore, hied off to Sierra Leone to improve sanitary conditions, attack mosquitoes, remove garbage, provide piped water, develop sewerage systems, drain ponds, and clear undergrowth. The different emphases of Manson and Ross led to a polarization between the science of tropical medicine and "the practical application" of tropical health which has continued to the present day (20).

Disillusionment with the failure of WHO's massive effort to eradicate malaria led to a revulsion towards "the technocentric approach to health (3)." This was reinforced by the ideas of Thomas McKeown, Professor of Social Medicine in Birmingham, UK, who, in his influential monograph The Modern Rise of Population (9), claimed that:

* the increase of population is due not to an increase in fertility but to a decline in mortality due to a
reduction of deaths from infectious diseases.

* since immunization and therapy had little or no effect
the explanation for the decline in mortality was
improvement in the environment, particularly the
availability of greater food supplies.

McKeown then concluded that "Medical measures of immunization
and treatment were relatively ineffective; they were also
unnecessary." Unfortunately, he extrapolated the results of
historical data from the North to the problems and control
strategies of the South of today (22).

The Expanded Programme in Immunization

The unprecedented success of the smallpox eradication campaign
clearly validated the value of immunization. This led the WHO to
initiate the Expanded Programme on Immunization (EPI) in 1974 (5). From the time of Jenner's discovery of the smallpox vaccine in the 18th century through the late 1980s less than 20 vaccines had been
developed against major infectious diseases (Figure 1) (18). Of these, 6 were adopted by EPI for dissemination throughout the
developing world - tuberculosis (BCG), diphtheria, pertussis, tetanus, measles and polio. With these alone, UNICEF has estimated
that several million deaths could be averted annually. While EPI
had done an outstanding job in providing the basic underpinning for
campaigns to immunize all of the world's children it took the "Children's Revolution" (19) and a consortium of five agencies, WHO, UNICEF, the United Nations Development Programme (UNDP), the World Bank, and the Rockefeller Foundation, coordinated by the Task Force for Child Survival, to take the global immunization level from 15% in 1984 to 80% in 1990. The remarkable support by Rotary International for polio immunization has provided the impetus for the next campaign of eradication; poliomyelitis has now been virtually eliminated in the western hemisphere and work is well underway in the eastern half of this globe.

Several of the EPI 6 are undergoing improvement: tuberculosis because of erratic effectiveness (10), pertussis because of potentially severe side effects, and measles for ineffectiveness below nine months of age. Other vaccines have not been added to EPI because of side effects and relative ineffectiveness, e.g. typhoid and cholera; ineffectiveness below the age of 2 years, pneumococcus; localized distribution, yellow fever and meningococcus; presumed relative unimportance, mumps and rubella; and new vaccines, hepatitis B and H. influenzae b. The use of the hepatitis B vaccine, the first with a major anti-cancer (hepato-cellular carcinoma) activity, is being promoted in Asia and Africa via a remarkable campaign led by the Program for Applied Technology for Health (PATH) with major support from the James McDonnell Foundation. The addition of both new vaccines to the EPI 6 is being planned. The positive outcome of a recent controlled trial
of a formalin-inactivated hepatitis A vaccine which was "well tolerated", and with a single dose was "highly protective against clinically apparent hepatitis A" (23), is a major new development.

A recent compilation of estimates from WHO and UNICEF by the Task Force for Child Survival has revealed that only 21% (2.82 million) of the approximately 15 million deaths in children under five can be prevented with our present vaccines, while 43% (6.2 million) die from diarrheal diseases and acute respiratory infections, for which there are presently no effective vaccines (12,13). One million children die from malaria, and, as mentioned above, there are no vaccines available for any of the human infections caused by the protozoan and helminth parasites. Another 4.2 million deaths are listed as "other." It is clear, therefore, that the development of new vaccines can more than triple the reduction in child mortality achieved by the present EPI vaccines, while also reducing morbidity and increasing growth and development.

The Biotechnology Revolution

The opportunity to both improve old vaccines and develop new ones has been greatly enhanced by the development of the science of molecular biology and its manipulation through genetic engineering (18,4). Ada (1) has recently described the variety of means by which new and more specific vaccine can be produced:
1) the synthesis of oligopeptides representing particular epitopes of antigens; 2) the production of anti-idiotypes; 3) the transfection of cells with DNA coding for protective antigens; and 4) the use of live agents, viruses or bacteria, as vectors for such DNA.

Oligopeptides are small bits of much larger proteins that can be derived from the active areas that stimulate protective immune responses; they can easily be manufactured chemically, in large quantities. The anti-idiotypes that are stimulated by the unique structure of the protective antibodies themselves, may function as antigens for highly specific vaccines. Rapidly replicating cells, bacterial, yeast or mammalian, into which a gene (DNA) for protective antigens has been inserted multiply into massive numbers of individual vaccine factories; this technique led to the first genetically engineered vaccine, hepatitis B. Genes can also be inserted into living viruses, such as vaccinia (smallpox vaccine) and bacteria such as BCG (tuberculosis vaccine) or Salmonella (typhoid vaccine) to immunize against a wide variety of other infectious diseases. Furthermore, a great variety of adjuvant systems are being developed to stimulate the response of the immune system to all of the types of vaccines described above.

The Children's Vaccine

All of these scientific developments and the great global
programs to apply them have led to the so-called Declaration of New York of 10 September 1990 which announced the Children's Vaccine Initiative. "Universal immunization will be facilitated by accelerating the application of current science to make new and better vaccines, benefitting children in all countries. These include vaccines which:

1) require only one or two doses; 2) can be given earlier in life; 3) can be combined; 4) are more heat stable; 4) are effective against the major causes of child mortality for which vaccines are not currently available and 4) are affordable.

To conclude with the words of Geoffrey Edsall, "Never in the history of human progress has a better and cheaper method of preventing illness been developed than immunization at its best (19)." The Biotechnology Revolution should give us the tools to provide immunization at its best, and the Children's Revolution resulting in "universal childhood immunization" has given us the means to rapidly and effectively apply these remarkable new tools for the well-being of children throughout the world.
References


Table 1. Prevalence, Mortality and Morbidity of the Major Infectious Diseases of Africa, Asia and Latin America, 1977-1978.*

<table>
<thead>
<tr>
<th>Infection</th>
<th>Infections (Thousands/Yr)</th>
<th>Deaths (Thousands/Yr)</th>
<th>Cases/Yr</th>
<th>Disease (Thousands of cases/Yr)</th>
<th>Average No. of Days of Life Lost (per case)</th>
<th>Relative Personal Disability</th>
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<tr>
<td>Diarrheas</td>
<td>3-5,000,000</td>
<td>5-10,000</td>
<td></td>
<td>3-5,000,000</td>
<td>3-5</td>
<td>2</td>
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<tr>
<td>Respiratory infections</td>
<td>4-5000</td>
<td>800,000</td>
<td>1,200</td>
<td>200,000</td>
<td>10-14</td>
<td>2</td>
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<tr>
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<td>85,000</td>
<td>80,000</td>
<td>500-1000</td>
<td>20,000</td>
<td>600-1000</td>
<td>3-4</td>
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<td>Measles</td>
<td>120-150</td>
<td>250-450</td>
<td>1,000</td>
<td>7000</td>
<td>21-28</td>
<td>2</td>
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<tr>
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<td>120-150</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Neonatal tetanus</td>
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<td></td>
<td></td>
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<td>Malaria</td>
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<tr>
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<td>Low</td>
<td>2-3000</td>
<td>100</td>
<td>7-10</td>
<td>3</td>
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<td>Low</td>
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<td>Very low</td>
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<td>5-800,000</td>
<td>Low</td>
<td>2000</td>
<td></td>
<td></td>
<td></td>
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</table>

*Based on estimates from the World Health Organization and its Special Programme for Research and Training in Tropical Diseases, confirmed or modified by extrapolations from published epidemiologic studies performed in well defined populations (see references). Figures do not always match those officially reported, because under-reporting is great.

11 denotes bedridden, 2 able to function on own to some extent, 3 ambulatory, & 4 minor.
Titles For Figures

Figure 1  - Vaccines since Jenner
H. influenzae b
Hepatitis B
Pneumococcus
Meningococcus
Rubella
Mumps
Measles
Polio (Sabin)
Polio (Salk)
Yellow Fever
Influenza
Pertussis
Cholera
Tetanus
Tuberculosis
Diphtheria
Typhoid
Smallpox
Rabies
TRANSFER OF VACCINE TECHNOLOGY TO DEVELOPING COUNTRIES
THE LATIN AMERICAN EXPERIENCE

Dr. Akira Homma, Regional Advisor in Biologies
Dr. Robert F. Knouss, Deputy Director
The Pan American Health Organization
525 Twenty-third Street, N.W.
Washington, D.C. 20037
Tel.: 202-861-4304
Fax: 202-861-8472
The transfer of the technology of vaccine production has been taking place over the last five decades in some developing countries, and has made possible their participation in the production and supply of essential biologicals required for immunization programs. Examples of successful transfer of technology, the decisive elements and factors contributing to the transfer, as well as the major obstacles that have been faced are analyzed. The technological advances currently being made by developed countries will result in safer, more potent vaccines. In order to facilitate development and production of a new generation of vaccines in Latin America and the Caribbean, the Pan American Health Organization launched, in 1991, a Regional Vaccine System (SIREVA). This project encompasses all the steps required for vaccine development, such as epidemiological surveillance, research, pilot plant production, quality control, field trials and transfer of technology, and takes into account the existing niches of scientific and technological capabilities in the Region.
TRANSFER OF VACCINE TECHNOLOGY TO DEVELOPING COUNTRIES

THE LATIN AMERICAN EXPERIENCE

The World Health Organization estimates that five million children die each year in the developing world from infectious diseases, many of which would be preventable with the timely administration of existing vaccines. Through the expanded program on immunization (EPI), affordable vaccines against diphtheria, tetanus, pertussis, measles, poliomyelitis and tuberculosis are being delivered to the vast majority of the world's children. Although the successes of the EPI have reduced the risk of contracting the target diseases, many other infectious diseases, potentially preventable if effective vaccines existed, remain a serious threat to the people, and most particularly the children, of the less economically advanced countries. Unfortunately, most of the technology to develop new vaccines exists only in the developed world as does a large part of the installed capacity to produce vaccines that meet internationally accepted quality standards.

The needs for universal coverage underscore the importance of improved planning of vaccination programs and of increased production capacity, especially in close proximity to third world markets. Countries with emerging economies, such as Mexico, Brazil, China, India, and others, are already investing in their
own production capacity to meet national and, in some instances, export requirements. According to one World Health Organization estimate (Dr. P. Evans, Expanded Program on Immunization), sixty percent (60%) of the world's requirements for tetanus toxoid vaccines are now being supplied by producers in the Third World, although some products do not consistently meet acceptable standards for efficacy. Thus, greater attention must be paid by some of these laboratories to the adoption of international standards on Good Manufacturing Practices, systems for quality control and assurance, and facility modernization. Facilitating the transfer of modern production technology would enhance the role these producers could fulfill in meeting global requirements for vaccines and could result in spinning-off related technologies.¹

I. Historical Notes on Vaccine Production in Latin America

Historically, several public health and biomedical research institutions in Latin America, founded around the turn of the century, contributed enormously to the development of public health in general and to the development of vaccines and other biologicals in particular. European and North American trained scientists carried out research as well as supervised production of biologicals such as anti-venom, anti-diphtheria, anti-tetanus and anti-rabies sera, as well as vaccines for example, against yellow fever.
Reputations were frequently established because of the leadership provided by strong individual scientists and their success in contributing to significant disease control efforts. However, some of these laboratories and institutes gradually declined as governments were hard pressed to make the financial investment required, as political interest in continued disease control activities waned, and as existing scientific leadership was not replenished. At the same time, during the 1960's and 1970's, the industrial world was investing mainly in the development and production of new vaccines, modernization of production facilities and development of new production technologies, especially fermentation processes.

In the past, international producers of human vaccines and biologicals established production facilities in Latin America, taking advantage of low wages to manufacture products at internationally competitive prices. All have since ceased their operation. Government interest in supporting public research laboratories has also declined. Diminished budgets have resulted in lack of facility and equipment modernization, shortages of supplies, and insufficient salaries to encourage and attract promising scientists from the Region.

When the EPI was launched in the mid 1970's, almost all of the laboratories in Latin America (most of them public) were using obsolete technologies and hence were not able to meet the new
demand created by the intensification of vaccination programs. The establishment of a vaccine purchase revolving fund by PAHO benefited most Latin American and Caribbean countries by providing them with good quality vaccines at low prices. However, the small vaccine producers in the Region could not compete in providing vaccines of high quality at prices offered by larger manufacturers. Only a few countries, thus, have remained self-sufficient for one or more of the EPI vaccines.

The majority of producers of human vaccines in Latin America are public institutions. Several reasons might be cited for the limited role of private production:

1. Vaccine production requires investment in complex technology yet yields only marginal profits;

2. National governments are the principal purchasers of vaccines for free distribution through national programs. Direct purchase of vaccines, as with other public procurement, has been troubled by payment delays and sometimes cumbersome government procurement procedures.

3. The PAHO vaccine revolving fund was able to obtain more favorable prices through competitive global markets. Additionally, both private and public producers have had to face other difficulties such as the lack of national public
policy favorable to vaccine production, the risk of changing policies with changing government administrations, a relatively low institutional priority assigned to development and maintenance of in-country production capacity, and diminished national experience in vaccine production. Following years of declining investment, production technology becomes obsolete and requirements to maintain good manufacturing practices are by-passed for economic, if not technical, reasons.

Notwithstanding these trends, several countries in the Region are deciding that human vaccine production merits national investment in laboratory and production modernization. Mexico and Brazil foresee national self-sufficiency in human vaccine production, especially for EPI. The size of their populations have made this investment economically justifiable. Export capacity may eventually be achieved. Cuba has decided that international marketing of vaccines and other biologicals can be a significant source of export revenue and has invested substantial sums in vaccine development, production and distribution. Venezuela is investing in production facilities and is discussing the organization of a consortium for vaccine production with its Andean counterparts, utilizing a public/private mix of funds. Meanwhile, international vaccine producers are expressing renewed interest in investing in joint vaccine production ventures in Latin America.
given the improving economic situation and the heightened level of political interest in disease control.

II. The Successful Transfer of Vaccine Technology with Latin America

For successful transfer of vaccine technology to take place, several important preconditions must be met: 1) the vaccine must be of high public health importance; 2) a local or regional demand must exist; 3) supplies must be readily accessible; 4) a scientific-technical infrastructure capable of absorbing, adapting, and improving technology must exist; 5) government must be politically and financially supportive; and 6) local, national or regional institutions should be involved in the process. Following the experience of the last five decades, one can find various examples of the successful transfer of vaccine technology in Latin America. In some instances, the technology was developed outside the Region and transferred into it; in other instances, it was developed within the Region and transferred to other regional countries. The examples provided below are not intended to be exhaustive, only illustrative.

An excellent example of the transfer of technology from a developed country to Latin America is the production of a yellow fever vaccine. Urban yellow fever was one of the most important tropical diseases in the Region at the beginning of the century,
and was eradicated from cities by the control of the A. aegypti vector. However, due to the existence of sylvatic yellow fever, the development of an effective vaccine was critically important for protecting populations in rural areas where the vector could not be controlled and epidemics still occurred.

Yellow fever vaccine, strain 17D, was developed by Theiler and Smith in 1936 at the laboratories of the Rockefeller Foundation in New York. The entire existing supply (200 doses) was brought by Dr. H. H. Smith to Brazil for testing in 1937. There, Rockefeller and Brazilian experts established yellow fever vaccine production facilities which, following successful field trials, began producing thousands of doses per month. By the end of 1938, over 100,000 doses of yellow fever vaccine had been administered. Since then, some important technological improvements have been incorporated into the production procedure. For the first time, in a vaccine production, the seed-lot system was introduced by which it was possible to maintain the same virus passage level for a given period, hence avoiding the possibility of changing viral genetics. Initial problems related to standardization of vaccine production and thermostabilization were overcome.

Since then, modern production facilities, utilizing specific pathogen-free embryonated egg techniques have been introduced and continue to yield high quality, thermostable vaccines. More than 100 million doses of the Brazilian yellow fever vaccine have been
administered while less than 20 cases of serious neurological sequelae have been registered.

In 1980 a follow-up study in the interior of the State of Minas Gerais in Brazil found that 80% of those vaccinated 45 years earlier were still protected against yellow fever. Today, Brazil has the capacity to produce up to 50 million doses of yellow fever vaccine each year, enough to meet regional needs as well as to supply other continents, if required.

Yellow fever vaccine technology was successfully transferred to Brazil because the prerequisites cited at the beginning of this section were met. In addition, the potential for success of this technology transfer was strengthened by the organizational capacity, work discipline, and dedication of the Rockefeller Foundation, as well as by the technical, scientific, and managerial skills of the leaders of the laboratory. Through the years, the three directors of the yellow fever laboratory have been able to continue to motivate the technical personnel and maintain a low turnover rate.

Furthermore, the high quality of the 17D seed virus and the use of relatively simple technology to produce a vaccine from local supplies that confers very high and long lasting protection has yielded sustained production of an effective vaccine. Production has been maintained by a cadre of specialized technical personnel.
This success has stimulated interest in the field of vaccine development and production as a whole.

Other examples of successful vaccine technology transfer have been the transfer, in 1976, of a polysaccharide *N. meningitidis*, serogroup A and C vaccine from the Mérieux Institute and the transfer, in 1980, of measles vaccine, strain CAM-70, from the Biken Laboratory of the Osaka University to the Oswaldo Cruz Foundation.8

In the first case, the decision to initiate vaccine production in Brazil was stimulated by epidemics of *N. meningitidis* in 1971 and the following four years. While the first epidemic of serogroup C was still in progress, another epidemic with serogroup A occurred. At its height, the incidence of C serogroup meningitis reached 50 cases per 100,000 and of A serogroup 500 cases per 100,000, resulting in a high mortality rate. These epidemics had an enormous impact on the population and created public demand for action.

At the time those epidemics occurred, the federal government had already launched a national policy for the strengthening of scientific and technological institutions, and the Oswaldo Cruz Foundation was considered a priority. This fact and the possibility of incorporating the same type of fermentation technology used for production of an *N. meningitidis* polysaccharide vaccine for the
production of other bacterial vaccines contributed strongly to the decision in 1976 to begin production of the *N. meningitidis* vaccine, the first human bacterial vaccine to be produced by fermentation technology in the country.

Measles vaccine production was established during the same period. The existence of a virology infrastructure as well as trained professionals enormously facilitated the organization and implementation of measles vaccine production in the country.

Although the transfer of technology for measles vaccine began under the auspices of a large European private manufacturer, it was switched during the process of implementation to the Biken Laboratory of Osaka University because of a more favorable agreement reached with the Japanese Government for the transfer of technology for the complete cycle of production. Under the umbrella of technical cooperation, the Japanese Government decided to support and finance the transfer of technology based on the following elements: a) measles was an important public health problem; b) both a well-defined technology and a Japanese institution willing to collaborate existed; c) the receptor government was strongly committed to the project; and d) an institution with appropriately trained professionals was prepared to carry out production.
Examples of the transfer of production technology from one developing country to another also exist. If vaccine development requirements can be met and official institutions approve, technology transfer can be encouraged as a means to lessen dependency on external sources of supplies and materials.

Possibly the most important example of intraregional vaccine technology transfer is that of rabies vaccine. A vaccine against rabies, using an inactivated virus produced in the cerebral tissue of newborn mice, was developed in 1954 by Fuenzalida and Palacios at the Bacteriologic Institute of Chile for human and canine use. The Pan American Health Organization (PAHO), through a center located in Argentina, played a significant role in assisting in the standardization of production and quality control procedures and in facilitating the transfer of the technology to countries willing to initiate production. Eleven countries in the Americas are still producing the vaccine using the Fuenzalida and Palacios technology. This successful technology transfer was possible largely because there was no requirement for sophisticated (i.e., expensive) equipment nor for the importation of production supplies.

In the intervening years since its initial development, production and quality control procedures have been improved until now there are very few reports of adverse reactions largely due to the strict use of newborn, instead of suckling, mice as the primary
source of vaccine virus. This vaccine product is now invariably safe, efficacious and relatively inexpensive. Use of this vaccine has aided in the control and even elimination of rabies among canine populations in several areas of the hemisphere.

At the beginning of the twentieth century, Dr. Vital Brazil, at the Butantan Institute, São Paulo, developed an efficient production technique for heterologous anti-snake venom serum. Since the introduction of this biological, the Institute has transferred the technology to many other countries in the Region where it is still in use.

The transfer of technology for the production of diphtheria, pertussis, tetanus, and BCG vaccines has been made through public institutions and has not necessitated special agreements to protect intellectual property or patent rights.

In the near future, the technology to produce the new vaccine against Argentine hemorrhagic fever (AHF) virus is expected to be transferred from the United States of America to Argentina. If successful, this transfer will open new opportunities for establishing the production of other vaccines using similar technologies. In recent years, the Argentinean government has moved toward self-sufficiency in vaccines. The AHF virus vaccine will also enable them to produce measles vaccine in the same plant,
using the same infrastructure, thereby minimizing production costs.

III. Problems Related to Vaccine Technology Transfer

Technology transfer has been recognized as an essential tool for enhancing technological capability and capacity, especially in developing countries.2,3,4,10,15

As noted in the previous section, the transfer of vaccine production technology has been occurring for the last twenty years or more (almost fifty-five years in the case of yellow fever vaccine) until now a few countries are self-sufficient for the EPI vaccines. Yet problems remain in the Region.

In some instances, the transfer of production technology has been a one-time event. As technology has continued to advance in more economically advantaged countries, it has remained fixed in developing ones. Vaccine products from outdated technology are less competitive on world or even national markets and the advantages from the investment in the initial transfer can eventually be lost or at least minimized. For example, Brazil was the recipient of second-generation measles vaccine production technology from Japan. Since 1980, Brazil has produced measles vaccine for its national EPI use. In the interim, however, measles vaccine technology has progressed through a third into a fourth
generation, leaving further development in Brazil an unanswered question. Recently, Brazil had to enter international markets to procure enough vaccine to launch a national campaign which has as its ultimate objective the elimination of the disease.

Local production of vaccines for national use requires adherence to rigorous quality control to assure the safety and efficacy of the products. In the campaign to eradicate the transmission of wild poliovirus in the Americas, Type III cases were still being found in previously vaccinated populations in a Latin American country. Epidemiologic investigation led to examination of their national production. What was eventually learned was that the concentration of type III virus in the vaccine was approximately one hundredth of the desired level. A comprehensive quality control system, rigorously applied, will now detect this type of production deficiency prior to the occurrence of failures in protective immunity in the population. Other examples of inadequate quality control can be cited such as that which led to the decertification of Colombian yellow fever vaccine.

Quality control is not an issue just for vaccine production. The field testing of candidate vaccines requires that internationally accepted standards are applied in order to assure the safety of test participants and the acceptability of test results. Inexperience in the design of clinical and field trials has led to skepticism as to the validity of the trial as well as
the need to repeat work, leading to unnecessary delays in bringing potentially sound vaccines to production, as in the case of a new malaria vaccine developed in Colombia. Inexperience in designing field trials has also led to criticism about the adequacy of human subject safeguards. Uncertainty about ethical review procedures has, at times, delayed the initiation of clinical trials while detected deficiencies in the protection of human subjects has required informed consent procedures to be redesigned. The premature breaking of trial codes can also jeopardize rigorous scientific analysis of trial results as occurred in rotavirus vaccine trials in Peru. Hopefully, training matched with more supervised field experience will overcome reluctance by vaccine developers to participate in such studies and will lead to more reliable and acceptable results from these expensive trials.

Even when well designed field trials have been initiated, the outcome may be far from certain. Misdirected competition among vaccine laboratories can lead to unexpected conflict among various commercial interests. A planned field trial of a South Korean produced hepatitis B vaccine, just as it was to be started in a Caribbean country, had to be canceled after competitive manufacturers brought political pressure on the Government to stop the activity. Although the issue was framed as a question about the quality of the vaccine to be tested, at stake were the markets for their own products.
For vaccines that have been developed in third world countries where adequate protection of intellectual property is still in its formative stage, developing world investigators have been reluctant to divulge the formulation of their vaccine in order to protect their own scientific and commercial interests. On the other hand, first world vaccine firms have been reluctant to manufacture new vaccines in these countries because of fear of inadequate protection of intellectual property and patent rights.

IV. Intellectual Property and the Transfer of Technology

The average period required for new vaccine development is eight to ten years. Because the long term investment is high risk (a marketable product may not result) and usually requires the application of expensive technology, most vaccine development is being done in research laboratories in the developed world. Once developed, production of a new vaccine often requires overcoming substantial patent barriers. An example of how complex arrangements can be is the production of a newly developed recombinant hepatitis B vaccine manufactured by a well known European vaccine producer which had to assemble fourteen different patents to be able to produce the vaccine. Thus the global strategy for the development of new vaccines will also depend on the development of policies for the transfer of technology, the protection of confidentiality and the management of patents. A system for the rapid negotiation of the transfer of intellectual property while protecting such property rights is urgently needed.
The marketplace requires strong multilateral rules that can be uniformly applied and enforced. These include common standards for protecting intellectual property, a compatible international (rather than national) patent system and a mechanism for the timely resolution of disputes. Without the development of such a system, artificial barriers will remain a serious impediment to efficient technology transfer and vaccine development.

Several Latin American countries are now in the process of developing or revising intellectual property and patent legislation with a view to encouraging technology transfer and commercial development.

Patent protection is not the only barrier to technology transfer. Successful transfers depend on governmental commitment, the availability of appropriately trained and experienced personnel, sufficient financial resources, and a capacity to incorporate the technology to be transferred. In the case of vaccine production technology, several conditions must be met, including strong quality control and quality assurance mechanisms which must be put in place. For developing countries, even the importation and maintenance of equipment and supplies requires careful planning and government support to overcome usual bureaucratic delays.
Finally, production must be at industrial level to achieve economies of scale and to obtain a consistent product of acceptable quality at a price competitive with similar products produced elsewhere. Part of the challenge is continued technological innovation to remain competitive and to avoid obsolescence.

V. The Latin American and Caribbean System for Vaccines

Various global efforts, such as WHO's Program on Vaccine Development and more recently, the Children's Vaccine Initiative, provide a stimulus for increasing activity in vaccine development, much of which will address diseases which are most prevalent in developing countries. This effort holds enormous promise for communicable disease control on a global level and has the capacity to stimulate new investment in vaccine development, testing, production and marketing. Realistically, most of the capacity to respond to these challenges rests in Europe, Japan and North America, in established laboratories and enterprises where the scientific, technological, financial and marketing resources already exist. Although highly skilled scientists continue to be productive under difficult circumstances in the developing world, they are limited in number, work with minimum, unpredictable resources, and do not have support from the required, sophisticated systems to bring their discoveries from the bench to the market. Moreover, competitive advantage of their colleagues from developed countries for available financing is so strong that they risk
remaining dependent on continued technology transfer instead of
being able to concentrate on contributing independently to the
science of vaccine development. Until a system is developed that
can strengthen the scientific and technological capacity of the
Third World, these research scientists will not be able to be
active participants in the vaccine development process and
developing regions will at best be merely passive beneficiaries
from the sale and distribution of the final products developed
elsewhere. Even national and local vaccine production capacity can
become rapidly outdated or yield suboptimal products because of the
lack of adequate quality controls sustained by a vigorous
scientific endeavor.

To overcome the institutional, technological and economic
barriers to full participation in vaccine development and to
unleash the scientific potential of Latin America and the
Caribbean, the Pan American Health Organization, Regional Office
for the Americas of the World Health Organization, has initiated
development of a Regional System for Vaccines (SIREVA).5,14 Through
this system, investment can be attracted to support the required
epidemiological studies, laboratory research, pilot plant
production, and field testing of new vaccines designed to prevent
and control selected diseases important in the Americas.

The objectives of the system are to promote the adequate
transfer of technology and to develop and strengthen national and
Regional capabilities: 1) for identifying, developing, and evaluating immunizing agents for the prevention and control of communicable diseases of public health importance in the Region and 2) for improving existing vaccines and fostering their use in these countries. To achieve these objectives, SIREVA is intended as a system that can support the epidemiological, biomedical and operational research necessary to develop new or improved vaccines that can coordinate and integrate basic and applied research for vaccine development to be conducted by institutions and groups within the Region; and contribute to the development of the scientific and technical human resources needed for effective vaccine research, development, and production.

During the first three years, it is envisioned that a) the systems for epidemiological surveillance and the programs for epidemiological research and development of selected vaccines will be initiated and organized; b) the infrastructure of laboratories and pilot plants will be strengthened and the affiliated laboratories involved in research and development will be identified; and c) systems for quality control, information, technology transfer and human resource development will be designed and implemented. During subsequent years, candidate vaccines will be evaluated for tolerance, antigenicity, immunogenicity, and safety by means of small and large scale clinical and field trials. Thereafter, as production is scaled up, the vaccines should be available for use, especially by the EPI.
During the conceptualization of SIREVA, a decision was made to focus efforts on the development of a few vaccines which were of epidemiological importance to the Region and for which laboratories in the Region had already been actively working. The target diseases appear in global disease control priority lists such as those developed by the Institute of Medicine of the United States National Academy of Science\textsuperscript{11} and the World Health Organization\textsuperscript{18}. From the ensuing analysis, initially selected vaccines were those against \textit{S. pneumoniae}, \textit{S. typhi}, and \textit{N. meningitidis}, serogroup B. To assure the strengthening of regional capabilities and the coordination and integration of SIREVA projects into an ongoing regional system, the target diseases were also selected to build on the capacity already well routed in the Region. The shared characteristics of these bacterial diseases and of the promising approach of conjugate vaccines offer significant advantages to regional scientific development and to creation of a system to promote and sustain vaccine development while making the ultimate products available to the benefit of all countries in the Region. The same infrastructure can be used to develop and produce test batches of all three bacterial vaccines for field evaluation.

Subsequent to the selection of these diseases for vaccine development, opportunities arose for the inclusion of dengue and \textit{V. cholerae} vaccines in the list of diseases for which vaccine development would be incorporated in the system.
All the diseases to date offer opportunities for creation of related parts of the envisioned system, accompanied by transfer of technology, as required. With the support of the Canadian International Development Agency (CIDA), epidemiologic studies to map the prevalence of the most pervasive serotypes of *S. pneumoniae* are being launched. This information will be provided to laboratories already developing new vaccine candidates which ultimately will be tested in field trials which can be conducted in the Americas. A new inactivated oral cholera vaccine is being tested in field trials with the support of the Swedish International Development Agency (SIDA). Collaboration has already been provided by several agencies in the United States Government for the development of vaccines against typhoid fever (with Mexican laboratories), meningococcal B caused meningitis and dengue fever (with Brazilian laboratories).

The feasibility of organizing SIREVA has been analyzed with support from the Rockefeller Foundation, the Government of Mexico, the Inter-American Development Bank, International Development Research Center (Canada), and the Pan American Health Organization itself. The study concluded that establishing the system was politically, economically, scientifically and administratively feasible. Even though the cost for developing and implementing SIREVA over the first ten years is estimated to be around $143 million, detailed studies have found that the proposed approach is cost-beneficial. One third of these costs relate to investment in
technology transfer, capacity building and technical cooperation; the remaining cost relate directly to vaccine research development and testing.

Centers to be established in Mexico and Brazil will coordinate a network of laboratories in Latin America and the Caribbean, develop training opportunities, mobilize resources to support the development of the vaccines incorporated in the system, and facilitate pilot plant production and eventual scale-up of vaccine production. A coordinator for the Mexican center has been appointed, and work is progressing to create the system.

The concept of SIREVA expressly seeks support for accelerating scientific and technological development as part of a commitment to foster regional approaches to the development and production of vaccines in Latin America and the Caribbean. It is offered as a response to an increasingly difficult task, that is for the countries of an emerging Region to increase scientific and technological development as a means of assuring access to, and the benefits of, modern science for disease prevention and control. The system is also proposed as a strategy for attracting collaboration from developed countries to strengthen and reinforce institutions in the Region.

The major problem still not solved in order to fully implement SIREVA is the assurance of a reliable sustainable source
of financing from both national and international sources.

VI. Concluding Observations

The science and technology for vaccine development offer an ideal means for promoting institutional development and technology transfer in the Region of the Americas. Following decades of contributions to vaccine technology, the research institutions of Latin America have not yet been able to create a self-sustaining capacity to develop new vaccine products and make them available for widespread use. Frequently participating in aiding others accomplish their goals, the institutions of Latin America and the Caribbean have not been able to realize their own potential, even being vulnerable at times to deficiencies in their own systems or worse to unscrupulous practices by others seeking purely economic gains.

Even though problems have existed, there are outstanding examples of the transfer of vaccine technology to Latin America and the Caribbean and among the countries of the Region, as highlighted previously. Centers of scientific and technological capabilities in the Region have been able to develop in part as a result of these successes. Barriers to vaccine development and production have also arisen and those that remain must be successfully addressed, building on past experience and regional expertise.
To address the need for continued technology transfer and for further strengthening of regional institutions, SIREVA, as described in previous sections, is intended to be a tool for focusing epidemiological, biomedical and operational research on the solution of public health problems. It holds the potential for increasing cooperation among individuals and institutions in the Region in order to produce a greater impact than that associated with the initial projects alone. Once the system has been established, has produced its first products and has contributed to regional scientific development, the benefits should continue far into the future.
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COMBINATION VACCINES

Ronald W. Ellis
Merck Research Laboratories
West Point, PA 19486
Telephone No. (215) 652-7545

and

R. Gordon Douglas
Merck Vaccine Division
Rahway, NJ 07065
There has been an intensified interest during the last decade in the development of new vaccines, especially for pediatric use. Recently developed vaccines which have been licensed and recommended for general pediatric use are those for hepatitis B (HepB) and Haemophilus influenzae b (Hib). Other vaccines currently in clinical trials for potential widespread pediatric usage include those for varicella, Bordetella pertussis (acellular vaccine), hepatitis A, Streptococcus pneumoniae (pneumococcal), and nontypable H. influenzae. The availability of these vaccines is providing for an unprecedented opportunity to prevent serious infectious diseases and their associated morbidity and mortality. However, each new product entails the need for additional injections which could become so numerous as to discourage the administration of new vaccines. This challenge can be overcome by mixing individual vaccines before delivery such that multiple vaccines are administered in individual injections as combination vaccines. Such mixing can occur at the time of manufacturing, by combining vaccines within a syringe, or by mixing vaccines at the time of vaccination within a vial for administration in a single shot.

This paper will review several aspects of combination vaccines. Existing combination vaccines as well as new combinations based on current and potential future vaccines are presented. Technical, clinical, manufacturing and marketing issues then are discussed relative to combination vaccines in general as the major challenges for ongoing and future development (Table 1).

There are two combination vaccines which currently are recommended for routine pediatric administration (Table 2). One of these, measles-mumps-rubella [available in the United States as M-M-R® II (Merck Sharp & Dohme)] (13), is a mixture of live attenuated vaccines as defined by the ability of the vaccine virus strains to replicate within humans. The other, diphtheria-tetanus-
pertussis (DTP), is a mixture of killed or inactivated vaccines (4). These two vaccines form the basis for future combination vaccines, one live and the other inactivated. It is noteworthy that a 23-valent pneumococcal (Pn) vaccine indicated for adults has been demonstrated to be efficacious (8).

The first issue that must be considered in creating new combination products is that the component vaccines must be indicated for administration at the same ages. These indications are established through clinical trials of each vaccine. For DTP vaccine, each of the three components was developed as a separate vaccine. It was demonstrated that a 3-dose primary series at 2, 4 and 6 months of age (other ages have been used for infants in other countries) elicits immune responses associated with protection against clinical disease. A subsequent booster dose is indicated at 15-18 months of age. The DTP combination vaccine became available in the 1950s and has become a required pediatric vaccine (2). As a result, when other inactivated pediatric vaccines are developed for young infants, it is perceived as desirable to test the vaccines at 2, 4, 6 and 15 months of age for ultimate combination with DTP.

The recombinant-derived hepatitis B vaccine was developed as a second-generation product succeeding the plasma-derived hepatitis B vaccine (8). Universal vaccination of infants is considered the most effective strategy for reducing the incidence of hepatitis B in the United States as well as many other countries. Two schedules are being recommended in the United States (Table 3). 0, 1, 6-12 months and 2, 4, 6-15 months of age (3). While the former schedule affords an earlier dosing regimen, the latter is compatible with the DTP schedule at the 2- and 4-month and booster doses.

The Hib conjugate vaccines are second-generation products for the prevention of invasive Hib diseases in infants and young children. The first-generation PRP vaccine was indicated for vaccination of children greater
than 18-24 months old; however, most Hib disease occurs in children less than
18 months of age. Four Hib conjugate vaccines have been developed (11). Each
of these consists of the Hib capsular polysaccharide (PRP) of different sizes
conjugated to different carrier proteins; diphtheria toxoid (PRP-D), mutant
diphtheria toxin (HbOC), tetanus toxoid (PRP-T), and meningococcal outer
membrane protein complex (PRP-OMPC). HbOC and PRP-OMPC have been licensed for
infants in the U.S. (Table 3). PRP-OMPC is indicated at 2, 4, and 12-15
months of age, and the HbOC vaccine is indicated at 2, 4, 6 and 15 months of
age. With licensure expected soon, PRP-T would be indicated at the latter
schedule. Therefore, these vaccines are compatible with the DTP schedule at
the 2-, 4-, 6- and 15-month doses.

Based on these schedules and given that Hib conjugate vaccines were
licensed for infants (1990) before the recommendation for universal hepatitis
B vaccination in the U.S. (1992), DTP/Hib combination vaccines have undergone
extensive clinical evaluations. DTP/HbOC has been combined in a single vial
(6). Likewise, DTP has been used to rehydrate the lyophilized PRP-T vaccine
(12). Both combination vaccines have been shown to be immunogenic for all
four component vaccines and are expected to be licensed soon for 2-, 4-, 6-
and 15-month old infants. A PRP-OMPC/HepB bivalent vaccine has been developed
and evaluated clinically in infants. With these vaccines as stepping-stones,
DTP/Hib/HepB vaccines are being formulated as pentavalent vaccines for routine
immunization of infants.

The different indications among Hib conjugate vaccines (2, 4, 12-15 months;
2, 4, 6, 15 months) may create some confusion when these vaccines are combined
with DTP and HepB. In regimens of these combinations, an infant receiving
DTP/HbOC at 2 and 4 months will need to receive the same product at 6 months,
while another receiving DTP/PRP-OMPC/HepB at 2 and 4 months will receive DTP
only at 6 months. Furthermore, there has been much discussion of immunizing infants with a mixed regimen of Hib conjugate vaccines, given that PRP-OMP is most effective at eliciting antibodies in the first dose, while PRP-T and HbOC may have superior boosting characteristics. This would extend likewise to combination vaccines with different Hib components. If such a mixed regimen is adopted, this will create additional challenges from the perspectives of clinical testing and marketing, as described below.

Both live and inactivated polio vaccines have been licensed for the immunization of infants. While some countries mandate the use of the live attenuated oral vaccine at 2, 4, 6 and 18 months of age, other countries have adopted a mixed schedule utilizing the inactivated polio vaccine (IPV) for at least the first two doses. Placing IPV in a combination vaccine may lead to its increased utilization. Hence, a DTP/IPV tetravalent vaccine has been tested clinically in infants. It also will be a stepping-stone toward a potential pentavalent DTP/Hib/HepB/IPV vaccine.

It is difficult to project with any certainty as to which other inactivated vaccines might be licensed for widespread pediatric use. However, one could speculate that a cocktail of DTP-Hib-HepB-IPV may eventually be supplemented with hepatitis A, pediatric Pn, and nontypable N. influenzae (ntHinf) vaccines (Table 4). It is tempting to think that such a multivalent cocktail could be technically feasible by the end of this decade, subject to some of the challenges elaborated in the rest of this paper.

The measles, mumps and rubella vaccines were each clinically tested and licensed in the 1960s before they were combined into a single vaccine in the 1970s, which is administered at 12-15 months of age (13). Recently a live attenuated varicella vaccine has been developed and tested in similarly aged children (10). This vaccine is expected to be licensed in the near future.
Given the same age indication, a combination measles-mumps-rubella-varicella vaccine has been tested clinically as well. This tetravalent cocktail may represent the most complex cocktail of live vaccines which may become available in the foreseeable future.

The second issue that must be considered in creating new combination vaccines is that the final product must be stable and pharmaceutically acceptable in terms of physical interactions, excipients, preservatives, adjuvants and presentation.

Different vaccines in combination may physically interact with one another in terms of aggregation or noncovalent binding. This issue can be further complicated by the presence of aluminum adjuvants, as described below. Such interactions can affect the stability and consistency of the product. If there is an excessive amount of physical interactions among component vaccines, in particular inactivated ones, then the final combination product may appear nonuniform, clumpy or discolored. It may require vigorous mixing to assure accurate uptake into a syringe and delivery. It is desirable to achieve a final suspension as uniform as possible for the medical practitioner, who otherwise may be reluctant to utilize the product for aesthetic reasons. Appropriate modifications of the formulation of individual vaccines or buffers may minimize such interactions and assure uniformity.

The issue of physical interactions among component vaccines becomes more technically challenging as the number of vaccines to be combined increases. If one considers some of the multivalent inactivated vaccine cocktails alluded to above, there may be a limitation to the number of individual vaccines which can be combined in a physically stable form due to the need for each component to occupy a minimum volume to retain its stability. This creates a particular challenge for achieving a stable combination vaccine.
There are several kinds of excipients in vaccines which may be part of the composition of component vaccines such that they are brought along into the final combination. Lyophilized vaccines contain bulking agents such as lactose which confer sufficient physical mass upon the lyoplug so as to render it intact and visible at the time of reconstitution. The vaccine solution may contain buffers which assure the stability of the component vaccines during long-term storage. Preservatives are used in multi-dose vials to assure that contaminants inadvertently introduced into the vial by multiple needle-punctures of the stopper cannot grow. Alternatively, preservatives may be needed to assure the sterility of vaccines which cannot be attained otherwise. There may be low levels of process intermediates or chemicals in the final product which are not essential to the efficacy and stability of the final product. Finally, adjuvants often are added to inactivated vaccines to enhance their immunogenicity, as described below. The presence and concentration of all such excipients should be justified for the final combination vaccine in terms of all its components. For example, a buffer constituent which assures the stability of one component vaccine may destabilize another component vaccine. In that sense, a combination vaccine is treated by the manufacturer and by regulatory agencies essentially as a new product.

A very important excipient in some inactivated vaccines is the adjuvant, which is defined as a nonantigenic component which enhances the immune response to the vaccine. The only adjuvants currently used in licensed vaccines are aluminum salts, although many experimental adjuvants are under clinical evaluation (1). The aluminum salts, such as hydroxide and phosphate, physically bind to inactivated vaccines by means of noncovalent ionic bonding and influence the presentation of the vaccine antigen to the immune system.
Depending upon the composition of the aluminum salts used for different component vaccine antigens in a combination, there may be physical interactions between different adsorbed antigens which may affect the stability efficacy. This issue can be minimized through the use of identical or similar aluminum salts for all components and by stably adsorbing each vaccine component per se before mixing into a combination vaccine. If a component vaccine which is not aluminum-adsorbed as an individual vaccine is mixed with other adsorbed vaccines, the particular antigen would be expected to become adsorbed to aluminum in the course of mixing; the implications of this for the stability and efficacy of that component must be defined. Aluminum salts also can adjuvant any contaminating proteins in the preparation, as would be the case if an aluminum-adsorbed inactivated vaccine were mixed with a live vaccine containing protein excipients in its stabilizer. Such adjuvantation can lead to inappropriate immune responses, with concern for tolerability.

It is necessary to define the expiry date or end of dating period of the final product, during which the vaccine is expected to remain stable and efficacious at the indicated storage temperature. A minimum of 2-year dating is ordinarily desirable from a marketing perspective. A change in the process for making the final vaccine which results in a change in its composition may entail the need to reestablish its dating period. In addition, since the manufacturing process entails producing each component vaccine prior to making a combination, the dating period for storing each component within the production facility prior to combination needs to be determined. The dating period is established by stability testing, i.e., analytical testing of the vaccine by assays which measure its stability. The ideal stability-indicating assay is one which would anticipate changes in the clinical performance or efficacy of the vaccine.
The desired outcome of stability testing of a new combination vaccine is that all component vaccines can be stored stably in a single vial for up to two years. While this is often the case, there are examples where there is an inherent incompatibility between vaccines. For example, thimerosal present in DTP vaccines gradually destroys the potency of XFPV. To overcome this problem, separate components of a combination vaccine can be loaded into separate parts of a dual-chambered syringe, as is being done for DTP/IPV, in order to prevent the components from mixing together before administration. If stability can be demonstrated in a single vial, then different sized vials can be made available: single-dose, 3-dose, 5-dose, 15-dose, etc. For each different vial or preloaded syringe, a separate stability study is required to establish its dating period.

The third issue that must be considered for combination vaccines is clinical evaluation, which includes study design, tolerability, immunogenicity, and efficacy.

Clinical studies on combination vaccines should be well controlled, with study arms which look separately at the components which are the previously licensed vaccine products, e.g., evaluation of DTP/Hib would have DTP and Hib as study arms rather than D, T, P and Hib. This type of controlled study, which typically is randomized, prospective and multi-center in design, affords the opportunity to look for both safety and immunogenicity interactions among component vaccines in the combination.

The adverse experience profile of a combination vaccine is expected to be no better than that of the particular component which is least well tolerated. Therefore, as the number of vaccine components in combination increases and creates the potential for interaction in vivo, special attention should be paid to safety-related issues. Appropriate study designs as described above
will facilitate the observation of such interactions. There may be rare new adverse experiences, e.g., 1 in 50,000 vaccinees, which cannot be observed in clinical trials involving thousands of vaccinees prelicensure. Post-marketing studies may enable such events to be noted.

A pivotal issue regarding combination vaccines is their immunological interaction. It is undesirable that the immune response to a particular component when in combination be significantly lower than that to a previously licensed vaccine. It is optimal that the immune responses in such a case be comparable. However, there may be some leeway in terms of whether a slightly lower immune response in a new combination is of clinical significance in terms of an actual increased risk of infection; the degree of leeway depends upon the vaccine in question. Such inhibitions or interferences have been observed in several clinical trials, yet the immunological basis for interference is not well understood. For example, in a trial where PRP-T was coadministered in the same syringe as DTP vaccine, the anti-pertussis antibody responses were significantly lower in vaccinees receiving DTP/PRP-T than those receiving DTP (5). This empirical observation was not predicted by preclinical studies and underscores the challenge of combination vaccine development. Conversely, it is possible, although not well documented, that there might be an immunological interaction between different vaccines in combination that results in enhancement of an immune response. It is not known whether it is possible to continue to combine component vaccines without limit and still achieve satisfactory immune responses to each component vaccine. Such a limitation can only be discovered empirically through controlled clinical trials. In this regard, the efficacy of the 23-valent Pn vaccine is noteworthy.

Once a vaccine has been proven efficacious in a placebo-controlled
clinical trial for preventing disease, it is not ordinarily possible for ethical reasons to perform another controlled efficacy trial on the same vaccine. Therefore, the predicted efficacy of a particular vaccine in a new combination product must be judged on the basis of a serological assay, the results of which have been shown to correlate with clinical efficacy. Such an assay is known as a surrogate assay for efficacy. One of the best known of such assays is the anti-HBs assay for antibodies to hepatitis B surface antigen, where a post-dose 3 response >10 mIU/mL has been shown to correlate with clinical protection against hepatitis B infection (9). The surrogate assay is the basis for assuring that the immune response to a component vaccine is satisfactory in a new combination product relative to the vaccine in a previously licensed product.

The fourth issue for combination vaccine relates to manufacturing. Each component vaccine in a combination is manufactured separately before being mixed together. There are numerous quality control assays which are performed to evaluate the safety, potency and consistency of manufacture of each vaccine, which must pass all such assays before it can be mixed into combination. After component vaccines are mixed, additional quality control assays are performed on the final bulk product and upon the final filled vials. In addition, the stability of the final formulated individual vaccines must be established so that there is a flexible manufacturing operation where vaccines are stored for varying periods of time prior to blending into a combination. There are many regulatory issues regarding manufacturing of a combination vaccine during clinical trials, licensure of the process and manufacturing facility, and amendments to a license which cover changes in the process or facility; these issues are reviewed in a separate chapter (New Challenges in Quality Control and Licensure-Regulation).
The final general issue for combination vaccines is marketing. As more vaccines are developed for pediatric use, the opportunity increases for confusion in the marketplace. More products will need to be administered. Dosing schedules may be different from available products and for different products within a class of vaccines, e.g., Hib conjugate vaccines. Combination products offer the opportunity for simplifying the administration of vaccines by decreasing the number of injections per visit.

In order to be as user-friendly as possible, vaccines will need to be presented to medical practitioners in both the private and public sectors in the simplest possible form. This means that the products will need to be clearly labelled with respect to the identity of component vaccines, age of administration, and compatibility/incompatibility with other single-component vaccines. The latter two issues are especially important, since within a sequence of well-baby visits at 2, 4, 6, and 12-15 months there may be different combination vaccines formulated for administration at different ages. It will need to be clear to the medical practitioner that a particular product can be administered at, e.g., 2 and 4 months only, 2, and 4 and 6 months, 12-15 months only, etc., and which other vaccines would be used to fill out the schedule. Simplicity of presentation and schedules will be of the utmost importance.

As discussed above, combination products can be available in different presentations: single-dose vials, multi-dose vials, and preloaded syringes. Different medical practices may have different needs for such presentations. It will be important for manufacturers to assure that they anticipate these needs through the proper marketing surveys, given the long lead time (usually at least 3 years) for developing a new presentation. In addition, there may be an opportunity to present combination products in volumes larger than the
currently utilized 0.5-ml volume per injection. For example, the dual-chambered syringe mentioned above for DTP/IPV would deliver a 1.0-ml volume of injection based on 0.5 ml of each of the licensed DTP and IPV component vaccines. As the number of inactivated vaccines in a potential multivalent cocktail increases to the point where constraints on formulation compromise vaccine stability, increasing the volume to greater than 0.5 ml may enable a stable formulation to be achieved.

Given the number and complexity of potential combination vaccines (Table 5), especially from inactivated component vaccines, there may be no single manufacturer which would have developed all component vaccines itself. This means that alliances would be required between manufacturers to share their respective component vaccines for creation of the ultimate combination products. Such alliances already are being formed, e.g., Connaught-Merck, SKE RIT-Michigan Department of Health. Finally, combination vaccines represent a vehicle through which it is possible to secure more widespread use of vaccines. While there are some new vaccines which would be recommended immediately for universal vaccination of infants, other new vaccines might not get such a recommendation immediately. However, if the new vaccine can be offered as a combination product with another existing pediatric vaccine that is recommended for universal vaccination, e.g., DTP, Hib, HepB, measles-mumps-rubella or polio, then the new vaccine is much more likely to be recommended for universal vaccination in such a combination.
References


Table 1

Challenges for the Development of Combination Vaccines

I. Age of Administration.
   - Compatibility of component vaccines.
   - Flexibility of dosing schedules.

II. Technical Development.
   - Physical interactions.
   - Excipients.
   - Adjuvants.
   - Dating period.
   - Stability testing.

III. Clinical Evaluation.
   - Controlled studies.
   - Safety.
   - Immunological interactions.
   - Surrogate assays for efficacy.

IV. Manufacturing.
   - Separate manufacturing.
   - Quality control.
   - Regulatory.

V. Marketing.
   - Simplification.
   - Different images.
   - Alliances among manufacturers.
Table 2

Current Combination Vaccines

- Diphtheria-Tetanus-Pertussis (DTP)*
- Measles-Mumps-Rubella*
- 23-valent Pneumococcal (Pn)

* pediatric
### Table 3

**Alternate Dosing Schedules (Months of Age) for Pediatric Vaccines**

I. Hepatitis B

<table>
<thead>
<tr>
<th>Schedule 1</th>
<th>Schedule 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>dose 1</td>
<td>0</td>
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<tr>
<td>dose 2</td>
<td>1-2</td>
</tr>
<tr>
<td>dose 3</td>
<td>6-18</td>
</tr>
</tbody>
</table>

II. Haemophilus b

<table>
<thead>
<tr>
<th>Schedule 1 (PRP-OMP/C)</th>
<th>Schedule 2 (HbOC*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dose 1</td>
<td>2</td>
</tr>
<tr>
<td>dose 2</td>
<td>4</td>
</tr>
<tr>
<td>dose 3</td>
<td>NA*</td>
</tr>
<tr>
<td>booster dose</td>
<td>12-15</td>
</tr>
</tbody>
</table>

*expected to be recommended for PRP-T  
†not applicable
### Table 4

<table>
<thead>
<tr>
<th>Component Vaccines for Potential Future Pediatric Combination Vaccines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatitis B</strong>&lt;sup&gt;1&lt;/sup&gt; (HepB)</td>
</tr>
<tr>
<td><strong>Haemophilus b Conjugate</strong>&lt;sup&gt;2&lt;/sup&gt; (Hib)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Acellular pertussis</strong>&lt;sup&gt;2&lt;/sup&gt; (to replace current cellular pertussis in DTP)</td>
</tr>
<tr>
<td><strong>Hepatitis A</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Varicella</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Pediatric Pneumococcal</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Nontypable H. influenzae</strong>&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. approved and recommended for routine pediatric use
2. approved for children
3. approved for children and adults
4. under clinical evaluation
### Table 5

**Potential Future Combination Vaccines**

- Measles-mumps-rubella-varicella
- DTP/Hib
- DTP/HepB
- DTP/Hib/HepB
- DTP/IPV
- DTP/Hib/HepB/IPV
- DTP/Hib/HepB/IPV/HepA/Pn/ntHinfl
LIVE ATTENUATED VACCINE VECTORS

John J. Mekalanos

Department of Microbiology and Molecular Genetics
Harvard Medical School
200 Longwood Avenue
Boston, Massachusetts 02115
617.432.1944
ABSTRACT

There are currently several different live attenuated vaccine vectors under development. These vaccines are composed of living viruses or bacteria that are innocuous but can replicate in host tissues and induce immune responses. The genes encoding foreign antigens can be inserted into these vectors to produce multivalent vaccines that promise to induce immunity to more than one target disease after administration of a single dose of vaccine.
ACKNOWLEDGMENTS

Research in the J.J.M. laboratory is supported by grants AI-18045 and AI-26289 from the National Institutes of Health. J.J.M. is pleased to acknowledge the help of Ms. Jill Gray in the preparation of this manuscript.
INTRODUCTION

Immunization offers one of the best means we have to control the incidence of infectious disease. The use of vaccines is also one of the most cost effective measures in part because a minimum number of visits to a clinic can in theory lead to life long protection against many pathogenic agents. In the best case, a single dose vaccine may lead to not only protection of the individual but also to the eradication of the agent if it has an obligate human host and no other animal or environmental reservoir. Such success has been so far achieved with only a single human pathogen (small pox virus) and was accomplished through an extraordinary worldwide vaccination effort combined with an effective, live attenuated viral vaccine (vaccinia). Live attenuated polio vaccine could in theory lead to elimination of polio if a similar worldwide effort could be mustered but political, economic, and logistic barriers make the success of such a campaign less likely today particularly when focused on a single infectious disease. The successful elimination of smallpox as a threat to mankind, underscores the potential that live attenuated vaccines offer and has inspired hope among researchers that genetic engineering may convert some effective live vaccines into multivalent vehicles that protect against several diseases in a single dose. In this chapter I will discuss the live attenuated vaccine vectors that make the "carrier vaccine" approach possible.

LIVE ATTENUATED VACCINES: WHY?

Vaccines can be divided into three types: subunit vaccines, inactivated microorganisms and live attenuated microorganisms. Subunit vaccines (composed of purified proteins, carbohydrates, peptides, or conjugates of these) are generally considered the most safe because of their well-defined biochemical makeup. The ability to manipulate these vaccines chemically also provides a means of altering the immune response in specific ways (e.g., addition of adjuvants, chemical crosslinking to improve immunogenicity, or to provide "T-help" to carbohydrate antigens of poor immunogenicity in neonates)(14;69). However, for some diseases it is often difficult to define a single immunogen that provides solid immunity of long duration. For example, subunit vaccines typically do not induce cell-mediated immunity that is essential for combating certain viral and parasitic infections. Similarly, mucosal immunity cannot be induced by a subunit vaccine unless effort is made to deliver the antigen to mucosal M-cells (55) in a pharmocologically acceptable vehicle (e.g., microspheres, liposomes, etc.)(8;15;47). Additional drawbacks of subunit vaccines fall under the categories of cost and technology (i.e., production, purification, and formulation may be beyond the financial and technological capabilities of many potential vaccine producers).

In contrast, inactivated microorganisms can be among the most inexpensive vaccines to manufacture requiring only the cultivation and recovery of the infectious agent followed by simple chemical inactivation which itself tends to enhance the thermostability of the preparation. Because
they are composed of whole bacteria or viruses, vaccines of this sort are composed of more than one immunological target. This same advantage is a drawback when such a vaccine shows even mild reactogenicity (e.g. the whole cell pertussis vaccine) since the offending component can usually not easily be defined biochemically (7). Like subunit vaccines, inactivated microorganisms seldom induce cell-mediated responses and will induce mucosal immune responses only when delivered in sufficient quantity through multiple doses (11).

Live, attenuated vaccines are composed of living viruses or bacteria which are avirulent because of either attenuating mutations or because of replication restrictions associated with host range. In theory a single inoculation with a small dose of a live vaccine can expand by replication to a larger and therefore more immunogenic dose of vaccine within the vaccinee. During the course of this replication, the live vaccines presumably expresses all or most of the important target immunogens that ordinarily lead to protection after the natural infection. The infection with live vaccines can induce cytokine production which in turn can recruit elements of the immune system (e.g., macrophages and other antigen presenting cells, lymphocytes, etc.) that might not ordinarily respond to subunit and inactivated vaccines. The immunostimulation through these cells can lead to markedly higher immune responses. When the live vaccine is a virus or intracellular microorganism, they are capable of inducing both humoral as well as cell-mediated responses (e.g. cytotoxic T lymphocytes; CTL). Because the live vaccine replicates in a manner analogous to the target agent, they promote the processing and presentation of antigens in a way that is most similar to the nature infection. This includes the induction of a mucosal immune response if the live vaccine is appropriate. For a live vaccine to induce a mucosal immune response, not only must it be administered on a mucosal surface (i.e., oral, nasal, rectal, or urogenital surfaces) but it also must adhere to mucosal M-cells (55). The fact that some viruses and bacteria naturally "target" to lymphoid tissue of the mucosal epithelium, adhering to, entering, and multiplying within these sites, suggests that attenuated mutants of these agents might make excellent mucosal vaccine carriers (47). Thus, live, attenuated vaccines provide in the best case, a replicating antigen delivery vector, capable of stimulating mucosal immune responses but also capable of inducing systemic humoral and cell-mediated immune responses all after administration of a small dose of vaccine on an easily accessible host surface.

VIRAL VECTORS

Vaccinia  The oldest and to date most successful viral vaccine is the small pox vaccine based on the cow pox virus, vaccinia. The eradication of small pox through the prodigious vaccination efforts of the World Health Organization demonstrated that a vaccine with properties such as heat stability, simple one shot administration with subsequent evidence of successful immunization, and low cost, could indeed be effectively used on a worldwide scale and yield spectacular results.
These same properties have led investigators to use vaccinia virus as vector for the expression of other antigens with the goal of producing multivalent carrier vaccines (42;52;57;58). Because vaccinia multiplies within the cytoplasm of host cells, such recombinant carrier vaccines are capable of generating cytotoxic T-cell responses (i.e., mediated by MHC class I restricted antigen processing and presentation) as well as humoral responses to expressed heterologous antigens (i.e., mediated by MHC class II restricted antigen processing and presentation). This spectrum of immunological responses is quite complete, and can even include a secretory immune response if the virus is administered on the respiratory or intestinal mucosal surface (30;67;71). Given the wide variety of potential immune responses that vaccinia is capable of eliciting, it is a near ideal candidate as a vaccine vector from an immunological point of view.

Vaccinia virus is a pox virus that contains a 200 kilobase pair, double stranded DNA genome. Because it replicates in the cytoplasm of host cells, foreign DNA inserts must be inserted as DNA copies that require no post-transcriptional splicing. Foreign DNA is inserted in the viral DNA by recombination with plasmid vectors and it has been estimated that vaccinia can accommodate at least 25 kilobase pairs (72).

Expression of antigens within the vaccinia system can be modulated by fusion of antigen encoding constructs to either early or late viral promoters (52). Because a cytopathic effect can interfere with effective processing and presentation through the MHC class I pathway, early promoters are used to target this arm of the immune system. However, larger amounts of antigen expression can be obtained with late promoters or through the use of heterologous promoter systems. Once expressed the modification of antigens by normal pathways of proteolytic processing, glycosylation, myristylation, membrane incorporation, and assembly into particles appears to be unaltered until late in the replication cycle of the virus. Accordingly, it is not surprising that vaccinia vectors have under experimental conditions allowed the demonstration effective immune responses to a variety of viral, bacterial, parasitic, and tumor antigens. For example, humoral or cell mediated immune responses to over 30 different viral proteins have been detected after expression in vaccinia vectors (52;58).

The carrier vaccine concept has been tested successfully for vaccinia recombinants expressing in several different animal disease models (52;58;73). Indeed, veterinary vaccines for rabies, based on vaccinia vectors have been successfully field tested (6;67). In terms of potential human vaccines, a few studies in lower primates have been performed to date that have demonstrated immune responses but no protection against malaria (62), and poor immune responses to HIV-1 antigens in several others (28). Nonetheless, human phase I studies with vaccinia recombinants expressing HIV-1 envelope protein are proceeding and so far have yielded some encouraging results (13).
The major obstacle to the use of vaccinia as a vaccine vector for expression of heterologous antigens is safety. Besides the accepted reactogenicity seen during vaccination (i.e., pustular skin lesion, fever, lymphadenopathy, local pain, and scaring) occasional severe reactions occur (52,58). These range from transfer of the virus to secondary sites (e.g., the eyes, vulva, or perineum) to severe often fatal infections in individuals suffering from immune dysfunction or skin conditions like eczema. While some variation has been noted between the reactogenicity of different strains of the virus, most investigators considering the use of vaccinia as a vaccine vector have sought to either delete certain non essential genes (e.g., thymidine kinase or hemagglutinin) or insert heterologous gene constructs into such genes as a way of further attenuating the virus. However, these modifications often reduce the replication and immunogenicity of the virus (33). An interesting new approach to attenuation of vaccinia has been the insertion of genes encoding lymphokines such as interleukin-2 (66) and γ-interferon (82). These constructs have shown reduced virulence for both normal as well as immunocompromised animals and, in the case of the IL-2 recombinant, displayed the same immunogenicity as nonattenuated constructs.

While we know much about vaccinia, indeed its entire nucleotide sequence (22), the future of vaccinia as a carrier of heterologous antigens remains somewhat in limbo. Although studies have demonstrated its usefulness as a vector for veterinary vaccines, the long term goal of human multivalent vaccines have yet to be realized because of problems with safety and immunogenicity. Because the use of vaccinia may preclude its reuse as a vector for subsequent immunizations (due to anti-vaccine immunity)(13), considerable thought will need to be given to which antigens should be prioritized for vaccinia based vectors (i.e., CTL targets, antibody targets or both?). These problems can only be solved by more experimental studies in humans performed to carefully assess issues of safety, route of administration (dermal or mucosal), immunogenicity, and protection.

Fowl Pox Virus Concern over the potential for adverse and severe reactions with vaccinia has prompted investigation into other animal pox viruses for use as vaccine vectors(58). Avian pox virus (fowl pox) is similar in many way to vaccinia in terms of its molecular biology and ability to be manipulated genetically. However, the wild type form of this virus does not grow productively in mammalian cells but instead undergoes an abortive replication cycle. Expression of heterologous antigens under early fowl pox promoters can nonetheless lead to significant antigen expression in infected mammalian cells (5;78;79). Induction of immune response against heterologous antigens expressed in fowl pox vectors has advanced to animal work with encouraging results but their use may be limited to induction of primary immune responses.

Adenoviruses The adenoviruses represent a diverse serological group of viruses that share a common molecular biology (23). All viruses contain a single, linear, double stranded DNA genome of approximately 30-40 kilobase pairs in length enclosed within a stable protein capsid.
Because the virus will package only about 105% of its genome, these viruses are limited to about 2 kilobases of heterologous coding sequence that can be inserted without producing defective viruses that replicate only on "helper" cell lines expressing adenovirus functions. Defective viruses may still make reasonable vaccines when combined with helper virus and this strategy could push the upper limit of foreign DNA that could be accommodated to about 8 kilobase pairs.

Adenovirus offers a number of advantages as a vector system for expression of heterologous antigens(23). First, is the fact that two adenovirus strains Ad4 and Ad7 have been used with 80% efficacy as oral vaccines against the respiratory illness, epidemic acute respiratory disease (ARD). This result suggests that this virus may induce effective respiratory mucosal immunity while replicating at a distant gastrointestinal site. Second, the virus is easy to manipulate genetically and can be engineered to express foreign DNA inserts at comparable high levels. Third, the virus grows to high yields in a variety of different types of cells and can stably incorporate its DNA into the host cell chromosome (a property that may be useful for life long antigenic stimulation). Finally, there exists at least 50 human serotypes and countless more animal serotypes making possible manipulation of multiple vectors that will not be restricted in their use by "vaccine immunity" or could be used as replication deficient, nonpermissive vaccines in heterologous hosts.

The disadvantages of adenovirus as a vector in some cases mirror its advantages. It is possible that only nondefective adenoviruses will make effective vaccines (39;41). Thus, gastrointestinal shedding of the virus could lead to outbreaks by horizontal transfer and some serotypes do cause severe infections in infants and immunocompromised adults. Its permissive replication in a variety of cell types combined with its ability to incorporate its DNA into host cell chromosomes, raises the question of the oncogenic potential of adenovirus. While it has been shown to transform human cells in culture, there is little evidence that an adenovirus is involved in the biology of any known human tumor. Furthermore, the fear of oncogenesis has not precluded the use of Ad4 and Ad7 viruses as vaccines in adults (although it might prevent their use in neonates). Finally, human adenovirus have a very restricted host range and this property makes evaluation of vaccine constructs in animals very difficult. Preexisting antibody to adenoviruses may also limit its use as a general vector in adults.

Nonetheless, a number of antigen constructs in Ad5 vectors have been evaluated in animal models and have shown promising protective responses against proteins encoded by herpes virus (45), VSV (60), human hepatitis B virus (41), and HIV-1 (61). This success continues to support the study of adenoviruses as vaccine vectors.

Poliovirus The use of live attenuated oral polio vaccines in developed countries has led to the near eradication of this virus. Because rare cases of poliomyelitis are known to occur due to reversion of attenuating mutations in the vaccine strains, work has proceeded on making these strains safer by introduction of additional mutations (63). Expression of intact foreign proteins by
poliovirus has been problematic due to the life cycle of this virus which involves synthesis of a polyprotein that must be correctly processed (56). However, insertion of small peptide epitopes into the capsid protein has led to promising recombinants. One of these poliovirus chimeras, carrying an HIV-1 pGP41 epitope, was capable of inducing broadly neutralizing antibodies and is currently under investigation as an oral mucosal vaccine for AIDS (16).

**Herpes Simplex Viruses** The use of herpes viruses as vectors has also been considered in part because of the capacity of their large DNA genomes but also because of their potential as mucosal vaccines vectors (81). Progress is being made on the attenuation of these viruses by deletion (46). Several recombinants have been constructed and characterized that express antigens of hepatitis (70), Epstein-Barr (29), and HIV (65) viruses. As with adenovirus and vaccinia, the use of herpes virus as vectors may be limited by preexisting antibody in the population and by the potential for severe infections in immunocompromised individuals.

**BACTERIAL VECTORS**

Bacterial vectors offer several advantages over virus vectors as live attenuated vaccines. The most important is the fact that adverse reactions (e.g., disease in an immunocompromised person) can be presumably treated with antibiotics. Bacteria have virtually an unlimited capacity to encode heterologous antigens. Methods also exist for the expression of heterologous antigens on the surface of bacterial cells (54). Disadvantages include the fact that some eukaryotic and viral proteins are not folded, assembled, or modified correctly in bacterial cells.

*Salmonella typhi* Attenuated species of the genus *Salmonella* are currently the most well developed bacterial organisms capable of delivering of heterologous antigens as a live, attenuated vaccine (35). *Salmonellae* are efficient enteric pathogens that invade the intestinal mucosal tissue by direct interaction with the follicular lymphoid tissues of the gut (55). The multiplication of salmonellae in the gut-associated lymphoid tissue (or GALT) leads to a strong humoral immune response dominated by secretory IgA (47). However, salmonellae are also facultative intracellular parasites, and can therefore induce a vigorous cell-mediated immune responses as well (1;47;48).

Attenuation has been studied primarily in the species *S. typhimurium* using the highly susceptible BALB/c mouse. Many mutations have been used to attenuate *Salmonella* species including SmD (producing streptomycin dependency), *galE* (producing galactose toxicity in vivo), various *pur* and *aro* mutations (producing auxotrophy for compounds not available in animal tissues), *crp* and *cyc* (producing global changes in gene expression under catabolite control), *phoP* and *phoPC* (producing global changes in the expression of virulence genes), and *pagC* (producing a macrophage survival defect) (9;10;12;24;26;27;48;49;50;51;53). It is clear that different attenuating mutation affect immunogenicity more dramatically than others. Due to the potential for
reversion (perhaps through recombination), it seems likely that more than one mutation will be needed to attenuate *Salmonella typhi* for use in humans (26;49).

The live, attenuated *Salmonella typhi* vaccine Ty21a has been licensed in many countries as a typhoid vaccine (34). This strain is a galE mutant of *S. typhi* which also carries other undefined auxotrophic mutations. Because Ty21a is a relatively poor growing strain and requires large multiple doses to immunize, considerable effort is being made to construct new *S. typhi* vaccine candidates. Human studies will be required on these strains to determine what is a safe but effective combination of attenuating mutations (9;26;27;35).

Many different heterologous antigens have been expressed in attenuated salmonellae including toxin subunits, fragments, or fusion proteins (10;12;74), lipopolysaccharide (20;21;76), parasite proteins (1;68) and viral antigens (74). In general, a immune response to these "carrier antigens" can be demonstrated in animal models suggesting that this approach is of considerable merit. This response includes the generation of CD8+ cytotoxic lymphocytes (1). Human studies have so far been restricted to measuring the immune response to heterologous lipopolysaccharides expressed in Ty21a (20;21;76). Most antigen constructs that have so far been examined have been plasmid-encoded (10;20;53) and include expression by both constitutive (20;68;76) as well as regulated (10) promoters.

**Vibrio cholerae** The effort to produce an effective cholera vaccine spans over a century of research (77). This effort contributed significantly to our understanding of the importance of the local immunity in combating mucosal infections. The fact that patients who convalesce from cholera have long lasting immunity, has prompted the development of several generations of live, attenuated oral cholera vaccines (25;31;36;37;38;43;59;80). The most promising candidates have been those carrying a deletion of the cholera toxin A subunit gene (ctxA) and many of these have undergone various degrees of clinical testing in volunteers (25;37;38). Volunteer studies with strains JBK79, CVD101, and derivatives of these strains, showed that a single dose of a genetically engineered strain could induce immunity to challenge (37) that is reflected in a vigorous IgA mucosal response as well as a strong systemic IgG response to bacterial antigens.

However, these recombinant vaccines also caused reactions (e.g., moderate diarrhea, cramps, and fever) in the volunteers. Volunteers that ingested one of these strains, 0395-N1, showed significantly less reactions, particularly diarrhea (25), that other isogenic derivatives such as CVD101 (37) and this insight led to the discovery of a new toxin named "ZOT" for zonula occludens toxin (17). ZOT may be responsible for the residual reactogenicity seen in strains such as CVD101 and the recent identification of the zot structural gene (4) should allow testing of this idea. Currently, only one recombinant strain, *V. cholerae* CVD103-HgR, when given in a single large dose of $5 \times 10^9$ bacteria has been shown to induce high levels of seroconversion and
protection with little if any severe side effects (38). Given that CVD103-HgR does produce ZOT, an unknown property is responsible for its apparent attenuation.

Concern must also be raised concerning stability of live attenuated cholera vaccines. Both cholera toxin and ZOT are encoded by a large genetic element that undergoes duplication and amplification in *V. cholerae* (4;44;59). The CTX genetic element is a transposon that can insert into the *V. cholerae* chromosome by an illegitimate recombinational event (59). A live attenuated cholera vaccine such as CVD103-HgR can in theory regain the capacity to produce cholera toxin by re-acquiring the CTX element from toxigenic *V. cholerae* that carry DNA mobilization systems (59).

Recently, new *V. cholerae* mutants have been described which carry deletions that both remove *zot* and prevent the insertion of the CTX element, as well as a second deletion that removes *recA* (required for homologous recombination) (64). These strains carry the *ctxB* gene (encoding cholera B subunit) under the control of either a *ctx* or heat shock promoter. Testing of these strains in volunteers is underway. These studies may show that live attenuated *V. cholerae* vaccines can be used to deliver antigens to the mucosal and systemic immune system and which in vivo regulated promoters provide the best means of expressing these antigens in humans.

**Bacille Calmette-Guerin (BCG)** BCG is a strain of *Mycobacterium bovis* which continues to be used as a live vaccine for tuberculosis although its effectiveness is less than clear (3). It remains the most widely used vaccine in the world with a good record of safety. Originally used as an oral vaccine, BCG is now used as an injectable vaccine because of its tendency to cause lymphadenitis in children. When used as an oral vaccine, much higher doses were needed because of the acid sensitivity of the organism. Enteric coating BCG may solve both oral delivery problems and provide a formulation of the vaccine that would be both inexpensive and potentially capable of inducing a mucosal immune response.

The potent adjuvant properties of BCG together with its ability to induce cell-mediated immunity, might make BCG exceptional as a carrier vaccine for heterologous antigens (3). Progress has been made in engineering the expression of heterologous antigens in BCG via heat shock promoters (2). Such recombinant BCG strains have been shown to induce in animals a response to HIV-1 proteins that includes both humoral as well as cell-mediated activities. Whether BCG can be used as an effective live delivery system for inducing local immune responses on mucosal surfaces remains to be determined. Questions concerning the safety of BCG in immunocompromised subjects also need to be answered.

**Shigella** A variety of attempts have been made to construct live vaccines effective in controlling shigellosis. These studies began with *E.coli-Shigella* hybrid strains which were reasonable vaccines but were also somewhat reactogenic at high doses (32). More recently, *aroA* mutations in *S. flexneri* 2a have shown promise as attenuating mutations in studies involving
monkeys and volunteers (40). As the virulence factors of this invasive pathogen have been defined, additional attenuated mutants have become available for evaluation as live vaccines. Derivatives of several *Shigella* species have been constructed that carry mutations in genes encoding cell to cell spread (*icsA*), iron uptake (*iuc, iut*), catalase (*KatF*), virulence regulation (*ompB*), and shiga toxin production (19). After evaluation of these mutants in volunteers it should be possible to engineer the appropriate strains to express heterologous antigens.

*Yersinia enterocolitica* Recently *Yersinia enterocolitica* has been proposed as a candidate live, oral vaccine capable of delivering heterologous antigens. Genetically engineered strains of *Y. enterocolitica* have been shown to express cholera B subunit and to induce antibody responses to CT-B after gut infections (75). The gene for a heat-stable enterotoxin produced by this organism has been defined so its should be possible to attenuate this organism for gastroenteritis in humans. However, it is hard to know whether vaccination against carrier antigens alone would justify the expense of using *Y. enterocolitica* as a vector. Certain sertoypes of *Y. enterocolitica* also induce a reactive arthritis after infection and this may preclude the use of the organism as a carrier vaccine.

**CONCLUSIONS**

The challenge of developing a single dose multivalent, live vaccine is indeed a major one. We must solve the problem of reactogenicity in some individuals within humans populations that are immunocompromised. The stability of vaccines must be considered in light of changes that may occur in the vaccine on mutation or on acquisition of virulence genes encoded by transposable or other genetic elements (plasmids and bacteriophage). We need to determine how heterologous antigens should be expressed (i.e., which promoters are best in which carrier organisms; at which stage in the infection cycle should the antigen be expressed; should the antigen be displayed on the surface of the vector). We need to understand which carriers will do the best job of inducing systemic, cell-mediated and local secretory immune responses. Finally, because what we learn in animal models is not always directly applicable to what occurs in humans, we will need to do many more human studies in both young and older subjects of different genetic and geographical origins. Hopefully as this information is accumulated, a rational approach to the development of multivalent live attenuated vaccines will become clear and will ultimately be put in motion.
REFERENCES


SYNTHETIC PEPTIDES AND PURIFIED ANTIGENS AS VACCINES

Fred Brown
USDA, Plum Island Animal Disease Center
P.O. Box 848, Greenport, NY 11944-0848
Introduction

Vaccination against infectious diseases of man and his domestic animals is one of the great success stories of human and veterinary medicine. The greatest achievement has been the eradication of smallpox, but many other virus diseases afflicting man such as measles, mumps, poliomyelitis, rubella and yellow fever, and bacterial diseases such as diphtheria, tetanus, tuberculosis and whooping cough have been largely controlled by vaccination. In veterinary medicine, vaccines to control foot-and-mouth disease, Marek's disease and Newcastle disease have been equally successful.

All these vaccines were prepared somewhat empirically, embracing only the principles which evolved from the work of Jenner (12) with smallpox, Pasteur with chicken cholera, anthrax and rabies (20, 21, 22), Salmon and Smith (27) with Salmonella, and Roux and von Behring with diphtheria and tetanus (2, 26). These were either (a) selection of a variant which infected the host without causing clinical disease to provide the so-called "attenuated vaccines"; (b) growth of the agent in large amounts followed by its inactivation with a chemical such as formaldehyde, or a physical agent such as heat or ultraviolet light without damaging its immunogenic properties to give the so-called "killed vaccines" or (c) inactivation of toxins secreted from the infectious agents to give toxoids.

Although these vaccines have been highly successful, they have some disadvantages (Tables 1 and 2). Moreover, there are still some diseases for which no satisfactory vaccine exists, either because the agent cannot be grown in the quantities required to produce an inactivated product or because it has proved impossible to obtain the necessary level of attenuation while still retaining its ability to replicate without causing clinical disease.
At the more fundamental level there is also the challenge to understand how vaccines protect us against infection. With live vaccines, it is easy to accept that, since infection with the natural agent usually leads to long lasting immunity unless there is antigenic variability, infection with the attenuated agent will evoke immunological responses which are the same, or at least similar to, those found in clinical infection. With non-replicating vaccines, however, the situation is different. It has been known since 1886 that heat inactivated cultures of Salmonella cholera suis protected experimental animals against infection (27), and shortly afterwards killed vaccines against cholera, plague and typhoid were prepared on a small scale, although these were not entirely successful (10, 11, 23, 30, 31). We also have the evidence, on a major scale, for the success of inactivated vaccines against influenza, poliomyelitis and, despite frequent widespread adverse publicity, whooping cough. A highly effective rabies vaccine has also been used but on a much more limited scale. But certainly of much greater importance as we consider the possibility of making vaccines based on sub-units of micro-organisms was the demonstration that the secreted toxins of the organisms causing diphtheria and tetanus elicited antibody which, when passively transferred, protected patients suffering from these diseases (2). The sequel to these discoveries was the demonstration that prophylactic immunization against the two diseases could be achieved by inoculation of the appropriately de-toxified toxins (8,24).

**New Vaccines**

A major objective in developing new vaccines has been to identify in complex molecules such as bacteria and viruses those functional units which elicit protective immunity, and which are amenable to high resolution analysis and rapid modification. A second and more demanding objective is to synthesize those units and present them to the host in a functional configuration. What is often overlooked by those sceptical of this approach is that two of our highly successful vaccines, those against diphtheria and tetanus, are in fact sub-units of the causal agents. This means that the complete organism is not essential to evoke the protective immune response.
Dissection of micro-organisms into biologically functional sub-units started in the 1960s as the molecular approach to biology began to make its impact on virology and bacteriology. Because of their relatively simple structures compared with bacteria and parasites, the early inroads were made with viruses. It was shown that viruses with a lipid coat could be readily dissected into subunits which had biological activity. In particular, the surface projections of influenza, measles, rabies and vesicular stomatitis viruses were shown to evoke neutralizing antibody (4). With the exception of the hemagglutinin of influenza virus, these sub-units have not been used directly as vaccines. One reason is that, compared with the intact organism, the level of protective antibody evoked is much less unless the method of presentation is correct (see below). In recent years, the ISCOM (Immuno Stimulating COMplex) method of presenting the proteins as cage-like structures resembling virus particles has gone a long way towards solving this problem, only to be met by questions about its safety and acceptability. (18)

Consequently, when methods were developed for expressing genes in attenuated bacterial and viral vectors, there was already considerable information available on proteins which could evoke protective immune responses. Two main routes are available: (1) expression in a bacterium such as *Escherichia coli* or *Bacillus subtilis*, in yeast (*Saccharomyces cerevisiae*) or in a baculovirus such as *Autographa californica* to produce antigens which can be formulated as inactive vaccines; (2) presentation in an already accepted live vaccine such as vaccinia virus, adenovirus, or BCG. Safety considerations with non-living antigens can be met by applying rigorous purification procedures. However, with the live vector method of expression and presentation, there has been considerable debate about whether insertion of foreign genes into a safe vector converts this to a pathogenic agent (5). Clearly this question cannot be resolved without extensive trials and it is on this issue that acceptance or rejection of the approach depends. These regulatory demands are the subject of many debates which need to be borne in mind but which will not be considered further in this presentation.
Production of non-replicating vaccines

(a) Proteins

Efficient methods for expressing foreign genes in several micro-organisms have been devised. The major problems which have been encountered are twofold: (i) isolation of the required protein product from the mixture of host cell proteins and (ii) expression of the protein in a configuration which evokes the required immune response. The first of these problems can usually be overcome by the application of different purification procedures, although considerable difficulties are often encountered in expressing the required product in a soluble form. The second of the problems, the so-called protein folding problem, is critical if the relevant immune response is to be obtained and it is still far from being solved.

The only successful recombinant vaccine is that against hepatitis B (29). This owes something to good fortune because the immunogenic surface protein of the virus, expressed in yeast cells, forms particles which closely resemble the natural antigen. The immune response to these particles is far greater than that obtained with the protein itself, probably because the relevant immunogenic site is being presented in a multimeric form on the particle. The success of this vaccine has also led to it being studied experimentally as a carrier for foreign epitopes by incorporating the genes coding for them into the gene coding for the surface protein. As with the native protein, these hybrid proteins form particles spontaneously.
The concept of using peptides as vaccines is a logical extension of the process whereby the sites (epitopes) of a micro-organism essential to the protective immune response are identified. Anderer (1) demonstrated 30 years ago that a sequence corresponding to the six terminal amino acids of tobacco mosaic virus protein will elicit antibody which neutralizes the virus. The subsequent work of Sela’s group (see for example, 13) provided much of the basic information which persuaded several scientists that peptides could function as immunogens. With the exception of tobacco mosaic virus and MS2 bacteriophage protein, however, the amino acid sequences of immunologically important proteins were not available until the DNA sequencing methods of Maxam and Gilbert (16) and Sanger et al (28) became available. The publication of these methods in 1977 heralded a new era in biology, including the ability to derive the amino acid sequences of these proteins and the tracts which elicit protective immune responses.

Epitopes are usually defined as continuous or discontinuous. Continuous epitopes comprise linear sequences of amino acids which are specifically recognized in their free form by the antibody against the protein. Discontinuous epitopes are made up of residues that are not contiguous in sequence but are brought together at the surface of the protein or infectious particle either by the folding of the polypeptide or by the constraints imposed by the architecture of the virus.

Immunogenic sequences have been identified by several methods (Table 3). Clearly the most critical criterion to be met is that the epitope should react with the antibody which neutralizes the infectivity of the organism. It is somewhat surprising, therefore, that the random expression of sequences to produce peptides which react with neutralizing monoclonal antibodies has not been explored more extensively.
Clearly the possibility of designing an effective immunogen when the relevant epitope consists of discontinuous sequences will present as many problems as protein folding. However, with continuous epitopes the problem is simpler and considerable headway has been made with a sequence from influenza hemagglutinin. Taking advantage of the x-ray crystallographic structure of the hemagglutinin, van Regenmortel and his group (19) cross-linked the two ends of the peptide sequence of the chosen epitope so that the distance between them mimicked that on the protein itself. Using this approach they were able to protect mice against a lethal challenge with the virus.

The great advantages of a peptide vaccine are summarized in Table 4. Probably the more important advantages would be: (1) there would be no need to rely on a biological vector to express the peptide, thus avoiding the vagaries of such systems, and (2) their stability, thus allowing delayed release mechanisms to be used provided that they were protected from hydrolytic enzymes.

**T cell epitopes**

Because of their small size it had been predicted that peptides would behave like haptens and consequently would need to be attached to a carrier protein to enhance their immunogenicity. It is now clear, however, that peptides are highly immunogenic in their free form provided they contain T helper sites which can elicit help for antibody production in addition to the specific antibody recognition sites (B cell epitopes). The T cell epitopes must be able to bind class II major histocompatibility complex (MHC) molecules on the antigen presenting cells of the host and the B cells and subsequently to interact with the T cell receptor in order to
induce the B cells to proliferate. In simple terms, for a peptide to elicit a satisfactory antibody response it must possess a T cell epitope appropriate for the recipient species. T cell epitopes may come from the same molecule as the B cell epitope or from a foreign protein.

This realization has prompted considerable interest in the prediction of amino acid sequences in proteins which can elicit a T cell response. For example, Berzofsky and his colleagues (15) have proposed that T cell determinants of a protein correlate with regions which have a propensity to adopt amphipathic alpha-helical structures, i.e., one face of the alpha-helix, such as the hydrophobic face, may interact with the Ia molecule and the hydrophilic face may bind to the T cell receptor.

In another approach Rothbard and his colleagues (25) have suggested that a common motif is found in the majority of helper and cytotoxic T cell determinants represented by a 4 or 5 residue sequence in which a charged residue or glycine is followed by two or three hydrophobic residues and ends with a polar residue.

Several examples have been reported which show the value of T-cell help, such as those of Good et al. (9) with a malaria circumsporozoite epitope, Borras-Cuesta et al. (3) with a rotavirus peptide, Leclerc et al. (14) with an epitope from hepatitis B virus, and Francis et al. (7) with epitopes from ovalbumin and sperm whale myoglobin. The last two examples provided the help necessary to elicit the formation of neutralizing antibody against foot-and-mouth disease virus in $H_2^d$ mice when synthesized co-linearly with a B cell epitope which did not elicit neutralizing antibody when inoculated alone.
These examples have demonstrated clearly that B and T cell recognition sites can be mimicked by small peptides and studies aimed at identifying potent T cell epitopes for individual species would provide valuable information, not only academically but also in practical terms. Moreover, such studies may provide information on the generally poorer response of older individuals to antigens. This subject has been reviewed in detail by Milich (17).

**Presentation of proteins and peptides to the host**

Almost without exception immunogenic proteins separated from the intact organism have a much lower activity than when presented *in situ*. The configurational alterations which occur when the proteins are released from their natural environment are likely to diminish their immunogenic activity or at least to modify the response qualitatively. Several methods have been described for increasing the immunogenicity of proteins since Glenny and his colleagues first described the use of aluminium salts for this purpose in the 1920s. Surprisingly, adjuvants based on aluminium salts are still the only ones which are accepted for use in man. To be optimally immunogenic it is now appreciated that antigens should be presented in several copies on a microscopic or submicroscopic particle. Such a multimeric structure mimics that of the micro-organism itself. As referred to above, Morein and his colleagues (18) have described an effective system in which saponin, which has a well-documented adjuvant activity, cholesterol, phosphatidyl choline and the antigen are combined in equinolar amounts to form a spherical, cage-like structure (ISCOM) held together by hydrophobic interactions.

In a detailed study of ISCOMs with a wide variety of antigens, this group has studied clearance from the injection site, uptake in macrophages, transportation and prevalence in lymphatic organs. They have shown in extensive animal studies that protective immunity can be
induced against several pathogens. Not only do ISCOMS induce the formation of antibody but cellular immune responses are also stimulated. Of particular interest is their demonstration that protective immunity against influenza can be induced by intranasal presentation.

Peptides can also be presented by this method, for example by combining them with a carrier protein such as influenza virus hemagglutinin and then incorporating the peptide-protein complex into an ISCOM. Possibly greater activity would be obtained by expressing the peptide sequence in one of the antigenic regions of the hemagglutinin molecule before incorporation into the complex.

An ideal vaccine

With our current knowledge of the structure of antigens and the way in which they are processed by the immune system, is it now possible to construct an 'ideal' vaccine? During the past few years several authors have made a list of the attributes of such a vaccine. Clearly this is a laudable objective but it is naive to define the properties of an ideal vaccine which would be applicable to all diseases, because the protective immune responses we are seeking to elicit will differ for individual diseases.

Nevertheless there are some goals which apply to all vaccines, whether attenuated or killed. They must be safe and cause no serious side effects. We should also aim for one inoculation, preferably by the oral route, to provide long lasting immunity. If the vaccine is attenuated it must not revert to virulence.
What are the best candidates for such development? Clearly a live vector such as Salmonella typhimurium would meet these requirements (6). It is also particularly noteworthy that in an experimental mouse model protective immunity against influenza was achieved by intranasal presentation of an ISCOM carrying the hemagglutinin of the virus. But clearly such an approach would not be appropriate for a vaccine against malaria where the route of infection is via the blood, unless it can be demonstrated that oral delivery will provide systemic immunity.

The clear initial objective must be to determine the immune response which is protective for each disease. The lack of knowledge is well illustrated by a disease which has been studied intensively, namely poliomyelitis, where two quite different vaccines have provided protective immunity in many millions of people worldwide. Do we know even now what the essential common feature is which affords protection?

In conclusion, however, there is now so much information available regarding the production and presentation of immunogenic proteins and peptides that many choices are available for several diseases. The great need is to focus on the route to take.

The Future

Molecular biology and in particular our understanding of the immune response at the molecular level have provided us with the opportunity to design vaccines which will elicit the appropriate response for many diseases. The crucial issue is to identify the immune response which correlates with protection. It is remarkable how well the empirical approach has worked so it is important that we should not ignore the lessons to be learned from these successes. In particular it is essential that each disease and the immune response which affords protection should be studied individually so that the appropriate responses can be induced.
**Table 1.** Disadvantages with live attenuated vaccines

1. Possible presence of adventitious agents in the cells and medium used for growth
2. Reversion to virulence which causes a small but significant number of clinical cases each year
3. Refrigerator temperatures are required for storage and transport
4. Limited shelf-life

**Table 2.** Disadvantages with killed vaccines

1. Hazard to personnel working with large amounts of human pathogens (e.g., rabies virus)
2. Hazard to the environment when working with large amounts of virus which will infect livestock (e.g. foot-and-mouth disease virus)
3. Need to ensure complete inactivation of infectivity
4. Presence of considerable amounts of cellular material, leading to side-effects (e.g. the rabies vaccine produced in the brains of sheep and goats can produce neurological problems in man; foot-and-mouth disease vaccine produced in tissue culture cells can cause hypersensitivity and anaphylaxis in cattle)
5. More than one injection usually required
6. Refrigerator temperatures are required for storage and transport
7. Limited shelf-life
### Table 3. Identification of immunogenic sites on proteins

1. Measure biological activity of fragments
2. Identify regions accessible to water
3. Interpret secondary structure (e.g. amphipathic helical regions, B-turns, flexibility)
4. Relate antigenic variation to amino acid sequence variation in naturally occurring and laboratory derived mutants
5. Measure antigenic activity of polypeptides expressed from fragments of the coding region
6. Map synthetic fragments of the protein with neutralizing antibody

### Table 4. Advantages of a peptide vaccine

1. Product chemically defined
2. Stable indefinitely
3. No infectious agent present
4. No large-scale production plant required
5. No downstream processing required
6. Can be designed to stimulate appropriate immune responses
7. Provides opportunity to use delayed release mechanisms


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31. Wright, A.E. & Leishman, W.B. (1900). 'Remarks on the results which have been obtained by the antityphoid inoculations and on the methods which have been employed in the preparation of the vaccine', British Medical Journal, 1, 122-129.
NOVEL APPROACHES TO CONTROLLED RELEASE ANTIGEN DELIVERY

Smadar Cohen¹,², Maria J. Alonso¹,³, and Robert Langer¹,⁴.

¹Department of Chemical Engineering, Mass. Inst. of Technology, Cambridge, MA 02139, USA.
²Present address: Department of Chemical Engineering and Program of Biotechnology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel.
³Present address: Department of Pharmaceutical Technology, School of Pharmacy, Santiago de Compostela 15706, Spain.
⁴To whom correspondence should be addressed (Tel: (617) 253-3107).
Abstract

Two strategies for vaccine delivery systems, both relying on concepts of controlled release technology, are described in this review. The first strategy uses biodegradable polymer microspheres for parenteral and oral delivery of antigens. The other strategy combines two technologies, the encapsulation of antigen within liposomes and liposome encapsulation in hydrogels, to protect them from a rapid degradation, in vivo. Both strategies have shown promise in terms of increasing the immunogenicity of poorly-immunogenic peptides and protein vaccines. Issues such as the microencapsulation process, antigen stability, mechanism of antigen release, and optimal release kinetics for vaccine delivery are reviewed along with a discussion on the strengths and weaknesses of each approach.
Introduction

Many of the difficulties associated with the production of live or attenuated vaccines and their application in human medicine could be overcome if long-term immunity was achieved by simply vaccinating with subunit or peptide-based antigens. At present we are living through a biotechnology revolution that promises to provide a plethora of new peptide-based vaccines. The practical application of such vaccines depends, however, on their ability to successfully mimic naturally occurring antigenic determinants present in the infectious agent and on their capacity to stimulate the appropriate response to induce immunity to the natural infection. Unfortunately, most of these synthetic peptides are poor immunogens and require the use of adjuvants to increase their immunogenicity (24). This limits their application to human medicine, where the selection of adjuvants is very narrow. Thus, the prospect of using synthetic peptide antigens in vaccines will become a reality only if safe and effective adjuvants will be developed.

An approach, advanced by ours and several other laboratories, to adjuvant design employs concepts from the area of controlled release technology where therapeutic molecules are delivered from vesicles or polymeric systems at a predetermined rate for a definite time period. The rationale for using this approach for adjuvant design evolved from the widely held view that adjuvant activity is partly due to their 'depot' effect, whereby an antigen is adsorbed onto the material and is slowly released to the surrounding medium over a certain time period (15). Controlled release systems provide advantages over conventional adjuvant systems.
For example, they can be constructed to deliver the vaccine at a constant rate for prolonged times, or to deliver two distinct pulses, or more, of antigen. Other potential advantages of controlled release systems include: (i) localized or targeted delivery of the vaccine to antigen presenting cells and the lymphatic system (ii) preventing the peptide-based vaccine from being rapidly destroyed by the body, and (iii) improved patient compliance. If successful, this approach will not only advance the use of peptide-based vaccines but will have a great impact on national and international immunization programmes as it offers the prospect of reducing the number of injections of vaccines to a single shot regime. Such a regime would greatly increase the percent of immune coverage of the population, particularly of children, in developing areas, where five million deaths per annum have been reported to occur in children who remain unimmunized against measles, polio, tetanus, pertussis, and diphtheria (18).

This review highlights the advances we and others have made since controlled release technology was first applied for immunization (23). These first studies showed that a single micropellet containing bovine serum albumin (BSA) embedded within an inert, non-degradable ethylene-vinyl acetate copolymer (EVAc) matrix induced antibody responses in mice for more than 6 months, comparable to the antibody levels obtained after 2 injections of the same amount of BSA in complete Freund's adjuvant. The sustained immune responses were of the IgG class and tolerance was not exhibited. The prolonged production of antibodies was consistent with the prolonged in vitro delivery of BSA from these matrices. Recognizing the potential of controlled release technology in immunization programs, and the need of simple, safe, and economic
method of immunization, the non-biodegradable EVAc was later replaced by biodegradable polymers, thus obviating the need for retrieving the vaccine-depleted device.

Biodegradable Microspheres for Protein and Vaccine Delivery.

Among the various available biodegradable polymers, the polyesters of lactic and glycolic acids and their copolymers were selected as the material for adjuvant constructs. These polymers have the advantage of being FDA approved for a number of clinical applications, e.g., surgical sutures (17;25) and controlled release microspheres (e.g. Lupron Depot). Long clinical experience with these polymers has shown that they are biocompatible in physiological environments and degrade to toxicologically acceptable products that are eventually eliminated from the body (16;28). In vivo, the polymer undergoes random, nonenzymatic hydrolysis of its backbone ester linkages at a rate that is controlled by the polymer molecular weight, polymer matrix surface area, monomer stereoregularity, and the ratio of lactic to glycolic acid. Poly (lactic/glycolic acid) (PLGA) can be prepared in any molar ratio of lactic to glycolic acids. The proportion chosen is important in determining the crystallinity, solubility, and water uptake of the final polymer, and most importantly it determines the in vivo degradation rate. Copolymers prepared in a 50:50 proportion of lactic and glycolic acids are hydrolyzed much faster than those which have a higher proportion of either monomer. For use in peptide or protein delivery, lactic acid is usually selected as the predominant species because it is more hydrophobic than glycolic acid, thus allowing better control of the diffusion rate of hydrophilic molecules such as proteins through the polymeric matrix. In addition, the use of D,L-lactic acid is preferred over
L-lactic acid since it leads to an amorphous polymer, thus allowing a more homogeneous dispersion of the protein within the monophasic matrix.

Vaccine delivery with PLGA presents important challenges such as the development of gentle and efficient methods of encapsulation. Proteins are highly sensitive to organic solvents, the only solvents for PLGA polymers. In the case of vaccines, this can be detrimental to their native antigenic properties, leading to changes in their immunogenicity and the production of undesired immune responses upon immunization. In addition, since many of these proteins are not soluble in organic solvents, their dispersion, as a powder, within the polymer solution is often not efficient, leading to a nonhomogeneous distribution of protein islets within the polymer matrix. The uncontrolled distribution of proteins may produce variable and nonreproducible release kinetics, making the design of controlled release systems a difficult task. To overcome these problems, the multiple emulsion method was developed (8). The method combines the use of both water and organic phases, where protein is dissolved in the aqueous solution and is dispersed in the PLGA solution, using ultrasonic radiation, to create the first inner emulsion of water in oil (W1/O). This emulsion is then emulsified again in a second water phase containing the surface active material, polyvinyl alcohol. Upon contact with the second water phase, PLGA precipitates to create embryonic microspheres, that are further solidified when the organic solvent is removed by evaporation. The method produced spherical PLGA microspheres with diameter sizes between 1-100 \( \mu m \), a parameter which is easily controlled by the formulation conditions. These microspheres can be injected through a 25 G needle, thus obviating the need for surgery for implantation. The protein encapsulation yields were greater than 90%. Most importantly, proteins,
enzymes (e.g., horseradish peroxidase), and several vaccine candidates (e.g., tetanus toxoid, and malaria synthetic peptide) retained their solubility, activity, and immunogenicity following this microencapsulation procedure (7;8). Other advantages of this method are simplicity, no need for complex equipment, speed (it takes approximately 30-40 minutes, including solvent removal by extraction), and amenability to scale-up.

Protein release from biodegradable polymer matrices can occur by two main mechanisms: diffusion through a tortuous, water-filled path in the polymer matrix and by matrix bioerosion. The latter occurs when the release of the protein from the matrix follows erosion of the polymer surface and/or the bulk matrix rather than simple diffusion (21). In the case of PLGA, the mechanism of protein release is very complex. At present, even for small peptides, there is no single model which is capable of correlating the impact of polymer formulation, drug properties, drug loading profiles and dosage form on the release rate(s). Thus, the design of PLGA systems with desired protein release kinetics has to be based mainly on empirical principles and common sense.

When trying to design vaccine delivery systems based on PLGA microspheres we realized that it would be difficult to manipulate and predict apriori protein release kinetics if the release from these systems was purely diffusion-controlled. Thus, we exploited an approach where protein release is regulated mainly by matrix erosion, i.e., chemical reaction, that can be better controlled by parameters such as polymer molecular weight and comonomer ratio. For example, by simply changing polymer molecular weight and/or comonomer ratio one can obtain systems that degrade within one week or as long as 1 year. To examine this approach we needed 1) to minimize protein release by diffusion and 2)
PLGA polymers with degradative properties that will allow the release of high molecular weight protein within an appropriate time period (1-3 months). In order to minimize protein release by diffusion through preformed pores, we decreased the protein loading ratios to 0.3-1 wt% (protein/polymer) and created very dense polymer matrices by working with high concentrations of PLGA (1 gr. per ml methylene chloride). By adjusting these technological parameters and selecting PLGA of low molecular weights (5-10,000 daltons) we were able to design controlled release PLGA systems that deliver proteins continuously or with biphasic patterns at rates that were controlled mainly by polymer degradation (8).

**Optimal Release Patterns for Vaccine Delivery**

While a relatively large amount of data concerning the physico-chemical characteristics of microspheres and the mechanism of protein release was accumulated, little is known about the type of release patterns which will maximize the immune responses to a given antigen, and most importantly, will induce long-lasting immunity and protection. Currently, two release patterns have been considered as appropriate for vaccine delivery: one is a constant antigen release pattern; the other is a pulsed release pattern. Very recently the suitability of these release patterns to immunization was challenged on the basis that they do not take into account the phenomenon of affinity maturation which occurs during an immune response; when the concentration of antigen becomes limiting, cells with high affinity receptors are selectively stimulated (1). According to this model, an ideal vaccine delivery pattern should mimic the profile of antigen concentrations seen in the course of natural infection, namely delivering a high dose of antigen within a few days of injection followed by
a period of delivering decreasing amounts of antigen. The initial high load of antigen will influence the extent of memory T-cell formation, while the subsequent steady decrease in antigen load will enhance the development of antibody affinity maturation. The versatility of views reflects, in our opinion, the need for a better understanding of the nature of immune responses generated by vaccines and their role in preventing infection by the wild-type agent. It is possible that controlled release technology, with the availability of systems with different release patterns, could aid in answering some of these questions.

Application of PLGA Microspheres for Tetanus Toxoid Delivery

Nevertheless, the potential of controlled release technology in immunization is being established by the increasing number of reports on the capability of biodegradable microspheres to increase the immunogenicity of poorly-immunogenic peptides and vaccines, such as tetanus toxoid, diphtheria toxoid, and Staphylococcal Enterotoxin B toxoid (13;14;22;27). Recently, our group has shown that the controlled delivery of tetanus toxoid in PLA or PLGA microspheres greatly enhanced its immunogenicity (2). The immunization study was performed with mice and guinea pigs and the results are presented in Figure 1. Two types of microspheres were tested: one made of PLA, with a small size and relatively rapid vaccine release (9 μm, 50% released in 10 days) and the other, made of PLGA, with a larger size and slow release (PLGA, 50 μm, 30% released over 30-day period). Overall, both formulations produced high levels of antitoxin neutralizing antibodies for more that 24 weeks. The levels were 4-5 times higher than those obtained after the administration
of the same amount of fluid tetanus toxoid (data not shown).

Two findings which, in our opinion, demonstrate the impact of antigen release patterns and kinetics on the profile and type of antibody production are noteworthy; the first is that at earlier times (e.g., in mice, 4 weeks after immunization), the levels of neutralizing antibodies are higher in mice receiving tetanus toxoid in PLA, than those receiving the antigen in PLGA. This behavior correlates with the fast *in vitro* release kinetics of the toxoid from these formulations, and the theoretical provision of early high antigen load *in vivo*. The other is the finding that while the levels of neutralizing antibodies (as measured by the toxin neutralization assay) increased with time, the levels of IgG antibodies to tetanus toxoid measured by ELISA remained constant or decreased with time (data not shown). Such a pattern could be explained by the phenomenon of affinity maturation, where cells with high affinity receptors are selected when the antigen load is decreasing. This pattern correlates, again, with *in vitro* release studies that showed decreasing rates of tetanus toxoid release over time (2). Many studies have yet to be done, including the characterization of the *in vivo* release behavior of these formulations, before any conclusion regarding their suitability in clinical immunization can be reached. Nevertheless, the findings that these formulations induce high quality antibody (i.e., neutralizing antibody) responses, and that the levels of these antibodies are sufficient to provide protection against the infectious agents, indicate that the approach of controlled release may play an important role in future immunization studies.

**Biodegradable Polymers with built-in adjuvanticity**

The concept of using biodegradable polymeric systems for vaccine
delivery is particularly attractive if the polymer degradation products are intentionally designed to have adjuvant properties. This would permit the design of a system that could stimulate the immune response while simultaneously releasing antigen over long periods. Because of the adjuvanticity of L-tyrosine and its derivatives, a polymer consisting of tyrosine or a tyrosine derivative connected by hydrolyzable iminocarbonate bonds was synthesized (19). When this polymer was converted into small pellets, this system provided sustained adjuvanticity while simultaneously serving as an antigen repository. The release of antigen from a single tyrosine-based iminocarbonate pellet gave rise, in mice, to higher antibody titers than release of the same antigen dose from a polyiminocarbonate (not containing tyrosine) pellet or from two injections of the antigen over 1 year (20).

**Liposomes and Microencapsulated Liposome Systems**

The rationale for using liposomes for carrying antigens for immunization was originally based on the known preferential uptake of parenterally injected liposomes by macrophages (3;9). Macrophages are an important component of the antigen-presenting system and play a fundamental role in the production of T-cell-dependent humoral immunity. Thus, the natural targeting of vaccines to macrophages by utilizing liposomal carriers was expected to induce the immunogenicity of small peptides and evoke a suitable immune response. This approach has been utilized by several groups for several subunit and peptide-based vaccines (e.g., tetanus toxoid, cholera toxin, human malaria sporozoite antigen) and generally the immune responses elicited by the liposomal vaccines were higher than those obtained with fluid vaccines (4;9).
However, the immune responses were almost always lower than those obtained when immunizing with peptide in complete Freund's adjuvant. Moreover, at least two injections of antigens in liposomes were generally needed to elicit an adequate immune response (9). This is partly due to the inherent instability of liposomes in the host where they are rapidly destroyed by high density lipoproteins and destructive cells (26). Thus, although the approach of targeted delivery of antigens to macrophages seems attractive, until very stable liposomes can be constructed, other approaches to extend antigen delivery need to be explored.

We attempted a different approach to extend antigen delivery while still utilizing liposomes as the basic encapsulation procedure (5;6). Liposomes, containing the antigen of interest, were protected from the host by encapsulating them within a hydrogel matrix composed of Ca-crosslinked alginate and an additional coat of alginate-poly(L-lysine). This approach was successful not only in extending the delivery of antigens from the encapsulated liposomes for more than 80 days, but also produced high levels of antigen-specific antibodies that persisted for more than 150 days (Figure 2). The antibody levels induced by BSA delivered by the microencapsulated liposome systems (MELs) were 3-4 times higher than those induced by BSA in liposomes or saline and 1.3 times higher than those induced by BSA in complete Freund's adjuvant, at their maximal levels. More significantly, while antibody titers dropped by day 50 in rats injected with BSA in complete Freund's adjuvant, in rats injected with BSA in MELs the titers remain high for more than 150 days (6). The prolonged production of antibodies was consistent with the prolonged presence of BSA at injection sites, suggesting that MELs can be used in long-term single-step immunization strategies.
Oral Immunization With Biodegradable Microspheres

The approaches discussed so far involve polymeric systems or vesicles that are injectable. Recent studies have shown however, that such systems, particularly the biodegradable microspheres, may also induce immunity when administered through the oral route (10-12). Oral immunization with microspheres containing 0.23 wt% Staphylococcal Enterotoxin B (SEB) toxoid, not only induced circulating IgM, IgG, and IgA anti-toxin antibody, but also a disseminated mucosal IgA response in mice (10-12). In contrast, oral immunization with the same amount of fluid antigen resulted in minimal to absent antibody titers.

The immunity conferred by these microspheres was related to their preferred uptake by the Peyer's patches of the gastrointestinal tract. Peyer's patches are specialized lymphoid tissue, and participate in the process of antigen sampling and presentation to the immune system. The initial uptake and cellular trafficking of the encapsulated antigen was determined by the size of the microspheres. Fluorescence microscopy analysis of histologic sections revealed that orally administered microspheres with diameters higher than 10 μm were not absorbed, while microspheres > 5 μm were taken up by the Peyer's patch lymphoid tissue of the gut and remained detectable there for up to 35 days. Particles with diameters of less 5 μm, however, were found in draining mesenteric lymph nodes and the spleen (10;12).

Another parameter which seems to determine the Peyer's patch uptake of microspheres is their effective hydrophobicity (11). Similar sized biodegradable microspheres made of ethyl cellulose triacetate or cellulose
acetate hydrogen phthalate were not taken up by Peyer's patches, whereas the more hydrophobic poly(hydroxybutyrate) microspheres were absorbed more efficiently than the lactide/glycolide copolymers.

The brief experience with oral immunization using biodegradable microspheres has shown proficiency in terms of inducing secretory and, to a certain degree, circulating antibodies. There have not yet been any long-term studies with this approach to probe the degree of protection conferred by this route of antigen administration, and there are still many issues to be resolved regarding the mechanism of uptake of microspheres, quantification of the process, and their destiny. It is expected that a better understanding of the mechanism of microsphere uptake by Peyer's patches and the parameters affecting this process will enhance the application of this technology in the clinic.

**Microencapsulation Process and Storage—Implication in Future Vaccine Design**

Several issues, including the gentleness of the microencapsulation process, the desired conditions for vaccine storage, and the distribution process in the field, of these formulations, are expected to have an impact on the choice of the vehicles in immunization programs. With respect to the presently available techniques for antigen entrapment in synthetic polymeric systems, such as those composed of polyanhydrides or polyesters, liposome encapsulation is advantageous since it does not involve the exposure of antigen to organic solvents such as methylene chloride, or to lyophilization. The latter process is responsible for the phenomenon of reduced solubility of many antigens, leading, in many cases, to a decrease in their immunogenicity. Even methods which are
intended to minimize the exposure of antigen to organic solvents, such as the modified evaporation method using a multiple emulsion (2), are hazards in terms of maintaining the immunogenicity of certain antigens. Use of organic solvents milder than methylene chloride, such as ethyl acetate, might provide a gentler method of encapsulation, however, their use limits the types of polymers to only those which are soluble in these solvents.

From a storage and distribution point of view, the use of polymeric systems for vaccine delivery is advantageous over the use of liposomes because the former formulations maintain the peptide in the dry state, while the latter require aqueous conditions for maintaining their carrier characteristics. Using dry vaccine formulations will simplify the storage and distribution of vaccines especially in places where power supplies for refrigeration do not exist and ice is unavailable, and will enable rapid expansion of immunization programs.

Conclusions

The idea of using controlled release technology in immunization is becoming a reality and progress in this area suggests that this concept may play an important role in future vaccine design. There are many questions to be answered and many parameters to be studied before this approach will be used clinically. However, the importance and the need to design better adjuvant systems for existing and new vaccines makes it likely that research in this area will continue to grow.

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Figure Legends

Figure 1: Immune responses to encapsulated tetanus toxoid. (A) in mice; (B) in Guinea pigs. Toxoid was delivered s.c in controlled release systems composed of ( PLA or PLGA microspheres. Amount antigen injected was 5 and 15 LF, for mice and Guinea pigs, respectively. Antitoxin neutralizing antibodies titers are in international units/ml serum.

Figure 2: Immune responses to encapsulated BSA, in rats immunized with antigen in Freund's adjuvant liposomes composed of egg hydrogenated phosphatidylcholine and cholesterol, and MELs of the same lipid composition. The data points are the average of five animals, at a serum dilution of 1:100.
Figure 1

A

Neutralization titers (AU/ml)

Time, weeks

4 6 12 24

B

Neutralization titers (AU/ml)

time, weeks

6 12 24
THERMOSTABILITY OF VACCINES: TECHNOLOGIES FOR IMPROVING THE THERMAL STABILITY OF ORAL POLIOVIRUS VACCINE

Stanley M. Lemon, M.D. 1
Professor of Medicine, Microbiology and Immunology
The University of North Carolina at Chapel Hill,
Chapel Hill, North Carolina 27599-7030 USA

Julie B. Milstien, Ph.D.
Scientist, Biologicals Unit
World Health Organization
1211 Geneva 27, Switzerland

TEL (919) 966-2536; FAX (919) 966-6714

"Vaccines and Public Health: Assessing Technologies and Global Policies"
International Journal of Technology Assessment in Health Care
Editors: A. Robbins and P. Freeman

DRAFT
ABSTRACT

The heat stability of vaccines administered through the Expanded Programme on Immunization varies widely, with oral poliovirus vaccine (OPV) being the least stable. Technologies which promise to enhance the stability of vaccines are likely to be determined by the physical structure and biological functions of specific vaccine immunogens, and thus may be very product-specific. A Product Development Group of the Children's Vaccine Initiative is encouraging research to define the extent to which the stability of OPV may be improved by the addition of antiviral compounds which bind to the poliovirus capsid, and/or the application of novel drying technologies.
A considerable percentage of the total cost of immunization programs relates to the creation and maintenance of "cold chains", continuous refrigeration systems designed to ensure that vaccines retain potency to the point of administration. These costs include not only the capital costs of refrigeration equipment, but also expenses for the management and distribution of the systems. In countries where vaccine procurement is financed through the public sector, the costs of an effective cold chain represent a greater proportion of the total costs of immunization systems than in countries with a private sector vaccine production and procurement market. By substantially enhancing the stability of vaccines at ambient temperature conditions (which may exceed 40 °C in the tropics), cold chain requirements and costs may be reduced, leading to financial savings which could be applied to the purchase of additional vaccines or improvements in the vaccine distribution system. Enhancing the thermal stability of vaccines would also have the major benefit of providing greater reliability of potency at the time of administration, especially in countries where problems have been documented in establishing and maintaining effective cold chains.

Vaccines which are currently included in the Expanded Programme on Immunization (EPI) vary widely in terms of their thermostability (Fig. 1) (1). In general, the nonreplicating antigens which comprise the DT and pertussis vaccines are relatively stable at 37 °C, but undergo significant degradation at higher temperatures. The least stable EPI vaccines are oral poliovirus vaccine and measles vaccine (live, attenuated viruses, which must retain infectivity in order to replicate within the vaccine recipient), and perhaps BCG which is also a replicating antigen. Available data concerning the thermostability of BCG are difficult to interpret as they suggest substantial differences among different vaccine preparations (1). Measles and BCG vaccines are both distributed in lyophilized forms which greatly enhance their stability under ambient temperature
conditions; the stabilities of the reconstituted vaccines are much less than those depicted in Fig. 1. Unfortunately, it has not been possible to lyophilize poliovirus vaccine due to large loses in vaccine potency which accompany drying.

Cold chain conditions are defined by the requirements of the vaccine which is least stable (OPV). Thus it is clear that efforts to improve the thermostability of vaccines in general must first be focused on OPV.

Significant improvements in the thermostability of OPV would have benefits that go well beyond the potential savings in cold chain costs, including increasing the probability of achieving the global eradication of poliomyelitis. The primary strategy for global eradication is to interrupt the transmission of the wild-type virus through effective immunization programs. This will require supplemental immunization efforts in addition to routine immunization as developed by EPI, including extensive house-to-house immunization by health staff and community volunteers in areas at high risk for continued transmission of the wild-type virus. The mobility of immunization workers is a key factor in these supplemental immunization strategies, and this could be greatly increased if it were possible to develop a thermostable OPV which does not require continuous refrigeration in the field. In addition, poor seroconversion rates to type 3 poliovirus have been repeatedly observed in a number of tropical countries following routine immunization with multiple doses of OPV. The cause of these poor seroconversion rates is not known (3), but enhancing the thermostability of OPV would help to eliminate lingering doubts about cryptic thermal inactivation of the vaccine, and would help to ensure that vaccine potency is maintained all the way to the end user.

The EPI has placed the development of a more thermostable OPV at the top of its list of priority research needs related to the eradication of poliomyelitis, and has suggested as a goal an OPV formulation which has the ability to retain potency (less than 0.5 log₁₀
drop in the titer of each of the 3 poliovirus serotypes) following exposure for 7 days at 45°C. Such a level of thermostability represents a quantum increase over the stability of currently manufactured OPV formulations which contain magnesium chloride, sucrose, or other empirically derived stabilizers, and is certain to require the application of novel technologies. To determine whether newly available technologies would permit this level of thermostabilization of OPV, a Product Development Group for a Thermostable Poliovirus Vaccine has been established under the aegis of the Children's Vaccine Initiative. For a variety of reasons, many of which are imperfectly understood, it may well be that this level of stabilization is not biologically possible. However, less impressive stabilization (such as retention of potency at 42°C for 7 days) may still provide significant advantages to immunization programs, and thus is not being overlooked by the Product Development Group.

In addition to better stability at temperatures expected in the field, a thermostable poliovirus vaccine must remain at least as stable as current OPV when held at 4°C or when frozen. The vaccine must also be as immunogenic, as safe, and as easy to administer as the OPV used currently in EPI programs. If it is to make a practical impact on the control of poliomyelitis, it must be possible to make the stabilized product available to immunization programs at only a small, fractional increase in price. However, a significantly more expensive thermostable vaccine might still be useful in special situations, such as supplemental immunization activities in particular countries with significant barriers to access, or in developed countries where cold chain problems have been documented. Ideally, the technologies that result in stabilization of the vaccine virus should be readily transferred to lesser developed countries that are currently manufacturing OPV.

A number of obstacles stand in the way of the development of a thermostable OPV. These include the fact that OPV is a trivalent vaccine, containing attenuated virus strains
representing each of the 3 poliovirus serotypes. These 3 viruses may have significantly
different thermostabilities, and thus somewhat different requirements for stabilization. As
important, the physical basis of inactivation at 37-45°C remains poorly defined. It is not
certain whether loss of vaccine potency relates to degradation of the virus capsid,
destruction of the RNA genome (possibly by encapsidated RNase activity), or both.
However, on the positive side, we have a uniquely detailed view of the physical structure of
poliovirus which derives from studies involving X-ray crystallography of the virion. This
elegant work has defined the structure of the virus capsid at a 2.9 Å level of resolution, a
level at which the orientation of the side chains of individual amino acids is readily
apparent (2). In addition, this knowledge has come on the crest of an explosion in our
understanding of the mechanisms of replication and attenuation, and the overall molecular
genetics of poliovirus.

Given these considerations, there are three technologies which appear at present to
have the potential for producing a thermostable OPV. These include genetic manipulation
of the virus to arrive at more thermostable seed virus stocks, the use of novel, capsid-
binding antiviral drugs to stabilize the virus, and the application of specialized drying
techniques to remove water from vaccine and thereby reduce the probability of chemical
reactions involved in virus inactivation. Each of these technologies has potential advantages
and disadvantages.

Genetic approaches to stabilization of this positive-stranded RNA virus are possible
because virus can be recovered from recombinant cDNA (4). This allows the poliovirus
genome to be manipulated as DNA, and specifically mutated in ways which might enhance
the stability of the virus. This general approach, coupled with our detailed knowledge of
the three dimensional structure of the poliovirus capsid and recent advances in
understanding the molecular basis of poliovirus attenuation, represents a very powerful
technology. Advantages include the fact that recombinant cDNA "seed" material can be
replicated with significantly greater biological stability than RNA seed viruses. However, it seems very unlikely that mutations could be made in the virus which would result in increases in thermostability approaching the magnitude desired by EPI without significantly handicapping the virus in terms of its ability to replicate. Moreover, the overwhelming disadvantage to this approach is that any genetic change in the Sabin vaccine strains will raise serious questions about vaccine safety, as temperature sensitivity during virus replication ($t_s$ phenotype) is an important attribute of the attenuated Sabin vaccine strains and the interplay between $t_s$ phenotype and virion stability remains poorly defined. A genetically altered thermostable vaccine, even if biologically possible, would take many years to develop and thus would be unlikely to play any major role in the poliovirus eradication effort. The Product Development Group, after a careful review of the technology, has not embraced genetic strategies for enhancing the thermostability of OPV.

A second approach to vaccine stabilization is offered by the availability of a novel class of antiviral compounds which bind into a hydrophobic pocket located within capsid protein VP1 of poliovirus and related picornaviruses (6). Arildone is the prototype of these "WIN" compounds or "Janssen" compounds, which are often referred to according to the pharmaceutical companies seeking their development. A large number of congeners have been synthesized, some of which have undergone extensive evaluation as potential systemic antivirals, including use in humans. These compounds exert their antiviral effect either by stabilizing the capsid against uncoating reactions within the cell, or by creating subtle but effective changes in the conformation of the receptor binding site on the surface of the virus particle. It has been recognized for a number of years that these compounds enhance the thermostability of poliovirus and other picornaviruses, and there is now substantial interest in their use as potential vaccine stabilizers. Recent work indicates that at least one of these compounds is capable of stabilizing the antigenicity of Sabin type 1
poliovirus for 7 days at 42°C (5), and an intensive screening and testing program is now under way with the support and guidance of the Product Development Group.

Although prospects are hopeful, there are a number of uncertainties. First and foremost, it remains to be shown that the stabilization of potency (infectivity) will match that of antigenicity. It will be of little benefit to stabilize the capsid of the Sabin vaccine strains, if the RNA within is still degraded. A second problem relates to the antiviral effect of the compounds. To restore infectivity, successful stabilizing compounds must be capable of dissociating from the capsid under the concentration gradient established following oral delivery of vaccine. Thus, it will be critical to confirm the bioavailability of any vaccine stabilized with these compounds. Finally, the activity of these compounds varies among the individual poliovirus serotypes, making it important to identify a compound which has the appropriate properties of thermostabilization and reversibility of binding for each of the 3 vaccine strains. Recent efforts by Bart Rombaut and coworkers at the Vrije Universiteit Brussel suggest that the latter two problems may be overcome, but as yet there is no evidence suggesting that infectivity (potency) has been sufficiently stabilized.

Should one or more of these compounds ultimately prove to be an effective stabilizer, this approach would offer many advantages. First of all, it should be relatively inexpensive and very amenable to technology transfer. In addition, there should be no substantial safety concerns, as the total dose of antiviral administered in a complete immunization series would be many orders of magnitude less than the doses of these compounds which have been administered to humans in systemic antiviral trials. A serious consideration, however, is the nature of legal agreements which must be negotiated with the pharmaceutical manufacturers who have developed these compounds. Should a stabilized vaccine result from this work, the Product Development Group has strived to develop mechanisms to assure access to it by the public sector.
The third and final technology option is the application of novel drying techniques for preservation of OPV. At present, the outlook here must be viewed with caution if not outright pessimism. Although many vaccines, such as measles, are substantially stabilized by lyophilization (1), this is not the case with poliovirus vaccines. The major problem with freeze-drying of poliovirus is the loss of infectivity which occurs upon drying and which is usually greater than 3 log₁₀ in magnitude (that is, >99.9% of virus infectivity is lost). The mechanism underlying this inactivation is not well understood, but it is likely due to removal of water molecules which are involved in stabilizing the structure of the native virus capsid. While excipients such as trehalose (α-D-Glucopyranosyl-α-D-glucopyranoside) (7) have been used to stabilize biologically active molecules, including enzymes, during drying procedures, these excipients seem unlikely to work in the case of poliovirus because of the nature of the virus capsid. The capsid forms a tight shell, which is impermeable to molecules as small as neutral red (m.w. = 289), or single Cs anions (atomic mass = 133). Excipients with equal or larger molecular mass (such as trehalose, which has a m.w. of 342) are not likely to penetrate the capsid and thus will not stabilize internal protein-protein interactions. On the other hand, water molecules (m.w. = 18) are likely to diffuse freely through the capsid, and will "boil" out of the virus capsid upon drying of the vaccine.

Despite these uncertainties, it is reasonable to evaluate various excipients for their stabilizing effects during drying, as well as novel conditions for drying including the removal of water under ambient or even elevated temperature conditions. In addition, it may well be that one of the WIN or Janssen compounds is capable of stabilizing the virus capsid during removal of water. The major expected advantage of the drying approach is the potential for stabilization of the viral RNA as well as the protein capsid. Novel drying strategies are currently being tested in two laboratories under support of the Product Development Group. Should a drying strategy prove effective in stabilizing poliovirus vaccine, there will be a need to develop a simple rehydration process which could be used
on on a per-unit basis at the time of vaccine administration. This would significantly reduce vaccine wastage. Finally, it should be noted that while lyophilized measles and BCG vaccines are widely used in EPI programs, it is not yet clear that novel drying technologies would be amenable to transfer to lesser developed countries.

How applicable are these strategies to the stabilization of other vaccines? As indicated above, the stabilities of different vaccines vary widely under ambient temperature conditions (Fig. 1). These differences in thermostability are related to fundamental differences in the chemical structures and biologic functions of vaccine immunogens. Thus it is not surprising that efforts to improve the stability of any given vaccine must be tailored to the specific immunogen. The technologies being tested for stabilization of OPV may not be relevant to stabilization of other vaccines, such as measles or BCG for example. In particular, the capsid-binding compounds have relevance only to the potential stabilization of other picornaviruses. On the other hand, drying technologies are potentially applicable to a wide variety of biologically active molecules and thus perhaps should receive particular emphasis.

At this point in time, it is not at all certain that the Product Development Group will succeed in stabilizing OPV for 7 days at 45°C. Such a target may be biologically impossible. However, the Group has stimulated the creation of a multi-national research and development effort involving academic, industrial and government-based scientists which will rapidly test the available technologies in a thorough and well coordinated fashion, and which should determine whether or not these technologies will permit the production of a thermostable OPV. Should these efforts prove successful, the Group is prepared to guide the development of a product through the steps required to make it available to the children of the world.
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Legend to Figure:

Figure 1. Approximate $t_{1/2}$ of each EPI vaccine under different temperature conditions. The half-life ($t_{1/2}$) of a vaccine is defined as the length of time (days) required for loss of 50% of potency (infectious units for the replicating antigens shown with shaded symbols, or antigen units for nonreplicating antigens shown with open symbols). Above 37 °C, the potency of pertussis and BCG vaccines is rapidly lost. Data for measles and BCG vaccines are for lyophilized vaccines; the data for BCG suggest that the $t_{1/2}$ of different preparations may vary from 3 to >28 days. The $t_{1/2}$ of reconstituted measles vaccine is measured in minutes at ambient temperatures. Reconstituted BCG is also unstable at 36 °C. Data compiled from reference (1).
ABSTRACT

Immunizing pregnant women can increase neonatal antibody titers and provide new or prolonged protection against infectious diseases. This approach of passive protection of young infants can bypass the problems of immunological immaturity in the neonate, avoid or delay active immunization of the infant in the first year of life, and prevent transmission of an infection from the mother to the neonate. Optimal vaccines for this approach should induce high IgG antibody titers that reach quickly their maximum level after immunization and persist at protective levels for several years to provide passive protection in subsequent pregnancies. The theoretical possibility that maternal immunization may activate or suppress the infant’s active antibody response or alter the repertoire of that response by transplacental transfer of maternal antibody to the vaccine antigen, maternal anti-idiotypic antibodies, or the vaccine antigen itself, will require analysis with each vaccine. The beneficial effect of maternal immunization on transmission or carriage of pathogens as well as on augmentation of breast milk antibodies is under investigation. Specific applications of this approach include the world-wide practice of maternal immunization with tetanus toxoid vaccine, and ongoing studies of maternal immunization to prevent Haemophilus influenzae b, group B streptococcal, pneumococcal, meningococcal, and human immunodeficiency virus infection in the infant. Addressing the cultural, sociologic, and liability aspects of maternal immunization will be required to ensure the success of this approach.
The concept of preventing neonatal infections by immunizing pregnant women has been appreciated for over a century and was extensively studied in the past for the prevention of tetanus, diphtheria, and pertussis infections in infants (1-3,23,25,31). At the present time this approach is used routinely only with tetanus immunization, where it has proven to be the most cost effective means of preventing neonatal tetanus, a major cause of worldwide infant mortality (51). More recently, maternal immunization for the prevention of *Haemophilus influenzae* b (Hib), group B streptococcal (GBS), and meningococcal disease has been under evaluation (1,23,31). The possible application of maternal immunization for the prevention of influenza, respiratory syncytial virus, and human immunodeficiency virus (HIV) disease, has been promoted and, in some instances, is currently being or soon to be studied. In this review we will discuss the theory behind immunization during pregnancy, its potential effects on the fetus and on maternal colonization with and transmission of pathogens, and potential applications. Only active immunization with vaccines and not passive immunization with gamma globulin will be addressed in detail. Passive immunization with high titered antibodies could provide protection to neonates, but would be expensive and impractical and would need to be repeated with each subsequent pregnancy.

**Theory of Maternal Immunization.** Maternal immunization has the potential to induce protective antibodies in the pregnant woman that can be transferred across the placenta to the fetus, thereby transiently providing passive antibody protection from a variety of childhood infections. Most children in the United States are born with passive maternal antibody protection to tetanus, diphtheria, poliovirus, and measles. Immunity wanes during the first six months of life with catabolism of maternal antibody.

Actively immunizing pregnant women has the potential to increase specific antibody levels in her serum, and, thus, to generate in the offspring a greater amount of protective antibody at birth that will last for a longer period of time during infancy. Passive antibody protection could be conferred to neonates and infants throughout a period of susceptibility at an age when active immunization of the newborn or infant: (1) will not prove immunogenic because of immunological immaturity or inhibition by high titeres of
maternal antibody, (2) would require a period of time or multiple doses to induce protective responses; (3) can not be instituted or completed due to barriers to medical care. Active immunization of the infant who has been immunized passively by this approach may not prove necessary in some instances because natural immunity to some pathogens is acquired early in life or the pathogen is only infectious in the neonate or very young infant (e.g., group B streptococcus). In other instances, active immunization of the infant could be delayed until later in infancy with this approach. The ability to delay primary active immunization and/or decrease the number of active immunizations required for inducing immunity has important relevance for health care delivery in the Developing World as well as in the United States. Lastly, active immunization of pregnant women may have the potential of interrupting transmission of a pathogen from the mother to her offspring (e.g., HIV-1, group B streptococcus).

Transfer of immunity from mother to offspring has been well established for humoral immunity, but remains controversial for T cell or cell-mediated immunity (31). Transplacental passage of maternal IgG begins during the first trimester and increases markedly throughout the final trimester with the growing exchange area in the placenta (52). Transfer is initiated by binding of immunoglobulins to Fc receptors on the membrane of trophoblast cells in the placenta, followed by receptor-mediated endocytosis and active transport (46). IgG is the only immunoglobulin isotype transported. IgG1 subclass immunoglobulins are preferentially transferred compared to IgG2 immunoglobulins because of the binding specificity and affinity of the Fc receptor (21). Neonatal cord blood IgG1 and IgG4 levels reach maternal levels by about 35 weeks gestation, IgG3 by about 36 weeks gestation, while IgG2 seldom reaches maternal levels during gestation (19). Neonatal serum IgG levels correlate with gestational age. A 32-week premature infant will have an IgG level approximately one-half that present in the full-term neonate, whose level equals or exceeds their mother's. As a specific example of the efficiency of transplacental transport with fetal age, the ratio of cord/maternal IgG antibody to type Ia group B streptococcal antigen is 0.33 at 28 weeks gestation, 0.49 at 32 weeks gestation, and increases to 1.09 at 40 weeks.
gestation (11). This deficiency of passively acquired serum IgG in premature infants partially explains their increased susceptibility to infection.

Maternal IgG has a half-life of three to four weeks in the offspring, but this may be a slight underestimation (9). The duration of passive antibody protection is dictated by the protective antibody titer at birth. The duration of passive antibody protection also may be affected by differences in the half-life of passive antibody, as shown in some populations (9). Part of this difference may be a result of the frequency of infections, which can accelerate IgG catabolism in the first several months of life. It has been suggested that different populations may vary in their efficiency of transplacental transport of antibody induced by the same vaccine (9).

The humoral response to immunization in pregnancy is normal (31), but there is a delay between immunization and fetal acquisition of maternal postimmunization antibodies. This delay reflects both the time interval for the pregnant woman to generate an antibody response, which will always be shorter with a secondary or anamnestic response to a previously encountered antigen, and the necessity for the antibody to equilibrate with the circulating IgG pool and be transported across the placenta. Maternal immunization with tetanus toxoid vaccine is best if the primary dose is administered at least 60 days and the secondary dose at least 20 days prior to delivery (15).

The physiology of transfer of maternal antibody to offspring dictates some considerations of an ideal vaccine for use in pregnancy. The ideal vaccine should induce a high IgG1 antibody titer and the maximum IgG antibody response should occur with one dose and reach its peak within a short period of time so that antibody protection will occur even in prematurely born infants. This feature also would permit immunization to be performed later in pregnancy. Immunization probably should take place 4 to 6 weeks prior to delivery to allow adequate transplacental passage. Ideally, the vaccine should induce a high antibody titer in the newborn to allow persistence of passive antibody protection during infancy until active immunity can be provided to the infant. Vaccines that can induce very high protective maternal antibody titers that persist for years will result in passive antibody protection
during subsequent pregnancies. Live viral vaccines usually are avoided during pregnancy because of the risk to the fetus, although yellow fever vaccine and OPV can be given to pregnant women at substantial risk of exposure to infection. Although there are theoretical risks of immunizing with live rubella virus vaccine in pregnancy, a rubella syndrome in infants born to immunized, susceptible mothers has not been reported (13).

An alternate to immunization during pregnancy is immunization prior to pregnancy. Women of childbearing age who are likely to deliver newborns at high risk could be targeted for immunization or for certain diseases, such as rubella, all women would be immunized. This approach would require access to this population and possibly require multiple encounters if more than one dose of vaccine is required for immunity. In some situations, pregnancy is the only time when some women have contact with the health care system. In addition, the antibody response must persist for several years at a protective titer to prevent disease in future offspring. It should be noted, however, that even with immunization during pregnancy, protection for future offspring will be based on the antibody titer obtained with the initial immunization unless immunization is repeated with each pregnancy. Most importantly, though, immunization prior to pregnancy would avoid the potential liability problem of immunization during pregnancy, as discussed below.

Effect of Maternal Immunization on the Infant’s Immune Response. An infant’s immunologic response theoretically could be altered by transplacental transfer to the fetus of high levels of maternal antibody to the vaccine antigen, maternal anti-idiotypic antibodies directed to antibody to the vaccine antigen or the vaccine antigen, itself. Either suppression, activation or priming, or alteration of the repertoire of the active antibody response in the infant could occur.

Maternal antibody can interfere with immunization of infants with live-viral vaccines (66), and may suppress antibody responses to primary immunization with Hib conjugate vaccines (16), although the latter has not been a universal finding. In addition, high antibody responses to a protein carrier of any conjugate vaccine, such as diphtheria or tetanus toxoid, could theoretically interfere with active antibody responses after DPT immunization.
in the first six months of life. To date, these concerns have not been examined carefully. It has been shown that tetanus toxoid immunization during pregnancy does not interfere with subsequent active tetanus immunization of the offspring (26) and that passive tetanus antitoxin therapy administered simultaneously with active toxoid immunization does not interfere with seroconversion. The risk of suppression of responses to active immunization with polysaccharide antigen after immunization with polysaccharide vaccines during pregnancy has been evaluated to some extent. Infants born to women immunized during pregnancy with serogroup A and C meningococcal polysaccharide vaccines showed no obvious suppression after active immunization with these vaccines at six months of age (42). It should be noted, however, that active immunization of the young infant with high doses of meningococcal group C polysaccharide has been shown to produce transient immune suppression to later immunization (28) and that immunization of neonates with the Hib meningococcal outer membrane protein, conjugate vaccine can suppress antibody responses to immunization in infancy (62). Offspring of women immunized during the third trimester with the Hib PS vaccine that were then immunized actively at 18 months of age with the same PS vaccine had antibody responses indistinguishable from a control group (4). It has been shown recently that Apache infants receiving Bacterial Polysaccharide Immune Globulin (BPIG) (containing high titers of antibody to the Hib PS) at 2, 6, and 10 months of age did not have suppressed antibody responses to active immunization with tetanus and diphtheria toxoid; they did have interference with responses to live-virus measles vaccine but not rubella immunization (50). They also had a significantly reduced frequency of "high" concentrations of "natural" antibody titers to Hib compared to control infants. Nevertheless, antibody response after active immunization with the Hib PS vaccine at 18 months of age or to conjugate vaccine at younger ages was not impaired (50).

The possibility of activation or priming of the infant's immunity by maternal immunization should be considered. This could occur by transfer of the vaccine antigen or maternal anti-vaccine antigen or anti-idiotypic antibodies. The fetus can make an active antibody response with a congenital infection, or after maternal exposure to allergens, food
antigens, or drugs. Gill et al have published data demonstrating that infants born to women immunized between the fifth and eighth month of pregnancy with tetanus toxoid had been actively immunized to this antigen (26). Their studies showed that the infants not only had IgM antibody to tetanus toxoid but also increased lymphocyte proliferative responses to tetanus toxoid at birth and later during childhood. A state of priming for antibody responses to active immunization in the first year of life was not studied. The antibody titers after 3 doses of tetanus toxoid vaccine of offspring of these immunized women were similar to controls. Similar results were observed by Vanderbeeken (61) but have not yet been published or observed by other groups. Lymphocyte priming and neonatal IgM antibody, in fact, were not observed by other groups after maternal immunization with tetanus or influenza vaccines (24). Animal models, however, do support Gill's findings. Lee et al have shown that immunization of pregnant mice or monkeys with pneumococcal PS conjugate vaccines led to higher antibody responses to active immunization of the offspring (31,39). On the other hand, Martin and colleagues, observed neither enhanced nor suppressed responses to active immunization in the offspring of rhesus monkeys immunized during pregnancy with pneumococcal PS or Hib conjugate vaccines (31).

The possibility of suppression, priming, or alteration of immunologic repertoires of the neonate and infant by generation of maternal anti-idiotypic antibodies after immunization during pregnancy should be considered. The vertebrate immune system is composed of an interacting network of variable regions of antibodies and T-cell receptors that interact by recognition of V-region complementarities (18). A recursive pattern of autonomous or internally activated lymphocytes may show connectivity and be maintained. Immunization during pregnancy (by increasing serum antibody to the vaccine antigen or anti-idiotypic antibody) could alter the available or actual antibody repertoire of the offspring. In animals there is abundant evidence of changes of lymphocyte repertoires in the offspring of pregnant females whose immune system has been altered by active or passive immunization (5,38). In mice, immunization or passive transfer of antibodies to pregnant females can alter the establishment of repertoires in the offspring and affect their antibody responses
later in life. In the offspring, suppression of idiotype expression (63,65), favoring of expression of dominant maternal idiotypes (41), or alteration of the antibody repertoire after immunization with the same or with other antigens (59,60) can occur.

In man, relationships between the maternal and progeny's antibody repertoires remain largely unexplored. The fact that idiotypic interactions between mother and offspring can occur in man has been established. For example, cord blood mononuclear cells of children born to mothers with schistosomiasis or Chagas' disease showed specific T cell proliferative responses to idiotypes expressed on antibodies specific for Schistosoma mansoni or Trypanosoma cruzi (22). These findings suggest that infection led to transplacental passage of antibodies to these pathogens that then induced neonatal anti-idiotypic T cells specific for idiotypes on the maternal antibody. These T cells could act to enhance or suppress active immune responses in the young infant. Anti-idiotypic interactions to maternal antibody have been demonstrated only rarely in human neonates (30). Further studies of antibodies and immune responses in newborns and infants of women immunized during pregnancy are required to determine whether suppression, priming, or alteration of antibody repertoires occurs.

Effect of Maternal Immunization on Transmission or Carriage of Pathogens. Immunization during pregnancy may be warranted in a situation where a mother was at high risk for a particular infection for which a vaccine was available. Pregnancy may be the only time when some high-risk women have contact with the health care system. Maternal immunization could also affect maternal carriage or transmission of a pathogen to the fetus. Transmission of a blood-borne infection from mother to the offspring prior to or at the time of delivery could be prevented by maternal immunization. There is interest in preventing transmission of HIV-1 to the fetus with this approach (58).

Parenteral immunization with Hib, pneumococcal and meningococcal PS vaccines has, at best, only had transient effects on colonization rates (29,34,47,53). Parenteral administration with bacterial polysaccharide vaccines can induce mucosal IgA antibody but persistence appears to be short-lived (48). Mucosal antibody responses to conjugate vaccines
have not been studied very well, however, oropharyngeal carriage of Hib was shown to decrease relative to prior historical controls in 3-year-old children immunized parenterally with Hib conjugate vaccine (57). It should also be noted that Appache Indian infants receiving bacterial polysaccharide hyperimmune globulin had a slightly reduced carriage of Hib (50). It is possible that the Hib conjugate vaccines decrease colonization by inducing a high level of serum antibody which, by transudation, leads to high concentrations of mucosal sites.

Risk factors for early-onset GBS disease include heavy maternal colonization with GBS and the size of the bacterial inoculum transmitted to the neonate (10,20,37). Overall the ratio of maternal colonization to neonatal disease is approximately 100 to 1, but when the mother is heavily colonized, 10% of the newborn infants develop early-onset sepsis. Decrease in maternal carriage of GBS by maternal immunization could prevent GBS transmission to the neonate.

Effect of Maternal Immunization on Breast Milk Antibody. The beneficial effects of breast feeding and the protective factor(s) in breast milk are controversial. A supporting role for breast feeding in the prevention of RSV, rotavirus, Hib infections as well as otitis media, necrotizing enterocolitis and cholera has been suggested (32,54). Breast milk antibodies induced by parenteral immunization during pregnancy could provide or enhance protection against respiratory or enteric pathogens by preventing attachment of the microorganism on mucosal membranes. Others have shown that human milk blocks attachment of otitis media - causing strains of pneumococci and untypable H. influenzae to pharyngeal cells, possibly explaining why breast feeding may prevent otitis media (32).

Because of the compartmentalization of the systemic and mucosal immune systems, parenteral immunization with protein antigens generally does not induce an IgA immune response in external secretions (43,44). Both pregnancy and prior exposure to the antigen by a mucosal route appear to lead to exceptions to this observation (2,32). Breast milk may have high levels of antibodies to microorganisms that colonize or infect mucosal sites at respiratory and intestinal surfaces (32,54). Parenteral immunization during pregnancy has
the potential to increase antibody titers not only in serum, but in both colostrum and breast milk.

Pregnant women immunized with Hib PS vaccine have levels of antibodies to this antigen in their breast milk 20-fold higher than either unimmunized women or women immunized prior to becoming pregnant (36). Lactating Pakistani women immunized after pregnancy with cholera and, poliovirus vaccine show increases in antibody levels in breast milk (32,43,54). In pregnant animals, and probably in man, there is specific homing and retention of antigen-activated lymphocytes in mammary glands (64). This activation may occur at mucosal sites or after parenteral immunization during pregnancy. It is unclear whether breast milk antibody will be induced to all antigens administered parenterally during pregnancy or only to those antigens for which there is pre-existing mucosal priming prior to parenteral immunization.

Specific Vaccines for Maternal Immunization. Maternal immunization has been studied for multiple diseases early in this century (1,2) and more recently (Table 1). A few brief comments illustrates the potential of maternal immunization. Maternal immunization is practiced on a world-wide basis to prevent tetanus neonatorum, a major cause of neonatal mortality accounting for approximately 50% of all deaths and 80% of preventable deaths in the neonatal period in developing countries other than China (33). Immunization during pregnancy with tetanus toxoid vaccine has proven to be the most cost effective approach to its prevention (51). In spite of licensing of Haemophilus influenzae type b conjugate vaccine for active immunization of infants, studies of maternal immunization with these vaccines is warranted for certain high risk young infant groups (Native American, Canadian Inuit, New Guinea), because of a window of susceptibility that exists prior to induction of an antibody response by immunization in the first months of life. This approach would prove more cost-effective and easier to administer (2,27) than passive immunization with hyperimmune bacterial polysaccharide immune globulin (BPIG) (50). Maternal immunization with group B streptococcal vaccines is thought to be the most effective approach to prevention of GBS disease (7,8,17,37). Maternal active and passive immunization will be studied as a method to prevent transmission to the offspring of HIV-1 by inducing or boosting maternal immunity.
to HIV, decreasing plasma viremia, slowing disease progression, or inducing viral latency (58).

**Sociologic, Ethical, Legal Aspects of Maternal Immunization.** Several practical considerations need to be considered in any maternal immunization program. Pregnancy may be the only time when some women who are at highest risk for delivering offspring with infectious morbidity and mortality interact with the health care system. Obviously, high risk women who do not receive prenatal care prior to delivery will not benefit from a maternal immunization program. Additionally, some individuals or cultures may refuse participation in any immunization strategy, and still others may decline participation only during pregnancy. The possibility of immunizing the latter group of women postnataally at delivery to protect her as well as her offspring in subsequent pregnancies should be considered for some vaccines. Health education and attention to socioeconomic barriers must be an integral part of any maternal immunization program (14,35). Obstetricians and health care providers will need to be educated to the risks of the target disease, and the safety and efficacy of immunization during pregnancy. Liability issues for vaccine manufacturers will need to be addressed and even some type of indemnification policy arranged for maternal vaccine trials to be conducted. The background incidence of major congenital abnormalities and spontaneous abortions and stillbirths could plague any large-scale vaccine trials. Temporal relationships rather than a scientific rationale have often been the basis for court settlements in the past. Some vaccine manufacturers have stated they would need indemnification prior to participating in maternal immunization trials. Failure to address these sociological and legal aspects of maternal immunization will obviate this approach to prevention.
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The Global Capacity for Manufacturing Vaccines: Prospects for Competition and Collaboration Among Producers in the Next Decade

Anthony Robbins & Isao Arita

The Children's Vaccine Initiative will place heavy demands on the world's vaccine manufacturing capacity. The success of CVI depends on how people and institutions respond. Thus this paper, relying on history and on the current state of vaccine manufacture, attempts to assess how the world can respond to the production requirements of CVI. Three aspects of CVI's desiderata impinge on vaccine production capacity: the properties of the vaccines to be manufactured, the volume or number of doses needed, and affordability as reflected in the cost and price of the product.

CVI seeks an armamentarium of vaccines that together provide protection against a longer list of diseases while reducing the number of contacts required to immunize a child fully. Existing vaccines fail to meet the needs, thus CVI is developing new ones, applying new science and technology. New and improved vaccines will often require new or altered manufacturing technology. As CVI develops new vaccines, how they will be made becomes an important consideration. Can they be made in existing facilities? Can they be made under developing world conditions? These questions cannot be left until research and development work is complete.

The target population for CVI vaccines, estimated by the developing world's birth cohort, approaches 150,000,000 children per year and defines the global manufacturing capacity for any CVI vaccine to be used world-wide.

No one has defined affordability as clearly, despite a consensus that it is a CVI objective. At one theoretical extreme, the world should be willing to pay an amount for vaccines equal to their benefit to society. Yet political reality jerks us back to what is possible. The bidding begins with the meager amounts that developing countries, international organizations, and industrial world donors now spend on immunization. Moreover, who spends it and in what currency remains important. Thus after minimizing research and
development costs, CVI seeks to get an acceptable final product that is affordable and thus widely used because production costs are low.

Herein, we propose that thinking about vaccine production and supply not be separated from the research, development, clinical testing, and licensure that precede it in the natural sequence of events. Success, measured by the vaccines delivered and the diseases prevented, depends on completing the whole sequence. If that is true, then investment at any point in the system can improve chances of preventing disease.

Vaccine Production: history and status report

If vaccines were not such important tools for public health, vaccine manufacturing would be too small an activity to appear in any economist's view of the world. Total world vaccine sales have not reached $2 billion, and are dwarfed by pharmaceutical sales—at least 25 times larger—that accrue to a closely related industry. In the future, new vaccine production technologies may eliminate many of the differences that separate drugs and vaccines. Biologicals, whose manufacture depends on less predictable life processes, will certainly resemble the pharmaceutical products of chemical synthesis as scientists gain more precise control of the biological steps.

Marketing and regulatory similarities would link vaccines and drugs even if production shared few attributes. Drugs and vaccines target health professionals as their intended buyers. Both vaccines and drugs benefit from government regulation to assure safety and efficacy, often seeking licenses from the same agency.

Complexity was not always present. When Jenner introduced the first vaccine, the live virus of cowpox and later vaccinia, he was researcher, developer, manufacturer, distributor, and one of the users. During the nineteenth and early twentieth centuries, vaccine production capacity spread to every corner of the earth. As Pasteur Institutes proliferated, no single firm or institution dominated vaccine making. Vaccine production was an accepted part of health, scientific, and economic development. The technologies were often more like cuisine than science. The pathogen had to be grown and then attenuated or killed or the toxin rendered harmless but immunogenic. By World War II, vaccines were an effective part of public health in industrial countries and a principal strategy of the U.S. Army for protecting troops.
The invention of antibiotics dampened enthusiasm about vaccines. Sulfa drugs, then penicillin and streptomycin, opened a quest for an array of drugs that could treat every infection. Antibiotic sales fueled expansion of multinational pharmaceutical firms and left the small public and private institutes that made vaccine without the resources to continue research and development. When vaccine development resumed, spurred by the success of polio vaccine, the major commercial firms of the America, Europe, and Japan dominated the world's production. Some national vaccine institutes continued to make only simpler vaccines. Institutes in Eastern Europe and the Soviet Union continued to produce vaccines, suffering slow decline with the rest of the Socialist bloc's impoverished health system. Others shut down altogether. For industrial world commercial firms, vaccines were not an attractive market and fewer than half of those selling vaccines in 1970 remain in the vaccine business today. During the same twenty years world vaccine production increased over 10 fold*

Founded on scientific advances in the most basic aspects of biology, the biotechnology revolution spawned dozens of small firms seeking to exploit genetic engineering to design and make products for agriculture, food, energy, and health. The heaviest concentration of these firms resides in the United States. Despite a general preference for diagnostics which are less regulated and drugs which have a larger market, a few biotechnology firms have tackled the development of vaccines. The first HiB vaccine emerged from such a firm and the genetic engineering used in hepatitis b vaccine was developed in another biotech firm. Biotechnology firms have great intellectual resources, but rarely possess both the manufacturing and marketing capacity to establish a toehold in the vaccine industry. Either their intellectual property or the whole firm has been acquired by bigger firms already in the vaccine or pharmaceutical business.

Global use by the Expanded Program on Immunization provided a giant boost to production of vaccines containing six groups of antigens: for prevention of diphtheria, pertussis, tetanus, polio, measles, and tuberculosis. In the early 1980s UNICEF and PAHO

* Industrial world immunization rates doubled from below 50% to above 80% for one third of children born in the world. Developing world rates, now at 80% were less than 5% for the other two thirds of children born.
established tender and bid programs to buy large quantities of these vaccines for national immunization programs. UNICEF donated the vaccines it bought to the poorest countries and PAHO used a "revolving fund" so that countries in Latin America and the Caribbean could buy vaccines in local currency. Purchases rose rapidly to keep pace with immunization coverage rates. UNICEF bought 5.3 billion doses of vaccine for $298.5 million from 1982 to 1991, spending $46.9 million in 1991.1 Approximately 15 suppliers respond to one or more parts of the UNICEF and PAHO tenders, originally floated annually, now biennially. No firm supplies every vaccine product on the list and, to avoid dependence and monopoly, neither UNICEF or PAHO buys all of a product from only one supplier.

Vaccine needs are large when one compares the number of doses required today and the number used in the past. UNICEF estimates that by the year 2000, developing countries will require 1.7 billion doses yearly of the six EPI vaccines. New vaccines will increase total output. However these quantities alone do not require a large number of production facilities. We are far from realizing the full benefits of centralizing production. Economies of scale make the production of vaccines most efficient if executed in a handful of plants. Manufacturers prefer production units dedicated full-time to a single vaccine, obviating changeovers. Factors other than efficiency must explain the strong trend to local production.

Data needed to describe local production in detail are inadequate, but over half of the vaccine used in national EPI programs is manufactured in the country where it is used. Local production has grown more rapidly than UNICEF and PAHO purchases. Several of the largest countries in the developing world, India, China, Brazil, and Mexico, seem likely, with the help of CVI, to attain self-sufficiency for the six EPI vaccines. Local production is rarely based on new technologies and rarely produces new vaccines. Developing countries have employed existing technologies, developed and used in industrial countries, often transferred by established manufacturers.

UNICEF, which must raise funds to expand its vaccine purchases, currently about $50 million per year, strongly supports local production. Only when facilities in developing countries have been unable to turn out safe and effective products, has UNICEF become

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concerned about local production. If and when big countries like Bangladesh, Egypt, and Vietnam lose the capacity to make effective vaccine, UNICEF would be confronted with large new demands for donations.

Countries that do not manufacture vaccines buy their supply or receive a donation from UNICEF or other donors. Although the quantity of donated vaccine is rising, so is the use of funds from national health budgets. National EPI programs usually pay for personnel and resources that can be purchased with local currency. Many now buy vaccines as well, a hard currency import. In Latin American all countries buy vaccine, either directly or through PAHO's revolving fund. As countries in Asia and Africa have added hepatitis B vaccine to their immunization programs, they have been forced to use their own budgetary resources, because the vaccine is not yet donated by UNICEF.

Industrial countries account for only one fifth of births in the world and immunization plays a smaller role in public health strategies of these countries, because nutrition, shelter, and sanitation have already controlled many communicable diseases. Decisions about what vaccines to use and when to give them are made country by country, even in the European Community. In addition to the six EPI vaccines, many countries have introduced new vaccines: mumps, rubella, hepatitis B, HiB, hepatitis A vaccines. Buyers in industrial countries pay from ten to one thousand times as much for vaccines as do developing world countries. Vaccine prices, and the contrast with UNICEF/PAHO prices in particular, stimulate institutional buyers to seek better deals. In the United States many state programs are looking for ways to benefit from the price negotiated by the Federal government, approximately 40% below market prices. Because they sell vaccines to UNICEF at prices that are far closer to the marginal cost of production, European and Japanese manufacturers maintain greater differences in price between different customers. With increasing pressure on health programs to contain costs, how long will industrial countries tolerate tacit subsidies of third world vaccine supplies by domestic health budgets?

**Vaccine Production: future needs.**

We can catalogue the future needs for vaccines. The world will require large quantities of vaccine, new vaccines, multiple sources of
production, low prices or costs, and simplified production
technologies.

The required production of pediatric vaccines will be defined by the
birth cohort, (possibly doubled if strategies require immunization of
pregnant women). For new vaccines there will be "catch up" for
additional birth cohorts from the past to protect the full population
that can benefit from the vaccine. Moreover, effective vaccines for
adults may be developed, such as influenza vaccine effective against
most types of the disease, increasing the total quantity of vaccines
that must be produced.

Commercial manufacturers usually describe new vaccines as more
complex and sophisticated. This may be the case, but it undoubtedly
reflects their desire for high prices as well. For example, most of the
new acellular pertussis vaccines require the combination of purified
components, far more complex than making whole cell vaccine.
Prices for these new products as discussed by commercial
manufacturers more than compensate for increased complexity.
However, vectored vaccines, chemically synthesized subunit vaccines,
live vaccines and vaccines produced in bacterial, yeast, or animal cell
systems may be less complex to make and less costly than some
current vaccines.

The world will continue to need two or more sources for each vaccine
to protect against production failures. Large countries that rely on
domestic production may also seek redundancy in their own
production capacity and competition to keep prices down. Thus,
despite economies of scale, vaccine will be made in many countries
and in many plants.

If vaccines are to be made in developing countries, simplified
technologies will be easier to transfer. Complex technologies may
serve to restrict entry of competitors into commercial vaccine
production, but they have also made it difficult to copy good
production in developing countries. This is true for production, and
true for quality assurance as well. In many cases, the analytic
techniques required to assure safety and efficacy are more
demanding than the manufacturing methods.

CVI Strategies and their impact on production.
The global Children's Vaccine Initiative proposes to advance the control of infectious diseases by creating the ideal vaccines for the task and making them available to the world. First and foremost, it is a program of directed product development. Yet CVI will not succeed if newly developed products do not fit into immunization programs and if these products cannot be supplied in sufficient quantity and at low enough cost. The problem of compatibility with existing immunization programs is being confronted in the priority setting and planning stages of CVI. Reform of immunization programs may also be required.

CVI's Situation Analysis Task Force is analyzing vaccine production and crafting a strategy to assure future vaccine supply. Three emerging CVI strategies will affect production of vaccines directly: Self-sufficiency in vaccine supply in large countries; shared production, often the first step in strategy for achieving self-sufficiency; and "vaccine independence", a strategy to have all countries pay for the vaccines they use.

The self sufficiency in production strategy concentrates on a small group of countries with large populations and birth rates who currently possess the capacity to make vaccines. India, China, Indonesia, Pakistan, Brazil, Mexico, Iran, Vietnam, and Egypt account for 66 million of the world's 130 million surviving infants. All make polio vaccine, eight make DPT, seven make measles, and six make BCG. The goal is to assure that they can meet their national needs for EPI vaccines (the current six) and assure safety and efficacy. CVI's Task Force on Quality Control of Vaccines will assess regulatory procedures and production, propose remedies for observed deficiencies, and provide assistance for needed upgrades. The process has begun, with evaluations of production and regulation in China and Vietnam. The biologicals program at WHO will now seek participation from the other countries.

Because UNICEF's prime concern has been the current level of spending for donated vaccines, the self-sufficiency strategy focuses on the six EPI vaccines. However, a second approach, termed "shared production", may also be applied to assure local supply of new vaccines. Shared production usually begins with the importation of bulk vaccine from industrial country manufacturers to be formulated, packaged, and labeled in the country where it will be

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2Peter Evans
used. Shared production advances further toward new vaccines by exploiting technology transfer and "shared development".

In a discussion paper circulated to CVI colleagues, Dr. Philip Russell, summarized a major dilemma. Commercial vaccine makers in the industrial world are developing new combination products that will use a technically sophisticated acellular pertussis component. "The manufacturers in the developing countries which currently manufacture 60% of the DPT used by the EPI will not participate in the coming technical evolution in DPT based vaccines without a major CVI effort. A situation could very likely emerge in which the developed world will use the multi-component 'high-tech' vaccines and the developing world will continue to use mainly locally produced traditional DPT; additional vaccines added to the EPI schedule such as Hep B and HiB will have to be added as separate vaccines."³

Russell rejects the idea of waiting until the price of the high tech combinations comes down, proposing instead the "development of new DPT-based combination vaccines for the developing world." He would accomplish this by incorporating technology transfer into a cooperative vaccine development effort involving the developing country manufacturer from the start. He envisions collaboration with a highly competent vaccine developer in an industrial country. The result would be combination vaccines using a purified DPT and antigens such as HepB, HiB, S. pneumoniae, and N. meningitidis, purchased in bulk or made locally. Other shared development projects could be supported by CVI in the future.

Since 1978, the Pan American Health Organization has operated a revolving fund for vaccine purchases. Countries in the Americas region may take advantage of the good prices obtained in response to PAHO's tenders and they may pay for the vaccines in local currency. This is possible because PAHO can spend the local currency that it receives in payment back in the country, and vaccine purchases remain small compared to PAHO in-country spending. The Americas leads all other WHO regions in immunization coverage and virtually all vaccines are paid for from national health budgets. The revolving fund may have contributed to the success.

Strictly speaking, UNICEF's Vaccine Independence Initiative, modeled on the PAHO revolving fund, is not a production strategy. However, it's emphasis on local funding and low cost, quality vaccines will undoubtedly reinforce local production, technology transfer, shared development, and shared production. The program, now being pilot tested in Morocco, envisions careful planning for vaccine needs, purchase of vaccines with resources from the national budget, access to the low prices obtained by UNICEF's Supply Division, and payment in local funds on receipt of the vaccine. USAID has pledged to provide $1 million for initial capitalization of the revolving fund.

CVI's Dilemmas

CVI is principally a program of directed product development. The goal is to assure that new products meet public health needs. The biggest problem CVI faces is how to direct product development which, today, is largely guided by manufacturers. How can priorities established by analysis of epidemiology and assessment of technologies be imposed on developers?

Large firms, public institutes, and small biotechnology firms located in industrial countries develop most new vaccine products. Commercial entities look to industrial country markets for most of their return on investment. The remaining public institutes tend to be restricted to meeting national needs. Thus CVI faces a daunting task when it seeks to assure development of vaccines to meet developing world needs. Heat stable vaccines would help eliminate the "cold chain"—the continuous program of refrigeration whose links run from the manufacturing plant to the arm of the child. EPI's cold chain costs fully as much as the vaccines themselves. In industrial countries where refrigeration is taken for granted, there is little demand for more heat resistant vaccines. How can CVI create incentives to develop more heat stable vaccine or any similar product where effective demand is small because it comes principally from developing countries?

When trying to influence commercial manufacturers incentives must make a difference in the "bottom line". In the vaccine business, the rate of return on investment may not be equal to that of the pharmaceutical industry, but there continues to be sufficient overlap. When deciding where to put the next dollar of investment, only vaccines expected to produce a rate of return near the industry standard will receive attention. We do not understand all the
reasons and ramifications of commercial decisions, yet the example of hepatitis B vaccine is illustrative of the problem. Merck was the first firm to produce the vaccine and the first to bring a genetically engineered vaccine to market. For 10 years both Merck vaccines, first the plasma derived one and then the genetically engineered one, were priced at over $100 for a three dose series. No developing country could afford to immunize its population. The price was so high that the American Hospital Association called it unfair when it became clear that hospital workers exposed to blood products should receive the vaccine. The Federal government recommended the vaccine first for individuals at high risk and more recently as a universal vaccine, but has contributed little to paying for it. The U.S. price remains high.

Developing countries received a price break when the New York Blood Group licensed its ultracentrifugation method for producing plasma derived hepatitis B vaccine to a Korean firm with the promise that the product would cost one dollar per dose or less. Subsequently SmithKline Beecham brought another genetically engineered vaccine into the market, priced below the Merck product. And finally Merck is cutting its price and exporting its technology to compete in the global market. WHO has endorsed universal childhood immunization against hepatitis B, but UNICEF has not yet chosen to donate the vaccine to national EPI programs. The critical question for CVI is whether an early decision by national immunization programs and UNICEF would have induced the commercial firms that developed the hepatitis vaccines to set a price that encouraged universal immunization. Moreover, what is that price? Does development of vaccines for the third world depend on continued existence of high priced markets for the same vaccine in industrial countries?

If CVI must depend on high priced markets to stimulate development, production by the same makers of vaccines without industrial world markets will be hard to obtain. Recent workshops conducted by the Institute of Medicine of the United States National Academy of Sciences have provided a forum for large U.S. vaccine manufacturers to reassert how difficult it is for them to invest in and produce vaccines for developing countries. They left no doubt that it is the rate of return that discourages them, not rules and regulations. They discounted the effect of a legal requirement that the price that the Federal government pays be the lowest at which the product is offered.
Does CVI benefit from competition between developers and manufacturers? In the 1980s, competition magically delivered UNICEF vaccines at as little as one one hundredth of industrial world prices. In return for large orders that obviated marketing and guaranteed payment, manufacturers could confidently dedicate a portion of their capacity to making EPI vaccines and charge a modest amount more than the marginal cost of production. Competition between developers continues to be advantageous to consumers of new vaccines, but only when development occurs. It keeps price down, it increases the likelihood of making an effective product, and it assures backup producers in the event of a production failure. However the benefits of competition are obtained at a price as it results in duplicative investment—two or more companies develop vaccines against the same disease—requiring higher prices for the final product to recover costs. The competition also focuses attention on price, making institutional buyers in industrial countries increasingly intolerant of the multi-tier market and of subsidies of developing countries by industrial country health programs.

Domestic resistance to contributing to the developing world vaccine supply is accompanied by a preference among bilateral development assistance programs for tangible and visible contributions to developing countries. They prefer to donate or introduce vaccines than develop new ones. This appears to be a political partiality for alms. Investments in development or production of vaccines for developing countries produce health results more slowly. For example, USAID has expressed a preference for making its contribution to vaccines downstream, specifically introducing already existing products to national immunization programs. Other national development assistance agencies approach the manufacture of vaccines with caution, regarding them as overly sophisticated technology.

Conclusions and Prescriptions for CVI

CVI tries to remedy a defect in the market for vaccines by directing product development to assure that vaccine research is promptly applied and needed vaccines produced. What irony if CVI were to eliminate product development obstacles only to discover that manufacture and supply also need to be planned and managed. The work of CVI depends on manufacture and use of the newly developed products. Economic forces alone will supply the desired
vaccines for the developing world slowly or not at all. And every year of delay causes deaths of children, unnecessary spending to treat illness and disability, continued high birth rates, and retardation of economic development.

If these delays are to be avoided, the CVI must heed the manufacturing objectives at the time product development is planned. CVI planners must consider the technological requirements that limit how and where the new products can be made, the quantity that will ultimately be needed to immunize the full target population, and the final cost. Two CVI policies, described above—manufacturing self-sufficiency and vaccine independence—move in the right direction. Yet they have barely affected the desire of industrial world vaccine makers to develop CVI vaccines, because they have not altered effective demand. There is no guarantee that the funds invested in development can and will be recouped in final sales.

We believe that there must be a major commitment of funding on the front end. The process of vaccine development is linear and can be planned. Moreover CVI is ready to exploit shared development, shared production, and technology transfer. Through loans, grants, and contracts, funds must be put into development and production at the front end of the sequence. Money invested at the front end does not eliminate competition, rather it permits CVI to make the best use of competition. Front end funding encourages the CVI and its collaborators to strike a bargain about the final outcome—the vaccine, the quantity, and the price—while sharing the risks.

Investment early in the vaccine development sequence changes the calculations about return on investment. When payment comes only at the end, firms weigh large opportunity costs in choosing a CVI project over other commercial ventures. For the large commercial firms, the gold standard of pharmaceutical industry profits is in one pan of the balance. Smaller firms and institutes cannot contemplate large production runs and global sales, thus their participation can be elicited only by early payment.

With willingness to invest on the front end comes the potential for CVI to harness competition. Each step or group of steps in the sequence from product development to production can be planned in advance. For each step, CVI can competitively select a contractor or collaborator to execute it.
What are the cost implications of spending development costs on the front end rather than paying for development in the ultimate price? Very rough estimates may let us understand their magnitude. Let us make the following reasonable and very cautious assumptions. CVI will develop five new vaccines, investing $500 million in the next five years. This uses industry estimates of development costs—not likely to be understated. Half of the final products will be locally made and paid for by the country of manufacture. Half of the other countries will also buy the vaccine with their own health budgets. The remaining vaccine, for about 40 million children, will be donated and assumed to cost the donors one dollar per child—more than the per vaccine per child today cost today. Remember that this cost does not assume any recovery of the development costs paid by CVI. Moreover, assume there will be no new costs associated with adding the vaccines to EPI, not an unreasonable assumption if combination, oral, and heat stable vaccines are added to the existing delivery programs. CVI’s new vaccines will be used for ten years before being replaced. A very rough estimate suggests that over 15 years, the world will have invested one billion dollars to have five new vaccines in use. This is less per year than UNICEF projects it will spend for the current EPI vaccines next year. The health benefits can be estimated once CVI decides which vaccines to develop and produce. These benefits must be weighed against the list of benefits that would materialize if product development and production were left to the market alone.

We believe CVI can solve a problem inherent in the preferences of the firms and institutions that contribute to CVI, by being able to shift resources within CVI. As noted above, we propose that thinking about vaccine production and supply not be separated from the research, development, clinical testing, and licensure that precede it in the natural sequence of events. Success, measured by the vaccines delivered and the diseases prevented, depends on completing the whole sequence. If that is true, then investment at any point in the system can improve chances of preventing disease.

A CVI Fund will be essential. Research institutions want to play a role in CVI research. Other institutions may be able to contribute to product development, but lack the resources to undertake manufacture. Manufacturing firms can help transfer technology and produce vaccines. Many bilateral donors have selected particular diseases or countries to receive their help. The creation of a single
CVI fund can make more than currency interchangeable. Any and all contributions could accrue to the benefit of CVI.

Because CVI can attract funding directly to most of its projects, the fund need not be large. UNICEF has already recognized the advantages for the Vaccine Independence Initiative of capitalizing the revolving fund. For development, as well as for purchases, payment must be made in advance. The CVI fund would have no mission other than to assure prompt payment for each step in the development and production sequence and avoid delays. Time is money and lives. The CVI came into existence to eliminate the delays inherent in the market, now we believe it must live up to its intentions and eliminate unnecessary delays in its own process.

In the final analysis, vaccines are economically too small and for health too important to rely on market forces to assure their availability and use in developing countries. Thus CVI reflects an important premise in UNICEF's Report on the State of the World's Children 1992: "That the growing consensus around the importance of market economic policies should be accompanied by a corresponding consensus on the responsibility of governments to guarantee basic investments in people." CVI must create a planned global system for vaccines, relying on competition and on national efforts where they help meet the ultimate goal of disease prevention.

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A New Technologic Synthesis: The Children's Vaccine Initiative

Suryanarayan Ramachandran and Philip K. Russell

The Promise

The scientific and technologic advances discussed in detail in the preceding papers make it abundantly clear that great advances are possible in the use of vaccines for the further reduction of mortality and morbidity among children. Currently available and evolving technology has made it possible to envision the development of a new generation of vaccines and vaccine combinations which will greatly enhance the capability of the public health systems of the world to prevent and, in some instances eradicate, vaccine preventable disease. The Children's Vaccine Initiative grew from the belief that great advances are possible from a scientific and technical point of view and that a coordinated international effort would be needed to assure that the vaccination programs of both the developed and the developing world receive maximum benefits from the advances in science and technology.

The "Declaration of New York" (1) defined the objectives of the initiative which are based on the need for more efficient means of
vaccinating children against a broader range of diseases. The goals which have been established for the CVI (2) reflect the fundamental need to improve both the effectiveness and the efficiency of the vaccination programs. From a global perspective very impressive advances have been made in vaccination of children. National vaccination programs assisted by the World Health Organization's Expanded Programme on Immunization (EPI) and by UNICEF's Universal Childhood Immunization (UCI) program have succeeded in reaching the goal of 80% coverage worldwide. Poliomyelitis has been virtually eradicated in the western hemisphere by a vigorous campaign led by the Pan American Health Organization and the incidence of measles is has been dramatically reduced. As impressive as these achievements are, mortality and morbidity due to preventable infectious diseases in children remain at unacceptable levels in many parts of the world. The vaccines currently available, although effective, are a major hinderance to efficient vaccination programs. Multiple contacts with the children and an uninterrupted cold chain are needed, multiple individual vaccines must be given and several important vaccine preventable diseases not covered by current programs. Since the cost of delivery of vaccines is 80 to 90% of the total cost of vaccination and even higher in remote areas, new vaccines which improve the efficiency and effectiveness of the delivery system could be very cost effective as well as greatly reducing mortality and morbidity; in addition, improvements in efficiency of vaccine delivery may be essential in order to bring global vaccination coverage up to
acceptable levels and succeed in eradication of polio and measles. The goals set by the CVI reflect the importance of improving the efficiency of delivering the vaccines as well as increasing the extent of protection. New and improved vaccines should be developed which:

* Protect against a broader range of diseases
* Contain multiple antigens
* Require fewer doses
* Can be given by the oral route if possible
* Are heat stable
* Are safe and effective
* Can be manufactured in developing countries
* Are affordable

For each of the CVI goals there are a variety of technologic and scientific approaches which may provide a means of achieving the goal. Additionally, some fields of research especially immunology and biotechnology are rapidly progressing and with additional research effort could produce even more possibilities to improve the vaccines to be used in the future for vaccination of the world's children.

The CVI goal to broaden coverage to include diseases not currently targeted by immunization will be addressed through development of new vaccines based in large part on molecular biology including genetic engineering of vector organisms and production of protein
antigens as well as taking advantage of new methods for delivery of antigens. Among the most important targets are bacterial pneumonia and viral and bacterial diarrhea.

The CVI goal to develop multiple component vaccines combined in novel ways will rely on a variety of technologies including genetic engineering, new production and purification methods and novel antigen delivery methods such as microcapsule, microspheres, liposomes, novasomes and others. The goal of oral delivery is more remote for most vaccines but current research is actively exploring means for delivering inactive antigens through the intestinal mucosa using microspheres and ligands which may assist uptake of antigens by M cells. In addition, several vectors are being investigated as possible multivalent oral vaccines. The goal of thermostability to reduce the logistical burden of the cold chain is being addressed in an antigen or vaccine specific manner through novel drying techniques, new excipients and chemical compounds which stabilize viruses such as poliovirus by binding to capsid proteins. Improved efficacy and lowered reactogenicity will be achieve in part by improved purification of bacterial antigens. All of the CVI goals will require the application of advanced technology much of which is complex and will require an immense concerted effort to move from the research phase through vaccine development to global manufacturing and universal use.

The barriers.

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There is little doubt that the goals of the CVI are achievable from a technical perspective. However, the operational and management challenges the CVI are substantial even daunting. The resources needed to accomplish the goals are scattered widely around the world in both public and private sectors. For this endeavor needed resources include export personnel, knowledge and experience, intellectual property rights, organizational product development capability, and manufacturing capacity, as well as financial resources. Reliable and broad based epidemiologic data on disease burden will be essential. Substantial capital investment will be necessary in applied research and product development, and in manufacturing facilities. Provisions will have to be made to assure the availability and purchase of the vaccines for the entire world.

The funds needed to carry out the vaccine development activities are in private corporations, multilateral and bilateral funding agencies, private foundations and in national budgets. Mobilizing the necessary funds and promoting a internationally coordinated effort is a truly major (even heroic) undertaking requiring leadership and commitment by the major international and national agencies with responsibilities in the field of international public health.

If we are to achieve the visionary goals of the Children’s Vaccine
Initiative which were foreseen at its inception the CVI will have to be an initiating, integrating and propelling force in the complex field of vaccine development, manufacturing, and utilization. Success in solving the major technologic challenges ahead may depend first on solving the problem of integration of the CVI effort among the various major groups involved. Vaccine research, development, manufacturing, purchasing, delivery and final use have heretofore largely been separate and independent responsibilities of different segments of our immunization system. Research has been largely a public sector responsibility, especially the more basic aspects, with national agencies playing a dominant role in supporting investigator initiated research. Applied research is also heavily supported by the public sector but there is a substantial industrial investment with the small entrepreneurial biotechnology firms playing an increasing role. Vaccine development has been largely the province of the vaccine manufacturers with decisions being heavily influenced by market forces, patent positions, and other economic influences. Public sector funding has again been important in the clinical and field testing of new vaccine candidates. Purchase and utilization of vaccines by both the public and the private sector is in the province of the public health system and private medicine and principally deals with vaccines in the post-licensure phase.

The vaccines in current use have actually come about through a series of unconnected, for the most part uncoordinated, decisions by diverse organizations, mainly manufacturers, in the vaccine
field. The current international effort to develop and evaluate acellular pertussis antigens for incorporation in DPT is a recent exception and good example of how public sector initiative and coordination can accelerate, support and focus a broad based industrial development effort.

End users and the purchasers of vaccines for public sector use have generally had little or no say when the decisions were made regarding the specifications or characteristics of vaccines to be developed. Purchase of vaccines for public sector vaccine programs, both national and international has been done by competitive bidding for licensed vaccines. In the past there has been no integration of vaccine purchasing with vaccine development and the short term competitive bidding process for purchasing by the public sector is viewed by many as a disincentive to the development of better vaccines.

The founders of the initiative envisioned a major investment of resources over the next several years to develop and employ a new generation of vaccines which will greatly enhance the efficiency and effectiveness of childhood vaccination programs. This investment must come from a variety of public and private sources and to be effective the investment of resources must be channelled in mutually supporting directions through effective international coordination. To stimulate the investment of private sector corporate funds financial incentives will be needed. Incentives
such as co-investment by the public sector to share risks and long-term purchase agreement to ensure a market are possible mechanisms for providing incentive to industry. To stimulate the investment of public sector funds assurance of the value of the investment will be necessary in terms which are meaningful to both the public health and the financial communities.

The founders of the Children's Vaccine Initiative.... (brief description of the organizational structure)

The initial Product Development Groups have made substantial progress. (Brief description of the progress of the three PDG's with emphasis on the evolving relationships with industry)

From a broader perspective, the CVI has generated an international discussion and greatly increased interest in the various aspects of the initiative. The existence and initial activities of the CVI has stimulated corporations, institutions, foundations and national governments to address the merits of the initiative and in some instances appears to have resulted in increased investment in vaccine research, development, procurement, and delivery systems. In the U.S. the National Vaccine Program, NIH, CDC, FDA and USAID all have examined their programs and are endeavoring to make substantial contributions to the initiative.

Emphasis by the CVI on using economic analyses in determining
priorities has focused increased attention on the tremendous economic benefits of immunization in comparison with other interventions in public health as well as the economic benefits to be gained by investing in improved vaccines which will improve the efficiency and effectiveness of immunization programs.

The initial effects of the CVI are encouraging, especially the broad acceptance of the social and economic values of the initiative. Equally encouraging is an apparent evolving consensus regarding the technical feasibility of developing the vaccines needed to achieve the CVI goals. However, the progress so far is a very tiny fraction of what must be done to develop a whole new generation of highly effective and efficient vaccines and to find the ways and means of manufacturing the vaccines and making them available to the global vaccination programs at a cost which is affordable.

The CVI faces a series of critical challenges which must be addressed and overcome. Acquiring the necessary knowledge and technical capability which results from applied research in such fields as microencapsulation, other vaccine delivery systems, adjuvants, vector technology, and molecular biology has become very difficult because of financial interests in the information. In many areas such as microencapsulation most of the research has been done within industry and is not published in the scientific...
literature; even the results of publicly funded research are often withheld from publication to protect patent rights or to preserve a commercial advantage. Management of publicly funded efforts to develop new vaccines is hampered by the fact that a very large amount of information important to decision making is not available to the program managers. Further, the best options for development of a new product may be under the control of a single company. Finding and assessing technical options to assure the optimal technology is used for development of new vaccines will require innovative management on the part of CVI managers as well as effective means for communicating and working with the industrial sector. These are not new problems nor are they unique to the CVI, however the success of the CVI will be heavily dependent on the selection and application of the best technologies. When the best technologies cannot be applied either because they are not known due to secrecy or because they are not available because of patent rights the initiative will be jeopardized.

Intellectual property rights in general constitute a major management challenge to the CVI. It is quite conceivable that a future vaccine with multiple components will involve scores of patents related to the manufacturing and purification of individual antigenic components, as well as patents relating to formulation, stabilizers, and delivery systems. Developing the optimal
children's vaccines may well pose management challenges for the program managers and developers which are significantly greater than the scientific challenges. Multicomponent vaccines involving large numbers of intellectual property rights may be extremely difficult to produce at affordable prices.

In addition to promoting and managing the development of new vaccines, the mission of the CVI includes assuring the manufacture procurement and utilization of the new vaccines. The success of the CVI therefore depends on not only on successfully developing new vaccines but doing so in a manner that assures that the vaccines can be manufactured and made available to meet the global requirements. A very large proportion of EPI vaccines are manufactured in developing countries including at least 60% of the DPT used worldwide. For a variety of valid and understandable political, economic and social reasons a large part of the global vaccine supply will continued to be manufactured in developing countries in the future. Vaccine development sponsored by the CVI must therefore take into consideration long range manufacturing capabilities and strategies. Linking vaccine development efforts to a plan for manufacturing and assuring the global supply of improved vaccines will require long range planning and integration of vaccine development efforts with investment in improving the manufacturing base and regulatory capability in developing countries. The future manufacturing issue is an especially critical aspect of development of combination vaccines based on DPT.
Currently approximately 60% of the DPT used in the world is made in the country of use and manufacturing in several large developing countries will continue for the foreseeable future. The introduction of additional antigens in combination with DPT will require major modifications in the manufacture of the DPT as well as a technically complex product development effort by the manufacturers.

Several large multinational corporations which dominate the vaccine manufacturing in the developed countries will also continue to be the producers of vaccines for a substantial part of the developing world. These corporations are also the most effective developers of new vaccines. Their efforts will be focused on the developed world market and special efforts will be needed to make these products available for developing country use at affordable prices. The development of newer and more expensive vaccines by the major manufacturers may result in a widening gap between the vaccines in use in developed and developing countries. Providing incentives for the major manufacturers to work with the public sector in both developing and supplying improved vaccines to meet the worlds need will be critical to the success of the CVI. There is a very real danger of a widening gap between the developed and developing world in both the availability and the quality of vaccines.

The CVI Task Force on Situation Analysis on the Global Vaccine Supply has addressed these issues and a consensus is emerging among
Task Force members that a Global Vaccine Supply Plan is essential to guide investments in facilities by the private sector, national governments and development agencies.

Setting priorities and developing a comprehensive global strategy for the CVI is both an essential task and a very broad responsibility. The scope of the CVI extends from research on vaccines and related technology to product development, manufacturing, supply, and operational research, to the immunization of children. Factors to be considered in setting priorities for action and investment by the public sector include assessment of the preventable disease burden, potential impact of new or improved vaccine, feasibility of development, development costs and technical options, impact on immunization programs and cost effectiveness analyses. The ongoing development activities in the private sector are also a most important factor to be considered in conjunction with proposed investment by the public sector. Currently there are multiple private industry initiatives to develop new vaccines and combined vaccines, the development efforts in many cases are parallel and competitive e.g. DPT-HepB and DPT-Hib combinations but in some cases are single efforts due to patent position e.g. canary pox measles. In this environment with many options for public sector activity, setting of priorities becomes a very critical task. Because of the necessity to assure a global supply of the new vaccines, product development decisions must be integrated with a global plan for manufacturing and
supplying vaccines in the future. The global supply of vaccine is currently being met by a mixture of private corporate manufacturers and public sector manufacturers mainly in developing countries. This situation is predicted to continue for the foreseeable future although the introduction of new vaccines is expected to result in major changes in the overall system. Increased costs, intellectual property rights, and complex technology in manufacturing will put severe stress on the developing country manufacturers. Directing the CVI efforts to take maximum advantage of the private sector contributions to the field while assuring that the needs of the developing countries are met is a most difficult challenge.

The Children's Vaccine Initiative which began as a vision born of a social necessity is evolving into a series of parallel technical and organizational efforts toward a common set of goals. If well led and managed it will result in a new mutually beneficial relationship between national governments, international agencies and private industry. Well managed, the CVI can solve the technical, and economic, political and social issues and provide the maximum benefits of modern science and technology to the world's children.