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WORKING PAPERS, DISCUSSION AND SUMMARY

WORKSHOP SESSION III

PRACTICAL CONSIDERATIONS OF MALARIA VACCINES

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Presence of Multiple, Conserved and Non-Repeated Epitopes in Different Species of Malaria Parasites
Paper No. 28

* Paper Will Be Included In Final Publication *

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Abstract No. 29, page 37
Thomas McCutchan

PRACTICAL CONSIDERATIONS OF MALARIA VACCINES
A. Production of Plasmodia Antigens By
New Biotechnology
Paper 29 DISCUSSANT

*Paper Will Be Included In Final Publication *

Working Report - NOT FOR PUBLICATION

Production of asexual blood-stage antigens

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Introduction

Despite the ease with which *Plasmodium falciparum* can now be cultured *in vitro*, research on the antigens of *Plasmodium falciparum* has been hampered by difficulties involved in purifying parasite proteins by conventional biochemical means. The advent of recombinant DNA technology has solved this problem by providing the means of producing defined parasite antigens in large amounts. Several different expression systems have been used to express asexual blood-stage antigens of *P. falciparum*.

Expression systems

Systems used to express *P. falciparum* antigens are summarized in Table 1. Early work with low level expression systems, such as PBR 322 (1) has been superceded by more recently developed high level expression systems such as λgt11-Amp3 and its derivatives (2,3). Either complementary DNA (cDNA) prepared from messenger RNA or fragmented genomic DNA can be cloned in these various expression systems. Cloning genomic DNA has the advantage that a single library is capable of expressing sequences from any of the life-cycle stages. The resultant expression product may be either a protein fragment, as usually occurs in fused polypeptide expression systems or a full length polypeptide, for example in libraries derived from DNA fragments generated with mung bean nuclease. Less commonly, complete polypeptides can be generated from full length cDNA. Blood stage antigens have been detected in such clones using either monospecific probes such as monoclonal antibodies or rabbit antisera raised against purified proteins (3,4). Polyvalent sera derived from patients or from experimental immunized animals have been successfully used to detect antigens but for this purpose a high level expression system is mandatory (5-7). Expression systems in eukaryotic hosts are less advanced. Yeast systems and vaccinia virus are being used as second generation tools to express sequences previously isolated in prokaryotic systems.

Expression of specific *P.falciparum* antigens

Some properties of blood stage antigens expressed in *E.coli* are summarized in Table 2. Antigens from all stages of the asexual cycle have been expressed and they have been identified both with monospecific and polyspecific probes. Most examples of expression of asexual-blood-stage proteins use cDNA inserts expressed as fused polypeptides. In addition to those sequences summarised in Table 2, clones expressing an additional 22 distinct antigens have been identified in our laboratory. A striking structural feature of many plasmodium antigens is the presence of segments of extensive tandem sequence repeats and these will be discussed in detail.

Tandemly repeating peptide antigens

Details of some repetitive antigens of plasmodia are summarised in Table 3. This structural feature occurs in plasmodial proteins that interact with the immune system. For those antigens studied, it appears that much, if not all, of the naturally occurring antibody response is directed against the repeats. The function of the repeats is not known. It is possible that, in some molecules, repeats act as ligands leading to an increased affinity of binding to some receptor molecule. Alternatively, the repeats may be involved in immune evasion. For molecules that show great diversity in the repeat sequences among isolates, such as in the S antigens (8), this is most likely to be the case, but even where repeat sequences are conserved as in the RESA, the repeats may be involved in diverting the immune system from other portions of the same molecule (9). In *E.coli* expression systems there is a consistent tendency for recombination events to delete repeats from the DNA insert. This instability may make these systems unsuitable for expression of vaccine molecules if large numbers of repeats are essential for successful immunization.

Because repeats may have evolved as an evasion mechanism which suppresses immune responses to other parts of the molecule, we have expressed in the λAmp3 vector, repeat and non-repeat fragments of an antigen so that the immune response to these different regions of an antigen can be determined.

Antigenic differences between different strains of P.falciparum.

One consequence of the presence of multiple repeats is the ability to rapidly alter the predominant antigenic character of a protein. Gene conversion mechanisms allow the rapid spreading of a single change in sequence throughout multiple copies of the repeat and even relatively trivial changes in nucleotide structure can lead to markedly different antigenic properties. This is most strikingly seen in the S antigen system (8). Conversely, antigens that lack repeats, such as the CRA, appear to undergo less variation in sequence between different strains (4,21). One might postulate that the absence of variation within a repeat sequence among different strains suggests that the repeat is required for some purpose such as receptor binding.

Candidate vaccine molecules

Several of the antigens expressed in E.coli are potential protective immunogens:-

195K protein (Pf190)

The molecule with the most convincing credentials as a vaccine molecule is the merozoite surface coat precursor protein (3,10,11) (195K protein, Holder and Freeman protein, Pf190). Homologues of this molecule have been demonstrated to be effective vaccines in several experimental systems (12,13). It should be noted that protection has been best against homologous challenge suggesting variability in the epitopes against which the immune response was directed. In the case of P.falciparum, immunization of Saimiri monkeys with purified merozoite surface coat precursor gave some protection but it

is important to realize that this was a very small scale study. The sequence of a fragment of this molecule contains no repeat sequences (3). Data from our own laboratory on this antigen, from the FC27 strain, shows that it contains hexapeptide and tripeptide repeats. This molecule is antigenically complex with strain-specific and species-specific epitopes as well as epitopes that are common to several species of plasmodium (14,15). Detailed analysis of the sequence basis for this complexity may enable the expression of a molecule that immunizes against all strains of P.falciparum.

RESA (Pf155)

The ring-infected erythrocyte surface antigen, the 155 kd molecule present in merozoites is transferred into the membrane of the erythrocyte as or immediately after the merozoite invades. Experiments using antibodies affinity purified on the native molecule have demonstrated considerable inhibition of parasite growth in vitro (16). The repeat sequences in this molecule are conserved between at least two P.falciparum strains (9). The approach of expressing repeat and nonrepeat fragments of the molecule offers an opportunity to further fine dissect a potential protective immune response.

Several other molecules have been suggested as potential vaccine molecules. These include a rhoptry protein which protects in the P.yoelii system (17), the cytoadherence molecule responsible for parasite sequestration (18) and molecules where specific antibodies are correlated with growth inhibition (19,20). As yet there is little evidence for the utility of these molecules in inducing resistance to P.falciparum infection.

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Table 1 Systems used to express *P. falciparum* antigens

Vector	Insert	Product	Promotor	Inducibility	Expression Level
Prokaryotic					
λAmp3	cDNA	Fused polypeptide to β-galactosidase	Lac	Yes	High
pUK270	sonicated genomic DNA	Tribrid fused protein	Lac	Yes	High
pPL31A	cDNA	Fused polypeptide to MS2 polymerase	PL	Yes	up to 5% of cell protein
PATtrp	cDNA	Fused polypeptide to anthranilate synthetase 1	trp	Yes	high
λgt11	mung bean nuclease digested DNA	Native protein	phage or *P. falciparum*	No	Low
pBR322	cDNA	Fused polypeptide to β-lactamase	β-lactamase	No	Low
Eukaryotic					
pGS62 (vaccinia virus)	cDNA or genomic DNA	Native protein	7.5K late	No	High
pTT5 (S.cerevisiae)	cDNA or genomic DNA	Fused polypeptide to β-galactosidase	PGK	No	Intermediate

Table 2 Antigens expressed in E.coli

Name	Probe	MW (kd)	Location	Repeats
S-antigen	Polyspecific	200	Parasitophorous vacuole of schizont	Yes
RESA	Polyspecific	210,155	Membrane of ring infected cells	Yes
pPF-11.1	Polyspecific	~250,000	Membrane of schizont infected cells	Yes
PF190 195kd	Monospecific Monspecific	195	Merozoite surface	Yes
FIRA	Polyspecific	300	Extraparasitic areas of schizonts	Yes
CRA Ag5.1	Polyspecific Monospecific	23	Ring surface and parasitophorous vacuole	No

Table 3 Repeat portions of *P.falciparum* antigens

Antigen	Repeat size	Repeat Number	Canonical Sequence
FC27 S-antigen	11	>100	AKASQGLENP
NF7 S-antigen	8	35	A$^{L}_{R}$KSNEAE
RESA	10,11	5	DDEHVEEPTVA
	8	6	EANVEHDA
	4	36	EEN$^{V}_{Y}$
pPf11.1	9	22	$^{L}_{V}$EEIVEEVVP
FIRA	6	32	VTTQEP (extremely variable)

WEDNESDAY, April 24, 1985
Abstract No. 31, page 38
Robert T. Reese

PRACTICAL CONSIDERATIONS OF MALARIA VACCINES

A. Production of Plasmodia Antigens by New Biotechnology

Paper No. 31 DISCUSSANT

Dr. Reese---

Before jumping into what I believe the role of molecular biology, or should I say, biotechnology, has played and will play in the future in providing solutions to immunization against malaria, I'd like to take a step backwards and first review the kind of thinking that we had some years ago and try to build on those concepts which we developed sequentially from that sort of thinking, and show how we accumulated data first with polyclonal sera and how the molecular biological techniques have helped us really to confirm and to more precisely, specify and dissect those things which we began to learn, let's say, in 1980, 81. Which, in this field, might even look like the Dark Ages.

Now, in the first slide what I've outlined for you is what my concept was of the ways one might attack the blood stages when I first got into the field some seven years or so ago. Now, as I have outlined it, you can see a merozoite entering the erythrocyte. We have the 24 hours when it is silent as far as the immune response goes. That is to say, we can't detect any changes during this early development. And the, you will note that I have, after 24 hours, the production of knobs on the outside of the cell, or, projections, the morphologic changes, if you will, in the cell, and as we talked extensively about yesterday, the attendant antigens one can associate with these changes. And what I want to do at this point is remind you that these antigens and the antibody which can bind to it and kind of provide immunologic flags to tell us which of the cells are parasitized. These cells are marked and accessible to the immune response for 24 hours - for half of the parasite's cycle. So, in fact, they are highly accessible not only to the effects of antibody in some sort of direct killing effect but through all kinds of secondary phenomena, as I mentioned yesterday -fixation of complement, bringing in attracting cells as well as the direct cellular immune response. So, it is my opinion that during the second half of this immune response that this is a good place in which cellular immunity may play a role in inhibiting or killing the parasite, either blocking its development or lysing the cell, or phagocytizing the cell. And all of these are mechanisms for which there are some data which suggest they are very real possibilities. Today I'm going to concentrate my attention on the extracellular phase of the parasite, the merozoite, which actually, is not all that different from the sporozoite in that it is transient and there are the same problems associated with it. That is to say, if we expect to block merozoite invasion of an erythrocyte, it is highly likely that it's going to be dependent on having factors already present in the serum around that merozoite in the appropriate concentration, such as antibody, which can immediately neutralize, lyse, or have its effect on the merozoite to a limit penetration. And, in fact, if we look at and compare this penetration time-frame with what happens with

the spirozoite, we're really talking about the same time frame, particularly if we look at the suggestions from the time-lapse photography work which has been provided to us by the NIH group - that is to say, merozoites can achieve penetration of a new erythrocyte somewhere in the neighborhood of 10 to 20 seconds, but more often, it is achieved in the neighborhood of 5 to 10 minutes, which is basically the sorts of numbers that people use to explain sporozoite penetration.

In the second slide, I simply want to review for you the molecular changes which we know of that are associated with alterations in the erythrocyte membrane. And if I were to be asked at this point in time what I might select on the basis of the monoclonal data which are currently available to us, from this list I would have to select the two molecules that, those being the very high molecular weight protein which ranges somewhere between 3 and 400,000 and the low molecular weight proteins, having molecular weights of, let's say, 40 and 80,000 as the best candidates at this point in time.

Now, let's jump to the merozoite. And before we center our attention totally on the merozoite, I'm going to go back to an old experiment. The work was published in either 1980 or 81. And in this group of experiments, what we did was we took schizones or trophozoites, solubilized them in nonionic detergents and radioidinated with iodine. We then took a series of immune sera, some of which were from monkeys, some of which were from humans. The three that I'm going to compare for you here were three human sera, all of which had reasonably decent fluorescence titers which varied appreciably in two things: 1 - their ability to inhibit in vitro growth of the parasite and 2 - in their precipitation patterns of these radiolabeled schizont proteins. What we were able to learn, however, or at least what we suggested from comparing these data were that it was likely that molecules of three different molecular weights were going to be important in immunity. That is to say, we suggested that molecules of approximately 45,000, 70 - 85,000 and approximately 200,000 were likely to be important in providing protection. And we couldn't say more than 75-80,000 because different immune sera appear to bind different proteins in this region, and it was difficult for us to say a particular protein was more or less important than others. I think it's important to remember these data because they help to link us with the immune animal.

Now, as I mentioned, we used whole schizones and we couldn't say from those data whether we were looking at proteins which were associated with the erythrocyte membrane or potentially those which were associated with the merozoite. So we did another group of experiments. Harold Stanley had just developed another method of purifying merozoites. He provided the merozoites to me and I used an iodigen procedure and we surface labelled them with I125. Susan Langreth took an aliquot of those same merozoites and tried to determine:

1 - Was there erythrocyte contamination? The answer was

little or none and

2 - Were they intact and in good shape? Could we to some degree appraise how good or what was the likelihood that we were going to get surface labelling? And the answer is yes, the merozoites were in excellent condition. That profile is on the right. We then repeated the immunoprecipitation studies. Normal control shows no binding. The three immune sera on the right here are three human sera, each of which varied appreciably in their ability to inhibit in vitro growth. Now, although various proteins which they bound are very similar to what we found with the immune monkey serum, I would rather not spend as much time talking about the human sera as I do about the profile precipitated by the immune Aotus monkey serum because

1 - The in vitro inhibition of the human sera was considerably lower in all of the cases and more importantly, we know nothing about their clinical immunity. The Aotus monkey was highly immune clinically and by that I mean, if we were to challenge him with parasites of the homologous isolate, he showed virtually no parasitemia even when given an exceedingly high challenge dose, and he exhibited that same sort of immunity if we took a parasite from a completely different area of the world. I think it's important to note that this immune serum did not see a wide variety of proteins. In fact, it selected a 34K protein, a 45 or 46K protein, a protein of approximately 75,000, a protein of approximately 83,000, a protein of approxim tely 140,000 and something larger. Perhaps in the 2-300,000 range.

Recently, many laboratories have acquired a new set of tools by which we could more precisely ask and more precisely answer some of the questions I tried to address in those early group of experiments. That is to say, can we learn something about the structures on the merozoite surface and where they derive from, and something about the physical biochemical structures. The advantage of the early experiments, however, were that they gave us a link with immunity. And particularly, the Aota serum, not only was the monkey immune, but that immune serum could provide passive immunity to another Aotus monkey. So we knew there were the antibodies could identify proteins which were clearly important in providing immunity to the animal. That should be borne in mind when I talk later about the recombinant proteins which we have chosen to examine, because the same immune Aota sera were what we chose to screen our library with because we feel they provide us with a link to the real world and immunity in a living host, not just with some in vitro assay. One of the most studied proteins now in a number of different laboratories has been the high molecular weight glyco-protein, which has been attributed molecular weights of between 185 up to approximately 200,000. The difference in molecular weights can be attributed to a number of things as much as any, the different isolates that people work with and the gels that they use as well as the markers that they use to estimate the size of the molecule that is being studied. The important point I want to make about this particular

molecule is that it can probably be divided into two domains. This is based on the work of Randy Howard, and by using a whole battery of monoclonal antibodies which Harold Stanley has produced in our laboratory, Randy and Harold together as well as with Susan Langreth and Gary Campbell have been able to dissect this molecule, as I was saying, into two epitopically defined regions. One region gives rise to a series of smaller peptides, the smallest of which is 83,000 and is believed to be the molecule which is on the merozoite surface, and which is likely to be the same protein we saw immunoprecipitated by the immune monkey serum when we surface labelled the merozoite.

A second subgroup of monoclonals which we refer to as the Group A monoclonals, give rise to a group of glycosylated polypeptides, the smallest of which are in the range of 40 - 45,000, and, as perhaps some of you will remember, this protein, this 45 or 46K protein is likely to be the merozoite surface protein which is glycosylated which Randy Howard, about a year and a half ago, showed, was carried into the merozoite as the merozoite invaded and became a ring.

Now, the next group of molecules I'm going to talk about, and in fact, the two previous molecules, the 83 and the 46 all have been tested in the following way and they have the same following characteristics. If you produce an antibody which is specific for them, be it a monoclonal antibody or a monospecific polyclonal antibody against a cloned fragment, a recombinant protein, if you look by immunofluorescencwe with the pattern which these antisera generate on acetone-fixed schizones, you can see they give a rimlike fluorescence over each individual merozoite. In Panel B, I show that in fact, if you allow these merozoites to burst out and you examine living merozoites and ask the question, can you show that these antibodies can bind to the surface, the answer is again yes. And you will note in this particular case that the antibodies not only are causing the whole surface of the merozoites to glow but have caused them to aggregate, to agglutenate. There are some data which suggest this agglutenation itself may act as an immune mechanism by blocking penetration.

A third criterion which is really an extension of the previous and has not been done for all of them, I might add, is to examine the binding characteristics of these antibodies using immunoelectronmicroscopy. You'll note the negative control on the bottom and we find virtually no binding on the surface here when we use a monoclonal to an internal antigen, such a an antibody against rhoptry proteins. But, when we use one of these monospecific reagents which detect one of the proteins on the surface of the merozoite, you can see a very highly specific binding around the surface. And let's examine more closely what I mean by around the surface. Note here, that in this really very well preserved merozoite, we can detect three lipid bilayers, which some authors refer to as the polycular coat. External to that, you wil note, there is a loosely attached group of proteins. And it is this loosely attached group of

proteins which form the outer coat of the merozoite to which the antibody is bound. This is an important point because if one were to look by immunfluorescence and see a rim of fluorescence around the parasite, it would be very difficult to tell whether it was something associated with the inside of the merozoite membrane, the membrane itself, or, in fact, this outer coat, and it is the outer coat which is the only thing we can, a priori, expect antibody to have access to.

In this slide I've tried to provide a diagram. Perhaps some of you will find it too simplistic, in which I've tried to color-code and tried to select sizes which are at least compatible with the molecular weights of the molecules, so we can get some sort of visual idea of the number of proteins we can account for at the moment, or should I say, rigorously account for, as being on the merozoite surface. As I have already mentioned, there is an 83K protein which I have encoded in blue. This rectangular structure, which has the sugar on it, this glycoprotein is the 46K protein which we believe shares epitopes with the same high molecular weight 185K glycoprotein from which 83 is perhaps derived. There is 130K protein which is believed to bind to glycophorin, which Jeff Rafitch has cloned. Therre is 140K protein, which Forosa Ardishir and Jan Flint have studied in our laboratory. There is another protein of 56,000 which Harold Stanley and Randy Howard have studied in our laboratory, and is the first protein that we know of so far which also appears to have at least some lipophilic characteristics and may provide some of the glue, if you will, to help bind this outer coat into the lipid bilayer. And there must be something. Harold Stanley has also discovered a third glycoprotein of 34,000 and this 34,000 molecule as well as a 75K protein which has again been cloned by Forosa Ardishir and Jan Flint. These molecules, as well as the 140K proteins may well be the same proteins implicated by Michelle Jungery et al as being merozoite glycophorin binding proteins.

On the next couple of slides I've tried to organize for you, first the glycoproteins and the characteristics of them that we know, and then the nonglycosylated proteins. As I've just mentioned, there is likely to be a 46 and perhaps a 40K glycoprotein on the merozoite surface which is derived from, or at least shares epitopes with, the 185K glycoprotein. Dr. Randy Howard in our laboratory does not believe it is an integral membrane protein, but it does have certain characteristics which suggest that it may not necessarily just be the typical peripheral membrane protein. It is found in virtually all of the isolates we have looked at. It is prevalent on the surface of the merozoite. The second glycoprotein that we know is on the surface of the merozoite is this 56K protein, which has been described by Harold Stanley in our laboratory. It, in fact, at least a subset of the molecules which we can detect, are lipophilic. It is not found in all isolates. In fact, you will note in parenthesis I mentioned a 50K protein, and from the isolates that Harold has looked at, it would appear that one has either one of these, i.e. GP56 or one has the other,i.e. GP50.

It appears to be an either/or. And the monoclonal against the GP56 does not appear to cross-react with theGP50. There is also, I've noted, a 34K glycoprotein. And at this point in time, we know somewhat less about it. We should have this better characterized int he next month or so. I should make a point here relating to the sugar that these molecules contain on their surfaces. The evidence that we have accumulated suggest that most of the sugars that merozoite proteins have are O-linked. That is to say, they are linked to the peptide chain through either serine or threonine, not through arginine. The evidence that we have also suggests that these are unusual in that they are not sialyated. We have some evidence suggesting we may know what it typically uses as the terminal sugar, but more importantly, we have accumulating evidence that suggests that if the sugar is not present, that the antibodies, even polyclonal antibodies against the structures may not bind. As yet, we don't know whether the carbohydrate itself constitutes the epitope or whether the sugar simply stbilizes the peptide structure. But, whichever is the case, it is very clear that until we develop better systems for either synthesizing glycoproteins or having recombinant systems which can glycosylate and in this case, glycosylate in a very specific fashion, this particular group of molecules may be out of the real of providing good immunogens in the immediate future.

The second group of proteins which we know are on the surface of the merozoite are nonglycosylated, and I've listed them on this table. We can start with the 83K protein because, (1), it's been studied by a whole series of different laboratories: John Scaife's laboratory, Luc Perrin's laboratory - in fact,there are two or three British groups - the Wellcome group, and there are several groups in this country. This protein has a wide distribution, even though there is some size and antigenic variation within it. We know nothing about its capacity to bind to glycophorin or other specific proteins on the erythrocyte surface. It is quite prevalent on the surface of the merozoite from the work of John Scaife's laboratory, that is to say, the Roger Hall paper, which was published in "Nature" recently. There is evidence suggesting that at least the parent molecule, the 185k glycoprotein which was purified by affinity chromophotography, that molecule in its native state can produce immunity and we know, in fact, that a number of laboratories have clones of this molecule, or at least, clones of portions of the molecule. It is likely that its structure will be known reasonably shortly. Certainly, within the upcoming year or two. Ferosa Ardeshir has evidence that a 180K protein also either it, or a 59K protein which contains the same epitope, are also on the surface of the merozoite. It has been found of every isolate of P.falciparum they have examined. They know nothing of its ability to bind to glycophorin, but it is quite prevalent on the merozoite surface. There is little known about its ability to stimulate immunity, and I mentioned, it has been cloned by Ferosa Ardeshir and Jan Flint. There is 140K protein which has been cloned in our laboratory and may well be the same protein which Luc Perrin has studied and has used to immunize

monkeys. It is quite prevalent on the merozoite surface and it may also be the 140K protein which has been implicated by Jungari et al as being as binding to glycophorin or at least to N-acetylglucosamine. There is 130k protein which appears to be on the merozoite surface. It binds to glycophorin. We don't know how prevalent it is on the merozoite. We know nothing about its ability to stimulate immunity. This protein has been cloned by Jeff Ravitch at Sloan-Kettering.

And finally, there is a 75K protein which is probably one of the most prevalent proteins that the parasite makes. It is very prevalent on the surface of the merozoite. If, in fact, it is the same protein which Jungari has also implicated, it does bind to glycophorin. Again, if it is the same 75K protein which the group at the Pasteur has studied, DuVois et al, then it also has the capacity to stimulate immunity in monkeys. This protein has been cloned in our laboratory as well.

Finally, one of the things which we are hoping to do in our laboratory is not stop at simply producing an amino acid sequence deduced from a DNA structure, but finally, we would like to use some of the newer technology as well as some of the physical-chemical approaches that are available to us nowadays to see if we cannot generate an approximation of the three dimensional structures of these molecules. Now, our studies are just getting under way, but it is our hope that structures such as the one which I've displayed on the screen in the next two slides. This is a side view and this is looking down the peptide chain. This is a computer simulation in which the computer has tried to, beginning at a linear structure, has tried to calculate a lowest energy structure for us. We believe by using a series of different tools as well as those such as this, we may some day be able to even without having to go to crystallographic procedures, may be able to define in a three dimensional sense some of these molecules, and in doing so, may be able to put our studies on a somewhat more solid ground.

PRODUCTION OF PLASMODIA ANTIGENS BY NEW BIOTECHNOLOGY

Rapporteurs: Ronald Kennedy and Karen Yamaga

Dr. Victor Nussenzweig reviewed the relevant features of the circumsporozoite protein (CSP) which included the fact that Fab McAb fragments inhibited sporozoites *in vivo* and *in vitro* and that the CSP covered the entire surface of mature sporozoite. He discussed three regions of the CSP: N2 which flanked the repeats in the direction of the N-teminal; the C2 region which was situated between two pairs of cysteine residues close to the C-terminal end of the molecule; and the repeat area. The two flanking region showed considerable conservation when compared with different Plasmodium species. Both were exposed on the molecule and were potential targets of the immune system. Antibodies raised to the C2 peptide of *P. knowlesi* did not neutralize *P. berghei* sporozoites wherease antibodies to the N2 peptide showed partial neutralization. As shown before, antibodies to the repeats did neutralize. Dr. Nussenzweig reviewed some of the properties of the repeat region: they occupied 40-50% of the molecule; the repeat sequence were more conserved at the level of amino acid than at the nucleotide level; the sequence was conserved in isolates of the same parasite species from around the world; the sequence was less conserved than the rest of the molecule. The possible function of repeats include: structural but this seems unlikely because they varied too much; a ligand for a liver receptor; to enhance or deviate the immune response but antibodies do neutralize. The second and third possible functions were possible. It is also possible that repeats were responsible for concomitant immunity. Perhaps they were preserved to enhance the immune response to sprozoites and to prevent superinfection without affecting merozoites. If a parasite species wss in equilibrium with its host, those variants bearing CSP with different repeats have a selective advantage.

A synthetic vaccine composed of the repeat sequence may be ideally suitable because it contained only three amino acids--proline is found in the bends of the sequence; and asparagine is found in beta bends. It was found that 3 repeats was the minimum number needed to react completely with the McAb to the CSP. In addition all antibodies from adults or from rabbits reacted with three or more repeats. An antigen composed of 3 repeats coupled to tetanus toxoid in Freund's incomplete adjuvant elicited antibodies in rabbits at high titer. The rabbit antibody when absorbed with P. falciparum extract removed 70% of the repeat reactivity.

The arguments favoring the developing of a sporozoite vaccine composed of repeats included: (1) the $(NANP)_3$ repeat was found in strains from all endemic areas of the world; (2) $(NANP)_3$ was repeated at least 10 times in each molecule; (3) McAb neutralized infectivity; (4) population in endemic areas have a B cell repertoire capable of recognizing $(NANP)_3$. The problems were listed as well. The synthetic vaccine may not elicit sterile immunity. If it did not produce sterile immunity, it might still be useful to reduce the parasite load. It is not known if the vaccine will function with alum adjuvants. It is not known if the immune response will be boosted with natural parasites.

Dr. McCutchan reviewed four approaches to producing plasmodia antigens by new biotechnology methods. Chemical syunthesis has the following advantages: it is free of biological contaminants; it is less expensive; response can be targeted to a single region of a protein. It has the following disadvantages: appropriate carrier and adjuvant must be found; and short peptides may not accurately mimic immunodominant regions. The advantages of bacteria and yeast host vector systems is that a large insert can be incorporated; and that the material produced may be very immunogenic. The disadvantages are that the cost is high; biological contaminants must be removed; carriers and adjuvants may be required. The vaccinia system has the following advantages: the virus is stable; it can be administered by untrained personnel; the genome may accomodate 15-20 foreign genes; it elicits both cell mediated immunity and humoral antibody responses; and it may provide long term protection. The disadvantages are: serious complication may occur in 1-2 per million; there may be loss of control over live vaccines; and its use may be limited in infants.

Dr. Coppel emphasized the fact that there are a large number of antigens in the asexual blood stages and that antibodies react with many antigens. However, the immune system may have more than one target to attack. Expression libraries have been generated from cDNA producing fused polypeptides or full length products or from genomic DNA using mung bean nuclease cleaved genes or sonicated genes. He reviewed the lambda gt Amp sytem used by his laboratory which is screened with human antisera using a colony immunoasaay. Dr. Coppel reviewed the system that has been used to express P. falciparum antigens (see working paper) and noted one change. The vaccinia virus seems to express only low amounts of gene product like the lambda gt 11 system. The repetitive sequence of P. falciparum seem to be a frequent feature of the antigens. Two types of repetitive sequences have been noted. Type I antigens have 4, 8 or 10 amino acid repeats in tandem (CS protein, S antigen and histidine rich protein). Type II antigens have repeats interspersed with non-repeat sequences (RESA, FIRA, and 21K histidine rich protein). Of the more than 400 antigen positive clones, 25 yielded characterized gene products, 121 unassigned clones, and 13 of these clones have been sequenced. Of these, 12 have tanden repeats. Repeat and non-repeat fragments of RESA have been expressed and antibodies generated. Antibodies were formed against those antigen fragments containing the repeat sequences. Repeats have not been made easily in E. coli and have had a tendency to be unstable in bacteria. Dr. Coppel presented data that located the different genes on one of seven chromosomes found in P. falciparum. It was estimated using the highest expression vector possible that 5-10 people would be immunized with 1 liter of E. coli in broth culture medium.

By using immune Aotus serum which could inhibit the in-vitro growth of P. falciparum, had been shown by electron microscopy to contain antibodies against the surface of both the infected merozoite and schizont and which could passively protect other monkeys, a series of surface antigens were identified on purified ^{125}I surface labeled merozoites. Monospecific reagents, either mcAb of polycolonal antibodies produced against cloned expressed fusion proteins, were then used to demonstrate first by immunofluorescence and then in several cases by immuno-electron microscopy that the merozoite surface was composed of three glycoproteins, gp56, gp46, and gp34. The sugar moities on these structures were found to

be important in maintaining the appropriate conformations of these molecules. A second group of non-glycosylated moleclues having molecular weights of 140k, 130k, 83k, 75k and 59k were also shown to be on the merozoite surface. Several of these molecules have been implicated as having the capacity to bind to glycophorin and to stimulate immunity in monkeys when purified in their native states by affinity chromatography. Methods were suggested which would allow approximation of three dimensional structures to be produced using physcial techniques such as high resolution NMR and computer alogrithms which will allow lowest energy states to be calculated.

Dr. da Silva presented immunogenicity studies with 2 fusion polypeptides (one containing a nonapeptide repeat, the other from clone 2L) When SDS treated, denatured forms were used to immunize monkeys, the antibodies were directed to the denatured forms but gave very little reactivity to the native protein. Therefore, the use of DNA recombinant gene prooucts may not elicit antibodies that react with native protein.

Native Proteins for Malaria Vaccine Produced by Large-Scale In-Vitro Culture Systems

Kevin Palmer, Goerge Hui and Wasim Siddiqui
University of Hawaii, Honolulu, Hawaii

The development of all four types of potential malaria vaccines have proceeded along basically the same path. First, parasite derived polypeptides have been shown to be antigenic in small animals and then to be protective in either the Saimiri or Aotus monkey model system. The work has then expanded into identification of specific polypeptides using monoclonal antibodies or hyperimmune serum. These have then been purified, and finally either sequenced and produced synthetically as in the case of the circumsporozoite protein or a cDNA library has been produced and inserted in a number of host cell systems. Some laboratories have gone directly to the cDNA library, or have chosen to skip one or another step. But in general the assumption has been that if the whole parasite, be it merozoite, sporozoite, gametocyte, or culture supernatant, is protective then the genetically engineered or synthesized polypeptide will likewise confer protection.

Any vaccine to be practical, will have to be produced in bacteria, or yeast or else be totally synthetic. The key question will eventually be does the polypeptide or do the polypeptides protect as well as the parasite derived "native" proteins?

There are at least two possible answers to this question. First, that the genetically engineered or synthesized polypeptide or polypeptides protect as well or better than the native proteins. This would of course be the outcome we have all been looking forward to. On the other hand, these polypeptides may not protect at all or not as well as the native protein. If not why not?

Let me reiterate the obvious differences between native and genetically engineered or synthesized polypeptides. First there are numerous possible post-translational modifications that may take place within the living parasite, especially those affecting tertiary structure, which may not necessarily be duplicated in the bacteria or yeast host system and which may not be reproducible in the process of peptide synthesis. These modification may play a major part in the protective nature of the native proteins.

The importance of post translational modification was seen in developing the second generation of HbsAg vaccine currently undergoing human testing. When the viral genome fragment was placed in bacteria the resulting polypeptide did not protect as well as the native protein. When placed in yeast, however, the necessary glycosylation took place and the HbsAg like polypeptide did protect in animal systems. Whether correct post translational modifications leading to the full protective nature of the antigen in question takes place will obviously depend on the host system used as well as the unique nature of the antigen itself.

With such limitations of the new biotechnology in mind our laboratory has maintained the philosophy that at each step in vaccine development, the protection of purified, or genetically engineered polypeptides must be compared directly against parasite derived antigens. Immunization studies in Saimiri monkeys, Actus monkeys and finally in humans using "native proteins" should precede or at least parallel studies using genetically engineered or synthetic antigens.

This has required that we develop the capacity to produce large volumes of parasite material and a methodology for isolation and purification of the crude parasite antigen using monoclonal antibodies. Our colleagues working with the circumsporozoite protein have not had available to them a means for producing sporozoites in-vitro. This has necessitated the rapid development of the synthetic CSP protein which they are now testing.

Methods for large scale production of gametocytes are available and serve as the source of material for the work already described.

In terms of the erythrocytic stages of the parasite, numerous laboratories have devised methods for the production of quantities of the parasite to meet their needs. All these methods have been based on the original method for in-vitro cultivation described by Trager and Jensen.

When we looked toward the prospect of producing sufficient quantities of material for full scale immunization studies in Actus monkeys and

eventually a limited human trial we made the decision to undertake the development of a semi-automated culture system that would be capable of producing large amounts of crude parasite antigen.

Very briefly, the system is based on large plastic culture vessels, each holding a total culture volume of 500 ml, which is contains 25 ml of packed erythrocytes. The culture medium is RPMI 1640 supplemented with 7% reconstituted human plasma. Medium is withdrawn and replaced at preset intervals automatically. The intervals between changes, decrease as parasitemias rise.

Starting at approximately 1 to 2% parasitemia we obtain final parasitemias of 18 to 22% with total parasite yields of 10^{10} parasites per vessel.

The system can handle up to 12 culture vessels. We are currently operating with only 4 vessels.

In looking toward human trials the key question will be how much material will be needed to vaccinate one human? At this point there is no data on which to base an answer. Doses of purified antigens in published experiments using Saimiri monkeys have ranged from 100 ug to 30 ug. A major determining factor will be the nature of the adjuvant finally selected for use in humans.

I have arbitrarily chosen a total of 100 mg protein as a working assumption for a small scale clinical trial. Based on that figure and using the methodology briefly described by Dr. Kan for isolating specific protein bands identified by our monoclonal antibodies 219.5 and 5.2; together with data from the immunogenicity experiment described by Dr. Siddiqui I have made the following calculations.

In that experiment we started with 21.9 ml of saponin lysed, crude parasite antigen. This was extracted to yield 250 mg of protein which was divided into two lots of 125 mg each. One lot was used for isolation with mcAB 5.2 and the other with mcAb 219.5.

The yields for each were 312 ug using mcAb 5.2 and 113 ug with mcAb 219.5. Immunizing doses given the experimental monkey was 80 ug of 5.2 purified antigen together with 30 ug of 219.5 purified antigen to the second monkey.

Each large scale culture vessel yields 18 ml of packed, infected erythrocytes which when saponin lysed yields approximately 2.6 ml of crude parasite antigen. The 250 ug of extracted antigen therefore represented the production of 8 large-scale culture vessels or two weeks production with four boxes in operation. Full scale immunization studies using a minimum of six immunized Aotus monkeys and the same inocula will therefore require six weeks of production.

Further immunization studies comparing genetically engineered or synthetic polypeptides against native polypeptides would require a similar amount of production time.

To produce enough material for a small human trial totaling 100 mg of 5.2 purified antigen using current methods would take approximately 1.6 months and of 219.5 purified antigen approximately 4.4 months. when expanded to 12 culture vessels the system could producce 100 mg of 5.2 purified antigen in two weeks and of 219.5 purified antigen in approximately 6 weeks.

Judging from these figures limited human trials using parasite derived antigens are feasible. We strongly believe that parallel immunization be studies be carried out first in Aotus monkeys using both native and synthetic or genetically engineered polypeptides and that a small scale parallel trial is feasible and should be carried out in humans.

RHÔNE MÉRIEUX

WEDNESDAY, April 24, 1985
Abstract No. 32, page 38
Yves Moreau

INTRODUCTION

Successful experimental immunization with P. falciparum blood forms, crude in vitro supernatants and merozoïtes in Aotus Trivirgatus and in Saimiri Sciureus and the recent availability of P. falciparum blood forms from continuous in vitro culture have encouraged research on the development of Malaria vaccines for use in man.

The ideal situation, as it has been pointed out by Dr PERRIN, Dr SIDDIQUI and Dr RISTIC, should be the use of vaccines based on defined parasite antigen(s).

As at the moment, it remains difficult to choose and to produce the best candidate antigens prepared by DNA-recombinant technology, we feel that a pragmatic approach would be the use, of semi-purified native antigens for preliminary field human trials.

Such blood stage antigens, while providing total immunity only in exceptionnal cases, result in a much less severe course of infection on challenge.

If a total safety of these antigens can be warranted, preliminary human trials should be engaged in order to enlighten the opportunity of this blood stage vaccine choice.

1. LARGE SCALE "IN VITRO" PROPAGATION

1.1. Automatized - cultures : (K.PALMER)

1.2. Large flasks method : (My.CAGNARD, Rhône-Mérieux)

After the beautiful but now old- fashioned method described by TRAGER and JENSEN, many improvements have been made by different group In our laboratory, we are now using. Large sovirel flasks which are filled with 250 ml of RPMI 1640 medium supplemented with 2 g per litre of glucose and 10 % A^+ serum. The working hematocrite is 6 %

A continuous gas flow is provided which composition is 8 % CO_2, 2 % O_2 and 90 % N_2.

Medium is changed every day a subculture is made twice a week.

Various strains have been multiplied : Geneva I, Indochina I, FCR 3 and Palo Alto.

A yield of 220 ml per flask of crude supernatant is available each day.

2. PURIFICATION

Once these crude supernatants are collected, centrifuged and filtered, they will be purified.

They are now many prooves that there are protective antigens in the crude supernatants.

Doctor PERRIN and Doctor RISTIC group in collaboration with us have several demonstration on this question.

In our conditions we are following two methods of ions exchange and affinity chromatography.

2.1. Two kinds of ions exchange chromatography, were tested first the QAE gel involving an anionic exchange and then the SP Trisacryl gel involving a cationic exchange.

With the QUAE column, (figure 1)

Crude supernatant is concentrated 10x by ultrafiltration then applied on this anionic exchange column. Protein elution and ELISA value are followed for each fraction. As it has been described at Annecy Congress by AMBROISE-THOMAS,such a profile (figure 2) was obtained. A group of fractions called "E" antigen corresponding to a specific ELISA activity were pooled, characterized and used for monkeys trials.

Such a purification seems not sufficient as it does not eliminate all the components of the culture medium.

SP Trisacryl column, (figure 3)

The purification has been much more improved with a cationic exchange chromatography. As seen on the preparative flow sheet. Crude supernatant are filtrated and concentrated 10 x after dialysis and x centrifugation, they are applied on an SP Trisacryl Column (diapo) and (Figure 4), SPE antigens, were also used for monkeys trials.

SPE antigen were characterized by SDS Page and autoradiography (diapo)

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2.2. Affinity chromatography (Figure 5)

As specific antibodies, we are using a polyclonal IgG preparation extracted form human placentas collected in endemic areas (Western Africa). After elution with an acid buffer, specific antigenic activity was checked by ELISA . Such antigens were characterized by SDS-PAGE, autoradiography and Western blotting. (diapo)

3. CONTROLS OF THESE PREPARATIONS

3.1. Row material

on the medium

Nothing else than all the classical chemical and physical controls (pH, osmolarity.....) and bacterial and fungal sterility tests are applied.

on sera and red blood cells:

Here also, the usual tests of official blood banks are performed (Hepatitis, siphilis, Aids.....).

3.2. On the purified products

- Sterility tests : according to the European Pharmacopea recommandations are performed.
  - . bacterial and fungal sterility
  - . Absence of Mycoplasma
  - . Absence of viral contaminations (MRC_5 and Vero cells).

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- Safety tests : according to the European Pharmacopea the purified products are inoculated on mice and guinea pigs. A search for pyrogens is performed on rabbits.

- Research of other contaminants :
  - . Albumin and hemoglobin titration
  - . Blood group antigens : A and B
  - . Rhesus substance : "D" antigen
  - . Silver stain SPD-PAGE gels.

- Physico chemical controls :
  - . Protein titration
  - . Nitrogen dosage
  - . Aluminium hydroxyde titration which is used as an adjuvant.

- Activity tests :
  - . ELISA
  - . Neutralisation

- Identity tests :
  - . SDS-PAGE in comparison with a standard antigen
  - . Western blotting with polyclonal or monoclonal antibodies.

Antigenicity and Immunogenicity controls

- Antigenicity in vitro

An ELISA test was developed and allows an antigenic evaluation of each antigen preparation.

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- Immunogenicity in vivo

Monkeys trials initiated by University of Illinois (Pr RIST group) in collaboration with us. Soluble P. falciparum exoantigens contained in crude culture supernatant fluids induced protective immunity against experimental falciparum malaria in the Boliviam Saimiri sciureus monkeys. The SP immunogen conferred significant clinical protection of monkeys when combined with an aluminium hydroxyde adjuvant (homologous challenge) (diapos).

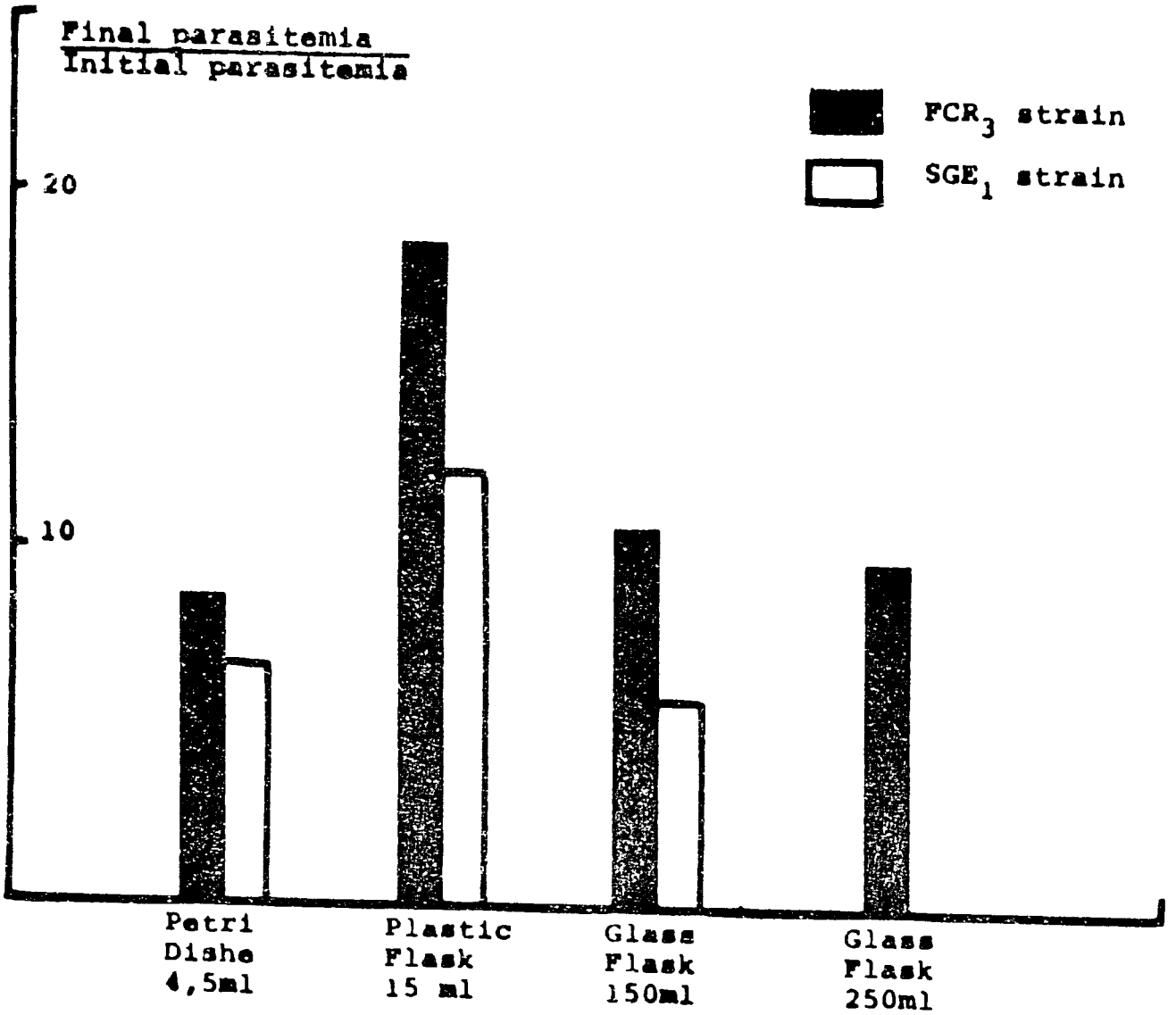
As this meeting is about Malaria vaccines and the type of clinical testing that will be required, we have to considered, that there is a considerable body of experience in the development of vaccines of good purity and with a high degree of chemical and physical characterization.

Do the Malaria vaccines prepared with native protein : present special consideration ? The answer in our opinion is no-

The first step is to devise a production system that gives the antigens in good yield of reproducible level of purity. Conventional Industrial vaccine producers are more and more used to the protective antigens being a minor component of the final product.

Waiting for DNA recombinant vaccines in the future, we feel that purified preparations from native antigens, should be used in limited field trials just to confirm that asexual blood stage proteins are good candidates for real human immunization.

COMPARISON OF PARASITEMIA



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SUPPORT MATERIALS FOR ION EXCHANGE CHROMATOGRAPHY

* SP TRISACRYL M

composition
- Strong cation exchanger
- Matrix : beads of acrylic copolymers
- Functional group : Sulfopropyl, $-CH_2-CH_2-CH_2-SO_3^{\ominus}$

* Q A E SEPHADEX

composition
- Anion exchanger
- Matrix : Dextran
- Functional group : Diethyl (2-hydroxy-propyl) aminoethyl,

$$OCH_2 - CH_2 - \overset{\oplus}{N}\begin{matrix} C_2H_5 \\ C_2H_5 \end{matrix} - CH_2 - CH\ (OH) - CH_3$$

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FLOW SHEET FOR "E" ANTIGEN PREPARATION

CRUDE SUPERNATANT

↓

CONCENTRATION 10x

↓

QAE A50 SEPHADEX COLUMN

↓

PROTEIN ELUTION
Tris HCl 50mM Buffer pH 7.6
SALT GRADIENT OM→0,5M

↓

ELISA

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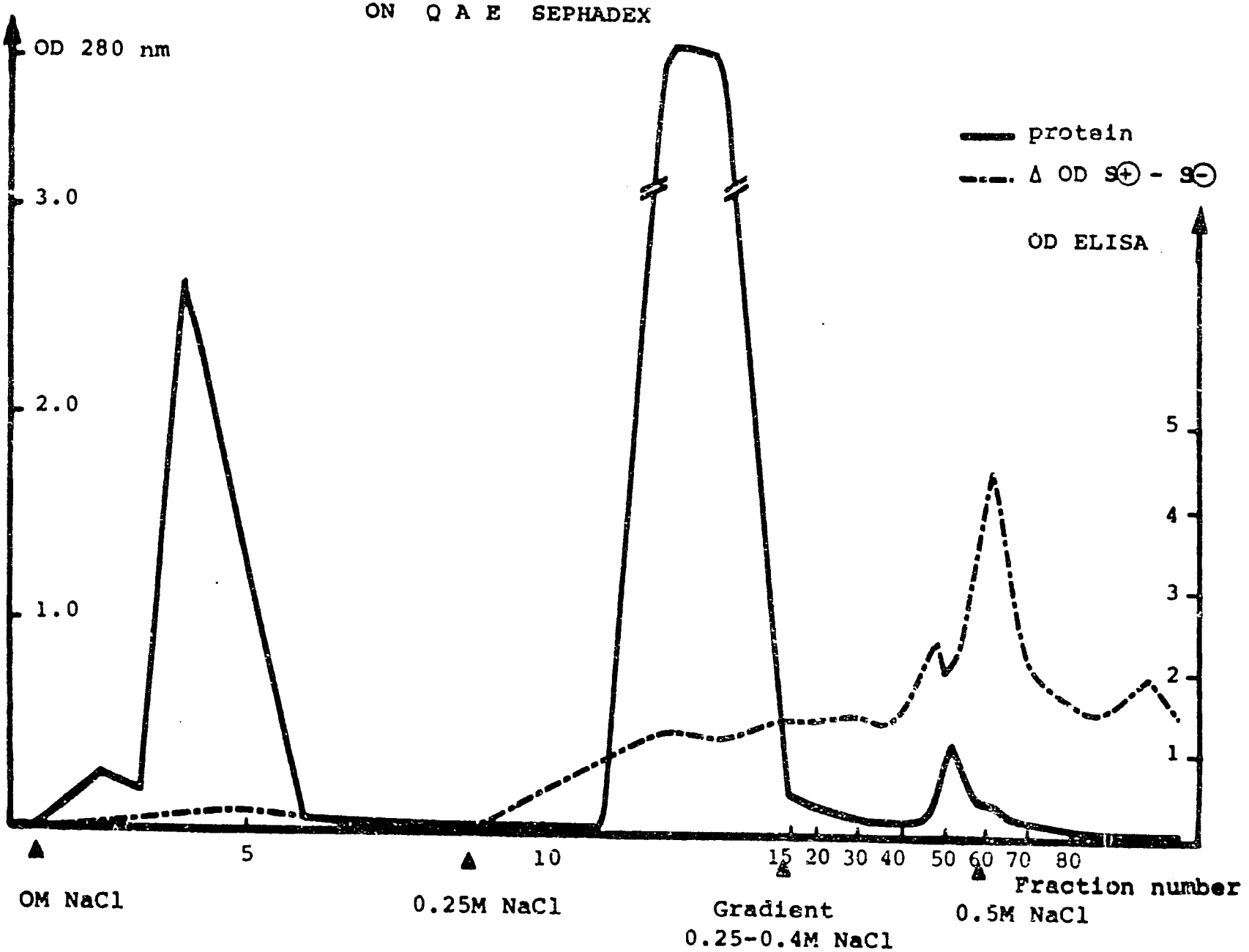
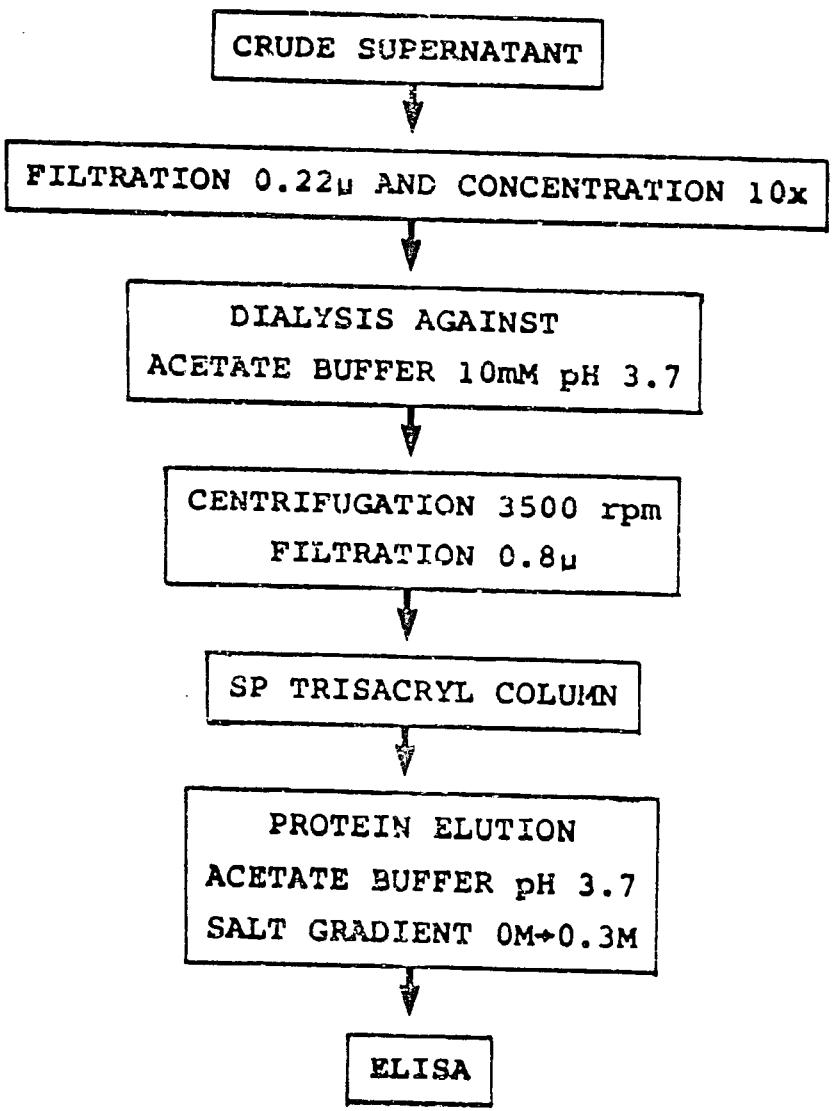
FRACTIONATION OF CRUDE SUPERNATANT
ON Q A E SEPHADEX



FLOW SHEET FOR "SPE" ANTIGEN PREPARATION



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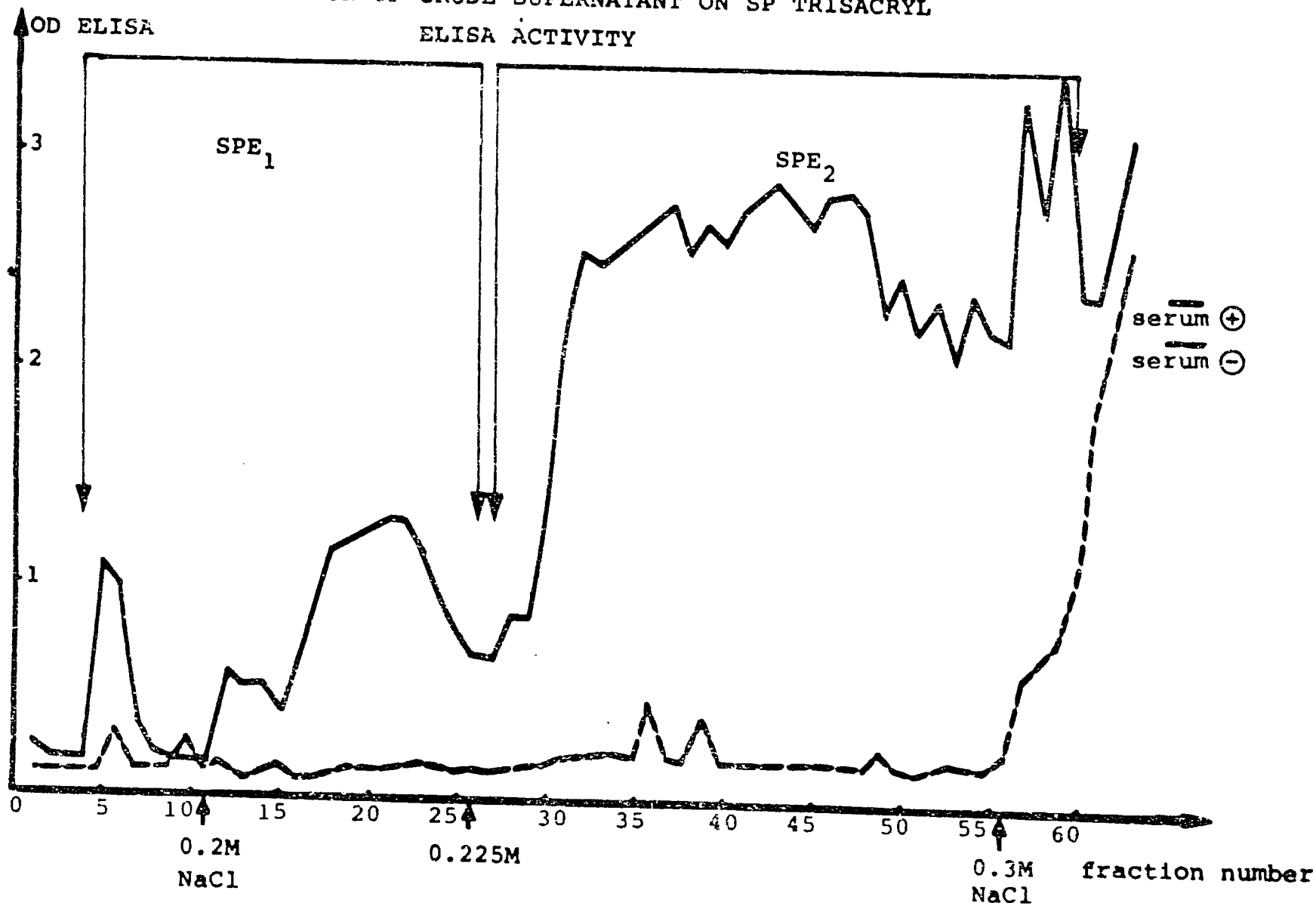
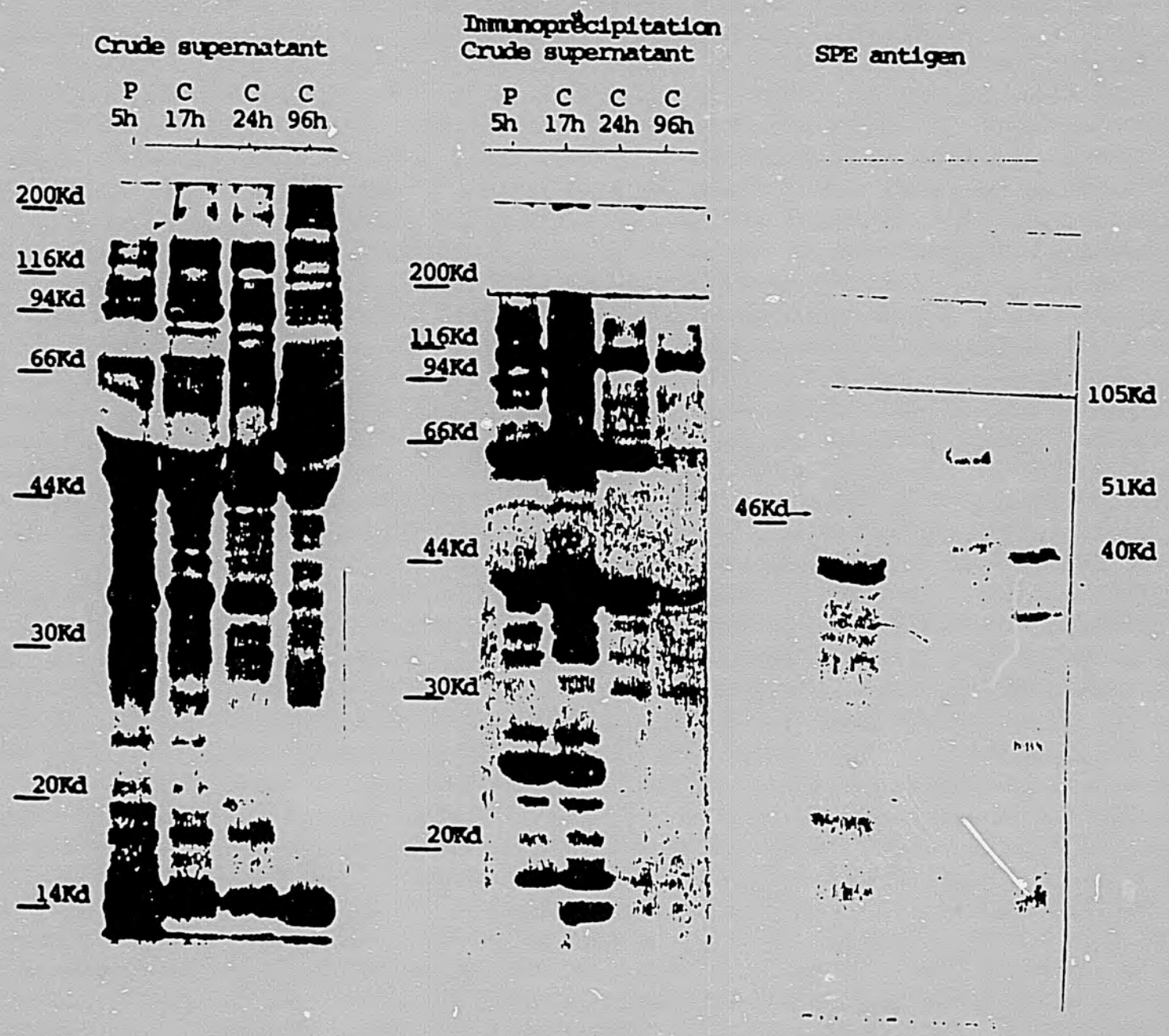
FRACTIONATION OF CRUDE SUPERNATANT ON SP TRISACRYL



^{35}(S) METHIONINE - LABELED CULTURE (SGE I Strain)



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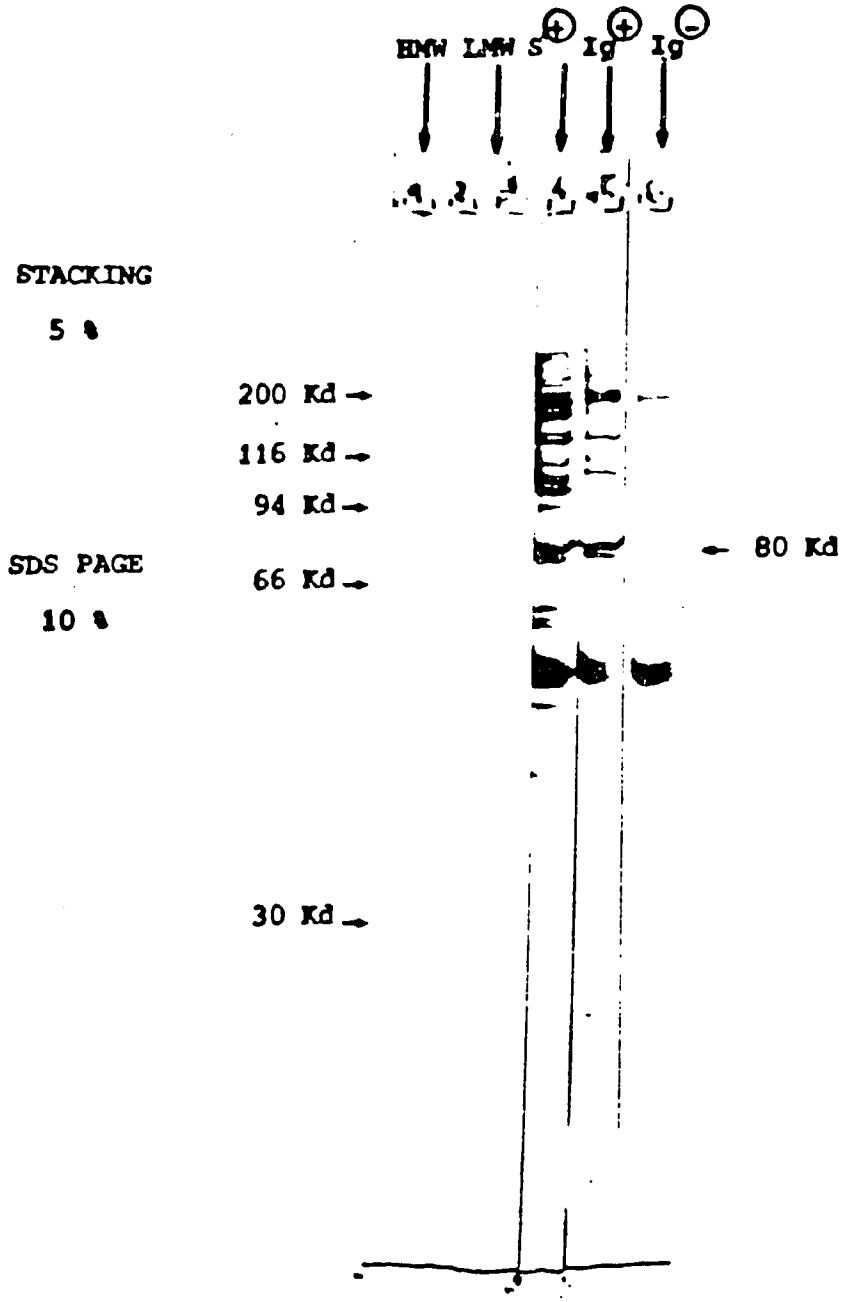
ANTIGEN PURIFIED BY

AFFINITY CHROMATOGRAPHY



WESTERN BLOT

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AFFINITY CHROMATOGRAPHY

ADSORBANT USED

CNBr - Activated Sepharose 4B

IMMUNOGLOBULIN USED

IgG positives in MALARIA purified from placenta

ELUTION BUFFER

ACID BUFFER pH 2.5 (+ Dioxane 10 %)
DIETHYLAMINE BUFFER 50mM pH 11.5

ANTIGEN DETECTION

SDS PAGE ELECTROPHORESIS
WESTERN BLOTTING
AUTORADIOGRAPHY

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TESTINGS ON THE PURIFIED PRODUCT

1. STERILITY TEST (According to the European Pharmacopea)

   - Bacterial and fungal sterility
   - Absence of mycoplasma
   - Absence of viral contamination performed on MRC_5 and Vero cells.

2. SAFETY TEST (According to the European Pharmacopea)

   - Inoculation of the product to mice and guinea pigs
   - Research of pyrogens on rabbits.

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TESTINGS ON THE PURIFIED PRODUCT

3. RESEARCH OF CONTAMINANTS

- Albumin and hemoglobin titration
- Blood group antigens - A and B
- Rhesus substance : antigen D

4. BIOCHEMICAL TESTINGS

- Protein titration
- Nitrogen dosage
- Aluminium hydroxyde titration

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TESTINGS ON THE PURIFIED PRODUCT

5. ACTIVITY TESTS

- ELISA
- SERONEUTRALISATION

6. IDENTITY TESTS

- SDS PAGE - Comparaison with a standard
- COUNTER IMMUNOELECTROPHORESIS
- WESTERN BLOTTING with polyclonal or monoclonal antibodies

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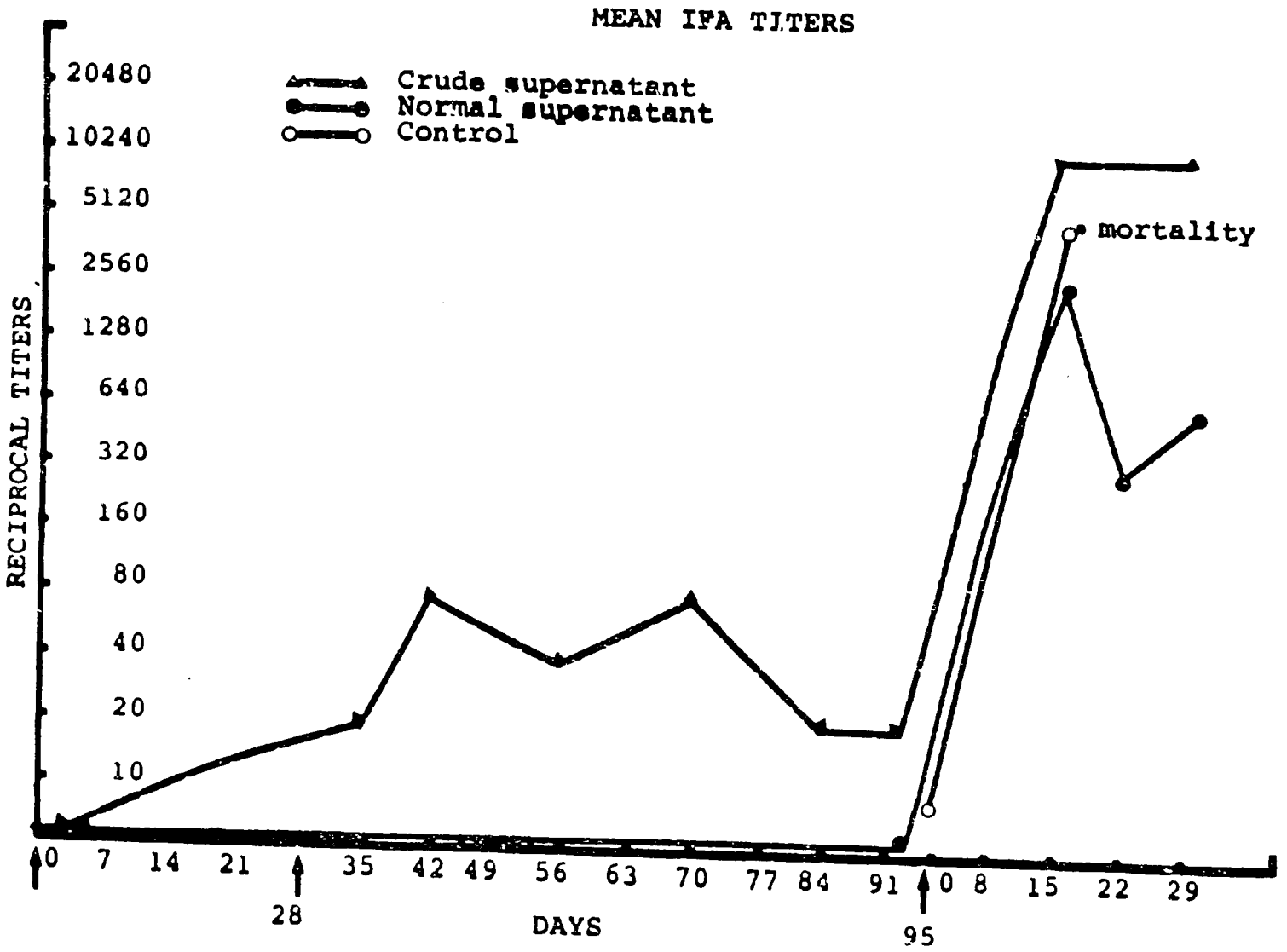

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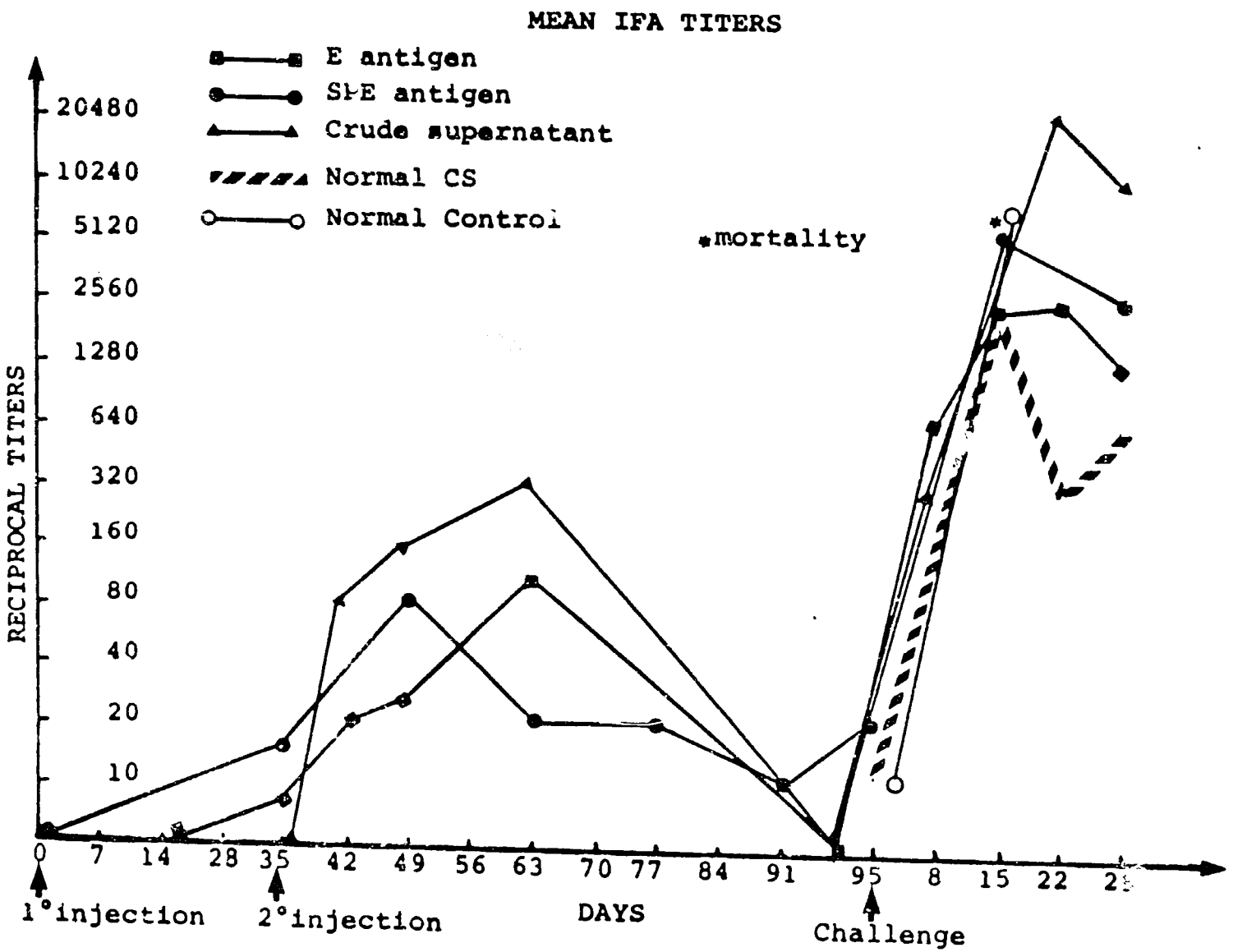

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SYNTHETIC PEPTIDES AND CARRIERS
Paper No. 33

* Paper Will Be Included In Final Publication

WEDNESDAY, April 24, 1985
Abstract No. 34, page 40
Ronald C. Kennedy

INTERNAL IMAGE BASED ANTI-IDIOTYPE VACCINES -
MAKING ANTIBODIES WITHOUT ANTIGENS
Paper No. 34

* Paper Will Be Included In Final Publication *

Asia and Pacific Conference on Malaria

Honolulu, Hawaii April 21-27, 1985

Virus Vaccines as Examples for Development of Malaria Vaccines

Bernard Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20205

Introduction

Recent progress in the identification of genes that encode Plasmodial antigens has raised the possibility of developing an effective malaria vaccine. Choices need to be made not only with regard to the antigen but to the type of vaccine. Although the ability to protect against disease is the paramount consideration, cost and method of delivery also must be taken into account. It seems appropriate at this juncture, to consider previous experience with virus vaccines as well as new emerging technologies.

Live Vaccines The majority of virus vaccines consist of live viruses that have reduced pathogenicity. The first vaccine was directed against smallpox. Initially variola virus, the causative agent of smallpox itself was found to be protective when inoculated in the skin. However, this procedure which was was called variolation was abandoned when Jenner demonstrated that vaccinia virus effectively protected against smallpox without the risk of spreading the disease itself. Vaccination, a term originally used to signify inoculation with vaccinia virus but now expanded to mean immunization against any infectious agent, proved highly successful and resulted in the total eradication of smallpox. The development of smallpox vaccine in the late eighteenth century was based on the fortunate existence of a virus of low pathogenicity that was closely related to the disease agent. In general, however, the development of live virus vaccines has depended on attenuation of viruses. This has been usually carried out empirically by repeated virus passage in tissue culture. In this manner, safe and highly effective vaccines against poliomyelitis,

measles, rubella, mumps and yellow fever were produced. Typically live vaccines are effective after a single dose and provide both humoral and cell-mediated immune response of long duration. Usually, live vaccines are relatively inexpensive because of the low cost of production, and small inoculum size. The stability of live vaccines varies: in some cases, such as measles, the vaccine must be refrigerated whereas smallpox vaccine can be stored in freeze dried form at elevated temperatures for long periods. The production of adverse side effects particularly in immunodeficient individuals, virus spread to susceptible contacts, and reversion to virulence are possibilities that have to be evaluated for each vaccine. In some instances, e.g. poliomyelitis, spread to contacts may be considered advantages since it contributes to the immunity of the population as a whole.

Killed vaccines. A second type of vaccine consists of killed or inactivated virus. There are important differences between live and killed vaccines and only a few of the latter type e.g. poliomyelitis and rabies vaccines have been used successfully on a large scale to prevent virus disease. Of practical importance are the usual requirement for repeated immunization with a killed vaccine to obtain protective antibodies, the low stimulation of specific cell mediated immunity, and the need for careful safety testing to ensure that the vaccine is free of infectious organisms. Occasionally, important antigens have been destroyed during the inactivation procedure leading to partial immunization that has actually increased the frequency of severe complication following subsequent infections. This probably occurred during trials with killed respiratory syncytial virus and measles virus vaccines.

Natural subunit vaccines. Subunit vaccines, so-called because they contain only a subunit of the virus particle, share some characteristics with killed vaccines. Repeated immunization are usually essential and specific immunity is probably limited to the humoral type. At present, the hepatitis B virus vaccine is the only licensed virus subunit vaccine. The inability to grow hepatitis B virus in tissue culture prevented the development of either live or killed vaccines. However, the presence of 22 nm lipoprotein particles consisting of excess hepatitis B virus surface antigens in the blood of chronically infected individuals provided an alternative material. Rigorous procedures have been used to remove or inactivate infectious particles and the vaccine is safe as well as efficacious. Unfortunately the limited quantities, requirement for multiple inoculations, and expense has thus far prevented wide use of the subunit vaccine in areas of the world where hepatitis B is highly endemic.

Recombinant DNA derived subunit vaccines. Recombinant DNA technology has provided powerful methods for the identification and expression of potential antigens to be used as vaccines. The most straightforward approach is to clone the gene of interest into a high expression vector in order to synthesize the protein subunit. Prokaryotic systems, currently based on *Escherichia coli*, are the best understood and were the first to be used. In order to obtain high expression and to ensure stability, fusion proteins that contain both prokaryotic and viral sequences are frequently formed. Provided the product retains immunogenicity, the bacterial vector system has great potential. However, viral proteins made in prokaryotes are not

glycosylated and are frequently not in a native configuration so that protective antibodies may not be induced.

For the above reasons, eukaryotic expression vectors may have advantages. Yeast systems are particularly attractive since they can be grown in large fermenters. Yeast recombinant that expresses hepatitis B virus surface antigen (HBsAg) and influenza virus hemagglutinin (HA) have been constructed. Although glycosylated, the recombinant yeast derived HBsAg forms lipoprotein particles that are both immunogenic and antigenic. It seems likely that this yeast recombinant HBsAg will ultimately replace plasma derived vaccines. The yeast recombinant influenza HA protein is glycosylated and is antigenic, although its ability to induce neutralizing antibodies has not yet been determined.

Mammalian cells also can be used to produce virus subunits that are properly processed and glycosylated. Several different approaches have been developed. The desired gene can be introduced into a defective virus (e.g. SV40, adenovirus, herpesvirus, retrovirus) which is then used in conjunction with a helper virus to infect cells. Many viral proteins including HBsAg and influenza HA have been expressed in this manner. Alternatively, cells may be permanently transformed and the desired gene greatly amplified. With such systems large quantities of HBsAg and herpes simplex virus type 1 glycoprotein D (HSV-1 gD) have been made. Although in the past, vaccines could not be made from continuous cell lines, modern methods of protein purification may eliminate concern about contaminating DNA with transforming potential.

Live virus recombinants. Genetic engineering also can be used for the construction of live virus recombinants that may be suitable as vaccines. Such recombinant viruses would retain many of the features associated with a live vaccine. To serve as a vector, the virus should have a DNA genome large enough to accommodate additional genes without losing infectivity. There are three major families of viruses that may meet these requirements: adenoviruses, herpesviruses, and poxviruses. Of these three, poxviruses have a number of advantages. They have the largest capacity for additional DNA, one member of this family--vaccinia virus--has been used extensively for mass vaccination, and natural infections with viruses of this family are now rare.

Initial experiment demonstrated the feasibility of using vaccinia virus as an expression vector and methods of inserting and expressing foreign genes and isolating recombinant viruses have been developed. One general method involved the construction of plasmid insertion vectors containing the vaccinia virus thymidine kinase gene interrupted by a segment of DNA containing vaccinia virus transcriptional regulatory elements (promoters) and multiple sites that can be cut with restriction endonuclease for insertion of the foreign gene. Homologous recombination is used for insertion of the foreign DNA and recombinants are selected on the basis of their thymidine kinase negative phenotype.

With proper engineering, it appears that virtually any gene can be expressed in a vaccinia virus vector. Moreover, the proteins an generally processed and glycosylated in an appropriate manner. Evidence has been obtained that vaccination leads to humoral and cell mediated immune response to the foreign proteins. Vaccination with

recombinants that express the HBsAg, the influenza virus HA and the herpes simplex virus glycoprotein D have been shown to protect animals against challenge with the corresponding infectious agent. Recently, the circumsporozoite surface protein of Plasmodium knowlesi and falciparum have been expressed in a vaccinia virus vector and vaccinated rabbits were shown to produce antibodies to the repeating epitopes. With further engineering it may be possible to obtain vaccinia virus recombinants that express antigens from multiple developmental stages. If effective, such vaccinia virus recombinants may be particularly suitable for prophylaxis of malaria since the production of the vaccine should be inexpensive, refrigeration is unnecessary and medically skilled personnel are not required for administration.

Acyl or Alkyl Derivatives of Muramylpeptides as a Potential Adjuvant Useful for Malaria Vaccine

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It is my greatest pleasure and honour to present a paper here. Many thanks for Professor Siddiqui for this invitation to this extremely well-organized conference. At the begining I would like to ask for your consent about that my talk will be limited to general and basic aspects of immunoadjuvant activities of muramyl peptides, because I am not a malariologist.

Since the synthesis of MDP as the active principle respondsible for the immunopotentiating activities of Freund's complete adjuvant, extensive studies have been made on the immunopharmacological activities of MDP and related compounds, in attempt to utilize the beneficial activities, such as the enhancement of antigen-specific and nonspecific host immune responses.

For the past several years, we have made comparative studies on a variety of MDP derivatives with the aim to obtain the compound with the maximum adjuvant activity and the minimum deleterious effects and to utilize it for enhancement of potency of vaccines. Chemical structures of representatives of those categories of best muramyl peptide derivatives are shown in this slide.

The first step of our studies was the selection of MDP derivatives that could exhibit powerful adjuvant activities without the use of highly irritative, Freund's incomplete-type water-in-mineral oil emulsion as an administrative vehicle.

The immunoadjuvant activities of a variety of 6-O-acyl MDP and 6-O-alkylphospho-MDP in combination with various vehicles are shown in this table. You can see that unlike MDP itself, L18-MDP and most of 6-O-alpha-branched higher fatty acid derivatives of MDP can work as a potent adjuvant in stimulation of both primary, humoral and cellular immune responses of guinea pigs against a test protein antigen, ovalalbumin. Above all, 6-O-(2-tetradecylhexadecanoyl)-MDP, abbreviated as B30-MDP, showed a strong adjuvant activities in combination with any of sequalene-in-water emulsion, liposomes and phosphate buffered saline. All of these vehicles are not irritative, easily metabolizable, and so applicable to human vaccines.

This table summerizes the assay results obtained with various N^{ε}-acyllysyl or -alkyllysyl-MDP and gamma-alkylamides of MDP. Some of them which were given with squalene-in-water emulsion or liposomes stimulated antibody production significantly, but could not induce DTH, a representative of cellular immunity so effectively. In addition, all of them were only slightly active when given as saline solution or suspension.

Among disaccharide peptides, namely GM peptide derivatives, the alpha-branched fatty acid derivative, B30-GMDP, seems to be

qualified because this compound, like B30-MDP, does not select administration vehicles for the manifestation of its adjuvanticity.

The second step of studies concerns with known deterimental activities of muramyl peptides to which attention should be given in attempt to use them as immunoadjuvants for human vaccines. Following two slides summarize the immunopharamacological activities so far observed, either beneficial or deterimental, when MDP and its derivatives were injected into laboratory animals. Possible deterimental activities which should be decreased as far as possible in use of muramyl peptides as adjuvants to vaccines will be pyrogenicity (no. 2), the induction of acute polyarthritis (no.11), the provocation of hemorrhagic necrosis at the primed site (no. 15), and the enhancement of the susceptibility to toxicities of LPS (no.13). The last activity was found by Dr. Takada with Dr. Chris Galanos.

This table summarizes in vivo immunopharmacological actives of several lipophilic derivatives of MDP that have been provisionally selected by their definite adjuvancies in combination with non-irritative administration vehicles. You can see that among selected compounds B30-MDP, unlike either L18-MDP or MDP-Lys(L18), is nonpyrogenic by intraveneous route, and does not provoke the hemorrhagic necrosis at the site, prepared by delayed type hypersensitivity when injected as a fine suspension in PBS. The harmful activity retained by B30-MDP is the arthritogenecity. However, as you can see in this table, the arthritogenic effects for rats was noticed only after repeated injections, that is to say, daily injections of as high dose as 100 micrograms per rat as a PBS suspension for 2-3 weeks. Twelve daily, subcutaneous injections of 20 micrograms MDP in PBS did not induce any detectable arthritis, though similar administration of B30-MDP as a solution in a detergent for medical use, NIKKOL HCO-60, resulted in mild arthritis. Therefore, we believe that the arthritogenicity of B30-MDP may not seriously distrub its use as an adjuvant for vaccines, because usual vaccination schedule does not need so frequent administration at short intervals of B30-MDP as mentioned above. In passing, B30-MDP in PBS was shown to cause acute arthritis in BALB/c mice by one intravenous injection of 100 microgram dose per animal. But the induced arthritis was very mild and transient, and completely subsided by the 3rd or 4th day after injection.

A compound, MDP-Lys(L18) whose L-alanine residue is methylated, does not show most of the deterimental activities such as pyrogenicity and arthritogenicity. Unfortunately, however, the adjuvant activities of this derivative was considerably inferior to those of the parent compound.

I will show you in the following slide the dose-response

relation shown by guinea pigs receiving intra-footpad injection of B30-MDP and ovalalbumin, in combination with various administration vehicles. The slide illustrating DTH induction in terms of positive corneal reaction shows us that the administration of B30-MDP with liposomes, PBS, squalene-in-water emulsion and Intralipid^{R} causes definite induction of DTH the intensity of which were dosage dependent, in a similar way to the injection of B30-MDP in water-in-mineral oil emulsion. Intralipid is an intraveneous fat emulsion for clinical use regarding the stimulation of anti-ovalalbumin precipitating antibody production, squalene-in-water emulsion and Intralipid^{R} serve as an effective administration vehicle as Freund's complete-type adjuvant, as shown in this slide.

Swelling of the injection site in test groups was neglegible as compared with the strong irritation observed in the control group receiving w/o emulsion. Undesirable effect of B30-MDP is the swelling of lymph nodes belonging to the injection site, noticed when high doses of B30-MDP was injected with liposomes or PBS. This is due to the inherent property of B30-MDP to induce epitheloid granuloma formation as an expression of macrophage activation.

Usefulness of B30-MDP as an adjuvant fo vaccines has been demonstrated with Plasmodium falciparum antigens derived from long-term culture parasites by Professor Siddiqui, and with purified influenza HA-NA vaccines in our laboratory. In this speech, however, I will talk quickly about our recent preliminary studies on application of B30-MDP to the potentiation of a subunit of Hepatitis B vaccines that was isolated and purified from a human plasma source, and inactivated by a combined heating and formalin treatment.

Comparisons were made on serum anti-HBs PHA antibody titers in groups of guinea pigs, each of which received plain HB vaccine, HF-2000 vaccine added with several to 50 micrograms of B30-MDPs and HF2000 added with aluminum hydroxide, namely TV-1 vaccine, respectively. You can see here that 12.5 to 25 micrograms of B30-MDP exhibits definite immunoadjuvant effects. The adjuvant effect of B30-MDP was comparable to, but to our regret, not more than that of albumin during 11 weeks after the primary immunization. The induction of cellular immunity was determined by antigen-specific stimulation of [^{3}H] thymadine uptake by peripheral blood lymphocytes. The last column of the table shows that B30-MDP can definitely induce the cellular immunity to HBs-Ag by this assay.

Now, I will briefly refer to our recent studies on the adjuvant activity on antibody production of lipophillic derivatives of MDP which were given with bovine serum albumin by oral administration, exactly speaking, by gastric intubation to BALB/c mice.

Not a few muramyl peptide derivatives have been disqualified to be an adjuvant for vaccines for human use, in spite of their high adjuvanticity, because of their pyrogenicity, arthritogenicity and other delertirious activities. We believe that most of the delertirious effects of these compounds that are noticed in parenteral administration can be avoided by oral administration.

This slide shows that the gastric intubation of a semi-synthetic, stearoyl derivative of disaccharide tetrapeptide (A_2pm type) with BSA in liposomes, to BALB/c mice, on day 0 and 1 as a primary immunization and on day 27 and 28 as a booster, markedly increased serum anti-BSA IgG, IgM and IgA levels. I do not know whether oral administration is applicable to malaria vaccination, because considerably larger quantities of immunogens are needed as compared with parenteral administration and because this route of administration may not induce cell-mediated immunity. I have no data on this point so far.

Lastly, I would like to touch on our recent studies on the adjuvant activity of synthetic lipid A analogs. Since 1980, Professor Shiba's group has carried out a series of synthetic studies on lipid A, that is to say, the active moiety carrying most of the immunobiological activities of endotoxic lipopolysaccharides, LPS. Several months ago, a compound called 50B was synthesized. The structure shown here. This compound has been demonstrated to have full endotoxic activity of E. coli type complete lipid. One of the purposes was to develop novel lipid A analogs on derivatives of lipid A with the maximum beneficial effects including immunogenicity and the minimum delerterious activities. This would be useful in clinical and preventive medicine. We proceeded in studies on muramyl dipeptide derivitives. Structures of lipid A analogs synthesized for this purpose are presented here these are bis-and 4'-monophosphate of glucosamine disaccharide carrying four myristic acid. As shown here, those that have two myristoyl groups at C-3 and C-3' positions and two p-hydroxymyristic acid at C-2 and C-2' amino groups. These compounds synthesized as the first step of studies with the aims mentioned above, since the sort of acyl groups attached to the disaccharide backbone have been shown to have big influences on the immunobiological activities of these molecules. These compounds were at least 100 to 1000 times less pyrogenic than compound 50B, a synthetic counterpart of E. coli type lipid A. Shown in this slide are the bis photo derivatives incorporated into liposomes with bovine serum albumin and subcutaneously injected into BALB/c mice twice, markedly enhanced anti-BSA IgG and IgA production. The potency was considerably less than that of a natural lipid A with IgG response. The study, just begun, and the above results are not satisfactory. Nevertheless, I hope that studies along this line will provide new types of

synthetic immunoadjuvants useful for potentiation of vaccines. I shall be very happy if our basic studies presented this afternoon serve to develope a potent malaria vaccine and ultimately contributed to the relief of malaria through out the world.

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WORKING PAPER

POTENTIAL MODE OF ACTION OF ADJUVANTS USED FOR MALARIAL VACCINES

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ABSTRACT:

Immunological adjuvants are compounds which potentiate the immune response to vaccine antigens. Purified malarial proteins are often not sufficiently immunogenic in animals unless administered with a powerful adjuvant such as Freund's complete adjuvant. Adjuvants not only reduce the amount of vaccine antigen required and the frequency of repeat immunizations, but may alter the relative balance between cell mediated and humoral immunity. Adjuvants appear to work by two major mechanisms. First, they facilitate contact between the vaccine antigen and the immune system. In its simplest form, this effect may be achieved by allowing for the sustained release of antigen from a depot site, as with alum-adsorbed vaccines. Additionally, adjuvants may attract inflammatory cells which in turn process and transport antigen to draining lymph nodes. Adjuvants may also enhance the expression of class II histocompatibility antigens on macrophages and facilitate vaccine antigen presentation to T lymphocytes. The second major funcion of adjuvants is to stimulate the synthesis of various immunoregulatory lymphokines. Initial, antigen-specific, T cell activation appears to depend on the availability of interleukin 1 (IL 1). IL 1 induces the synthesis of the T cell growth factor, interleukin 2 (IL 2), and causes the expression of IL 2 receptors on T cells. IL 1 also enhances the responsiveness of B lymphocytes to the T cell derived, B cell growth factor (BCGF). Many experimental adjuvants such as muramyl dipeptide (MDP) and peptidoglycan stimulate IL 1 production and the relative potency of various analogues of MDP and of peptidoglycan correlate directly with their capacity to stimulate IL 1 release.

The choice of an appropriate adjuvant for use with malarial vaccines

will require a detailed analysis of the rate limiting step restricting the induction of a protective immune response. In vitro assays of immune activation by malarial antigens will be extremely useful in this assessment and for the selection of an adjuvant for use in malarial vaccination programs. Such an adjuvant may be critical in the development of effective protocols for mass immunizaton studies with malarial vaccines.

INTRODUCTION:

Immunological adjuvants refer to compounds which, when mixed with an antigen, will enhance the immune response to the antigen either quantitatively or qualitatively. The prototype, and still the most effective experimental adjuvant, is that developed by Freund (Freund, et al., 1937). It consists of an aqueous solution of antigen emulsified in light mineral oil to which non-viable mycobacteria are added. The water in oil emulsion creates a depot effect allowing for the sustained release of antigen, while the mycobacteria activate the cellular elements of the immune system attracted to the site of antigen injection. This formulation enhances both the humoral and the cell mediated immune responses. Freund's complete adjuvant (FCA) has been used in many of the experimental studies demonstrating anti-malarial immunity in primate and non-primate species immunized with malarial antigens (Siddiqui, 1977; Mitchell, et al., 1977; Holder and Freeman, 1981; Perrin, et al., 1984). Its use in man, however, is contraindicated because of the intense granulomatous inflammatory response against the mycobacteria, leading to chronic ulceration and scarring. Other experimental adjuvants have tended to be less toxic, but also less effective, than FCA. Nevertheless, an analysis of the

various activities of these adjuvants has helped provide insight into potential approaches to immune augmentation.

OVERVIEW OF THE IMMUNE RESPONSE:

The immune response can be viewed as occurring in three stages (Figure 1). These stages are: 1) Localization of antigen to specialized "antigen presenting cells" (APC); 2) Presentation of the antigen, in association with major his compatibility complex (MHC) coded components, to T lymphocytes; and 3) Lymphocyte activation. Each of these steps is influenced by many variables which can have a profound effect on the nature and magnitude of the evoked immune response.

Localization is the process by which antigen makes contact with appropriate APC, such as monocyte/macrophages (Unanue and Allision, 1969), and various dendritic cells in the skin, and in the interfollicular and follicular regions of lymphoid tissues (Radzun, 1981). This step is generally achieved by the transfer of antigen, via the bloodstream or lymphatics, to the lymphoid tissues. Alternatively, APC may migrate to the site of antigen administration. Localization may be facilitated by accessory molecules which bind receptors expressed on certain types of APC. For example, antibody and activated C3 component of complement bind to Fc and C3b receptors, respectively. Fibronectin (Norris, et al., 1982) and platelets may also function in the transfer of antigen to APC. The physical state and route of administration of antigen are clearly important in antigen distribution and localization. Ingestion and processing of certain antigens may also be required for the appearance at the cell surface of the potential immunogen (Scala and Oppenheim, 1983; Jakway,

1983).

Presentation of cell surface bound antigen is required for the effective interaction between APC and helper T cells. T cells rarely, if ever, respond to free antigen. Rather, the antigen is required to associate with molecules coded for by the MHC. For T helper cells, the antigen-MHC association is with class II MHC molecules or Ia antigens (Paul, et al., 1977; Sredni and Schwartz, 1981). For cytotoxic/suppressor T cells, the association may be with class I molecules (HLA A,B and C coded) (Koszinowski and Ertl, 1975; Zinkernagel, 1976). The efficacy of antigen presentation is dependent on the quantitative level of Ia antigen expression on the APC (Yen, et al., 1981; Walker, et al., 1982; Beller, et al., 1984) and possibly also by interactive forces between the antigen and Ia molecules (Benacerraf and Rock, 1984). Genetic polymorphism exists among Ia molecules and certain examples of genetically determined immunological unresponsiveness can be attributed to a lack of specific antigen-Ia interaction (Benacerraf and Rock, 1984).

Lymphocyte activation can be conveniently divided into early and late phases. The initial activation of T helper cells for either antibody or cell mediated responses follows contact of T cells with antigen-bound APC. The T cells trigger the synthesis of interleukin 1 (IL 1) by the APC, possibly via the lymphokines colony stimulating factor (CSF) (Moore, et al., 1980) and macrophage activating factor (MAF). IL 1, along with specific antigen, will induce the T helper cells to release interleukin 2 (IL 2) (Larsson, et al., 1980; Smith, et al., 1980a; Smith, et al., 1980b). IL 2 acts as a second signal along with specific antigen for the activation of various effector T cells (Smith, 1980). These cells include cytotoxic cells which kill

by direct contact with a target cell; cells which kill by releasing various lymphotoxins and/or gamma interferon; cells which activate macrophages to become cytocidal; and cells which evoke granulomatous inflammation (Paul, 1984). Another class of T helper cells appear to interact directly with the Ia molecule on antigen bound B cells and to release B cell growth and differentiation factors which stimulate B cell proliferation and immunoglobulin synthesis. Both IL 1 and possibly IL 2 also act as immunoregulatory signals for B cell activation (Whisler, et al., 1982; Howard, et al., 1983; Giri, et al., 1984; Corley, et al., 1985). Gamma interferon is another important immunoregulatory molecule. Among its many actions, it can enhance Ia antigen expression (Steeg, et al., 1982a; Steeg, et al., 1982b; Wong, 1983), and stimulate IL 1 synthesis (Arensano-Nesdedos, et al., 1985) by APC. On the other hand, prostaglandins, released by activated macrophages, appear to dampen the immune response (Allison, 1978), possibly by down-regulating Ia antigen expression (Snyder, et al., 1982). Other suppressor mechanisms include antigen specific (Benjamin, 1975; Adorini et al., 1979) and antigen non-specific (Jandinski, et al., 1976) suppressor T cells, and possibly interferon induced non-T natural killer (NK) like lymphoid cells (Abruzzo and Rowley, 1983). Complex recipricol interactions also exist between the humoral and cell mediated pathways and within various components of each of these pathways. The schematic outline shown in Figure 1 is, therefore, oversimplified, but it does provide a useful framework on which to consider the potential mode of action of various adjuvants.

POTENTIAL MODE OF ACTION OF ADJUVANTS:

The immune response may be augmented by selective manipulation

of one or more of the three major stages outlined above. Thus, an adjuvant could facilitate antigen localization, promote antigen presentation or enhance lymphocyte activation. In this section we will review the properties of known adjuvants to help identify their site of action.

Antigen Localization

Injected antigens may be cleared so rapidly from the body that the period of effective contact with the immune system is inadequate. Therefore, considerable immprovement in immunogeneicity may be achieved simply by providing the antigen in a form from which slow and sustained antigen release will occur (Shroder and Stahl, 1984). Prominent examples of this approach are alum adsorbed antigen (Aprile and Wardlaw, 1966) and water-in-oil emulsions such as incomplete Freund's adjuvant (Freund, et al., 1937). Saponin is a mixture of triterpene glycosides extracted from the bark of the Quillaia tree. It is used in various veterinary vaccines and appears to work, in part, by promoting the aggregation of protein and polysaccharide antigens and facilitating their interaction with APC (Bomford, 1982).

More specific approaches to directed antigen localization have been investigated. Malarial protein antigens have been administered complexed with specific IgM antibodies to promote localization to macrophages (Harte, et al., 1983). The IgM antibody is likely to fix large amounts of the C3b component of complement and selectively bind APC via the C3b receptors on these cells. On the other hand, immunogenicity of malarial antigens may be impaired in the presence of specific IgG antibody (Harte and Playfair, 1983). Block copolymers, consisting of hydrophobic and hydrophilic arrays of surface acting molecules, have also been shown to fix large amounts of complement.

Antigen bound to these polymers has been shown to localize preferentially to the draining lymphoid tissue (Hunter, et al., 1984) The inherent affinity of APC for a wide variety of bacterial components may explain, in part, the adjuvanticity of antigen complexed to either tetanus, diphtheria or cholera toxoids. Cholera toxin consists of two polypeptide chains, one chain binds to cell surface receptors, while the second chain confers toxicity. Isolated cell surface binding chain of cholera toxin has been coupled to antigen and shown to enhance the immune response to the antigen. Concanavalin A has been similarly used, bound to tumor cells, to enhance tumor cell immunogenicity (Martin, et al., 1977).

Liposomes have been employed to focus encapusulated materials to macrophages (Fidler, et al., 1981). The composition of liposomes can have a significant effect on their ability to specifically localize to the macrophages in various organs (Raz, et al., 1981). The stability of liposomes can also be increased by exposure to osmium tetroxide and such osmicated liposomes have found use in experimental vaccines (New, 1984).

The various potential approaches to providing more effective antigen localization to the immune system are summarized in Table 1.

Antigen Presentation

As inidicated previously, a rate limiting step in antigen presentation is the density of surface Ia antigens on APC. An increasing number of substances have been shown to be capable of enhancing the expression of Ia antigens. These include gamma interferon, interferon inducers, and even simple compounds such as beryllium (Behbehani, et al., 1985). While adjuvants have not been specifically designed to selectively stimulate Ia antigen expression,

several of the more powerful adjuvants (e.g. FCA) certainly achieve this effect (Behbehani, et al., 1985). Interestingly, this function is not retained by muramyl dipeptide (MDP), a synthetic analogue of the major component of the cell wall of the mycobacteria used in FCA (Behbehani, et al., 1985).

A more significant problem exists if effective interaction between the vaccine antigen and Ia molecules fails to occur. This situation might, in part, explain the adjuvanticity of strongly immunogenic molecules coupled to a vaccine antigen. The immunogenic molecule may provide a substitute carrier capable of effective Ia interaction. Chemical coupling and even simple mixing have been employed to link vaccine peptides to tetanus toxoid, methylated bovine serum albumin, keyhole limpet hemocyanin and either influenza or vesicular stomatitis virus. Using recombinant technology, certain carrier determinants can also be synthesized as direct extensions to the specific vaccine antigen. One basic problem with this approach is that T cell memory would be specific for the substituted carrier rather than for the actual pathogen. Immunization might, therefore, not give long term memory or recall on re-exposure to the infection. In addition, the use of a carrier to which an individual is already immune can occasionally induce so called "epitope specific suppression" resulting in an inhibition of immunity to the attached vaccine antigen (Herzenberg, et al., 1980; Herzenberg and Tokuhisa, 1982). There is also concern that the use of a substitute carrier might adversely affect the immune response to the carrier antigen by either suppressing subsequent immunity or inducing an adverse hypersensitivity response.

An interesting approach to promoting physical interaction

between vaccine antigen and both normal cell surface antigens and immunogenic foreign viral antigen is the use of vaccinia viral vectors (Moss, et al., 1984). This approach may have an additional advantage if infection results in interferon secretion, but it also carries the risk of untoward infection in immune suppressed hosts.

A summary of some of the potential approaches to enhanced antigen expression by APC is provided in Table 2.

Lymphocyte Activation

The critical role of IL 1 in the early phase of lymphocyte activation has been discussed previously. Exogenously administered IL 1 has been demonstrated to potentiate priming to bovine serum albumin in mice (Staruch and Wood, 1983). In these studies, the timing of IL 1 administration was found to be important, with the maximum effect occurring when IL 1 was given two hours after antigen. The more usual approach to enhancing IL 1 synthesis is the use of agents which cause the release of IL 1. Prominant is the use of various analogues of MDP, peptidoglycan and Klebsiella lipopolysaccharide. Correlations can be shown between the IL 1 inducing activity and the adjuvanticity of these agents (Oppenheim, et al., 1980; Vacheron, et al., 1983; Kido, et al., 1984).

Late lymphocyte activation involves a complex network of cellular and soluble regulatory mediators. Some of the possible potentiators of this phase of the immune response are listed in Table 3. Enhancement of the late activation step could be achieved by the acceleration of antigen-specific lymphocyte proliferation. Mediators of T and B lymphocyte growth, such as IL 2 and B cell growth factor (BCGF), could be exogenously supplied, or their *in vivo* production could be induced. Pertussigen, derived from *B. pertussis,* induces

lymphocytosis and has been found to enhance cell mediated immunity as measured by delayed type hypersensitivity (Sewell, et al., 1983), whereas recruitment of additional B cells can be achieved by ribonucleosides such as 8-mercaptoguanosine (Goodman and Weigle, 1983; 1984). A wide variety of immunopharmacological drugs such as isoprinosine (Moya, et al., 1984) and cimetidine, can be shown to have immunomodulatory activity, possibly by affecting the metabolic pathways on which lymphokines exert their effects. Monoclonal antibody approaches to achieve proliferation of specific lymphocyte subpopulations have also been suggested. These include the use of antibodies specific for the IgD molecule on early B cells and for the Leu 3/T4 antigen on T cells. The actual requirement for antigen can also be replaced by using anti-idiotype antibodies reactive with cells expressing receptors for the vaccine antigen. These antibodies can induce proliferation of specific B and T cells (Marx, 1985).

An area of considerable uncertainty is how to preferentially elicit cell mediated immunity (CMI). The immunogenic requirements for CMI appears to be more stringent than for antibody production. The drug cyclosporin selectively inhibits CMI, and the development of drugs with opposing effects is to be expected. Viable organisms are generally more potent in eliciting cytotoxic T cells (CTL) than non-viable antigens, possibly because infection allows more effective association of the antigen with class I MHC molecules. CTL can be elicited, however, using non-viable vaccines as was shown with influenza (Ennis, et al., 1977).

All phases of the immune response are controlled by suppressor mechanisms. Inhibition of suppression may be a potentially useful technique for immunopotentiation. Thus, certain cytotoxic drugs, such

as Ara A, cyclophosphamide and colchicine, have been shown to enhance both humoral and cellular immunity by inhibiting suppressor mechanisms (Whek and Coons, 1977; Shand and Liew, 1980; Hirichs, et al., 1983). Dextran may augment the cellular immune response by specifically inhibiting the release of a macrophage derived suppressor factor (Battisto, et al., 1985).

It is clear from the forgoing that there is great potential to achieve immune enhancement to vaccine antigens. It is also apparent, however, that many areas of adjuvant activity have yet to be fully defined. The next section will identify the major issues of concern in the development of clinical adjuvants.

PRACTICAL CONSIDERATIONS:

An ideal adjuvant for widespread clinical use with malarial or other candidate vaccines has yet to be devised. Certain basic criteria can be defined for such a compound. For example, it should augment the development of protective immune responses, whether humoral or cell mediated or both. The immunity should be expressed at the site of potential invasion of the pathogen. The adjuvant should not disrupt normal immunoregulatory pathways for either normal self antigens or unrelated foreign antigens. It should be non-irritating to tissues, easy to administer and inexpensive. It should be structurally well characterized and evoke uniform responses in different individuals over a reasonable dose range.

Several important questions underlie the development and selection of an appropriate adjuvant for malarial vaccines. First, what type of response is most desirable? In the case of sporozoite immunity, antibody may provide sufficient protection, even in the

absence of complement fixation or opsonization. On the other hand, merozoite immunity is more likely to involve CMI mechanisms which, as yet, are poorly defined. Adjuvants are often judged on the basis of their ability to enhance the primary immune response. A more important action, however, is the achievement of more effective priming leading to an increased secondary response and to greater persistance of the memory response to rechallenge.

Given these goals, the next question is: What is the rate limiting step in the immune response to the malarial vaccine? Depending on whether this is localization, presentation or activation will determine the most appropriate type of adjuvant. In vitro assays will be needed to isolate and analyze each of these steps and to define the need for intervention with a given type of adjuvant.

Our laboratory is currently investigating the potential use of a human B cell derived IL 1 (George, et al., 1984) as a means to enhance the proliferative response of cultured human T lymphocytes to various mitogens and candidate vaccine antigens. The results from an assay demonstrating the immunopotentiating effect of IL 1 on the phytohemagglutinin (PHA) response of cultured human T cells are displayed in Figure 2. Antigen can be substituted for PHA and the priming effect can be measured by rechallenging the cultures with homologous or unrelated antigen 1-2 weeks later.

In general, we feel the use of IL 1 or its agonists may offer certain advantages over the use of IL 1 stimulating compounds, such as MDP. Specifically, these compounds may have actions apart from IL 1 induction and, in particular, may induce secondary immune suppression (Hang, et al., 1985). The response to these compounds in terms of both adjuvanticity and IL 1 release, varies significantly between mice

of different strains (Staruch and Wood, 1982; Abeshiri-Amar, et al., 1985). Recent findings indicate that genetic factors also play a significant role in the in vitro response of human cells to MDP (Bahr, et al., 1984). The responsiveness of macrophages to IL 1 inducers may also be greatly influenced by any pre-existing state of activation, as might be expected in individuals harboring chronic infections.

The exogenous administration of IL 1 is also, however, subject to several major concerns. The in vivo half life of IL 1 is short, it needs to be present at the same time as antigen and it may induce a range of non-immunological potentially toxic effects. Thus, IL 1 has a range of activities unrelated to adjuvanticity (Table 4). For these reasons, the development of approaches aimed at restricting IL 1 activity to the immune system is highly desirable. This might be possible by the use of structurally modified IL 1 or naturally occurring IL 1 sub-species which display only immunological activities. Of interest, in this regard, is the report that an MDP analogue elicited the production, in rabbits, of a non-pyrogenic IL 1 (Damais, et al., 1982). Interestingly, the IL 1 produced by a human B lymphoid cell line, established in our laboratory (George, et al., 1984), is also apparently non-pyrogenic. Compounds may also be produced which will act as IL 1 agonists. Towards this goal, we are undertaking studies to define IL 1 the receptors on human T cells. We plan to test for chemical compounds which are able to induce lymphocyte activation by specific binding to the IL 1 receptor.

A potential concern with the overall activation of the immune system is that response to self antigens may occur, leading to autoimmune disease (Hilgers, et al., 1984). The question of concomitant delivery of antigen and adjuvant to the specific site of

immune activation is therefore of considerable importance as a possible means to restrict the extent of immune activation. Among the several suggestions offerred are: a) the use of liposomes with entrapped antigen and lymphokines, b) the use of antigen-adjuvant complexes such as MDP coupled to antigen; and c) the use of adjuvant-acting polymers to which antigen is attached (Hunter, et al., 1984). We are pursuing the prospect of using antigen-bound platelets, pre-incubated with lymphokines, as a novel method of selective antigen delivery and lymphoid cell activation. The lymphokines will include IL 1 and/or gamma interferon. The initial test system will involve an assessment of the responsiveness of cultured T cells from either normal humans, vaccine injected human volunteers or vaccine injected primates. We plan to phenotype the responding cells, using a panel of monoclonal antibodies, to determine whether they are likely to have helper or suppressor activity. We then plan to test these cells in more specialized assays of CMI and humoral immunity. Similar in vitro assays should also be applicable to testing other proposed antigen-adjuvant delivery systems and in defining the rate-limiting step in immune activation to malarial vaccine antigens.

SUMMARY:

This paper has provided an overview of the pathways of immune activation and the potential mode of action of adjuvants for use with malarial vaccines. The rapid progress in the identification of various malarial proteins has outpaced the development of new forms of adjuvants. From a practical point of view, it is likely that initial clinical studies will simply involve the use of alum adsorbed antigen. However, if the levels of immunity evoked using this approach are

insufficient in intensity or duration, or if the evoked response is inappropriate for protective immunity, further trials involving new forms of adjuvants will be required. The development of a safe and effective new adjuvant for widespread clinical use may be yet another benefit to be derived from the Malarial Vaccine Development Program.

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TABLE 1

CONDITIONS WHICH EFFECT ANTIGEN LOCALIZATION

1. DOSE & ROUTE OF ADMINISTRATION
2. DEPOT ANTIGEN

   Alum Adsorbed, Saponin, Water in Oil Emulsion
3. ANTIGEN DELIVERY

   a) Natural Opsonins

      IgM Antibody, Fibronectin, Platelets

   b) Bacterial Opsonins

      Tetanus, Cholera, etc.

   c) Block Copolymers

   d) Liposomes

TABLE 2

ENHANCEMENT OF ANTIGEN PRESENTATION
MHC:AG ASSOCIATION

1. INCREASED EXPRESSION OF CLASS II MOLECULES
   a) interferon
   b) interferon inducing substances
   c) macrophage activating factors
   d) beryllium
2. SUBSTITUTE CARRIERS
   a) bacterial toxoids
   b) keyhole limpet hemocyanin
   c) influenza and vesicular stomatitis virus
   d) vaccinia viral vector

TABLE 3

AUGMENTATION OF LYMPHOCYTE ACTIVATION

1. EARLY ACTING

   a) Interleukin 1

   b) IL 1 inducers
      (e.g. MDP)

   c) IL 1 agonists

2. LATE ACTING

   a) Interleukin 2 and/or B cell growth factors

   b) IL 2 and BCGF inducers

   c) Immunopharmacological drugs
      (e.g. isoprinosine)

   d) Pertussigen

   e) Ribonucleosides
      (e.g. 8-mercaptoguanosine)

   f) Monoclonal antibodies
      (e.g. anti IgD, anti L3/T4, anti idiotype)

Table 4

POTENTIAL ADVERSE ACTIONS OF IL 1

1. Fever
2. Fibroblast and Smooth Muscle Proliferation
3. Procoagulant Synthesis
4. Collagenase Production
5. Neutrophilia and PMN chemotaxis
6. Alterations in plasma Fe^{++}, Zn^{++}, Cu^{++} levels
7. Synthesis of acute phase reactants

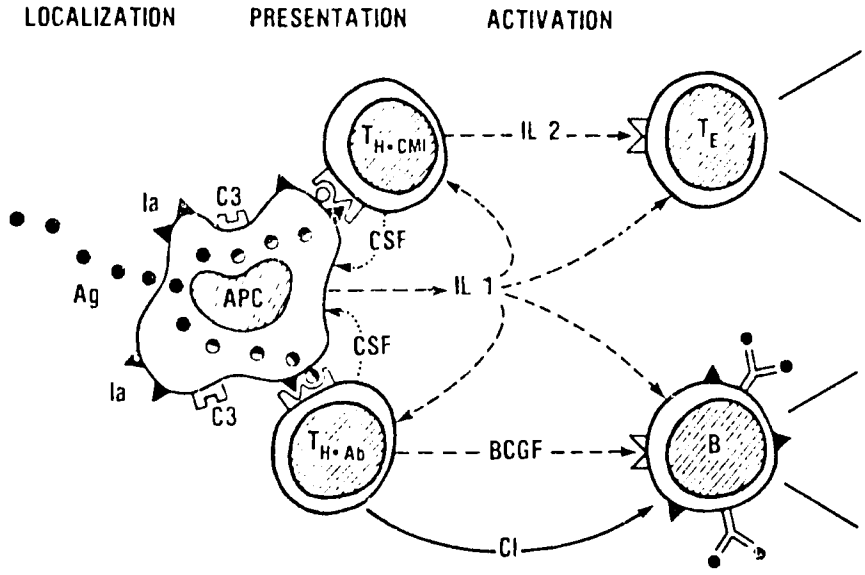

Figure 1: SCHEMATIC REPRESENTATION OF IMMUNE RESPONSE.

The abbreviations in the diagram represent the following: Ag = antigen; C3 = C3 receptor; Ia = class II MHC antigen; APC = antigen presenting cell; T_H-CMI = helper T cell for cell mediated immunity; T_H-Ab = helper T cell for humoral immunity; T_E = effector T cell; B = B cell; IL 1 = interleukin 1; IL 2 = interleukin 2; CSF = coloney stimulating factor; CI = cell interaction.

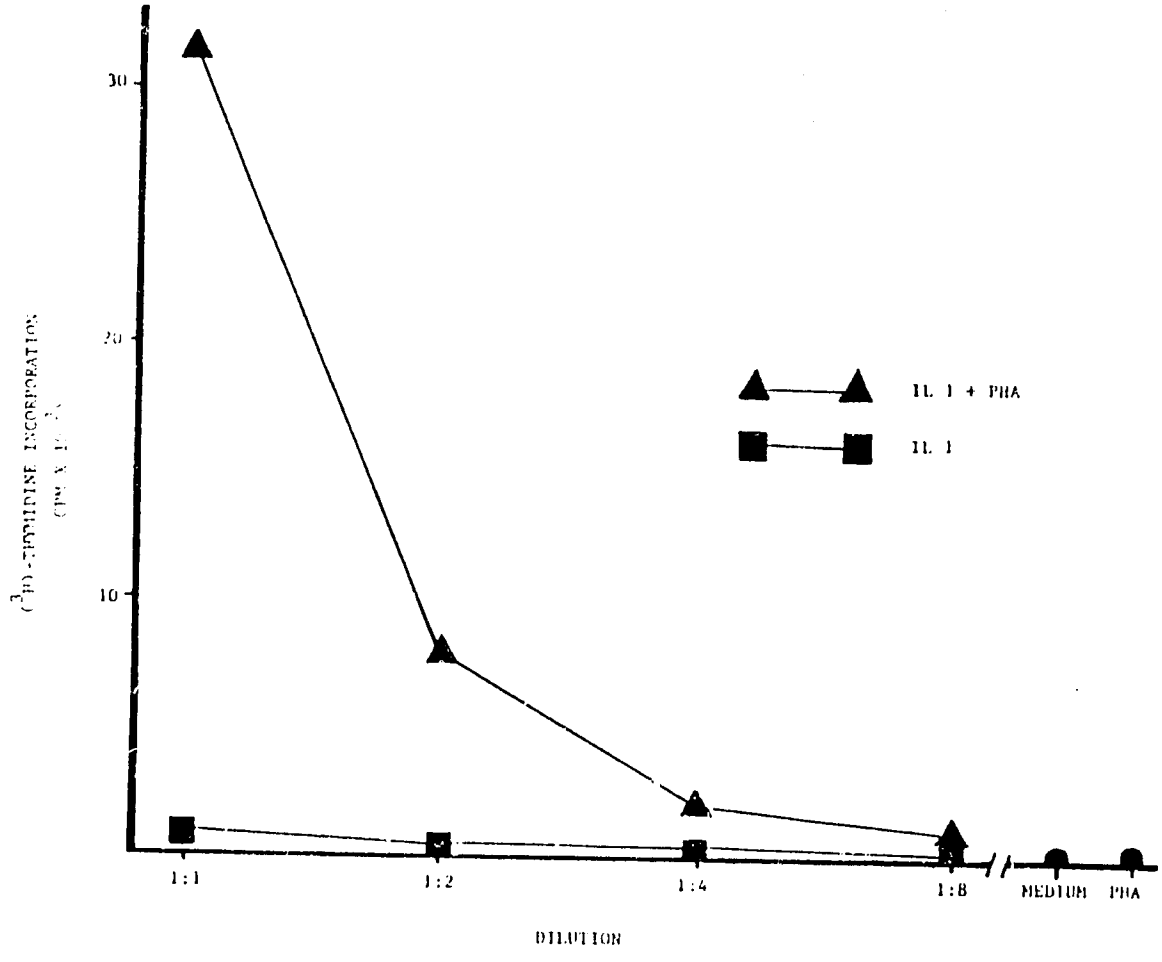

Figure 2: IL 1 ASSAY UTILIZING HUMAN CULTURED T CELLS.

Ficoll-Hypaque purified peripheral blood mononuclear leukocytes were activated with phytohemagglutinin (PHA) and cultured thereafter in lectin-free interleukin 2. After 10-14 days of proliferation, the cultured cells, which were ¶99% T cells by surface phenotype, were aliquoted into 96 well microtiter plates at 10^5 cells/well in 0.1 ml. Interleukin 1, at various dilutions, was added to the wells at 0.1 ml/well, plus 20 microliters of either plain medium or PHA (final concentration of 0.25 ug/ml). Cultures were incubated 72 hours, with 1.0 uCi ^{3}H-thymidine added, in 20 ul medium, for the last 6 hours.

WEDNESDAY, April 24, 1985
Abstract No.38, page 43
L. Chedid

PRACTICAL CONSIDERATIONS OF MALARIA VACCINES
B. Applicability Of Information On Modern Vaccine For The Production Of Malaria Vaccines
Paper No. 38

* Paper Will Be Included In Final Publication *

WEDNESDAY, April 24, 1985
Abstract No. 39, page 44

POLYVALENT MALARIA VACCINES

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Melbourne, Victoria 3050, Australia

For: Proceedings of the Asian and Pacific Conference on Malaria

Honolulu, Hawaii, U.S.A., April, 1985

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Abstract No. 39, page 44

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Polyvalence, as applied to future malaria vaccines, could refer to several things. A polyvalent vaccine may consist of a combination of stage-specific antigens, or portions of such antigens (epitopes), from the one Plasmodium species. Such a combination of antigens or epitopes from blood stages, sporozoites and gametes would be designed to effect anti-infection, anti-disease and transmission-blocking immunity. Alternatively, the polyvalent vaccine could consist of a defined mixture of antigens or epitopes from the one life cycle stage of the one Plasmodium species. Finally, it could be a combination of antigens or epitopes from different species of Plasmodium.

At the moment, most malariologists contemplating a hypothetical polyvalent malaria vaccine would probably have in mind a falciparum vaccine consisting of invariant portions of a CS protein, a major merozoite surface antigen (e.g. Pf190), RESA, cytoadherence protein, a rhoptry protein, Pf96, and 1 or 2 gamete antigens. They would also probably express some concern about antigenic competition, a possibility that will remain until data replaces conjecture.

In this presentation, and with scant attention to the all-important specifics of human-plasmodium relationships, I want to raise several issues relevant to engineered, molecularly-defined malaria vaccines. I hope that, because of the generalities, it is not too much a statement of the obvious. Particular attention will be directed to only one effector arm of host-protective immunity, namely antibody, and only one type of parasite molecule, namely protein.

No molecularly-defined vaccine based on protein antigens exists against any pathogen of man and even the bacterial toxoid vaccines now in use are in no way defined at the molecular level. Progress towards second generation "acellular" bacterial vaccines is well advanced as is progress towards an

entirely different entity - genetically engineered bacteria - that derives impetus from the proven efficacy of attenuated live bacteria that seem to best mimic solid infection-induced immunity (1). Meningococcal and (highly polyvalent) pneumococcal polysaccharide vaccines are chemically defined and their undoubted efficacy, at least in certain target populations, provides encouragement to pursue the same objective in parasitic diseases. Of course, all of the latter must be far more complex immunologically than bacterial pneumonias and meningitis.

Advantages of molecularly-defined vaccines have been outlined previously (2) but for this discussion, the one to emphasise is that the availability of pure substances will greatly facilitate the development of polyvalent vaccines. Incorporation of much higher doses of any one ingredient in the multicomponent vaccine can be envisaged with the likelihood of inducing high levels of immunity of longer duration. Attaining comparable levels of the one ingredient as a crude extract vaccine will be impossible or prone to dangerous or unpredictable consequences.

In the molecular vaccine era, the immunologist is confronted with the same problem that bedevils chemotherapy, chemoprophylaxis and the drug industry, namely, the selection of resistant organisms. The molecularly-defined vaccine will induce a restricted immune response that will enable organisms not expressing those antigens or epitopes to evade the consequences of vaccination. Because of population heterogeneity (i.e. molecular polymorphisms; antigenic variability), the more a molecularly-defined vaccine is restricted in its composition, the greater is the probability of emergence of parasites that are defective in expression of that antigen or epitope subset. Of course, the expectation that has dominated molecular immunoparasitology to date is that a <u>small subset</u> of epitopes will be found that

is highly immunogenic in hosts of all genotypes, that is available to immune effector mechanisms or indispensible to parasite physiology such that function is readily inhibited by antibodies, and that is not highly polymorphic within the parasite species (2).

Population heterogeneity must be extensive in any parasite species although some restraints, relative to the population heterogeneity of vertebrate hosts, must be imposed by genome size. High resolution methods such as 2D gel analyses of immunoprecipitates (3,4) and other serological techniques, nucleic acid probes (5,6), chromosome analyses (7), batteries of monoclonal antibodies (8), monospecific antisera raised to cloned antigens (9), and isoenzyme analyses (10) are only now beginning to reveal the extent, or allow quantitation, of this heterogeneity in Plasmodium falciparum. Whilst it is now easy to show heterogeneity in P. falciparum, restraints imposed by such heterogeneity on the vaccine objective are less readily assessed. One reason for this is the lack of a good mouse model of human falciparum malaria.

For some time now, attention in immunoparasitology has been moving away from the identification of "the host-protective antigen" in a parasite to identification of potential "host-protective epitopes" of several antigens in a parasite. Through synthesis of an entirely new antigen construct consisting of portions of various antigens, such a vaccine could in fact be molecularly defined to a greater extent than any native molecule of the parasite. It is in this endeavour that monoclonal antibodies and cloned portions of antigens are such powerful approaches and where immunoprecipitation and immunoblotting techniques with parasite extracts and polyclonal antisera can be less informative. A constructed vaccine antigen could consist of chemically or biologically-synthesised peptides and polypeptides conjugated together or to carriers such as tetanus or diphtheria toxoid.

Alternatively, the vaccine antigen (as a nucleic acid construct) could be delivered as a recombinant virus or recombinant attenuated gut microorganism. Vaccinia viruses in particular (11,12) provide the potential to deliver, as a soluble or membrane associated protein, relatively large numbers of epitopes as unimolecular or oligomolecular "unnatural" antigens. Time will tell whether such living antigen-delivery systems will herald in a new era of vaccines (see Moss, B., this volume).

This newer emphasis in the quest for molecular vaccines stems from the theoretical advantages of polyvalent versus monovalent vaccines and the increasing evidence for "suppressogenic" regions of antigens (13,14). Though in its infancy, the identification of epitopes that preferentially induce suppressor T cell (Ts) responses should lead to means of excluding them from an antigen construct. [Of course, it could be argued that induced antibody responses to such epitopes may inhibit the capacity to stimulate Ts cells. Fundamental studies in immunoregulation are required before further speculation on this is warranted. Responses induced by portions of a (soluble) native antigen during infection may be very different from those induced by these same epitopes conjugated to an immunogenic carrier molecule or presented on a cell membrane bearing class II MHC molecules]. In addition, the unnatural construct could be devoid of regions of antigens that are immunodominant, and that through whatever mechanisms are operative in intramolecular competition, inhibit high titered responses to relatively immunosilent host-protective epitopes. The constructed antigen would also be lacking epitopes that resemble human antigens with the potential for induction of autoimmunity.

The most remarkable finding from the sequencing of malaria genes coding for major antigens in blood stages of P.falciparum (and the use of synthetic

peptides and radioimmunoassays with sera from malaria endemic areas to confirm the reading frame), is the number that show tandem repeated short sequences of amino acids (see R.L. Coppel et al, this volume). Taking all malaria antigen sequences known to date, the repeat epitope antigens are of two types. Type 1 antigens contain a single block of repeats per molecule; Type 2 antigens contain at least two distinct blocks of repeats per molecule. As native molecules, and as judged by analysis of antibody levels or frequencies in sera from patients in malaria endemic areas, type 2 repeat antigens (e.g. FIRA and RESA) are usually more immunogenic than type 1 repeat antigens (e.g. S-antigen, CS-protein).

Are type 1 antigens potent stimulators of Ts cells in natural infection or are mechanisms such as effector cell blockade and B cell blockage with antigen fragments involved? Does the repeat structure reflect a strategy "employed" by the parasite to reduce the likelihood of inhibition of binding of the polyvalent molecule to a polyvalent receptor on or in the target cell be that erythrocyte or hepatocyte? Are the type 2 antigen repeats strong stimulators of irrelevant antibody production that through a swamping or smoke screen effect (i.e. antigenic competition) inhibit induction of other antibody specificities far more relevant to host protection?

Hypotheses on such matters have not yet been tested experimentally. Hopefully, studies in progress on in vitro responses to various antigens, repeat and non-repeat epitopes, and various combinations using limit dilution approaches will be instructive.

To supplement the multiple points of immune attack that are sought by way of a polyvalent vaccine, shared epitopes between life cycle stages are of importance. In falciparum malaria, the best example of a shared epitope is the target of the monoclonal antibody described by Hope et al. (15).

This monoclonal binds to sporozoites and a Mr 23,000 protein of blood stages. The gene for this protein has been cloned and sequenced in 2 laboratories (16,17) and the tetrapeptide Asn-Ala-Asn-Pro, the major repeat unit in the CS protein, and other closely related peptides have been identified in the Mr 23,000 protein. The immunological importance of this finding remains unknown as yet.

If a large component of antibody-mediated protection against blood stages of P.falciparum is variant specific then a strong case can be made for vaccination with any invariant portions of variant antigens in a manner that sensitizes helper T cells (i.e. T_H cells). In a host sensitized for accelerated antibody production because of the presence of a primed T_H cell population, variant specific antibody may appear earlier than normal in the course of a new parasitemic wave and abort this infection before the onset of severe disease. This approach to malaria vaccination was proposed many years ago by K.N. Brown (18) on the basis of work on P.knowlesi SICA variants in rhesus monkeys.

Some doubts about the supposition on which the approach is based have appeared in the immunology literature (e.g. 19). Thus mice preimmunized with carrier prior to hapten-carrier injection show reduced high affinity IgG (but not IgM) anti-hapten responses. This contrasts dramatically with the situation following transfer of cells to irradiated recipients (20) or if animals are preprimed to the hapten (on another carrier). Interestingly, anti-carrier antibody responses are not inhibited and analogies with the phenomenon of "original antigenic sin" in response to influenza viruses are discussed by Herzenberg et al. (19). Whether the immunology of new variants appearing in malaria-infected hosts will resemble the carrier pre-immunization model described above is unknown. Reduced parasitemias and

accelerated responses to late variants combined with evidence for variant-specific immunity in the rhesus/P.knowlesi model, would seem to be readily explained by T_H cell priming. In any case, present methods to identify T_H cell-stimulating epitopes of antigens are cumbersome and even when identified and produced, it is not clear how to go about injection in order to ensure T_H cell sensitization. It is likely that studies with malaria antigens will tell us more about immunoregulation under physiological conditions, and the immunobiological consequences of vaccination, than the multitude of haptenated antigens currently in use in cellular immunology.

Once an invariant epitope or subset of epitopes of an immunogenic variant molecule are identified, questions arise as to the reasons for that invariance. Is it the sought-after portion of the antigen in which polymorphism cannot be tolerated in order to preserve function? Or is this portion of the molecule simply not available to antibodies in the living parasite or intact parasitized cell? Is it recognized well by a mouse monoclonal antibody (i.e. a hetero-monoclonal) yet never recognized by human antibodies because it is an epitope that resembles a self epitope (21) to which effective tolerance is preserved.

The notion of immune responses in the vaccinee being necessary and/or sufficient is relevant in the context of polyvalent malaria vaccines. Very encouraging for the vaccine developer is any evidence that sensitization for an accelerated response of a particular type on subsequent encounter with the parasite is sufficient to effect solid resistance (22). Difficulties can be anticipated in inducing and maintaining high-titered responses with molecularly-defined vaccines in all individuals of the genetically-diverse host population. It would be highly desirable if constant exposure to the parasite boosts and maintains protective responses in hosts whose immune system has been "locked into" that particular response by vaccination. One

aspect, namely sensitization (clonal expansion?) of T_H cells with invariant portions of variant antigens, has been discussed above. This line of reasoning also lies at the root of the anti-idiotype approach (or, more particularly, the anti cross-reactive idiotype, IdX, approach) to vaccines (23,24). It is inconceivable that high titered responses of sufficient polyclonality and functional activity against natural pathogens in a field situation would be induced by the anti-Id vaccine; such responses may be measurable, however, after challenge of the vaccinee.

With malaria vaccines, the level of sensitization necessary to reduce morbidity and childhood mortality in human populations in endemic areas could be very different from that demanded by occasional visitors to the endemic area. In the former case, very detailed data will be required on populations prior to vaccine intervention in order to assess the overall efficacy of the vaccine. This sort of assessment is naturally very much more difficult and expensive than controlled challenge of human volunteers or Aotus and Saimiri monkeys. A very useful vaccine of value in an endemic area could be missed if profound effects on parasitemia in an acute challenge situation are considered as the only desirable consequence of a prophylactic malaria vaccine. More than ever, "control of disease" is the keypoint with "elimination of infection" being an unrealistic expectation, at least from the evidence available to date.

As a concluding comment, it is clear that artificial engineered vaccines of the polyvalent type are not just some armchair pipedream for another generation, another time. The ingredients and techniques are almost at hand, a statement that would have been fanciful just a few years ago. Agencies such as USAID, WHO-TDR, NIH and The Rockefeller Foundation can take much credit for the fact that this optimism regarding malaria vaccines abounds.

The creativity and skills of a new generation of biomedical scientists have added a new dimension to the endeavours of those individuals in tropical medicine who have pursued the vaccine objective for so long. Hopefully the polyvalence of the effort and approaches will culminate shortly in reagents of use for those most in need - the rural poor of the tropical less-industrially developed world. Population heterogeneity of *Plasmodium* spp. and their biological adaptability can be expected to be the most important stumbling blocks to rapid progress towards molecularly-defined vaccines. In all likelihood, such vaccines will require modification at regular intervals.

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Applicability of Information on Modern Vaccine For the Production of Malaria Vaccine

Rapporteurs: Ronald Kennedy and Leslie Tam

Three modern approaches applied to protective vaccine candidates based on experience with bacterial and viral vaccines were discussed. First, synthetic peptides, which mimic both the amino acid sequence and potential secondary structure of antigenic determinants, was discussed by Gordon Dreesman. He discussed the role of conformation in the induction of an antibody response and the necessity for carrier proteins and adjuvants for high titered antibody responses to the native molecule. For this approach, the complete amino acid sequence of the protective polypeptide must first be determined by the molecular biologists, and this was the case for the protective hepatitis B surface antigen epitope studied by Dr. Dreesman. The characteristics of a synthetic peptide containing a 14 amino acid loop were addressed, and the necessity of maintaining conformation to induce protective antibodies.

The analysis of other antigens and the location of T cell and B cell epitopes on these molecules were review. B cell epitopes contain beta turns, are largely hydrophilic, and can be sequential or conformational. On the other hand, characterized T cell epitopes have been less hydrophilic, have had alpha-helix, and have been linear. These characteristics may be useful in identification of candidate epitopes in malaria antigens once they have been cloned. Dr. Dreesman presented a modified Chow-Fasman prediction of the secondary structure of the CSP protein, which indicated that the repeat sequences, which have been shown to be a strong B cell region by the Nussenzweigs, may also have some T cell potential.

In the second approach, Dr. Kennedy revied the potential use of internal image anti-idiotype as an antibody vaccine without the direct use of antigen. This new and novel approach was defined and the evidence for potential applicability in trypanosomes glycoprotein, the hepatitis B surface antigen, rabies surface glycoprotein, reovirus hemagglutinin, sendai virus (T cell clone was used), phosphorylcholine of streptococcus, polysaccharide of E. coli, and poliovirus (B) systems were listed and the mechanisms of four outlined graphically.

Anti-idiotype approach has potential value when conformation dependent epitopes, such as the hepatitis B surface virus 14 aa loop peptide. Internal image anti-idiotype may also be used potentially in the priming of immune responses for a vaccine in infants and non-responding adults. Since it is a relatively new approach, the precise use and applicability have not been defined.

obtained with FCA, but these were still low relative to other antigens. In addition, the quality of the antibody response to native protective epitopes was also discussed. It was noted that high antibody titers to the native protein may not necessarily correlate with protective immunity. The exciting use of murabutide for producing a completely synthetic adjuvant with carrier funcrtion was shown by the completely chemical synthesis of a hormone linked in tandem with murabutide. This chemical sunthetic product was effective in the chemical castration of cattle. This suggested the possible use of murabutide in synthesizing a malaria synthetic peptide possessing both B cell epitopes, T cell epitopes and adjuvant activity.

The final presentation by Dr. Mitchell discussed the role of polyvalent vaccines for malaria. The ideal malaria vaccine might represent protective immunogenic proteins and or glycoproteins formulated by the methods described earlier. The role of T cell help and potential suppression in using a polyvalent vaccine was also discussed. The polyvalent vaccine for pneumococcal polysaccharide with regards to the findings of non antigenic competition. Potential antigenic competition would represent a problem for a putative single molecule vaccine containing several protective epitopes from different protective antigens.

The previous reports suggested the need for carrier protein that was licensed by the FCA, such as tetanus toxoid. However, the phenomenon of original antigenic sin suggested that prior immunization of the carriers might damp the induction of antibody to the desired epitope. This was also discussed with regards to the tandem repeats of CSP and a growing number of other malaria candidate antigens having the repeat characteristic. The potential problems associated with invariant hydrophilic regions along with variable tandem repeats with regards to the original antigenic sin were discussed.

In summary, CSP, pf190, RESA, cytoadherence antigen, rhoptry antigen, pf 96, and the glycophorin binding protein were listed as possible candidates protective antigens. For each of these molecules it is necessary to remove irrelevant and counterproductive (suppressorgenic) epitopes while retaining beneficial ones. The final analysis suggests that the search for putative epitopes associated with protective antigens on the malaria parasites should utilize the knowledge compiled by our colleagues working in bacterial and viral systems. In addition the success of the formulated vaccine will likely depend heavily on the development of strong but non-toxic adjuvants, which will be incorporated with the putative vaccine in the stimulation of long-lasting immunity.

In the third approach, Dr. Moss review exciting and elegant work where entire genes have been inserted into the vaccinia virus, used as a vector for recombinant vaccines. He discussed successes in the hemagglutinin and neuraminidase of influenze, glycoprotein D of the herpes simplex virus, the hepatitis B surface antigen and finally the exciting CSP gene of the malaria sporozoite, which has captured the attention of malariologists. The protein was expressed as a 56K doublet in vaccinia, and it probably is not processed as it is in the salivary gland of the mosquito, and is not expressed on the vaccinia surface membrane. Among many other advantages, the vaccinia vector is stable, does not require refridgeration and can be administered by the scratch alone. The stimulation of CMI, along with humoral immunity, has been documented and total efficacy of the vaccinia vaccine is known worldwide. The risk of complications looms as a disadvantage, but this may be short sighted in the face of increasing menace of malaria in the tropics. To date the vaccinia virus system appears to be most promising for a recombinant malaria vaccine.

In closing remarks to this session, Dr. Dreesman suggested that identified protective antigens or their epitopes might well be given first in the vaccinia virus, boosted with selected synthetic peptides, and tuned with selected tertiary anti-idiotypes.lria might be administered first in In the next group of sessions.

In the next group of sessions, the role and mechanisms in potentiating the immune response was discussed. The first presentation by Dr. Kotani discussed muramyl dipeptide and numerous synthetic analogs as potential adjuvants boosting both cellular and humoral immunity. In particular, B30-MDP is a strong candidate with minimal pyrogenicity and other adverse side effects associated with other derivatives. A newer study is the Compound 506 related to Lipid A. It has the full adjuvant acitivity associated with lipopolysaccharide, but is 5000 times less pyrogenic. It is now being investigated as a second potential synthetic adjuvant.

Dr. Martin discussed the potential of interlukin i in potentiating the immune response. An overview of the potential mechanism of adjuvants was also presented. It was also suggested that interlukin 2 may also be a potential new adjuvant.

Dr. Chedid discussed the role of a non-pyrogenic derivative referred to as murabutide, which has 1000x less pyrogenicity, does not induce either acute phase proteins, slow sleep wave activity, or leucopenia, among other detrimental effects. The role of this new adjuvant in potentiating the immune response to several synthetic antigens. Increased immune responses were demonstrated with antigens uncoupled or directly coupled to the synthetic adjuvant. Increased responses to the CSP protein were obtained. Dramatically, the responses were greater than those