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WORKING PAPERS, DISCUSSION AND SUMMARY

WORKSHOP SESSION II

CURRENT STATUS ON THE DEVELOPMENT OF MALARIA VACCINES

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Studies on the development of a P.falciparum malaria vaccine for the induction of transmission blocking immunity.

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1. Introduction.

The induction of transmission blocking immunity in avian malaria models as a potential tool in malaria control was reported by Gwadz (1976) and by Carter and Chen (1976) following earlier experiments by Huff et al. (1958). The efficiency of immunization with extra cellular gametes was also demonstrated in a P.yoelii rodent model (Mendis and Targett 1979) and in a P.knowlesi monkey model (Gwadz and Green 1978).

The introduction of methods for in vitro culture of P.falciparum (Trager and Jensen 1976) was followed by procedures for the production of gametocytes and gametes as well as the establishment of routine in vitro infection of mosquitoes (Ponnudurai et al. 1982a, 1982b, 1983).

The availability of gametocytes, gametes/zygotes and ookinetes were basic for biochemical and molecular biological studies of these developmental stages of P.falciparum. In the NIH research group Rener et al. (1980) were the first to demonstrate that monoclonal antibodies (MoAbs) against male and female P.gallinaceum gametes could interfere with the sporogony of the parasite in mosquitoes. In most experiments effective transmission blocking activity was achieved only in the presence of complement or by the synergistic action of two different MoAbs. In later studies in this avian model it was shown that the MoAbs prevented the fertilization of macrogametes by microgametes

(Kaushal et al. 1983). Similar results were obtained with P.falciparum. (Rener et al. 1983). A combination of two MoAbs against gametes of a Brasilian isolate 7G8 suppressed the infectivity of gametocytes in the absence of complement, whilst in the absence of complement (with one exception) individual MoAbs were not able to block transmission.

The MoAbs were used for the identification of target antigens of antigamete immunity. Three gamete proteins of P.gallinaceum of Mr 240, 56, and 54 k, were identified by specific MoAbs by the application of surface labelling techniques, SDS poly acrylamide gel electrophoresis of parasite extracts under reducing conditions and followed by auto- radiography. In the corresponding experiments with P.falciparum sexual stages a similar set of gametes surface proteins of Mr 260, 59, and 53 k, were precipitated by the available MoAbs. (Carter et al. 1984). As demonstrated in biosynthetic labelling experiments it was found that these gamete surface proteins were synthesized in gametocytes before gametogenesis. In the P.gallinaceum model it was shown that these surface proteins were shed into the surrounding medium (Carter and Kaushal 1984) and that a new set of proteins, Mr 26 and 28 k was synthesized and expressed on the surface of ookinetes. (Carter et al 1984).

Meanwhile similar studies on the sexual stages, zygotes and ookinetes of P.falciparum have been carried out independently at our institute. The results are reviewed in greater detail in order to indicate to what extent they confirm the above results and at what points new information can be added.

1. Surface labelling of macrogametes of a P.falciparum isolate NF 54 resulted in a number of a ^{125}I labelled bands after separation by SDS PAGE. The electrophoretic mobility of some proteins was different in the presence or absence of reducing agents like DTT. Usually four major polypeptides of 250, 58, 47 and 10 k could be detected under reducing conditions; under non

reducing conditions the above proteins demonstrated a Mr of 230, 48/45, 43 and 10 k. The 48/45 kD doublet did not separate under reducing conditions. Minor bands were seen at 33, 25 and 16 kD. The Mr under non-reducing conditions will be used for the rest of this review when reference is made to the various proteins.

Several MoAbs (32 F1, 32 F3, 29 F432, 32 F5) reacted with the 48, 45 kD doublet, some of these however co-participated also the 230 protein. Although a protein was labelled in the 25 kD region this could not be precipitated by any of these MoAbs.

2. Immunoblotting experiments using polyclonal as well as monoclonal antibodies showed a result somewhat different from the above. Polyclonal rabbit serum did not bind to a 230 kD protein whereas it precipitated this protein after surface labelling. On the other hand the serum did react prominently with a 25 kD protein which was not labelled in the surface iodination. Some of the MoAbs were obtained by selecting hybridoma lines that positivitely reacted to the blotted 25 kD protein. Similarly MoAbs reacting with the 10 kD protein were selected and these recognized also a 45 kD protein.

3. Seven transmission blocking and eight non blocking anti macro gamete antibodies were identified. The molecular weights of proteins of macrogametes/zygotes recognized by the MoAbs and polyclonal rabbit serum are given in table 1. Table 2 gives information with regard to the transmission blocking potency of the different blocking antibodies.

4. The strength of transmission blockade depends on two factors: the concentration of the specific antibodies and the quality of gametocytes that are involved. The intensity of the blocking effect appeared inversely related to the transmission potential of the gametocytes cultures. It should be

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

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

5. In biosynthetic labelling experiments (using freshly purified macrogametes incubated in methionine free RPMI 1640 medium supplemented with 35S methionine, for a period of three hours (at 25^0 C), it could be shown that predominantly 25 kD protein was produced. This protein could be solubilized almost completely (in 25 mM Tris 0,5% Triton X100 p H 8), and precipitated by blocking MoAbs 32F61, 32F81, 7F11 as well as by non blocking MoAbs 32F71 and 32F72. The 25 kD protein was not present in asexual parasites but it could be demonstrated in gametocytes and in much larger quantities on gamete/zygote surface. Using surface immune fluorescence with different MoAbs it was demonstrated that the surface of the zygote transforming into the ookinete in the mosquito midgut is gradually replaced by the 25 kD protein.

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

IRMA.

7. The SDS PAGE pattern of surface labelled proteins of P.falciparum NF54 macrogametes showed great similarity with that described by Rener et al. (1983). We confirm that some labelled proteins showed a difference in migration in SDS PAGE under reducing and non reducing conditions. Since all three proteins of 230-48-45 kD showed a higher Mr value in the reduced stage, they consist of no more than one polypeptide chain. The presence of intra-chain disulfide bonds probably influences the binding of SDS to the polypeptide backbone resulting in shift in the their relative molecular weights.

8. The three proteins of 230, 48 and 45 kD might possess a common antigenic site on account of their reactivity with a set of MoAbs (11C5-B10, 32F1, 29F432, 7F3; only MoAbs 28F1 was monospecific for the 230 kD protein). However the quantity of 230 kD precipitated relative to that of the lower Mr proteins, differed from one MoAb to another. This suggests that the 230 kD protein probably coprecipitated in a non-specific way. This is supported by the finding that in the immunoblotting experiments the 230 kD protein could not be detected. The 230 kD 48/45 proteins are no longer detectable on the surface of developing ookinetes. A precursor-product relationship of the 230 kD protein with any of the lower Mr antigens has not yet been demonstrated. The importance of the 230 kD protein in transmission blocking action is not clear. Only one MoAb (29F432), which recognizes the 230, 48 and 45 kD proteins blocked effectivitely , but it is not clear whether this was due to its reactivity with the 48, 45kD proteins as well.

9. The 48/45 protein doublet is present in the gametocytes and later expressed on the surface of the newly emerged macrogametes. Since transmission blocking MoAbs that are interacting with this protein doublet,

completely block formation of retort cells/ookinetes, it appears that these proteins play a significant role in the process of fertilization of the macrogametes. There was no indication in biosynthetic labelling experiments that one acted as a precursor for the other. This doublet, however, is no longer present on the intermediate stage (retort cell) between zygotes and ookinete. On account of similar findings in P.gallinaceum (Carter and Kaushal 1948) it seems likely that also the 49/45 kD proteins are shed from the furface during ookinete development.

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

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

The significance of these proteins remains unclear since none of these MoAbs block the formation of oocysts. The 10 kD protein is predominantly present with respect to the 45 kD one.

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

Reduction of this protein by the DTT resulted also in a shift in Mr to 58 k. The epitope present on both the 45 and 10 kD proteins, however, was not

sensitive to reduction.

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

Biosynthetic labelling experiments in in synchronized gametocytes cultures, containing gametocytes only (Ponnudurai et al. 1985 in preparation) indicate that the 25 kD protein is already being synthesized in small quantities in early stages of gametocytes development. MoAbs against this protein were selected on Western blots of macrogamete proteins. Some of these block transmission (7F11, 32F61, 32F81).

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

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

Isolates from Vietnam (NF 117), Ghana (NF 7), Central Africa (NF 115) and

Brasil (7G8) were probed with the anti-NF 54 MoAbs 32F3 and 32F81. All isolated demonstrated positive reactions with the MoAbs and no differences were found with regard to the Mr of the relevant proteins.

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

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

Positive reactions with 48/45, 25 and 10 kD protein were seen also with sera obtained from indigenous people from Burkina Fasso and Liberia. These preliminary results indicate that the development of transmission blocking antibodies might occur also in natural conditions. Further studies are needed to investigate under what circumstances this takes place and especially what the epidemiological significance is, of these antibodies.

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

as well as the 25 kD proteins.

Summary

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

References

1. Carter, R., Chen, DH. (1976) Nature 263:57-60.
2. Carter, R., Kaushal, DC. (1984) Mol.Biochem.Parasitol. 13:235-241.
3. Carter, R., Miller, LH., Rener, J., Kaushal, DC., Kumar, N., Graves, PM., Grotendorst, CA., Gwadz, RW., French, C., Wirth, D. (1984) Phil.Trans.Roy.Soc.Lond.B. 307:201-213.
4. Gwadz, RW. (1976) Science 193:1150-1151.
5. Gwadz, RW., Green, I. (1978) J.Exp.Med.148:1311-1323.

6. Huff, CG., Marchbank, DF., Shiroishi, T.(1958) Exp.Parasitol. 7:399-417.

7. Mendis, KN., Targett, GAT. (1979) Nature 277:389-391.

8. Ponnudurai, T., Meuwissen, JHETh., Leeuwenberg, ADEM., Verhave, JP., Lensen, AHW. (1982a) Trans.Roy.Soc.Trop.Med.Hyg.76:242-250.

9. Ponnudurai, T., Lensen, AHW., Leeuwenberg, ADEM., Meuwissen, JHETh. (1982b) Trans.Roy.Soc.Trop.Med.Hyg. 76:812-818.

10. Ponnudurai, T., Lensen, AHW., Meuwissen, JHETh. (1983) Parasitol. 87:439-445.

11. Rener, J., Carter, R., Rosenberg, Y., Miller, LH. (1980) Proc.Nat.Acad.Sci. USA 77:6797-6799.

12. Rener, J., Graves, PM., Carter, R., Williams, JL., Burkot, TR. (1983) J.Exp.Med. 158:976-981.

13. Trager, W., Jensen, JB. (1976) Science 193:673-675.

Table I

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

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

* rabbit polyclonal antibody.

Table II

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

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

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

* rabbit polyvalent antibody.

TUESDAY, April 23, 1985
Abstract No. 13, page 28
Ruth S. Nussenzweig

Characteristics of Sporozoite Antigens
and Evidence for Their Role in Protective
Immunity
Tuesday, April 23, 1985
Paper No. 13

* WORKING PAPER WILL BE INCLUDED IN FINAL PUBLICATION*

TUESDAY, April 23, 1985
Abstract No. 14
Thomas F. McCutchan

CANDIDATE ANTIGENS

Sporozoite antigens
Discussant

* PAPER WILL BE INCLUDED IN FINAL PUBLICATION*

SCHIZONT/MEROZOITE ANTIGENS OF P. FALCIPARUM

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The three major surface antigens of P. falciparum merozoites appear to be derived by proteolytic processing from a single precursor polypeptide synthesized in schizonts (Holder and Freeman, 1984). In the Wellcome (Lagos) strain of P. falciparum the precursor has an apparent molecular weight (MW) of 195,000. A polyvalent antiserum raised against this polypeptide immunoprecipitated labelled bands of 83,000 MW, 42,000 MW and 19,000 MW from extracts of ^{125}I surface-labelled merozoites. Peptide mapping indicated that the 83,000 MW and the 42,000 MW bands are non-overlapping fragments of the 195,000 MW precursor.

Satisfactory peptide maps were not obtained for the 19,000 MW band, and our conclusion that it is also a fragment of the 195,000 MW precursor is tentative. We have made three monoclonal antibodies that immunoprecipitate the 42,000 MW and the 19,000 MW fragments from extracts of merozoites (these also precipitate the 195,000 MW precursor from schizont extracts). However, these antibodies always precipitate both the fragments together.

The two are not disulphide-linked, and our working hypothesis is that our antibodies bind to only one fragment, the other being coprecipitated due to non-covalent association.

The 83,000 MW fragment seems to be shed from the merozoite surface. It can be recovered in soluble form from culture supernatant and cannot be detected in newly-invaded ring-forms. On the other hand, the monoclonal antibodies specific for the 42,000/19,000 MW complex react with ring-stage parasites in the immunofluorescence test using acetone-fixed cells.

The functions of these merozoite surface antigens are not known, but their abundance suggests that they may be constituents of the merozoite surface coat. As such they may be involved in the initial interaction of the merozoite and the red cell, or they may serve the function of protecting the merozoite from immune attack.

The 195,000 MW precursor of these three merozoite surface components shows considerable antigenic diversity between different isolates of P. falciparum, although some epitopes are conserved in all serotypes (McBride, Newbold and Anand, 1985). Where the variable epitopes are situated with respect to the 83,000 MW, 42,000 MW and 19,000 MW fragments has yet to be elucidated.

Whether the 195,000 MW protein is a protective antigen is not yet known, but it is interesting to note that young children with acute malaria may have a high titer of antibody against it, whilst it is not uncommon to find sera from immune adults that is low in antibody titer against the 195,000 MW antigen (Fig. 1).

We have recently identified an antigenic protein that is located within the rhoptries of P. falciparum merozoites (Holder, Freeman, Uni and Aikawa, 1985). Monoclonal antibody 61.3 stained free merozoites in the IIF test, apparently binding to internal paired organelles. The antibody immunoprecipitated a 140,000 MW protein and a 155,000 MW protein from schizont extracts. We purified the antigen from a detergent extract of schizonts by affinity chromatography using antibody 61.3 coupled to Sepharose. After washing away unbound material, the 155,000 MW band was eluted using 2 M KSCN. Then the 140,000 MW antigen was eluted using 50 mM diethylamine pH 11.5.

We raised a polyvalent antiserum in rabbits against the 140,000 MW protein, and this antiserum reacted only with the 140,000 MW band on Western blots of schizont and merozoite proteins. The antiserum bound to the rhoptries of cryosectioned merozoites, demonstrated by immuno-electron microscopy, indicating that the 140,000 MW protein is located within the rhoptries.

On the basis of immunoprecipitations using immune and non-immune sera, as well as monoclonal antibodies that inhibit the growth of P. falciparum in vitro, Perrin and co-workers have defined a subset of schizont/merozoite proteins that are likely to be protective antigens. Their sizes are 200,000 MW, 140,000 MW, 82,000 MW and 41,000 MW (Perrin and Dayal, 1982).

Finally, Perkins (1984) has identified two schizont/merozoite polypeptides, of 155,000 MW and 130,000 MW, that seem to have a high affinity for erythrocyte glycophorin. It is possible that these proteins may be involved in the initial attachment of the merozoite to the red cell.

References

Holder, A.A. and Freeman, R.R. (1984). J. Exp. Med. 160, 624-629.

Holder, A.A., Freeman, R.R., Uni, S. and Aikawa, M. (1985). Mol. Biochem. Parasitol., in press.

McBride, J.S., Newbold, C.I. and Anand, R. (1985). J. Exp. Med. in press.

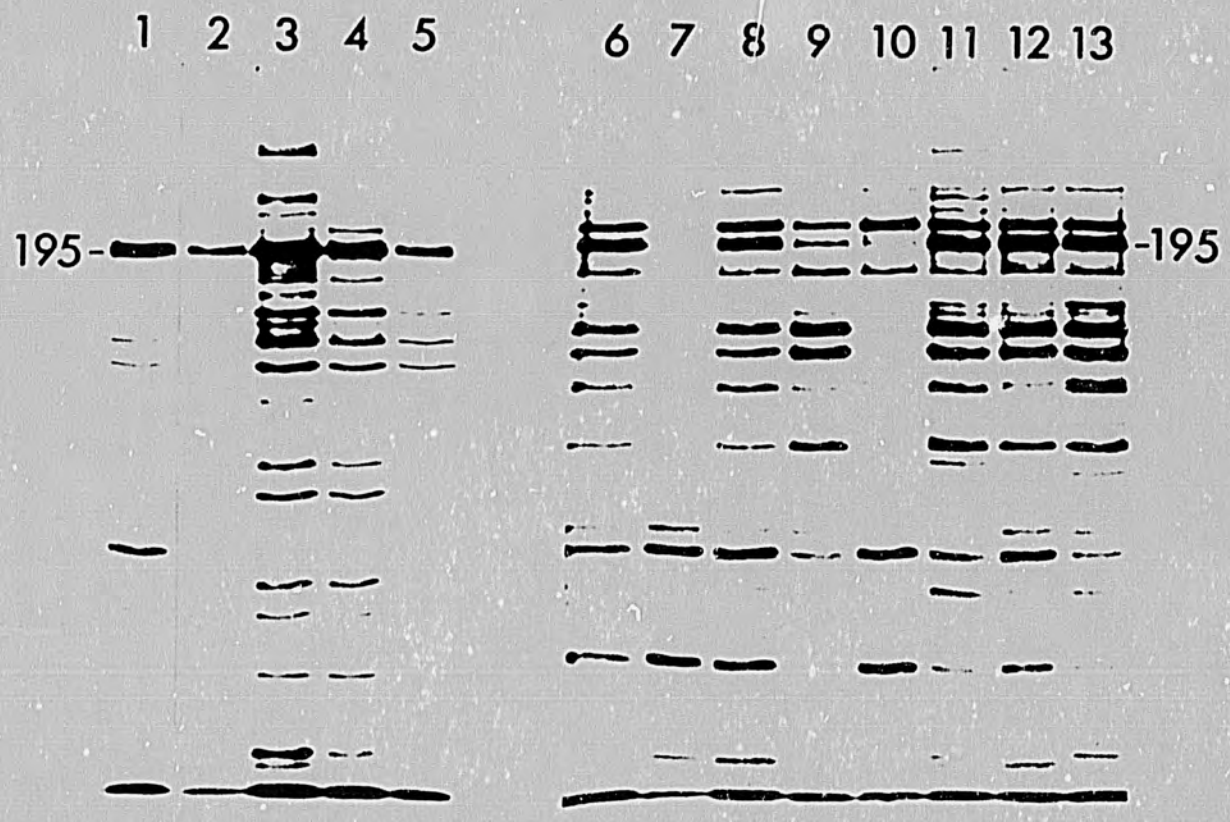
Perkins, M.E. (1984). J. Exp. Med., 160, 788-798.

Perrin, L.H. and Dayal, R. (1982). Immunological Rev., 61, 245-269.

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

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

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



Freeman and Holder Fig 1

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

by

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PARASITE-INDUCED CHANGES IN THE ERYTHROCYTE SURFACE

I. Alterations Due to External Parasites

   A. Merozoite surface proteins which bind to the red blood cell surface

   B. Internal merozoite molecules bound to the red blood cell membrane prior to or during endocytosis

II. Alterations Due to Internal Parasites

   A. Knob-related changes

   B. Non knob-related changes

PARASITE-INDUCED CHANGES IN THE ERYTHROCYTE SURFACE

I. Alterations Due to External Parasites

A. Merozoite surface proteins which bind to the red blood cell surface and remain accessible

Let us begin an examination of the effects of parasitism on the erythrocyte membrane at the point at which merozoites bind and begin entry into the cell. A number of merozoite surface proteins (Table I) have been purported to bind to glycophorin, to the erythroglycan of red blood cell band 3, or to N-acetylglucosamine which is found on both. The data have been difficult to reproduce suggesting many possible binding sites of similar energy and/or that the energy of interaction generally is low. As yet, there is no evidence that any merozoite surface molecules, which are often found free in the culture supernatant, bind to the surface of erythrocytes on their own. Whether they can be used as a means of identifying and attacking the parasitized erythrocytes is still unresolved.

B. Secretory molecules inserted into the red blood cell membrane as part of endocytosis

At present, there is no rigorous evidence that during endocytosis any proteins of parasite origin are inserted into the membrane of the erythrocyte. Nevertheless, electron microscopic studies beginning with those of Lauda *et al.* suggest that an electron-dense material normally contained in organelles found at the apical end of the merozoite (rhoptries and micronemes) may be secreted during the invasion process. These investigators suggested that endocytosis was driven by "membrane-active" materials released from these

organelles which caused the erythrocyte membrane to invaginate. This caused formation of a cavity within the erythrocyte into which the parasite moved. Consistent with such a hypothesis is the observation that whereas these organelles are easily identifiable at the segmenter through merozoite stages, they are no longer distinguishable once entry has been completed and "ring" morphology is assumed. Examination of time-lapse photomicrographs suggests that entry can be completed in under a minute; thus, invasion probably does not require the synthesis of new proteins. The electron microscopic studies of Ladda and Bannister suggest that the substances are first released from the micronemes and then from the rhoptries. This is understandable if the smaller micronemes are truly connected with the rhoptries as some studies suggest.

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

Evidence that the rhoptry material plays a role in generating the parasitophorous vacuole membrane comes mainly from electron microscopic data at this point. These studies suggest that although much of the material is secreted during invasion, the remainder is expressed into the parasitophorous vacuole, perhaps to enlarge it, and

then the organelles disappear. The rhoptry components have been observed outside of the merozoite by Bannister et al., who suggested that they condense into membraneous lamellae. However, we must await biochemical characterization of the proteins involved before we can determine whether these results are likely to be real or artifactual.

Group I Rhoptry Polypeptides

At present, two groups of polypeptides have been associated with the rhoptry (Table II). McAb against each of these groups of proteins detect their antigens in what appear to be paired organelles located in a region of the merozoite separate from the nucleus. Polypeptides in the first group characterized by lower M_r s (82K, 70K, 67K, 39K, and 37K) are found in merozoites and schizonts, but p67 and p70 are no longer detectable at the ring stage. These McAbs have not reacted with schizont proteins in Western blot analyses, and, as yet, we do not know if the polypeptides are organized into one or more protein complexes. These Group I rhoptry proteins do appear to fall, based on their ability to be immunoprecipitated by different McAbs, into two linkage groups so that if one member of a group of polypeptides is precipitated, all are likely to be, and if a particular McAb appears to be incapable of binding one member of a group, it does not bind any. This clearly argues against coprecipitation since if an antibody bound one member of the complex, the entire complex should be bound with all of its components. This, of course, assumes that antibody binding does not cause partial dissociation of the complex.

Group II Rhoptry Polypeptides

A second group of proteins, which appear to be found in the rhoptry during the merozoite stage, have molecular weights of approximately 150K, 105K, 100K, 60K, and 35K. At present, there is no evidence upon which to propose a specific role for these molecules, but data should be available shortly since several laboratories have reagents which detect them. The gene for what is likely to be the 155K Group II rhoptry protein has been cloned and partially sequenced. This protein first appears in trophozoites and then increases in concentration in schizonts and merozoites. Antibody to it has been reported to produce a generalized fluorescence associated with merozoites rather than the punctate fluorescence pattern observed with Group I rhoptry proteins. The 155K protein appears to bind to erythrocyte membrane protein(s), perhaps as part of the penetration process, and remain in a detectable form on the erythrocyte surface during at least part of the ring stage. Because of this property, one group has termed the protein the Ring-infected Erythrocyte Surface Antigen, or RESA ( ). The portion of this molecule which has been deduced from a cDNA library is made up of several different repeating units composed of the amino acids glu, asn, val, his, asp, and ala. This structure appears to be conserved among the four isolates from Papua-New Guinea, as well as the one from Thailand and another from Ghana, which were tested. Humans previously infected with malaria produce antibody to this protein.

As yet, it is unclear whether the proteins referred to as 155 by different laboratories are the same or not. This is confused even more by the realization that these molecular weights may in part be

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artifacts of the gel system or the molecular weight markers used in their determination. That is, at least one laboratory has relied on relatively high percentage gels to make their estimates, even though the proteins studied were greater than 100K; another has not provided the details or the data upon which their estimates were made. These problems, together with the real variance in molecular size which may come from isolate variance, are not likely to be resolved until protein and DNA sequence data are available or reagents to all of the proteins can be tested in a single laboratory. Nevertheless, based on the sequences of the DNAs encoding these proteins, it is likely that the two 105K proteins which have been studied are, in fact, different. Assuming that the oligo dT used to prime the synthesis of the first cDNA strand did not bind to some internal poly A stretch, then the reported DNA sequences should represent the carboxyl termini of two different molecules.

II. Alterations Due to Internal Parasites

A. Knob-related changes

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

deficiency and are also found in a series of disease states where protein and lipid metabolism are altered. Uremia can also induce them. Since the disease malaria often leads to uremia and metabolic disturbances, it is not surprising to find red blood cells undergoing such changes in infected individuals. Parasitism of an erythrocyte may also alter the potassium levels in the compartment outside of the parasite. This would become most pronounced beginning at the trophozoite stage as the parasite completes synthesis of new ribosomes and is able to greatly expand protein synthesis. Any lipids it might steal from the erythrocyte membrane might also contribute to induction of irregular erythrocyte morphology.

With these factors in mind, it is impossible to unequivocally link alterations induced in the surface of parasitized erythrocytes with the insertion of any parasite protein or proteins. That the membrane is altered has been undeniably established with a variety of independent techniques used in different laboratories (Table III).

Electron microscopic data clearly show the uninterrupted lipid bilayer covering electron-dense material localized just under the erythrocyte plasma membrane. This material was initially believed by some workers to be in the membrane, in regions of the cell where protrusions occur (Fig. I). These electron-dense deposits with the protrusions above were first described by Trager et al. and called knobs. Cloned cell lines have been derived which no longer produce knobs (K). The existence of such cell lines is the first proof that the changes in the erythrocyte morphology are not related to some indirect metabolic or ionic changes such as potassium levels but are more probably due to insertion of new parasite-derived components or

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parasite-induced modification of the structures already existing in the erythrocyte membrane.

When we compared a K^- clone with the K^+ clone from which it derived, a major change was observed. The K^+ clones had a histidine-containing protein of approximately 88K which was not detectable in the K^- progeny. With selection, it was possible to observe K^+ progeny arising from the K^- clones. These K^+ revertants contained a similar protein of M_r ~82K. The rates at which K^+ changed to K^- and K^- reverted to K^+ were of the same general magnitude, i.e., 5×10^{-6}. This is consistent with the rate at which mutational events often occur. That in going from K^+ to K^- and then back to K^+, there was a significant change in the apparent M_r of this molecule suggests loss of several amino acids which might best be explained by a recombinational event rather than a point mutation. Data such as these have been used to link this 85-88K protein with the occurrence of knobs.

It is likely, however, that there are at least three or four proteins made by the parasite which become associated with the erythrocyte membrane at approximately the trophozoite stage of development (Table IV). Of these, more than one may be lost in K^- cells making it inappropriate, based on currently available data, to suggest a single parasite protein is responsible for knob formation.

That K^+ organisms can directly give rise to K^- at a significant rate and vice versa suggests that during the course of most human infections an individual will be exposed to both. Nevertheless, organisms isolated from the blood are typically K^+, suggesting a strong selective advantage for these organisms *in vivo*. This selective advantage does not exist *in vitro* and may even shift in

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favor of the K^-. What happens to K^- organisms *in vivo* is at present unknown. It is likely that forms of the parasite which are K^- and perhaps incorporate other changes as well are also less lethal to their host. Recent work by Langreth *et al.* has demonstrated that the presence of knobs may well turn out to be a virulence factor. A K^- clone was found to cause a very limited infection in intact monkeys while splenectomized animals developed infections equivalent to those seen in animals infected with nonclonal K^+ organisms. Trager has data suggesting that K^- organisms may have the capacity to live for extended periods in intact animals. With the potential to do this as well as revert to its K^+ form as the *in vitro* studies demonstrate, the organism clearly has capabilities to exist in a subacute fashion which has not been widely appreciated. Thus, if we hope to effectively rid the body of malarial parasites, we must bear in mind that there may be the potential of clearing the body of one form of the organism only to create a less visible carrier state from which acute disease may arise later.

Is There a Knob Protein?

When it was first suggested that there might be a correlation between the presence of a 90K protein and the presence of knobs, the protein was believed to be localized in the erythrocyte membrane. This was inconsistent with the electron microscopic data showing a correlation of an electron-dense area in the cytoskeletal region with K^+ cells. It also does not take into account the biochemical data which found that the 90K protein was not easily extractible in nonionic detergents but was highly soluble in SDS, which is known to dissolve the cytoskeleton and other poorly soluble membrane proteins.

These biochemical and electron microscopic data have now been reproduced in at least three different laboratories. The important point which was used to localize this protein in the membrane was the apparent immunoelectron microscopic reaction of a rabbit antiserum produced against what was believed to be an avian analogue of this protein. The avian protein has been referred to as histidine-rich protein (HRP) because of its high histidine content. It is possible, therefore, that an 85-90' protein which is produced by the parasite is inserted into the erythrocyte cytoskeleton causing or contributing to dramatic changes in cell structure. It is also quite possible that this protein extends across the erythrocyte plasma membrane. There is precedent for this in such proteins as glycophorin and band 3. There is, however, no rigorous proof that it does extend through the membrane into the aqueous environment outside of the cell so that it would be accessible to the immune response, nor is there any proof that it is responsible for knob formation. More than one parasite-derived protein which is associated with the erythrocyte skeleton or membrane is present in K^+ organisms and absent in K^- (Table IV).

B. Non Knob-Related Changes

What evidence exists that parasitism really alters the surface of the erythrocyte membrane? Actually, a substantial amount of evidence exists, and, although most of it is immunologic, it derives from a series of different techniques; thus, the data from each reinforces the other.

The first study in which it was demonstrated that animals that could be infected with malaria could recognize parasite-induced alterations on the erythrocyte surface was done using a series of

immune sera from owl monkeys. Several important points were made in these studies. It was shown that serum from animals immune to *P. falciparum* always contained antibodies which could inhibit *in vitro* growth of the parasite and bind to the surface of erythrocytes containing live parasites. Such sera have also been found to provide passive protection to other owl monkeys. Some of the antibodies in these sera localized in the regions of the knobs and were low enough in concentration so that they could not easily be visualized by immunofluorescense but generally required immunoelectron microscopy. These studies were done at approximately the same time it was realized that K^- cells could develop during culture. Such K^- cells did not appear to bind antibody. When this was observed, K^+ parasitized cells were obtained directly from monkeys to see if a different picture was found. Again, antibodies from immune monkey serum appeared to localize in the regions associated with the knobs. Antibody binding was observed regardless of whether the cell examined was infected with homologous parasites or an organism from a different area of the world than that used to immunize the the monkey. Because electron microscopy is not easy to quantitate, no conclusions were drawn about degrees of cross reactivity, but it was clear that there was substantial conservation of certain epitopes on the surface of parasitized cells, and it was reasoned that this was highly likely if these antibodies were seeing a receptor-like structure which might play a role in allowing such cells to bind to endothelial cells. It became clear at this point that attention should be focused on those epitopes which were conserved among various isolates of *P. falciparum* since they could provide useful targets for a protective immune

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response; structures which the parasite could alter to the extent that many forms are permitted or so that substantial regions of three-dimensional structures are not retained are obviously poor candidates for immunologic protection. The parasite has already developed a method of modulating these and, thus, can readily protect itself against responses to them.

Inhibition of Intracellular Growth of the Parasite

The premise that antigens on the surface of the parasitized erythrocyte should be considered a target for any form of immunologic protection is supported by several groups of experiments. Firstly, they appear to be available as recognition sites for 24 hours in contrast with the relatively short-lived merozoite surface antigens: thus, several arms of the immune response can be expected to be effective against them. Secondly, evidence exists that an effective immune response to malaria is composed to varying degrees of many different factors. Specifically, preliminary data suggest that antibodies from immune human serum can react with the parasitized erythrocyte surface and alter the uptake and perhaps the disposal of essential metabolites such as the amino acid methionine and in so doing inhibit the intracellular development of the parasite (Figure II). The antibodies involved are not isolate-specific, which might be expected if they bind to the orifice of some channel involved in membrane transport. They are often found in individuals who have not yet produced high levels of antibody to internal schizont or merozoite antigens, suggesting that production of antibodies to these structures may be one of the earliest responses which is triggered. Thus, these

antigens may be among the first points at which the parasite is attacked.

Complement and the Red Blood Cell

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

In studying the interaction of parasitized erythrocytes with the complement system, two other things were learned as well. First of all, it was found that the parasite altered the erythrocyte surface so that even before significant antibody levels were stimulated, the complement system was activated by the "alternative" (nonantibody) pathway. This would suggest that trophozoites and schizonts (stages have not been examined) should contain some complement components on

their surfaces regardless of the level of immunity of the host at that point in time. Does the parasite subvert this basic protective mechanism in order to create channels through the erythrocyte membrane by which it can more easily obtain extracellular metabolites and discharge toxic by-products? This theory is consistent with information suggesting that beginning with the trophozoite stage, the outer red blood cell membrane becomes far more porous. Both complement fixation and permeability changes occur at a point in the parasite cycle when it escalates its metabolic level, but, as yet, there is no formal evidence to link them. This whole area clearly needs a great deal more study if we are to achieve any fundamental understanding of what occurs after parasitism of an erythrocyte.

<u>Morphologic Changes of Parasitized Erythrocytes Appear to be Independent of Surface Changes</u>

A second important point learned from the previous series of studies was that the dramatic physical changes in the erythrocyte structure, which we referred to as knobs, occurred independent of the expression of changes in the plasma membrane of the parasitized erythrocytes. This meant that they were likely to be under the control of different molecular events. The data demonstrating this are the following:

Rouleaux-inducing agents such as gelatin compounds have for some time been used to concentrate K^+ parasitized cells because the knobs physically preclude such cells from stacking, and they do not easily deform. Thus, these agents cause non knob-containing cells (normal RBCs, rings, or K^- cells) to stack in Rouleaux. This leaves the K^+ cells in suspension. When such a procedure was used so that concentrated K^+ cells could be studied, it was observed that with

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continued culture, the antigens on the surface of the parasitized RBC membrane were lost. This was demonstrated using an assay which showed that Ab-induced inhibition of intracellular development decreased with increased time in culture, even though the cells being assayed were always selected on the basis of having knobs (Table V). Another assay using the fluorescent-activated cell sorter suggested that there was a complete spectrum of levels of antibody binding to parasitized RBC cells.

These data all suggest that the parasite is contributing at least one molecule to the erythrocyte membrane, is altering existing erythrocyte membrane molecules, or both. That the lipid composition of the RBC membrane is changed by the parasite is well documented. That this could alter the relationship of normal erythrocyte proteins to one another is quite possible.

Binding of Trophozoites to Endothelial Cells

In addition to this type of study, an assay which may have considerable biological significance has suggested the independence of knobs and particular membrane changes. These experiments demonstrated that although trophozoites from infected monkeys could adhere in culture to endothelial and specific tumor cells, this capacity was rapidly lost during culture of the parasite. Significantly, the *in vitro* adherence could be blocked by immune owl monkey serum. This assay thus appears to provide an independent system by which the endothelial cell receptor on the trophozoite can be distinguished.

A very large protein of approximately 250K has been implicated as the possible parasite receptor for endothelial cells. This molecule is associated with the erythrocyte membrane as suggested by attempts

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

A 980bp cDNA clone which encodes part of a molecule which may be associated with the erythrocyte membrane has been examined. Antibodies to the protein, which were produced against the product of a cDNA clone expressed in E. coli, produced a speckled intracellular immunofluorescent pattern in both trophozoites and schizonts. On Western blots, it bound to a very large molecule of perhaps 300K and in a lesser degree to smaller peptides. This antigen reacted with 83% of the 65 human immune sera tested. It contains 13 hexapeptide repeats flanked by a highly charged region containing both acidic and basic residues. This structural unit is also repeated, giving rise to interspersed blocks of repeats and charged units. The sequences of the charged units are much more conserved than those of the repeats.

Summary

In summary, there are three groups of molecules which interact with the erythrocyte membrane or cytoskeleton as a result of infection with the human malarial parasite, P. falciparum. On entrance, molecules on the merozoite surface or those contained within the rhoptries and micronemes may bind to or become inserted into the erythrocyte membrane. Two families of molecules (Groups I and II) have been identified with MoAb as possible candidates. It is possible that more than one parasite-produced molecule is found in K^+ and not

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in K organisms and may be responsible for the production of the dramatic surface changes referred to as knobs. And finally, perhaps two or more proteins, one of which is too large to allow an accurate M_r determination by SDS PAGE, may be associated with the erythrocyte membrane. One or both may play a role in producing the receptor by which the trophozoite can bind to endothelial cells and sequester. There are, therefore, a series of parasite-induced alterations which appear in the host erythrocyte membrane. Further study is likely to identify specific structures which may be useful in providing immunologic protection for humans.

TABLE 1

ALTERATIONS IN THE ERYTHROCYTIC SURFACE
DUE TO BINDING OF EXTERNAL PARASITES

Possible Erythrocytic Receptor	$M_r \times 10^{-3}$ of Merozoite Protein	Reference
Glycophorin	(155?) 130	Perkins *et al.*
N-Acetylglucosamine	140, 70, 35	Jungery *et al.*
Erythroglycan of Band 3		Friedman *et al.*

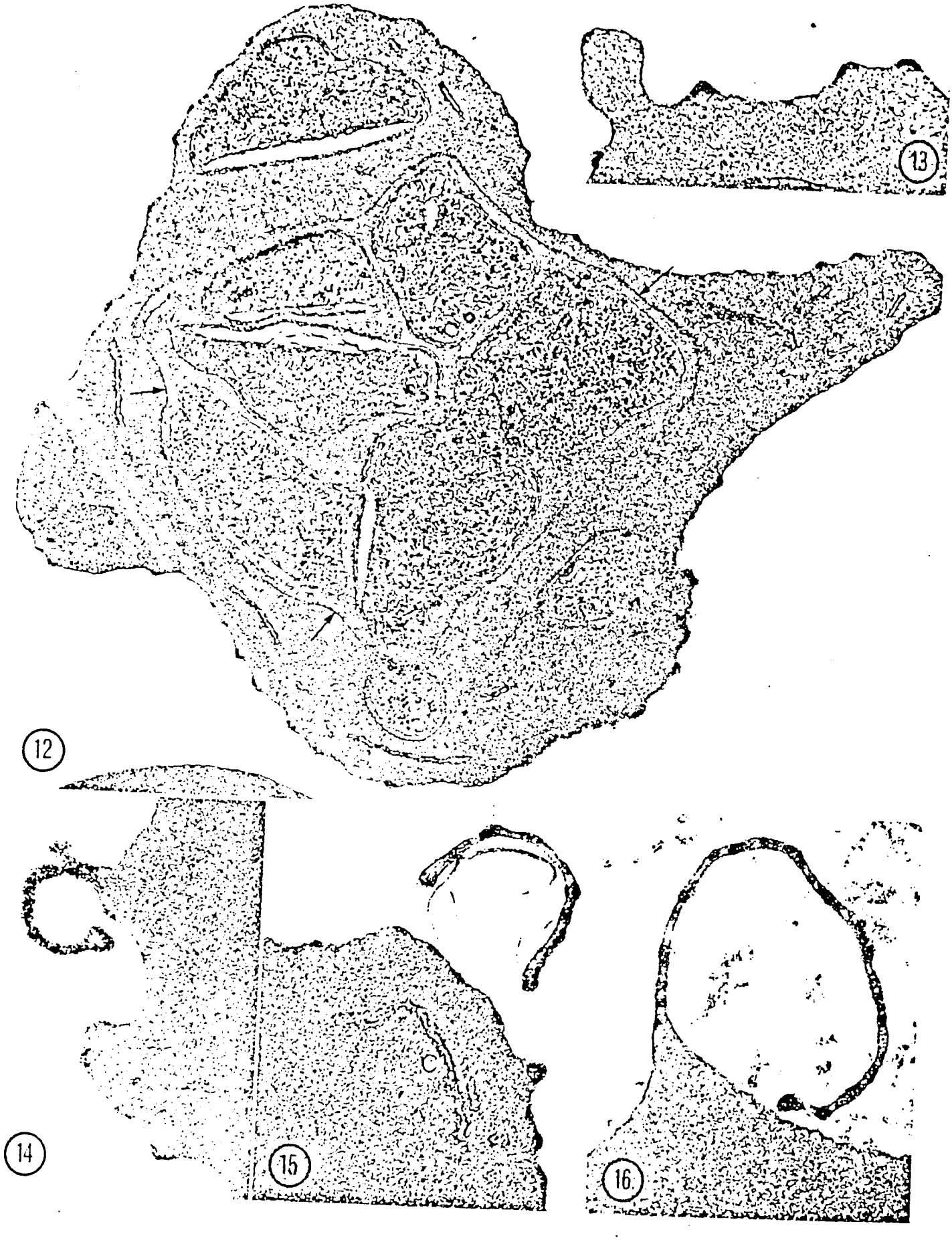
TABLE II

ALTERATIONS IN THE ERYTHROCYTE SURFACE BY PROTEINS SECRETED BY THE MEROZOITE DURING PENETRATION

Group	$M_r \times 10^{-3}$ of Rhoptry Proteins at Merozoite Stage	$M_r \times 10^{-3}$ of Rhoptry Proteins at Young Ring Stage
I Low Molecular Weight	82, 70, 67, 39, 37	82, 39, 37
II High Molecular Weight	150, 135, 100, 60, 35	?

FIGURE 1

MALARIA *in vitro*: FINE STRUCTURE



Langreth et al. 1978

TABLE III

EVIDENCE FOR NEW ANTIGENS ON THE SURFACE OF PARASITIZED ERYTHROCYTES

1. Antibodies from immune monkey serum have been demonstrated by immunoelectron microscopy to bind to K^+ parasitized erythrocytes; this binding did not appear isolate-specific, although quantitative differences among isolates would not have been detected.

2. Antibodies from immune monkey serum can block the *in vitro* binding of parasitized erythrocytes to certain culture cell lines.

3. Antibodies in immune human or monkey serum can block the uptake of ^{35}S-methionine into trophozoites and stop the intracellular development of the parasite.

4. Parasitized erythrocytes activate the complement system in the absence of immune serum; in the presence of antibody, the complement system is activated more efficiently.

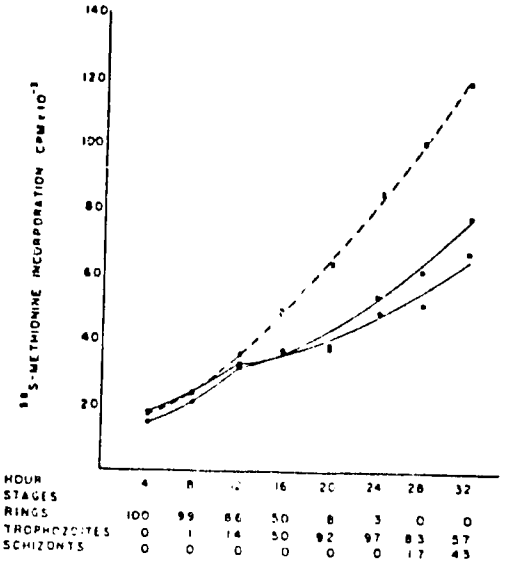
TABLE IV

ALTERATIONS OF THE ERYTHROCYTE MEMBRANE
CAUSED BY INTERNAL PARASITES

IFA Pattern	Distribution	K^+	K^-	Cytoskeletal Linkage	$M_r x10^{-3}$	Isolate Specificity	References
Confluent RBC Surface or Spotty*	Trophozoites, Schizonts	+	+	-	350-400	Broad	Stahl *et al.* Stanley and Reese
Confluent RBC Surface or Spotty	Trophozoites, Schizonts	+	-(?)	?	260-280	Limited?	Leech *et al.*
Confluent RBC Surface or Spotty	Trophozoites Schizonts	+	-	+	84-94 (Band Typically Indistinct)	Broad	Kilejian, Leech *et al.*, Stanley and Reese

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

FIGURE II



Incorporation of ^{35}S-methionine and stage distribution during growth of *P. falciparum*. Growth in the presence of control serum (×– – –×) versus immune serum 2 (○– – –○) or 36 (●– – –●). The experiment presented has been done three times using five individual replicates for each time point in each experiment. The standard deviation for each of the time points was < 10%. The parasite stage distribution was determined from the control cultures.

Stage distribution of intracellular parasites after growth for 32 hours in immune serum

Serum	% rings	% trophozoites	% schizonts
Control	0	57	43
2	2	59	39
36	11	80	9

TABLE V

% INHIBITION AS FUNCTION OF
LENGTH OF TIME PARASITES MAINTAINED IN CULTURE

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

Stanley, H.A. and R.T. Reese (1984) Am. J. Trop. Med. Hyg. 33:12.

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Abstract No. 17, page 30

Working Report - NOT FOR PUBLICATION

Development of malaria vaccines: Asexual blood stages:- soluble antigens

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Introduction

During schizogony, rupture of the infected erythrocyte releases many parasitic proteins into the blood stream. These include:- proteins secreted into the parasitophorous vacuole, proteins released from the secretory organelles of merozoites and soluble cleavage fragments (secondary to processing or degradation) of proteins that are not otherwise soluble. Many of these proteins will elicit immune responses. The term soluble antigen is therefore an imprecise one covering a wide spectrum of antigens. For example, the ring infected erythrocyte surface antigen (RESA) is transferred to the surface membrane of a newly infected erythrocyte during the process of merozoite invasion. A proportion of merozoites fail to invade and die, releasing RESA which can subsequently be detected as a soluble antigen. Antibodies against soluble antigens have been demonstrated to inhibit parasite growth *in vitro* but the target antigen has not been clearly demonstrated (12)

The S antigen system

An early attempt to classify soluble antigens of malaria identified antigens differing in their stability to heat (1). Those proteins that retained their antigenicity after boiling were designated S antigens. S antigens which are found in the plasma of infected individuals and in culture supernatants, exhibit extreme serological diversity. Although many different S antigens may be present in a localized region, individual S antigens are found in widely separate geographic areas (2,3). The S antigen serotype of a particular parasite strain appears to be stable, persisting unchanged after numerous in vitro and in vivo passages (3). Thus this system differs fundamentally from the variant surface glycoprotein of *Trypanosoma brucei*, as a cloned population of trypanasomes switches readily from one VSG to

another. As it has proved impossible to raise antisera against purified native S antigens (4) and the acquisition of anti-S-antigen antibody in man is age dependent (5), S antigens appear to be poorly immunogenic. Immunoblotting experiments using culture supernatants from P.falciparum lines maintained in vitro has enabled us to identify of the S antigens of several isolates (6). S antigens show remarkable size polymorphisms with apparent molecular weights ranging from less than 100kd to about 250kd. Even antigenically related S antigens from different clones of a single isolate may vary in size. Biosynthetic labelling experiments have demonstrated that the S antigen is a gene product encoded by the parasite (6). Use of ^{3}H-glycine as a biosynthetic label has been particularly useful for identifying S-antigens of different isolates (7). Localisation studies have demonstrated that the S antigen is synthesized in the mature parasite and released at the time of schizont rupture. Immunoelectron microscopy suggests that S antigen is located in the parasitophorous vacuole.

S antigen gene structure

The recent development of molecular cloning techniques have led to the construction of cDNA libraries that express the antigens of P.falciparum. One such expressing clone was found to produce a portion of the S antigen from FCQ27/PNG (FC27) a Papuan New Guinea isolate of P.falciparum (8). This fragment was entirely composed of a repeating 33 base sequence coding for tandem repeats of 11 amino acids. This 11 amino-acid sequence was found to be highly strain specific - the sequences were absent from other P.falciparum isolates that did not express that S antigen specificity. This cDNA was subsequently used to identify a genomic clone from the FC27 strain that encoded the complete S antigen sequence. Although the repeats were absent from other parasite isolates, flanking regions of the gene could be used to

find the corresponding S antigen in other isolates. Thus, the S antigen gene from NF7 a Ghanian isolate was also isolated. Both genes were completely sequenced and demonstrated a basic structure for S antigens (9). The structure of the S antigen gene is summarised in Figure 1. The central portion of the protein is composed of tandemly repeating oligopeptides, 11 amino acids in the case of FC27 and 8 amino acids in the case of NF7. The vast bulk of the natural antibody response to S antigens appears to be almost totally directed towards epitopes encoded by these repeat sequences. Variations in the number of amino acids in their repeats and their composition account for the serological diversity of the S antigens. Surrounding the repeat regions are stretches of highly charged amino acids, a structural feature also found in the circumsporozoite proteins of P.falciparum (10). The amino and carboxy terminal portions of the sequences are up to 70% homologous. There is a signal sequence present at the amino terminus. A mechanism of generation of the diverse S antigens appears to be by a process of point mutation, deletion and insertion throughout the repeat units. This has resulted in a series of genes that are maintained by selection as a polymorphism in natural populations of the parasite. The selection pressure that maintains this polymorphism would appear to be the human immune system.

S antigen families

The isolation of genes encoding the S antigen has enabled a subclassification of the S antigen family. The repeat portion of the gene can be used as a probe to identify cross hybridising S antigens, e.g. the repeats of the NF7 antigen will hybridise to the S antigen of K1, a Thai isolate. The corresponding S antigens from these isolates are not cross reactive at the protein level. Thus, the S antigens can be classified within subgroups which demonstrate nucleotide homology but not protein cross-reactivity. This may reflect the way in which the proteins have evolved.

Function of the S antigen

The function of the S antigen system is unknown. There are several striking and even paradoxical features about this system that are yet to be explained. These centre on the observations of extreme diversity coupled with low immunogenicity as native molecules (c.f. fused polypeptides). As has been mentioned there are very many S antigen types which have been maintained in the naturally occurring parasite population. This bespeaks some strong selective pressure. However, one of the main findings in the seroepidemiology of S antigens has been the observation that antibody responses to these antigens are rare. This is precisely what one might predict for a soluble antigen made up of repeating epitopes behaving as a T independent antigen and giving rise to short lived IgM responses together with suppression. Immunization with vaccinia virus expressing the S antigen has resulted in very low antibody responses in experimental animals (C.J. Langford, unpublished observation). It is difficult therefore to understand the need for such extreme serological diversity in a molecule that is so poorly seen by the immune system.

One seemingly obvious conclusion about S antigens is that because they are so variable they cannot be the target of protective immunity as there exist many strains that do not express a particular specificity. This conclusion seems less obvious after the discovery of conserved regions among S antigen molecules. However, the conserved regions have apparently been rendered invisible to the immune system as evidenced by seroepidemiological studies with cloned antigen fragments. That immune responses directed against S antigens can have some host protective value has been suggested by the finding of a monoclonal antibody directed against the repeats of the FC27 S antigen that inhibits the growth of the homologous strain in vitro (11).

Acknowledgement

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References

1. Wilson, R.J.M., McGregor, I.A., Hall, P., Williams, K. and Bartholomew, R. (1969). Lancet ii 201-205.
2. Wilson, R.J.M. and Voller, A. (1972). Parasitology 64, 191-195.
3. Wilson, R.J.M. (1980). Nature 284, 451-452.
4. Wilson, R.J.M. and Ling, E. (1979). Bull.WHO 57, Suppl.1.
5. McGregor, I.A. and Wilson, R.J.M. (1971). Trans.R.Sco.Trop.Med.Hyg. 65, 136-145.
6. Anders, R.F., Brown, G.V. and Edwards, A. (1983). Proc.Natl.Acad.Sci. USA 80, 6652-6656.
7. Winchell, E.J., Ling, I.T., Wilson, R.J.M. (1984). Molec.Biochem. Parasitol. 10 287-296.
8. Coppel, R.L., Cowman, A.F., Lingelbach, K.R., Brown, G.V., Saint, R.B., Kemp, D.J. and Anders, R.F. (1983). Nature 306, 751-756.
9. Cowman, A.F., Saint, R.B., Coppel, R.L., Brown, G.V., Anders, R.F. and Kemp, D.J. (1985). Cell (in press).
10. Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wortz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.O., Schneider, I., Roberts, D., Sandes, G.S., Reddy, E.P., Diggs, C.L. and Miller, L.H. (1984). Science 225, 593-599.
11. Saul, A., Myler, P., Schofield, L., and Kidson, C. (1984). Parasite Immunol. 6, 39-50.
12. Jepsen, S. (1983). Scand.J.Immunol. 18, 567-571.

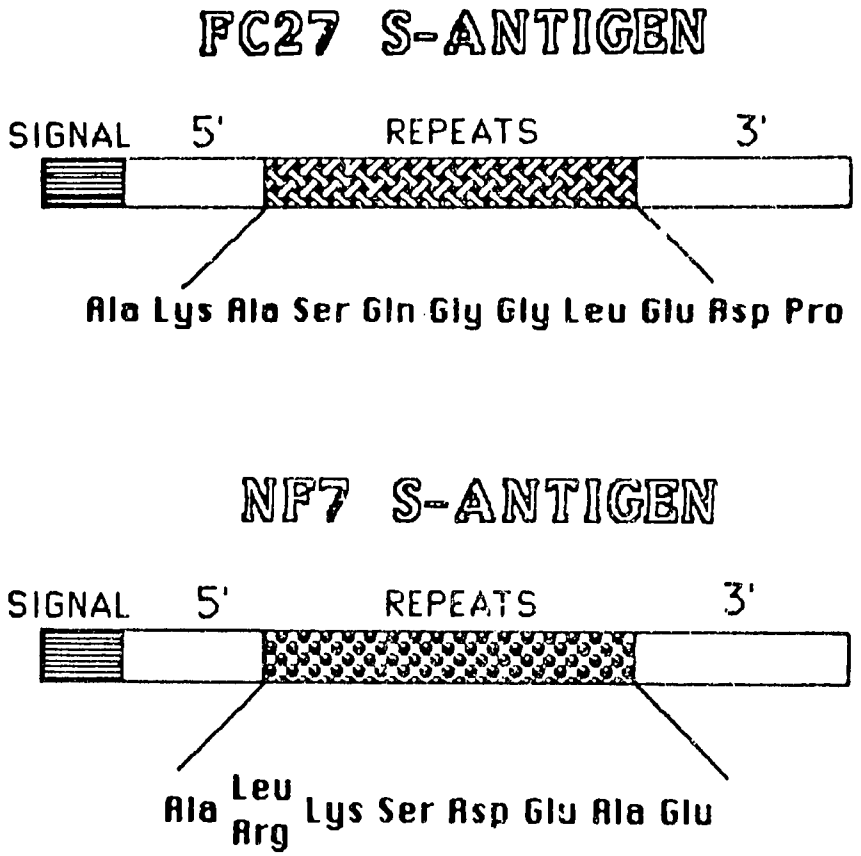

Fig. 1 Schematic of the structure of S antigen from two different strains of P. falciparum . The regions labeled 5' and 3' are more than 70% homologous between the strains.

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Progress Report

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Summary

The University of Illinois malaria vaccine program utilizes cell culture-derived soluble exoantigens of *Plasmodium falciparum* and the squirrel monkey (*Saimiri sciureus*) as its experimental model. Exoantigens are soluble proteinaceous moieties naturally released into the blood plasma of animals infected with *Plasmodium* and *Babesia* species or in the supernatant medium of *in vitro* cultures of these organisms.

In earlier studies, vaccination of squirrel monkeys with crude cell-culture supernatant containing exoantigens protected them from clinical reactions and death caused by *P. falciparum*. Challenge was administered 2 to 9 months after vaccination. The immunogen consisted of filtered, concentrated or chromatographically-purified supernatant fluid. Immunogenicity was enhanced by the use of saponin or aluminum hydroxide adjuvants. Strong humoral immune responses measured by the indirect fluorescent antibody (IFA) test were evident post-vaccination and challenge. No mortality occurred among 11 vaccinated animals, while 2 control monkeys developed typical signs of malaria following challenge and died. In addition to clinical protection evidence of protective immunity in vaccinated animals was revealed by stable hematocrit levels and

consistently lower parasitemias with structurally abnormal parasites situated intra- and extra-erythrocytically.

In more recent vaccine studies, susceptible squirrel monkeys were vaccinated with an aluminum hydroxide-fortified fraction purified from culture supernatants of the Indochina I and Geneve/SGE-1 P. falciparum strains by cation-exchange (SP-trisacryl) chromatography. Animals immunized with 'SP' and corresponding control immunogens were challenged with whole blood containing monkey-adapted virulent organisms of the Indochina I strain. Hematological, serological and parasitological profiles served as potential indicators of protection. This immunogen conferred significant clinical protection of squirrel monkeys against needle challenge with the homologous Indochina I strain, and a moderate degree of immunity to the heterologous strain.

Malaria antigens contained in the SP fraction have been identified by Western immunoblotting using rabbit anti-SP antiserum. This serum was highly schizont-specific and inhibited merozoite invasion of human erythrocytes in vitro. Examination of the SP fraction by crossed immunoelectrophoresis revealed an absence of erythrocytic elements and IgG immunoglobulins.

An 83,000 (83K) molecular weight protein has been purified from the SP fraction by use of conventional column chromatography, high performance size exclusion liquid chromatography, and high performance anion-exchange liquid chromatography. The 83K protein exists as a dimer in the native state and has been identified as a parasite antigen by Western immunoblotting and by its specific reactivity in the indirect enzyme-linked immunosorbent assay (ELISA). The N-terminal sequence of the first 35 amino acid residues has been determined. More recently, another 100K exo-antigen was isolated. Sequence data of this antigen were found to be identical with those of the 83K.

Sequencing of the N-terminal amino acids of the 83K polypeptide has led to the synthesis of a 20-nucleotide probe with only 4-fold degeneracy. Hybridization results show the oligomeric probe sequence to be specific to the P. falciparum genome. Since the probe does not cross-react with λ gt 11, λ 1059, PUC and E. coli DNA, a small genomic library has been constructed in the PUC 19 plasmid. Prospective clones are being screened by hybridization to the oligomer, and sequence analysis will be performed to confirm isolation of those fragments coding the 83K peptide. Isolated fragments will then be expressed by recombination to λ gt 11 for gene expression.

Report

I. Objective.

To develop a vaccine against malaria caused by *Plasmodium falciparum* utilizing exoantigens and the squirrel monkey (*Saimiri sciureus*) model.

II. Exoantigens.

Exoantigens are soluble proteinaceous moieties naturally released into the blood plasma of animals infected with *Plasmodium* and *Babesia* or into the supernatant medium of *in vitro* cultures of these organisms.

Our knowledge of these antigens commenced some three decades ago when we first observed their presence in the plasma and serum of several non-bovine animal species infected with *Babesia spp*. During these studies an interrelationship between parasitemia levels, concentration of plasma antigens, and the level of antibodies to these antigens was demonstrated. A direct relationship between the levels of parasitemia and plasma antigens was found while an inverse relationship existed between these two elements and serum antibody levels. Recognizing the immunogenic potential of these antigens, we initiated vaccination studies in dogs against infections caused by *B. canis* utilizing soluble exoantigens as immunogens.

It is in the course of these studies that we, for the first time, recognized the protective nature of these antigens. However, while such potential was clearly evident, it was also recognized that the immunogenicity of plasma-derived soluble antigens was low, probably because of immune-complex formation and enzymatic degradation.

The development of methods for *in vitro* propagation of *B. bovis* renewed our interest in soluble exoantigens as immunogens against bovine babesiosis. During the last six years, well-controlled babesiosis vaccination experiments using culture-derived soluble exoantigens of *B. bovis* were conducted in the United States, Mexico, Venezuela and Australia. The evidence of protection against virulent *B. bovis* challenge using infected blood or ticks was demonstrated in all of these studies.

Exoantigens of parasitic origin have been detected in the plasma or serum of rodents, chickens, monkeys and man with acute malaria. Several groups of plasmodial exoantigens from human plasma have been characterized according to heat stability, molecular weight, enzyme sensitivity, and antigenicity. In addition, exoantigens have been isolated from the culture supernatant medium of *P. falciparum* parasites propagated *in vitro*.

Partial protection against challenge infection has been demonstrated in chickens (Illinois) and monkeys (CDC) with blood plasma-derived exoantigens of *P. gallinaceum* and *P. knowlesi*, respectively.

Development of a method for continuous *in vitro* propagation of *P. falciparum* provided a means for the production of soluble exoantigens unaffected by the host's metabolic and immune systems. It appears that the antigen to which the host must respond if it is to develop protective immunity against the asexual blood phase of malaria, is a component of the surface coat covering the outer membrane of the merozoite. This surface coat appears necessary for attachment of the merozoite to erythrocytes and mediates the first essential step for entry of this organism into the erythrocyte. There is sufficient evidence that most of the exoantigens originate from the merozoite surface. Accordingly, the humoral immune response to these exoantigens should be capable of blocking an invasion of erythrocytes by the merozoite as recently demonstrated with *B. bovis* and *P. falciparum*.

III. Vaccination Experiments with Exoantigens Contained in the Crude Culture Supernatant: Homologous Challenge.

Squirrel monkeys (*Saimiri sciureus*) were protected from clinical reactions and death caused by *P. falciparum* by vaccination with a cell culture-derived immunogen. The immunogen consisted of filtered, concentrated (10x) supernatant fluid collected from *in vitro* cultures of *P. falciparum*. Challenge was administered 2 to 9 months after vaccination. Challenge material consisted of 2.0×10^7 *P. falciparum*-parasitized (Indochina I) erythrocytes contained in whole blood collected from an acutely infected monkey. Immunogenicity of vaccinal antigens was fortified by the use of saponin or aluminum hydroxide adjuvants. Strong humoral immune responses measured by the indirect fluorescent antibody (IFA) test were in evidence post-vaccination and challenge. No mortality occurred among 11 vaccinated animals which included 4 preimmunized and 7 malaria-susceptible monkeys, while 2 susceptible control monkeys developed typical signs of malaria following challenge and died.

Pathological and clinical observations demonstrated substantial protective immunity in vaccinated animals. The latter maintained stable hematocrit levels and

consistently lower parasitemias, accompanied by presence of crisis forms of this organism. Further corroborative evidence of the vaccine efficacy was the specific inhibitory capacity of sera collected from vaccinated animals prior to challenge. This was established by *in vitro* growth inhibition studies.

IV. Partial Purification and Characterization of Culture-Derived *P. falciparum* Exoantigens.

Previous work has led to the isolation and partial purification of an antigenic fraction from supernatant fluids of asynchronous cultures (average parasitemia= 2-3%) of the Geneve strain (Senegalese isolate) of *P. falciparum*. This fraction, referred to as the SP fraction, was purified by cation-exchange chromatography on a SP-trisacryl column (0.01 M acetate buffer, pH 3.7, with a step-wise NaCl gradient). Antigenic fractions in this and subsequent purification procedures were monitored by the indirect ELISA utilizing human anti-*P. falciparum* IgG and normal human IgG, both of placental origin. Antigenic activity was found in fractions which eluted between 0.2 M and 0.3 M NaCl concentrations. Malarial antigens contained in the SP fraction were then identified by the Western immunoblot technique using rabbit anti-SP

antiserum. This antiserum was highly schizont-specific, showed an IFA titer of 1:1280 and inhibited merozoite invasion of human erythrocytes *in vitro*. Analysis was also conducted with an appropriate control antigen and antiserum to a SP fraction from supernatant fluids of noninfected cultures (rabbit anti-negative SP fraction). Western Blot analysis revealed a major malarial antigen of MW 83K in the SP fraction. Subsequently, additional *P. falciparum* antigens of 100K and 70K were identified as being present in this fraction.

An examination of the SP fraction by crossed immunoelectrophoresis demonstrated that it was free of erythrocytic components and IgG immunoglobulin. We estimated that 80% of contaminating serum proteins was removed with the SP-trisacryl fractionation procedure.

V. Vaccination with Partially Purified Culture-Derived Exoantigens: Homologous and Heterologous Challenge.

In this experiment we investigated the induction of protective immunity with a SP-trisacryl cation-exchange purified antigenic fraction derived from supernatant fluids of asynchronous cultures of the homologous Indochina I and heterologous Geneve strains of *P. falciparum* in susceptible Bolivian adult male squirrel monkeys. This SP fraction is approximately 80% pure and contains *P. falciparum*

polypeptides of 100K, 83K, and 70K (see part IV above). A SP-purified fraction from noninfected cultures was was utilized as a negative control immunogen. This study involved duplicate experiments of identical design. In total, 4 monkeys were vaccinated with the homologous SP fraction, 8 animals were immunized with the heterologous SP immunogen, and 6 animals received the control preparation. Each dose consisted of 2 mg total protein in 1 ml volume. These preparations were gently mixed with 127.5 ul of 1.86% aluminum hydroxide adjuvant for 1 h at 25° C. Two subcutaneous inoculations were administered at a 4-week interval. Challenge exposure consisted of an intravenous injection of fresh infected blood containing 1 x 10^{7} virulent monkey-adapted Indochina I organisms approximately 2½ months following the second vaccination.

A complete hematological profile was considered essential for assessing potential indicators of protection post-challenge. In addition to hematology, blood chemistry and serology, parasitemia levels were monitored. Special emphasis was also placed on the development of parasite morphological alterations, termed crisis forms.

The critical period in the course of infection occurred 14 days post-challenge as decreases in hematocrits paralleled rises in parasitemia. Recovery to normal hematological levels was notably accelerated in the animals which received the homologous SP fraction, was moderate in monkeys immunized with the heterologous SP fraction, and was considerably delayed in the negative control animals. Mortality was observed with two monkeys of the control group on days 13 and 15 post-challenge. At this time, we felt that to provide an unbiased evaluation of protective immunity, treatment of experimental animals should be avoided. Significant differences in clinicopathological responses were seen, particularly with regard to animals immunized with antigenic fractions prepared from the homologous strain. Hematocrit decreases were limited, as well as the degree of parasitemia. Antibody levels in vaccinated monkeys increased rapidly and remained elevated 4 weeks post-challenge at the time titers in control animals began to decline. Crisis forms were first observed between 11 and 13 days post-challenge with mean parasitemias on those days notably lower in vaccinated monkeys. Three types of crisis forms were observed: (1) intracellular point or anaplasmoid forms, (2) intracellular forms with degenerative or 'stretched' morphology and (3) extracellular, apparently deformed organisms.

Gross and microscopic pathology in monkeys which succumbed to clinical malaria revealed hepato-splenomegaly with spleens 6 times normal size, splenic rupture accompanied by hemoperitoneum, jaundice and parasitic occlusions and microscopic lesions in major organs.

Potential criteria for protection against disease included parasitemia levels, appearance of crisis forms which would encompass parasite killing by both cell-mediated and humoral mechanisms, hematocrit and hemoglobin values, and importantly, SGPT and BUN levels as indicators of liver and kidney function, respectively. Fourteen days post-challenge, nonvaccinated animals showed dramatic increases in these specific parameters (SGPT=+119%, BUN=+58%). This is consistent with the hepatomegaly and proliferative glomerulonephritis seen upon necropsy.

In conclusion, this study demonstrated that purified P. falciparum exoantigens induce significant protective homologous and moderate heterologous strain immunity to clinical malaria caused by a virulent challenge exposure. Additionally, various clinicopathological parameters were found to be important indicators of protective immunity against disease.

VI. Isolation and Characterization of 83 AND 100K Exoantigens.

Fraction "SP", described in Part IV, was further purified by preparative gel filtration HPLC. Based on the ELISA monitoring of proteinaceous fractions eluted from the column, apparent molecular weights of antigenic moieties were 350 kd, 170 kd, 130 kd and 40 kd, respectively. However, subsequent characterization by SDS-PAGE and Western Blotting demonstrated that these proteins were actually being eluted in the dimeric form. Immunoblots of HPLC fractions showed unique bands representing major malarial proteins at 83 kd and 70 kd with minor antigens observed at molecular weights 185 kd, 100 kd, 43 kd and 20 kd.

Antigenic fractions from gel filtration HPLC which contained the 83K exoantigen were pooled and additionally purified by DEAE anion-exchange HPLC. ELISA reactivity corresponded to one major protein peak, demonstrated on Western blots to be the 83K antigen. This antigen was found as a single homogeneous protein by SDS-PAGE with Coomassie Blue staining. One liter of the original pooled supernatant fluid yielded approximately 50 micrograms of the 83K protein.

Automated Edman Degradation was performed on an Applied Biosystems, Inc. (Foster City, CA, USA),

Model 470A Protein Sequencer using 500 picomoles of purified 83K exoantigen. Phenylthiohydantoin (PTH) amino acids were detected by reverse phase HPLC on an Altex ultrasphere ODS column (Beckman Instruments, Inc., Palo Alto, CA, USA).

The N-terminal amino acid sequence of the P. falciparum 83K exoantigen was determined for the first 35 residues. There were no repeating units in the N-terminus, in contrast to that seen for some other P. falciparum antigens. Residues 16 through 22, Trp-Asp-Gln-Asn-Glu-Lys-Phe, contain the least codon ambiguity and represent a sequence appropriate for constructing a synthetic oligonucleotide probe. Tryptophan has a single codon and the other 6 amino acids, aspartic acid, glutamine, asparagine, glutamic acid, lysine and phenylalanine are only 2-fold degenerate.

The strong hydrophobic nature of the N-terminal sequence suggests that it is associated within an interior portion of the protein or alternatively it could serve as a membrane binding site. The possible existence of a membrane binding site on the 83K protein coupled with the schizont-specificity of the rabbit SP antiserum is suggestive that the 83K antigen may be derived from the 190-200K protein identified by other investigators.

A mixed 20mer derived from amino acid codons for residues 16 through 22, was selected as a hybridization probe on the basis of the relative stability of G . T versus A . C mismatches and the fact that Plasmodium DNA is very rich in A . T base pairs. Thus, a mixed probe with 4-fold degeneracy was synthesized on an Applied Biosystems Model 380A DNA synthesizer, having the composition: 3'ACC-CT$^{A}_{G}$-GTT-TT$^{A}_{G}$-CTT-TTT-AA 5'. The oligonucleotide probe was purified by reverse phase HPLC.

More recently, the 100K exoantigen has been isolated in pure form. Preliminary sequence data appear to be similar, if not identical, with those of the 83K polypeptide.

In summary, we have isolated and characterized a P. falciparum 83K exoantigen. The 83K protein was purified from a "SP" fraction that induces protective immunity in squirrel monkeys against a virulent challenge P. falciparum. This protein reacts with antibodies which are present in human placental immunoglobulin IgG from individuals who have recovered from P. falciparum infections. This observation suggests that the 83K

protein may be a good candidate for a potential host protective antigen in malarial immunoprophylactic regiments. We are currently preparing a polyclonal rabbit antiserum specific to the pure 83K protein. This antiserum should aid in the analysis of the 83K protein to determine whether it is a processed molecule produced from a larger precursor protein. Studies are also in progress in which the pure 83K exoantigen will be tested for its ability to induce protective immunity in experimental monkeys.

VII. Recombinant DNA Studies.

Sequencing of the 83K peptide N-terminus has led to the synthesis of a 20-nucleotide probe with only 4-fold degeneracy. This oligomer is being employed to search for sequences encoding the 83K protein by Southern blot hybridization to *P. falciparum* restriction digests.

The DNA was isolated from erythrocytes of *in vitro* cultures infected with the Geneve strain of *P. falciparum*. Infected cells with a 3-5% parasitemia were harvested and centrifuged to remove serum, buffy coat and the upper erythrocyte layer. Remaining erythrocytes were washed several times in PBS to remove any remaining serum contaminants. Cell-free parasites

were obtained by lysing infected erythrocytes in saponin and washing parasites 3x in PBS. The DNA was recovered by solubilizing the parasitic fraction in sarcosyl detergent buffer and repeatedly extracted with phenol, chloroform, and mixtures of the two. The DNA was then dialyzed, ethanol-precipitated, pooled and checked for purity from enzyme inhibitors by restriction digestion and agarose gel electrophoresis.

Preliminary hybridization results show the oligomer probe to be specific to the P. falciparum genome. Since the probe does not significantly cross react with λ gt 11, λ1059, PUC or E. coli DNA, a small genomic library has been constructed in the PUC 19 (plasmid). Prospective clones are being screened by hybridization to the oligomer, and sequence analysis will be performed to confirm isolation of those fragments coding the 83K peptide. Isolated fragments will be expressed by recombination to λ gt 11. Application of our ELISA detection system to colony screening has been tested, and no cross reaction to λgt 11 host strains using anti-exoantigen antibodies has been noted. Optimization of antigen detection by antibodies is currently being investigated in preparation for isolated gene

expression. Prospects for mRNA isolation are also being considered, but are awaiting further results from clone sequencing.

Addendum.

Pathology of Fatal Plasmodium falciparum Infection in the Squirrel Monkey (*Saimiri sciureus*)

Our laboratory has studied the pathological alterations associated with experimentally induced fatal acute falciparum malaria in mature Bolivian squirrel monkeys. Pathologic changes in the pulmonary, hepatic, renal, and hematopoietic systems suggest that the disease in this simian model closely resembles the human infection.

Marked splenomegaly with lymphoreticular hyperplasia was a constant feature of fatal infections in squirrel monkeys. Lymphoreticular changes were prominent in the liver, bone marrow, and lymph nodes and were identical to those described in human infections and other simian falciparum models.

Prominent microvascular sequestration of parasitized erythrocytes was present in adipose tissue and cardiac musculature. Occasionally, parasitized red blood cells were adhered to microvascular endothelial surfaces in the lung, liver, and adrenal gland. The capillaries of the brain were notably free of erythrocyte sequestration.

The animals which died had severe interstitial pneumonia believed to be associated with a pulmonary microvascular disturbance. This phenomena was not associated with sequestration of parasitized erythrocytes. Similar

pulmonary pathologic changes have been reported in a variety of hemotrophic parasitic diseases. The pulmonary disease in the squirrel monkey appears analogous to human "malaria lung" and may serve as a useful model in understanding the pathophysiology of this unusual and fatal complication of falciparum malaria.

Renal changes in the fatal squirrel monkey infections included tubular nephrosis and mesangioproliferative glomerulonephritis. Initial ultrastructural studies have shown that the glomerular changes are similar to those described in human and Aotus monkey falciparum malaria.

Retinal hemorrhages were present in a single animal. This is an interesting finding which has been described in human cerebral malaria and claimed to be of prognostic significance. Additional ocular studies are warranted in the simian model as the pathogenesis of the retinal lesions is unknown.

The similarity noted in the pathology of P. falciparum in the human patient and the squirrel monkey suggests that this simian model will prove useful in characterizing and understanding the sequential changes in uncomplicated cases of acute malaria.

Acknowledgment

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Characterization of Candidate *Plasmodium falciparum* Asexual Blood-Stage Antigens Recognized by Monkey Immune Sera

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Based on the evidence that antibody response plays an important role in resistance to malaria (2), extensive work has been carried out in various laboratories with polyspecific sera to identify and characterize *Plasmodium falciparum* antigens ever since an *in vitro* culture method of parasite became available (20). Some workers use human sera obtained from malaria patients and from people living in endemic areas (1, 5, 6, 7, 12, 15, 18). Others used either sera from *Aotus* monkeys (8,11,13,21) or from *Saimiri* monkeys (3, 4, 10, 21).

The *Aotus* sera used in our laboratory were from experimentally vaccinated monkeys (19). Serum samples from protected and unprotected monkeys were analyzed by immunoassays and immunoprecipitations of metabolically labeled parasite proteins. SDS-PAGE patterns of immunoprecipitates showed that sera from protected monkeys had a higher reactivity to all the identified protein antigens than those from unprotected monkeys (11). However when differences in antibody titer between protected and unprotected monkeys were negated by applying equal numbers of immunoprecipitated DPM, there was no evidence of a unique antibody response to an individual parsite protein in protected monkeys (Fig. 1). Twenty-one SDS-PAGE bands of polypeptide antigens, M_r ranged 180K-30K (designated *a* through *u*) were identified.

Synchronized cultures obtained by treatment with colchicine (9) were metabolically labeled with ^{3}H-isoleucine and labeled parsite proteins from various blood stages were immunoprecipitated with *Aotus* sera from protected monkeys. Fig. 2 shows that bands *a* through *g* (M_r 180K-102K) occur mainly at the schizont stage. Since the antigen we used in vaccination experiments was schizont-stage enriched, we concentrated our efforts mainly on such high M_r protein antigens, i.e. *a* (M_r = 180K), b (M_r = 152K), *c* (M_r = 143K) and *d* (M_r = 132K).

^{3}H-isoleucine labeled *P. falciparum* was released from red blood cells by saponin lysis, sonicated and separated into particulate and soluble fractions by ultracentrifugation. Fig. 3 shows SDS-PAGE patterns of the two fractions solubilized and immunoprecipitated by an *Aotus* immune serum. High M_r bands *a* (180K), *b* (152K), *c* (143K) and *d* (132K) are mainly found in the particulate fraction (A), whereas bands *g* (102K), l (75K) and *o* (53K) are relatively strong in the soluble fraction (B).

The SDS-PAGE pattern of immunoprecipitates of 14C-glucosamine labeled and solubilized *P. falciparum* extract shows that band *a* (180K) is heavily glycosylated (Fig. 4). In addition, bands *d* (132K), *j* (83K), *k* (77K), *n* (57K) and *p* (47K) are lightly glycosylated. Similar findings using ^{3}H-glucosamine were reported by other workers (8, 12, 14).

Since antigen bands *a*, *b*, *c* and *d* occur in schizont stage, and since most of them are presumably membrane-bound and some are glycosylated, they are most likely to be the candidate antigens for protection. Thus we have directed our efforts on the purification of these bands for further studies.

One approach is to use preparative SDS-PAGE. IgG from pooled *Aotus* hyperimmune sera was isolated and immobilized onto CNBr-activated

Sepharose 4B beads. NP-40 extract of ^{35}S-methionine trace-labeled schizont-enriched P. falciparum cultures reacts with the affinity beads. The antigens eluted from the beads are separated by slab SDS-PAGE (Fig. 5). Strips of visualized antigen bands in acrylamide gel are excised, electrophoretically eluted and concentrated. Fig.6 shows the individual bands prepared. Dubois et al (3) and Perrin et al (16) have used the similar methods to isolate antigens for their vaccination experiments with Saimiri monkeys.

Another approach is to raise monoclonal antibodies (McAB) to these high M_r bands which may then be isolated by affinity chromatography, with the use of appropriate McAB's immobilized on Sepharose beads. The McAB's we have raised give three different IFA patterns (Fig. 7). One set of McAB's, with linear IFA pattern, represented by McAB 5.2, recognizes bands a (180K), b (152K) and minor bands e and j. Another set of McAB's, with dotted IFA pattern, represented by McAB 219.5, recognizes bands c (143K) and d (132K).

Fig. 8 shows that bands a and b are depleted after passage of labeled P. falciparum lysate through McAB 5.2 affinity column; and c and d, likewise, are depleted after passage through McAB 219.5 affinity column. This experiment indicates the co-identity of bands a, b, c and d recognized by Aotus immune serum and monoclonal antibodies. McAB 5.2 immunoprecipitates a heavily glycosylated 180K band, whereas McAB 219.5 fails to immunoprecipitate a glycosylated antigen (Fig. 9).

Since antigens in SDS-PAGE bands a through d are of such high M_r, we would like to find out if they are composed of subunits linked by disulphide bonds. Diagonal SDS-PAGE has been carried out with immunoprecipitates from ^{35}S-methionine labeled lysate by both McAB's 5.2 and 219.5 and Aotus immune serum. It can be seen from both McAB and Aotus serum autoradiograms (Fig. 10) that no distinct spots fall below bands a through d. That such high M_r polypeptides are not formed by disulphide-bond-linked subunits are rather surprising.

Thus methods of isolating individual antigens recognized by protected Aotus serum have been worked out. By using McAB immunosorbants (McAB's 5.2 and 219.5), we have isolated these high molecular weight antigens for immunogenecity studies. That part of our work will be presented by Dr. W.A. Siddiqui this afternoon.

REFERENCES:

1. Brown, G.V. et al. Nature, 297 591, 1982.
2. Cohen, S. et al. Nature, 192 733, 1961.
3. Dubois, P. et al. Proc. Natl. Acad. Sci. 81 229, 1984.
4. Gysin, J. et al. Parasite Immunol. 4 421, 1982.
5. Heidrich, Hans-G. et al. Parasitenkd. 69 715, 1983.
6. Holder, A.A. and Freeman, R.R. J. Exp. Med. 156 1528, 1982.
7. Holder, A.A. and Freeman, R.R. J. Exp. Med. 160 624, 1984.
8. Howard, R.F. and Reese, R.T. Mol. Biochem. Parasit. 10 319, 1984.
9. Hui, G.S.N. et al. Am. J. Trop. Med. Hyg. 32 1451, 1983.
10. Jendoubi, M. et al. J. Immunol. 134 1941, 1985.
11. Kan, S.C. et al. Infection & Immunity 43 276, 1984.
12. Kilijian, A. Proc. Natl. Acad. Sci. 77 3695, 1980.
13. Leech, J.H. et al. J. Exp. Med. 159 1567, 1984.
14. Perkins, M. Mol. Biochem. Parasit. 5 55, 1982.
15. Perrin, L.H. et al. Trans. Roy. Soc. Trop. Med. Hyg. 75 163, 1981.
16. Perrin, L.H. et al. J. Exp. Med. 160 441, 1984.
17. Perrin, L.H. et al. Clin. Exp. Immunol. 56 67, 1984.
18. Reese, R.T. et al. Am. J. Trop. Med. Hyg. 30 1168, 1981.
19. Siddiqui, W.A. et al. Nature, 289 64, 1981.
20. Trager, W. & Jensen, J.B. Science, 193 673, 1976.
21. Yamaga, K.M. et al. Exp. Parasit. 58 138, 1984.

TUESDAY, April 23, 1985
Abstract No. 20
Karen Yamage

CANDIDATE ANTIGENS

Asexual Blood stage
Discussant

See Report by Rapporteurs Mitchell and Tam

TUESDAY, April 23, 1985
Abstract No. 22, page 32
Ruth S. Nussenzweig

Characterization of Sporozoite Antigens and
Evidence for Their Role in Protective Immunity
Tuesday, April 23, 1985
Paper No. 22

* WORKING PAPER WILL BE INCLUDED IN FINAL PUBLICATION*

IMMUNISATION OF EXPERIMENTAL MONKEYS WITH DEFINED SCHIZONT AND MEROZOITE-SPECIFIC POLYPEPTIDES OF *P. FALCIPARUM*

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Introduction

The development of acquired immunity to malaria in man depends on the frequency and duration of exposure to the parasite but sterile immunity is probably never achieved (1). To develop a malaria vaccine against asexual blood stages therefore seems to be a difficult task and, in the past, few people considered vaccination against malaria to be a reasonable objective. Pioneer experiments by several groups in the late '70s demonstrated, however, that immunisation of experimental monkeys with *P. falciparum* schizonts and merozoites may lead to a better degree of protection than that induced by natural infection (2-5). Despite the development of *in vitro* culture of *P. falciparum* asexual blood stages (6), this approach is inapplicable for large-scale vaccination in man.

Recent investigations have focused on the identification of *P. falciparum* antigens exposed at the surface of red blood cells containing schizonts and at the surface of merozoites, the invasive form of the asexual cycle. Such antigens are obvious candidates for the development of malaria vaccines based on asexual blood stages. One essential step in malaria vaccine development is the purification of defined antigens of the asexual blood stages followed by evaluation of the ability of these antigens to raise a protective immune response. The evaluation of the potential value of purified antigens can be carried out *in vitro*, either indirectly by checking the biological activity of antisera directed against the malaria antigens of interest, or directly using the antigens themselves in a variety of *in vitro* assays, e.g., the assay for inhibition of reinvasion and assays for measurement of cell-mediated responses. In a more direct approach, selected, defined antigens may also be tested for their capacity to elicit a protective immune response *in vivo* by immunising and challenging with asexual blood stages, monkeys susceptible to *P. falciparum* infection. In this short review, we will concentrate on this latter approach.

Criteria for the selection of "protective" antigens

The selection of P. falciparum antigens for experimental immunisation can be based on the following criteria:

a) synthesis of antigens late in the asexual cycle at the time of schizogony;

b) exposure of the polypeptides at the surface of erythrocytes containing schizonts and/or at the merozoite surface;

c) preferential recognition of these antigens by monkey sera which transfer protective immunity in naive recipients, or by sera of human individuals with a high degree of resistance to malaria;

d) monoclonal antibodies directed against these antigens inhibit the growth of P. falciparum asexual blood stages in vitro, inhibit binding of merozoites to erythrocytes, inhibit the binding of erythrocytes containing schizonts to endothelial cells;

e) involvement of these antigens in merozoite-erythrocyte interaction.

Several antigens or polypeptides meet some of these criteria (as mentioned in previous presentations on antigens of the asexual blood stages) but only four of them have been used for experimental immunisation:

1. 185-200 kd polypeptide which fulfills the following criteria: a, b (for a processed product of 83 kd), (c±), (d±), e? (7,8,9,10).
2. 140 kd polypeptide: a, (b±), c, d, e (7,8,11).
3. 96-100 kd polypeptide: (a±), c (12).
4. 41 kd polypeptide: a, b, (c±), d, e? (8,10).

Purification of these polypeptides

These four polypeptides have been purified by affinity chromatography (13,14) and/or electroelution from SDS PAGE strips identified by autoradiography. In the experiments concerning the 41 kd polypeptide, two preparations have been used, one purified by affinity chromatography (native 41 kd) and a second one which was purified by affinity chromatography

followed by electroelution from SDS PAGE (SDS 41 kd).

Immunisation and experimental protocol

Groups of four Saimiri monkeys, intact for the 200 kd, 140 kd and 41 kd and splenectomised for the 96-100 kd, were immunised at 2-3 week intervals with low amounts of purified polypeptides (less than 100 ug/injection) emulsified in Freund's complete adjuvant for the first immunisation and Freund's incomplete adjuvant for the others. Intact or splenectomised control animals were injected with NaCl or SDS mixed with adjuvants according to the same schedule. All monkeys were challenged by an intravenous injection of 5 $x10^7$ parasitised monkey erythrocytes (Palo Alto, FUP strain of P. falciparum). The course of parasitemia in individual monkeys was scored on Giemsa-stained blood smears. Immunofluorescence assay was carried out on acetone-fixed smears of schizont-rich preparations of various isolates of P. falciparum (13). Immunoprecipitation analysis of (^{35}S)-methionine-labelled P. falciparum culture by antisera of immunised monkeys was performed as indicated in reference 14.

Course of parasitemia

Nine out of 10 splenectomised monkeys and 6 out of 8 intact control animals showed the typical acute course of parasitemia which required antimalarial therapy to avoid death (treatment when parasitemia is higher than 20%). In all the immunised monkeys a delay in the appearance of patent parasitemia was observed; all these monkeys had at least a low level of parasitemia, and they all recovered spontaneously without antimalarial therapy. Peak parasitemia observed in the immunised monkeys is reported in Table 1.

Reactivity of the monkey sera

The humoral response of the control and immunised sera was tested by indirect immunofluorescence and immunoprecipitation. The antibody titers and specificity of the sera collected before the challenge infection

are reported in Table 2. The same sera were analysed for their capacity to precipitate (^{35}S)-labelled P. falciparum polypeptides (Table 3).

Synthetic peptides as immunogens

We have recently cloned a fragment of the gene coding for the 185-200 kd polypeptide and determined the DNA and the corresponding amino acid sequences of the inserted gene (Table 4) (15). Rabbits immunised with lysate of E. coli containing the plasmid coding for and expressing a portion of the 200 kd polypeptide, imunoprecipitated (^{35}S)-labelled P. falciparum polypeptides of 200, 155 and 83 kd. The same sera reacted with an 83 kd band of merozoites as shown by Western blot analysis (15) and with the surface of merozoites using indirect immunofluorescence. Three synthetic polypeptides were derived from the sequence presented in Table 4, coupled with tetanus toxoid by the glutaraldehyde method (ratio 1/10) and rabbits were immunised with these various reagents. All rabbits had the same type of reactivity as the rabbits immunised with the E. coli lysate (unpublished data), suggesting that the three peptides are present in the 83 kd polypeptide which is one of the main components exposed at the merozoite surface (16). The 43 aa peptide coupled to tetanus toxoid was used to immunise four Saimiri monkeys (3 x 1.5 mg/monkey in Freund's complete adjuvant the first time and subsequently with Freund's incomplete adjuvant). Four control monkeys were immunised according to the same schedule with glutaraldehyde-treated tetanus toxoid. All the control monkeys and one immunised monkey required antimalarial treatment when their parasitemia exceeded 20%. All the immunised monkeys presented a delay in the appearance of patent parasitemia and the peak parasitemia for the three monkeys who recovered spontaneously was 19%, 9.2% and 3.4% respectively. The prechallenge sera of the immunised monkeys had low antibody titres as measured by indirect fluorescence (between 1/50 and 1/200) and relatively good antibody titres against the purified peptide produced by the plasmid-

containing part of the gene for the 200 kd polypeptide, as measured by solid-phase ELISA test (1/5000 - 1/10000).

Discussion

The results reported here present several interesting features. (a) Immunisation with defined polypeptides of asexual blood stages induced a degree of protection similar to that induced by immunisation with intact merozoites and/or schizonts. (b) Protective immunity in the experiments reported here is not isolate-specific. It is possible, however, that better results may have been achieved by using polypeptides purified from the isolate used for challenge. (c) The question of relationship between antibody titres and protective immunity is still open. Rôle of cell-mediated mechanisms? (d) Experiments conducted with the 41 kd polypeptide suggest that the conformation of the polypeptides used for immunisation is an important factor. This may be of special relevance to the use as immunogens of peptides produced by DNA recombinant technology. (e) Additional experiments mainly in vitro or in cheap laboratory animals are necessary to check the potential of synthetic polypeptides. These experiments pose more questions than they resolve: (a) Did we select the right candidates? (b) Is it possible to obtain sterile immunity by immunisation? In the experiments reported here, we use a low amount of antigens diluted in a relatively large volume. In addition, antigenic diversity among isolates of P. falciparum may seriously limit the efficiency of immunisation. This may be of importance if the variant epitopes are those exposed at the surface of the parasites. (c) Can we improve the efficacy of immunisation by using a mixture of several putative "protective"antigens as immunogen? (d) Are Saimiri and Aotus monkeys good animal models for the testing of malaria vaccines? (e) Freund's adjuvant is not accceptable for human use. What is the best alternative? Other adjuvants? Insertion and expression of malaria genes in virus vectors?

REFERENCES

1. Miller, M.J., Observations on the natural history of malaria in the semi-resistant West African. Trans. R. Soc. trop. Med. Hyg. 52, 152 (1958).

2. Siddiqui, W.A., An effective immunization of experimental monkeys against a human malaria parasite Plasmodium falciparum. Science 197: 388-389 (1977).

3. Reese, R.T., Trager, W., Jensen, J.B., Miller, D.A. and Tantravaki, R., Immunization against malaria with antigen from Plasmodium falciparum cultivated in vitro. Proc. Natl. Acad. Sci. USA 75: 5665-5669 (1978).

4. Mitchell, G.H., Butcher, G.A., Richards, W.H.G. and Cohen, S., Merozoite vaccination of Douroucouli monkeys against falciparum malaria. Lancet I: 1335-1342 (1977).

5. Trager, W., Lanner, H.N., Stanley, H.A. and Langreth, S.G., Immunisation of owl monkey to Plasmodium falciparum with merozoites from cultures of a knobless clone. Parasite Immunol. (Oxf.) 5: 225-232 (1983).

6. Trager, W. and Jensen, J.B., Human malaria parasites in continuous culture. Science 193, 673-676 (1976).

7. Kilejian, A., Stage specific proteins and glycoproteins of Plasmodium falciparum : identification of antigens unique to schizonts and merozoites. Proc. Natl. Acad. Sci. USA 77 : 3695-3699 (1980).

8. Perrin, L.H. and Dayal, R., Immunity to asexual erythrocytic stages of Plasmodium falciparum: role of defined antigens in the humoral response. Immunol. Rev. 61: 245-270 (1982).

9. Holder, A.A. and Freeman, R.R., Biosynthesis of a Plasmodium falciparum schizont antigen recognized by immune serum and a monoclonal antibody. J. Exp. Med. 156: 1528-1538 (1982).

10. Asexual blood stage and transmission-blocking antigens of Plasmodia. Report of the Sixth Meeting of the Scientific Working Group on the Immunology of Malaria, Document TDR/IMMAL/SWG (6)/84.3, WHO, Geneva.

11. Jungery, M., Pasvol, G., Newbold, C.I. and Weatherall, D.J., A lectin-like receptor is involved in the invasion of red cells by Plasmodium falciparum. Proc. Nat. Acad. Sci. USA 80: 1018-1022 (1983).

12. Gysin, J., Dubois, P. and Pereira da Silva, L., Protective antibodies against erythrocytic stages of Plasmodium falciparum in the experimental infection of the squirel monkey (Saimiri sciureus). Parasite Immunol. 4, 421 (1982).

13. Dubois, P., Dedet, J.P., Fandeur, T., Rousilhon, C., Jendoubi, M., Pauillac, S., Mercereau-Puijalon, O. and Pereira da Silva, L., Protective immunization of the squirrel monkeys against asexual blood stages of Plasmodium falciparum by use of parasite protein fractions. Proc. Nat. Acad. Sci. USA 81: 229-237 (1984).

14. Perrin, L.H., Merkli, B., Loche, M., Chizzolini, C., Smart, J., and Richle, R., Antimalarial immunity in Saimiri monkeys: immunisation with surface components of asexual blood stages. J. Exp. Med. 160: 441- (1984).

15. Cheung, A., Shaw, A.R., Leban, H, and Perrin, L.H., Cloning and Expression in Escherichia coli of a surface antigen of Plasmodium falciparum merozoites, Embo J. in press. (1985).

16. Freeman, R.R. and Holder, A.A., Surface antigens of malaria merozoites. A high molecular weight precursor is processed to an 83 000 mol. wt. form expressed on the surface of Plasmodium falciparum merozoites. J.Exp. Med. 158: 1647-1653 (1983).

Table 1

Peak parasitemia observed in various groups of immunised monkeys*

Immunogens		Peak parasitemia (%)	Day
***200kd	1	13(intercurrent infection)	15
	2	6	13
	3	2	16
	4	2	16
***140kd	1	4	8
	2	2	15
	3	2	16
	4	1	11
96-100kd	1	19	10
	2	8	9
	3	6	13
	4	5	7
	5	5	14
***41kd native	1	1**	12
	2	0.2	34
	3	0.08	35
	4	0.05	28
***41kd SDS	1	13	14
	2	11	16
	3	3	16
	4	2	14

*9 over 10 splenectionised control monkeys had parasitemia higher than 25 % by day 8
6 over 8 intact control monkeys had parasitemia higher than 20 % by day 14, the two last monkeys had peak parasitemia of 11 and 12 % before recovery.

**Exclused following intercurrent infection unrelated to malaria infection.

***Purified from SGE2 isolate (Zaire), challenge with FUP Palo-Alto

Table 2

Prechallenge antibody titres measured by

indirect immunofluorescence.

****

Immunogens		Ab Titres Mear	Range	Specificity
200 kd		1/256	1/64 - 1/1024	Surface of schizonts Schizont diffusely Merozoite surface
140 kd		1/900	1/512 - 1/1024	Schizont diffusely Merozoite diffusely and spotts
96 - 100 kd		1/160	1/20 - 1/320	Parasitophorous vacuole
*41 kd	native	1/320	1/160 - 1/640	Schizont diffusely Merozoite: 2 spotts/merozoite
	SDS	1/320	1/160 - 1/640	

*By using a solid phase ELISA assay with plastic wells coated with either native or SDS 41 kd, it was found that both groups of monkeys have reciprocal Ab titres in the order of 1/5000 using wells coated with native 41 kd. On wells coated with 41 kd SDS the Ab titres increased up to 1/200'000 for monkeys immunised with the 41 kd SDS (Perrin et all in press 1985, J. Clin. Invest.)

Table 3

(35S)-Labeled *Plasmodium falciparum* polypeptides immunoprecipitated by prechallenge sera

****

Immunogens	MW of polypeptides precipitated (KD)
200	200, 155*, 83*, 31
140	140
96-100	110, 96-100, 90, 92
41	41 (105)

* Detected on western blott using a pool of sera of the four monkeys.

Table 4

Amino acid sequence of the peptide expresed
by the cDNA clone coding for part of the 200 kd.
****

1 15
Ser-Tyr-Gln-Glu-Leu-Val-Lys-Lys-Leu-Glu-Ala-Leu-Glu-Asp-Ala-

20 30
Val-Leu-Thr-Gly-Tyr-Ser-Leu-Phe-Gln-Lys-Glu-Lys-Met-Val-Leu-

43 45
Asn-Glu-Gly-Thr-Ser-Gly-Thr-Ala-Val-Thr-Thr-Ser-Thr-Pro-Gly-

46 60
Ser-Gly-Gly-Ser-Val-Thr-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Val-Ala-

75
Ser-Val-Ala-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Val-Ala-Ser-Gly-Gly-

79 90
Ser-Gly-Asn-Ser-Arg-Arg-Thr-Asn-Pro-Ser-Asp-Asn-Ser-Ser-Asp-

Ser-Asp-Als-Lys-Ser-

The amino acid sequence of the 3 synthetic peptides is as follow :

Peptide 1 : 43 aa no 1 - 43

Peptide 2 : 24 aa no 20 - 43

Peptide 3 : 34 aa no 46 - 79

The Induction of Protective Immune Responses in *Aotus* Monkeys

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Introduction

The successful vaccination of Aotus trivirgatus griseimembra (karyotype II and III) monkeys against a lethal challenge of the human malaria parasite P. falciparum (1,2) renewed hope that a human malaria vaccine might someday be a reality. The continuous cultivation of this parasite in vitro (3) signified to parasitologists that the means was at hand for the analysis of parasite antigens, and the advent of the new biotechnology promised a method for large scale vaccine production. But none of these developments have altered what malariologists have known for the last half century. In hyperendemic malarious areas, the aquisition of acquired immunity to P. falciparum requires years of repeated infections before clinical infection is reduced or minimized, and even then, the induced protective responses are neither long-lasting nor absolute. From the outset, it may safe to adopt a conservative approach, and expect that while the neutralization of a single epitope may be sufficient for vaccination against viruses, a great deal more will be required for the complex, well-adapted malaria parasites.

On the Role of Adjuvants

While the early vaccination studies in Aotus monkeys are now well known, the need for powerful immunopotentiating agents may be less well-appreciated. This is understandable since experiments defining the role of adjuvants are precluded by the scarcity of these animals. However, the need for anything less than a powerful adjuvant to ensure successful vaccination has never been dismissed, and as malariologists advance into new arenas with defined and synthetic antigens, this requirement should not be overlooked. The use of synthetic peptides (where carrier epitopes are scarce), and the growing evidence of repeat sequences (probably poorly immunogenic), cautions against use of anything less than powerful adjuvants. The not so obvious message from our past experience in vaccination of Aotus monkeys is that induction of protective immune responses requires strong adjuvants (Table I). In Aotus trivirgatus griseimembra (karyotype II and III), our best monkey model, the percent parasitemia often rises above 80 percent culminating in death; whereas, monkeys twice injected with intact, freshly passed K+ parasites develop parasitemias of less than a few percent recover thereafter.

Table I further reveals that successful experiments may correlate with the use of short-term, in vivo monkey-derived parasites. This may be because of antigenic (4) diversity and our use of uncloned isolates and the selection of parasites that may occur during long-term in vitro cultivation (5). In the studies of the ability of two sets of monoclonal antibody (McAb) defined of P. falciparum antigens to be described for the first time in this paper, we have used a conservative protocol in which the antigen was given not twice, but three times, each in Freund's complete adjuvant. Moreover, the antigen was from parasites never kept over eight weeks in continuous culture.

TABLE I
VACCINATION OF *AOTUS* MONKEYS WITH *PLASMODIUM FALCIPARUM* AND THE ROLE OF ADJUVANTS

ADJUVANTS	PROTECTION	REFERENCES
FCA, FCA	3/3	Siddiqui, W. (1977)
FCA, FCA	4/4	Mitchell, G. et al (1977)
FCA, FCA	2/2	Siddiqui et al (1981)
s-MDP (liposomes, 2x)	6/6	Siddiqui, W. et al (1978) Siddiqui, W. et al (1981)
CP-20,961 (intralip,2x)	4/4	Siddiqui et al (1981)
B-30-MDP (liposomes)	5/6	Siddiqui et al (1981)
MDP (FIA, 2x)	4/6	Trager et al (1983)*
MDP (2x)	1/3	Siddiqui, W. et al (1978)
MDP (FIA)	1/3	Reese, R. et al (1978)*
FCA, FIA	0/3	Reese, R. et al (1978)*

*All or some of the *Aotus* were immunized with antigens from long-term culture parasites.

Indirect Evidence of Protection

A criterion used to identify protective antigens is preferential reaction with sera from monkeys immune to parasite challenge. This approach, used with immune *Saimiri* and *Aotus* monkeys (through parasite-infection and drug-cure), has identified antigens associated with the parasitophorous vacuole (6) and a strain-specific antigen on the surface of infected erythrocytes (7), among others. In our laboratory, sera from vaccinated and protected *Aotus* monkeys immunoprecipitated minimally 21 metabolically labeled *P. falciparum* antigens (8), but because these animals were immunized multiply with Freund's complete adjuvant (FCA) it could not be decided which of these were candidate antigens. Similar to other groups, we have screened these *Aotus*-defined antigens for schizont-synthesized, membrane-associated, glycosylated surface polypeptides on the rationale that the candidate protective antigens should be anchored to the merozoite membrane and are exposed on the outside surface to protective immune mechanisms.

The most prominent antigen meeting these requirements was the high molecular weight schizont antigen (HMWSA). In the past, in vitro growth inhibition by McAb has weighed heavily in identification of candidate antigens. Is it paradoxical that McAb's which demonstrate in vitro inhibition have precisely the opposite characteristics? In our laboratory, McAb 219.5 gives a spot immunofluorescence pattern and appears to identify antigens associated with intracellular organelles. The 143K and 132K polypeptides are weakly membrane-associated and

non-glycosylated (9), while the HMWSA, so well meeting the surface membrane criteria, gives only weak inhibition in the presence of McAb (Table II). Or does this indirect test inappropriately weigh against protective cell-mediated immune responses (10), which are so efficiently potentiated by FCA? Are the malaria surface coats, whether sporozoite or merozoite, attacked by cell-mediated immune responses rather than humoral antibodies?

TABLE II
IN VITRO GROWTH INHIBITION BY SELECTED MONOCLONAL ANTIBODIES

mcAb	M_r	IFA Pattern	Inhibition	Reference
5.2	185K,152K,87K	surface	23[a]	(11)
219.5[b]	143K,132K	spot	75[a]	(11)
Mb31c12	41K	spot	>95[c]	(13,14)

[a]Percent inhibition at 1.5 mg ml^{-1}.
[b]Apparently similar to mcAb 61.3 (15).
[c]Percent inhibition at less than 16 ug ml^{-1}.

Direct Evidence of Protection

Despite the growing number of candidate antigens, only four have been directly tested in monkeys. None of these have been in *Aotus* monkeys. In each case, a highly adapted Uganda-Palo Alto (FUP) isolate was tested in splenectomized *Saimiri* monkeys (Table III). Except for the evidence to be presented in this review, no defined *P. falciparum* antigen or combination of antigens have been shown to induce protective immune responses in the *Aotus* monkey.

TABLE III
DIRECT EVIDENCE OF PROTECTION IN MONKEYS

M_r	Description	Protective Responses		Ref.
		Saimiri	*Aotus*	
185K-200K	HMWSA	+	+[a]	(16,17,11)
140K	mer/rhoptry	+	nd	(17)
143K, 132K	rhoptry-1	nd	+[a]	(11)
41K	rhoptry-2	+	nd	(18)
90K	PV	+	nd	(19)

[a]*Aotus trivirgatus azure* (karyotype VI); this paper.

Protective Immune Responses to Polypeptides Identified by McAb's 5.2 and 219.5 in Aotus Monkeys

In our laboratory, mcAb 5.2 identifies the glycosylated major high molecular weight schizont antigen and immunoprecipitates polypeptides having M_r 185K, 152K, 121K and 87K, while McAb 219.5 identifies antigens giving a punctate IFA pattern and immunoprecipitates non-glycosylated polypeptides having M_r 143K, 132K and 102K (variable). Together, the two mcAb co-identify and immunoprecipitate 33 and 13 per cent, respectively, of the total polypeptides immunoprecipitated by immune sera from protected Aotus monkeys (9).

To test the immunogenicity of these McAb-isolated polypeptides, three Aotus trivirgatus azure (karyotype VI) monkeys were immunized and the immune responses compared. Aotus 381 received 80 ug Ag5.2 combined with 30 ug Ag219.5 emulsified in FCA on days 0, 21 and 42. Aotus 391 and A390 received 240 ug immune Aotus IgG affinity-purified polypeptides and 1400 ug unpurified FUP antigen each time, respectively. By solid phase RIA, the 14d tertiary antibody titers were >31250, >31250, and <156250, respectively, for A381, A391 and A390. By SDS-PAGE, the tertiary sera of A381 immunoprecipitated polypeptides having M_r 185K, 152K (doublet), 141K, 132K, 121K and 87K. By IFA the punctate IFA pattern expected for two of the polypeptides was obscured by heavy schizont and merozoite staining of the HMWSA.

Since high titered antibody responses to the respective polypeptides had been evoked, the monkeys, along with an untreated control, A385, were given 1 x 10^6 freshly passed FUP K^+ and the course of infection monitored. As expected from previous studies, the unimmunized control A385 developed the 2-4 per cent parasitemia characteristic of FUP in karyotype VI Aotus monkeys. However, all immunized monkeys failed to reveal even a single parasite, scanning over 10,000 erythrocytes in thick blood films (Table IV) daily for 28 days.

These encouraging results are the first indication that McAb defined polypeptides are capable of inducing protective immune responses in Aotus monkeys. They appear to warrant a full vaccination experiment testing the efficacy of these polypeptides in Aotus trivirgatus griseimembra (karyotype II and III). This conservative approach, in which protective immune responses were first evaluated in karyotype VI monkeys, was mandated by the scarcity of karyotype II and III monkeys.

Discussion

It is the view from the past which has guided much of these findings. Was it necessary to ravage the monkeys three times with the polypeptides in FCA, even though the amount of Mycobacterium was successively halved? Would secondary and tertiary injections in FIA have been just as effective? Would the high molecular weight schizont antigen (Ag5.2) or the

TABLE IV
COURSE OF PARASITEMIA IN AOTUS TRIVIRGATUS AZURE[a] MONKEYS IMMUNIZED WITH DEFINED POLYPEPTIDES

	Percent Parasitemia						
Day	A360	A386	A371	A385	A381	A391	A390
1	neg	neg	neg	neg	neg	neg	neg
2	0.4	neg	neg	neg	neg	neg	neg
3	1.7	neg	neg	neg	neg	neg	neg
4	3.3	neg	neg	0.1	neg	neg	neg
5	2.6	0.1	0.1	0.1	neg	neg	neg
6	3.0	0.1	0.1	0.8	neg	neg	neg
7	3.9	0.7	0.1	0.9	neg	neg	neg
8	2.2	1.3	0.3	1.3	neg	neg	neg
9	1.8	3.8	1.0	1.9	neg	neg	neg
10	1.6	1.8	1.6	1.4	neg	neg	neg
11	1.7	0.8	1.7	1.5	neg	neg	neg
12	3.2	0.9	3.3	0.9	neg	neg	neg
13	1.6	0.7	2.4	2.2	neg	neg	neg
14	1.1	1.3	1.5	0.4	neg	neg	neg
15	0.5	0.4	2.7	0.4	neg	neg	neg
16	0.1	0.1	1.5	0.1	neg	neg	neg
17	neg	neg	0.9	neg	neg	neg	neg
18	neg	neg	0.5	neg	neg	neg	neg
19	neg	neg	0.3	neg	neg	neg	neg
20	neg	neg	neg	neg	neg	neg	neg

[a]Karyotype VI. Data for A360, A386, A371 were from experiments in previous years defining the course of infection of FUP K+ in Aotus of this karyotype.

high molecular weight rhoptry antigen (Ag219.5) been just as effective given alone, rather than in combination?
A conservative philosophy has merit until demonstrated otherwise: a) powerful adjuvants may be required; b) when clones are not used, antigens must be derived from short-term, in vivo-monkey derived cultures; c) protective immune responses, possibly cell-mediated as well as humoral, may be directed at multiple antigens, rather than a single key antigen or epitope.

References

1. Siddiqui, W.A. 1977. An effective immunization of experimental monkeys against a human malaria parasite Plasmodium falciparum. Science 197, 388-89.

2. Mitchell, G.H., G.A. Butcher, W.H.G. Richards and S. Cohen. 1977. Merozoite vaccination of Douroucouli monkeys against falciparum malaria. Lancet I: 1335-42.

3. Trager, W. and J.B. Jensen. 1976. Human malaria parasites in continuous culture. Science 193, 673-76.

4. McBride, J.S., C.I. Newbold and R. Anand. 1985. Polymorphism of a high molecular weight schizont antigen of the human malaria parasite Plasmodium falciparum. J. Exp. Med. 161, 160-180.

5. Yamaga, K.M., S.C. Kan, K.J. Kramer and W.A. Siddiqui. 1984. Plasmodium falciparum: comparative analysis of antigens from continuous in vitro culture and in vivo derived malaria parasites. Exp. Parasitol. 58, 138-46.

6. Gysin, J, F. Dubois and L. Pereira da Silva. 1982. Protective antibodies against erythrocytic stages of Plasmodium falciparum in experimental infection of the squirrel monkey, Saimiri sciureus. Parasite Immunol. 4, 421-30.

7. Leech, J.H., J.W. Barnwell, L.H. Miller and R.J. Howard. 1984. Identification of a strain-specific malarial antigen exposed on the surface of Plasmodium falciparum-infected erythrocytes. J. Exp. Med. 159, 1567-75.

8. Kan, S.C., K.M. Yamaga, K.J. Kramer, S.E. Case and W.A. Siddiqui. 1984. Plasmodium falciparum: protein antigens identified by analysis of serum samples from vaccinated Aotus monkeys. Infect. Immunity 43, 276-82.

9. Kan, S.C., K.M. Yamaga, K.J. Kramer, L.Q. Tam, S.E. Case and W.A. Siddiqui. 1985. Characterization of candidate Plasmodium falciparum antigens recognized by monkey immune sera. These proceedings.

10. Freeman, R.R. and A.A. Holder. 1983. Characteristics of the protective response of BALB/c mice immunized with a purified Plasmodium yoelii schizont antigen. Clin. exp. Immunol. 54, 609-616.

11. Siddiqui, W.A., et al. 1985. In preparation.

12. Perrin, L.H., E. Ramirex, L. Er-Hsiang and P.H. Lambert. 1980. Plasmodium falciparum: characterization of defined antigens by monoclonal antibodies. Clin. exp. Immunol. 41, 91-96.

13. Perrin, L.H., E. Ramirez, P.H. Lambert, and P.A. Miescher. 1981. Inhibition of P. falciparum growth in human erythrocytes by monoclonal antibodies. Nature 289, 301-303.

14. Perrin, L. H. and R. Dayal. 1982. Immunity to asexual erythrocytic stages of Plasmodium falciparum: role of defined antigens in the humoral response. Immunol. Rev. 61, 245-69.

15. Freeman, R.R. and A.A. Holder. 1985. Schizont/merozoite antigens of P. falciparum. These proceedings.

16. Hall, R., J.E. Hyde, M. Goman, D.L. Simmons, I.A. Hope, M. Mackay, J. Scaife, B. Merkli, R. Richle and J. Stocker. 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature 311, 379-382.

17. Perrin, L.H., B. Merkli, M. Loche, C. Chizzolini, J. Smart and R. Richle. 1984. Antimalarial immunity in saimiri monkeys. Immunization with surface components of asexual blood stages. J. Exp. Med. 160, 441-451.

18. Perrin, L. et al. 1985. J. Clin. Invest. in press.

19. Jendoubi, M., P. Dubois, L. Pereira da Silva. 1985. Characterization of one polypeptide antigen potentially related to protective immunity against the blood infection by Plasmodium falciparum in the squirrel monkeys. J. Immunol. 134, 1941-45.

NON-HUMAN PRIMATE MODELS FOR TESTING MALARIAL VACCINES

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With the adaptation of the human malarias to development in monkeys almost 20 years ago, it was recognized that a new system was available for the testing of anti-malarial drugs and possibly, for the development and testing of anti-malarial vaccines. However, although there has been considerable use of monkeys in the development and testing of drugs, there has been only limited testing of vaccines. With the rapid identification of the proteins which may be responsible for protection, these animal model systems may well play an important role in the development of vaccines for the control of malaria.

There are two basic approaches to the use of monkey models for the development of vaccines. The first is the use of parasites which are naturally infective to monkeys. These are biologically similar to human malarias and provide a natural host-parasite relationship. The second approach is to use human malaria parasites growing in certain species of susceptible monkeys. Here, immunogens specific to the human malarias can be examined. The investigator must realize, however, that monkeys are not natural hosts of human malaria and biologically, infections with human plasmodia in these animals are very dissimilar from that which occurs in humans. However, the use of such an animal model can provide important information regarding the protective effect of a vaccine and the immunogenicity/toxicity of the antigen involved.

We will present our proposals for the testing of both sporozoite and blood-stage vaccines in the different model systems as they are presently available.

SPOROZOITE VACCINES

Immunization against the sporozoite stages has the potential for prevention of penetration of the liver by normally infective sporozoites. Therefore, with an effective anti-sporozoite vaccine, complete protection of the host from any malarial infection either in the form of exoerythrocytic development in the liver or from subsequent infection of the red blood cells would be expected. Once the barrier had been breeched, however, protection against blood stage development would not be expected. Thus, trials which test the efficacy of these vaccines, should be straight-forward. An animal which has been immunized with an effective anti-sporozoite vaccine will fail to develop any infection. Those animals not protected will.

MONKEY MALARIAS:

Two species of monkey malaria, *Plasmodium knowlesi* and *P. cynomolgi* are the most likely candidates for the testing of sporozoite vaccines. Rhesus monkeys, *Macaca mulatta*, inoculated with adequate numbers of sporozoites can be expected to develop detectable parasitemias within a predictable time frame. With *P. knowlesi*, the prepatent period is between 5 and 9 days; with *P. cynomolgi*, it is between 7 and 15 days. Standardization in these studies has been based on the number of bites from a fixed number of heavily infected

mosquitoes. In our studies, we have used the bites of 4 or 5 mosquitoes; for the testing of antimalarial drugs in humans, the standard has been the bites of 10 heavily infected mosquitoes. An alternative method is the intravenous injection of known numbers of sporozoites from dissected salivary glands. From 10,000 to 20,000 sporozoites are usually inoculated.

The experimental animals (M. mulatta) needed for such trials with the monkey malarias are readily available. However, the housing requirements for these relatively large animals coupled with the current initial cost of from $600 to $1,000 per animal make these studies very expensive.

One major requirement in sporozoite vaccine trials is a ready supply of sporozoites to challenge the animals. Fortunately, both P. knowlesi and P. cynomolgi readily infect certain species of laboratory reared mosquitoes. In addition, the salivary glands normally contain massive numbers of sporozoites; it is therefore not difficult to obtain millions of sporozoites for challenge of large numbers of animals.

Since the peptide sequence of the P. knowlesi CSP has been identified, this parasite would appear to be a prime candidate for vaccine studies. Plasmodium cynomolgi would also appear to be of major importance since it is a relapsing malaria (as are the human malaria parasites P. vivax and P. ovale), and could be used in studies on the effect of immunization on the resting stages and the effect on relapse potential.

The proposed testing procedure for the sporozoite vaccines of the monkey malarias would be 1) to immunize intact M. mulatta monkeys twice using an effective adjuvant, 2) to challenge the animals either by mosquito bite or intravenous inoculation of sporozoites from dissected salivary glands at various time periods after immunization, and 3) to determine the antibody responses by various techniques to relate vaccine efficacy with one or more serologic results. It is considered to be very important to examine and select from one or more of the various serologic techniques available, the one(s) which can correlate best with protection.

HUMAN MALARIAS

The testing of sporozoite vaccines against the human malarias using monkeys is more difficult. A relatively few animals have been successfully infected via sporozoite inoculation. In fact, only the Colombian Aotus (karyotypes II, III, and IV) have been tested in sufficient numbers to establish a statistically valid base for the conduct of these studies. In addition, only the Santa Lucia strain of P. falciparum has been transmitted with enough frequency to establish transmission rates. In this model system, 25% of intact and 85% of splenectomized monkeys inoculated with relatively large numbers of sporozoites have subsequently developed patent parasitemias. Transmission rates in other parasite - monkey model systems have either been considerably lower or the attempts for drawing a sound conclusion are too few in number.

With the Colombian Aotus, the results of transmission studies with P. vivax are similar to those of P. falciparum. Preliminary studies with a limited number of Bolivian Saimiri monkeys indicates that sporozoite transmissions to splenectomized animals may be relatively high, and this host

may have a considerable potential for the testing of sporozoite vaccines against P. vivax. Studies are presently being made with other types of South American monkeys to determine their potential for sporozoite vaccine trials. To date, the results of these studies have been discouraging.

Although animal model systems will play an important role in the testing of vaccines, there are additional differences between these model systems and human infections. In addition to a relatively low transmission rate, the prepatent periods are greatly extended over those seen in humans or with studies of the monkey malarias. Thus, vaccine trials may be lengthy, extending over several months. In addition, the infection of mosquitoes with the Santa Lucia strain of P. falciparum from culture has been very low; it has also been very low from Aotus monkeys other than those from Colombia. Thus, the already scarce Colombian animals are also required for the infection of mosquitoes for challenge. Another compounding difficulty is that the number of sporozoites in the salivary glands of mosquitoes infected with P. falciparum and P. vivax are usually much lower than seen is with the monkey malarias; thus, many more mosquitoes must be fed to obtain adequate numbers of sporozoites for the challenge studies.

The proposed testing procedure for sporozoite vaccines to the human malarias calls for the immunization of animals twice using adjuvants. For the challenge, it is anticipated that a minimum of 20,000 sporozoites obtained at dissection will be injected into each animal. Since intact animals have such a low rate of susceptibility to infection, animals will be splenectomized, but only after the sporozoites have been injected and the antibodies have been allowed to exert their effect. Splenectomy will be performed 6 or 7 days after inoculation. Thus, in a vaccine failure, merozoites released from the schizonts maturing in the liver are in a splenectomized host and more likely to produce a detectable blood-stage infection.

In our view, the development or absence of detectable parasitemia is the only significant criterion for protection afforded by a sporozoite vaccine. Again, antibody tests would be required to correlate, if at all possible, protection with a measurable immune response.

Since not all animals injected with sporozoites, even after splenectomy, would be expected to develop a patent parasitemia, the number of animals in each test group must be large enough to detect differences between a vaccinated and control group at an acceptable of statistical significance. Using the best model system available -- the Santa Lucia strain of P. falciparum and Colombian Aotus monkeys -- a minimum of 10 animals would be required per test group.

BLOOD STAGE VACCINES

MONKEY MALARIAS

Immunization against the blood stages of the monkey malarias has been attempted on numerous occasions over a number of years. These parasites have an advantage in that their infections are biologically similar to human malarial infections. Although there are 10 species of monkey malaria which could be used as models for vaccine studies, most of the effort has been with P. knowlesi, P. fragile, and P. cynomolgi in rhesus monkeys. Plasmodium knowlesi is a quotidian parasite which almost always produces a fulminating

infection; death occurs in approximately 97% of the infections. With this parasite - monkey model system, it is very easy to establish a criterion for vaccine effectiveness - the survival of the host. Plasmodium fragile is a malignant tertian parasite with many biologic similarities to P. falciparum. The infections are fulminating and death is the result with about 30% of the animals. If an animal survives, the infection is characterized by long term persistence with frequent recrudescences. Plasmodium cynomolgi is a benign tertian parasite with maximum parasitemias of between 5 and 10%. Death is not a common occurrence.

Susceptible animals (rhesus monkeys) are readily available and the course of the parasitemia is predictable within certain limits of time and parasite count. Each of these parasites has been used in previous vaccine trials with crude antigens using a number of different adjuvants. Characteristically, all animals develop a parasitemia which is modified to a varying degree by the type of immunization and the challenge. Adjuvants have been required for successful immunization with these crude antigen preparations.

The proposed testing procedure for blood stage vaccines to the monkey malarias is to immunize at least twice with a vaccine containing Freund's complete adjuvant followed by Freund's incomplete adjuvant. Since challenge with large numbers of parasites often results in an abnormally rapid rise in parasitemia (especially with P. knowlesi), challenge is usually done with less than 10,000 parasites. The criteria for vaccine effectiveness are 1) extended prepatent period, 2) reduced maximum parasitemia, 3) shortened length of patent parasitemia, 4) presence or absence of recrudescences, 5) reduced infectivity to mosquitoes, and 6) protection against rechallenge with homologous or heterologous strains of the parasite. Serologic and in vitro tests are correlated with protection if at all possible. A minimum of 6 test animals per group is considered adequate for such trials.

HUMAN MALARIAS

The testing of blood stage vaccines against the human malarias in monkeys is considered to be a major part of the selection process for promising human malaria vaccines. Of the thousands of infections with the human malarias which have been studied in South American monkeys, most have been done with the Colombian Aotus. These animals, however, are now very difficult to obtain. Since they are the only monkeys which are suitable for the testing of sporozoite vaccines and the only ones which can used for mosquito infection with some strains of P. falciparum, the demand far exceeds the supply. Recent studies have indicated, however, that certain strains of P. falciparum can be adapted to development in other monkeys, many of which are available, at least in limited quantities. Among these are Aotus monkeys from Peru and Bolivia of karyotypes I, V, and VI, and Saimiri monkeys from Guyana, Peru and Bolivia. Several immunization trials have been reported using Saimiri monkeys and the Uganda Palo Alto and Indochina I strains of P. falciparum. We have taken the approach of adapting at least one strain of P. falciparum for development in all types of animals in order to obtain some uniformity of parasite challenge. In our studies to date, only the Indochina I strain has been adapted for growth in all of the monkeys examined. Other strains of P. falciparum will no doubt be added to the list once adequate numbers of linear passages in other monkey types have been made. Some strains of P. falciparum have failed to adapt to some of the animal systems. All of the parasites appear to lose certain characteristics upon passage; however, they appear to retain their high level of virulence once adapted to a particular type of monkey.

At present, based on studies in rhesus monkeys and limited studies in Aotus monkeys, adjuvants are required in order to produce a protective immune response in monkeys against the blood stages. It must be recognized, however, that these previously used vaccines were very crude mixtures and only limited studies have been made with purified, defined preparations. It is expected that all animals that are immunized with an effective vaccine will develop a detectable parasitemia with a mean maximum parasitemia somewhat less than that of the control animals and with parasitemias of a much shorter duration than do the control animals.

The proposed testing procedure for the blood stage vaccines to the human malarias would use only those strains of P. falciparum which have been adapted to produce parasitemias of 10% or higher within a period of 14 days following inoculation of approximately 500,000 parasites in homologous Aotus or Saimiri cells. This requirement should be met for any strain adapted for development in a particular model system. In our case, we anticipate that the Indochina I strain will be adapted to meet these criteria in all the animal systems in which the vaccines will be tested. Other strains from other geographic areas are being studied and can be used for heterologous challenge studies in certain animal systems. Since these animals are too valuable to purposefully allow to die, an infection which meets or exceeds the 10% level is treated.

Since P. vivax rarely produces high level parasitemias, a maximum parasitemia of 1% or higher within the same time frame would be considered sufficient. Since animals infected with P. vivax would not be expected to die, all of the control and immunized animals would remain in the study.

In the initial trials, animals will be immunized twice, using an appropriate adjuvant. Challenge is with adapted blood stage parasites growing in homologous RBCs. In order to correlate protection with serologic results, a number of different parameters are used as indicated in the testing procedure for the monkey malarias. To determine if protection is strain specific, secondary studies and rechallenges can be made with heterologous strains of the parasites. At present, it appears that for statistical significance, 6 animals would be required per test group. This number might be reduced as the results of additional immunization trials using standard inocula with adapted parasites are accumulated.

In summary, presented here are various model systems for the testing of sporozoite and blood stage vaccines against monkey malarias and human malarias. It is believed that such trials will help to determine the efficacy of different vaccine formulations and regimens which may also be effective in humans. The accumulation of these results using standard protocols and selected adapted strains of parasites should help to provide a logical basis for the selection of formulations and regimens for an effective vaccine against malaria.

EVIDENCE OF PROTECTION

Discussant: L. Pereira da Silva

To introduce a discussion on evidence of protection the first logical thing to do is to define protection. In fact, this term has been employed with different meanings. At an epidemiological level, it has been used to define an immune status of some human groups; in endemic areas, f.i. adults who develop immune resistance mechanisms upon repeated clinical and parasitological episodes of Malaria in the past.

However, these protected individuals often present parasites in the blood, especially gametocytes and represent the source of infection for maintenance of Malaria transmission in the area.

Would this be the aim of a vaccine against falciparum malaria? It would be certainly useful to induce in the risk population, children, migrants from non-infected areas, visitors, soldiers, etc. the same "immune status" of protected adults from endemic areas. Such an aim can be foreseen by the results of "partial protection" already obtained by some research groups working in asexual blood forms vaccines and which have been reported and reviewed by the different speakers in our section. However, any student of a Public Health School would be aware of the limitation of inconsistencies of such a solution: it would probably have no impact on the transmission, it would maintain the disease in an incubated "dormant" situation and it would expose the vaccinated population in the area (after the selection of variant strains) to epidemic outbreaks of vaccine-resistant malaria.

Consequently, we are obliged to be better than Mother Nature. We need to develop vaccines that will induce not only reduction (or elimination) of morbidity and mortality but also induce reduction of transmission. Let us call it "the ideal vaccine".

The corollary of this assessment is that we must introduce vigorous criteria of evaluation of protection. This raises a series of questions and I have selected five which have been discussed by the different speakers in the morning and afternoon sections:

1. Could we pretend to develop an ideal vaccine against only one stage of the parasite or would we be obliged to use polyvalent vaccines?
2. Have we already finished dressing the list of good candidates for protective antigens?
3. Do we dispose of the methodological tools to evaluate rigorously the protection? Which are they?
4. Do we already have a sufficient understanding of basic mechanisms of immune protection, parasite escape mechanisms, genetic polymorphism and variability which allow to predict eventual complications in the practical use of vaccines?
5. Have we already accumulated enough information to forsee into the near future, clinical trials in the field with one or other antigen?

Certainly, someone in the audience will argue that all the very efficient viral and bacterial vaccines (starting with the anti-rabies vaccine by Pasteur exactly 100 years ago) have been developed empirically without answering these questions.

I will respond to this argument that we must remember that malarial vaccines will be obligatory "2nd generation vaccines" produced by genetic engineering or chemical synthesis and that for developing 2nd generation vaccines, virologists and bacteriologists are faced nowadays with the same kind of problems.

The outstanding scientists selected by Dr.Sidiqqui and the Organizing Committee have brought to you previous information from their own labs and others concerning the current status of developmental vaccines and it is not in my purpose to repeat or to summarize their speeches. I will only try to discuss some points which have been raised.

It was very clear from what we have heard, that for the different vaccines - sporozoite, gametocytes and asexual blood forms - the amount of information accumulated is to variable to permit us to respond to these 5 questions.

Let us start with the more complex, or the more confused, if you will.

Asexual blood form vaccines. If we had to answer the questions - as it is a tradition in this country, by checking the box yes or no - we would have 5 no's.

Being more optimistic, we moved from a phase in the past, in which every laboratory had its own candidate to protective antigens to a period in which data starts to converge. A series of polypeptide antigens, as reviewed by the speakers have been identified by more than one laboratory and in several cases significant immunoprotection was obtained by injecting purified polypeptides in four different laboratories. The extremely positive aspect of these results is the persistence of the immunogenic properties of polypeptides purified under denaturing conditions, detergents and reducing agents. This is a promising situation if we remember that vaccinating molecules produced by DNA recombinant technology or chemical synthesis will probably not have the native configuration. However, two observations reported by one of the speakers today must be recalled: a native polypeptide (purified by immunoabsorption) showed to be more effective to induce protection than the denatured molecule. Synthetic polypeptides corresponding to fragments of a polypeptide molecule were much less efficient in induceing protection than was the entire molecule. Research directed to identify the relevant epitopes of different immunogenic molecules are in course in many laboratories. (And we had three examples in the reports we heard.)

This laborious approach is a prerequisite for the development of an "ideal" fusion polypeptides harboring antigenic determinants from different proteins and prepared by DNA recombitant technology.

Considering the complex mechanismsm of immune protection against the asexual blood forms of the parasite, evidence of protection cannot be deduced only from the development of neutralizing antibodies in mice or rabbits following the injection of the purified antigen, or even in primate models. The evidence of protection which is being utilized is the challenge with blood form parasites of homologous and heterologous strains or isolates. The existence of non-human primate models which had been expertly reviewed in our section is one of the privileges of the asexal blood form vaccine research groups. However, we must remember that this model has some limitations: I am not going to discuss the choice of the monkey's species, the viability on the use of splenectomised or intact animals, which have been very well reviewed

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and that, for this point, I think that is secondary. The "artificialty" is given by the fact that malaria is transmitted by a mosquito bite (and only rarely by blood transfusion) and the innoculated sporozoites arise after a sexual stage which provides the cellular and molecular basis for the genomic rearrangements we know to occur in the parasite. Therefore, the challenge by injection of infected blood and the evaluation of protection has only a limited significance. Furthermore, in the absence of a primate model for reproducing the entire cycle, we persist in the ignorance of the possible consequences of immunoselection in the survival fraction of the parasites, ouserved in the partial protection obtained to date by different laboratories.

In conclusion, asexual blood stage vaccines in spite of important progress reported recently, remains in a situation in which large amounts of research are still necessary in order to permit us to respond yes to most of the question. Paradoxically, two points would facilitate the progress in this area and they concern also the sporozoite and gametocyte vaccines:

1) The development of primate model allowing the reproduction of the entire cycle of the parasite;

2) The development of effective sporozoite and/or gametocyte vaccines which would potentiate the utility of a partially active asexual blood stage vaccine, eliminating the inconveniences which have been discussed.

A few words concerning the gametocyte vaccine. Considerable the progress that has been obtained recently by two laboratories and the excellent review of this progress by one speaker today. The situation is much more clear than the precedent. First, a gametocyte vaccine will be obligatorily utilized in association with others in the form of polyvalent vaccines. It would be, however, of great utility to potentiate a control of transmission of the other forms of vaccines and could eventually be by itself a guarantee in this sense. Since the antigamete, antizygote and/or antiookymete activity is certainly totally dependent on neutralizing antibodies, the description of monoclonal antibodies with transmission-blocking activity, is very significant. Immunochemical analysis seems to point out a limited number of target membrane polypeptides which are extremely stage-specific. The same target proteins have been identified for a series of monoclonal antibodies in different isolates of P. falciparum. This indicates a genetic stability of the target polypeptide antigens. The absence of a primate model has also been blocking the research on this point, since DNA recombitant methodology will certainly allow us in the future to provide a sufficient amount of antigen for immunization experiments. We would finally remark that inhibitory monoclonal antibodies seems to recognize a particular conformation in the structure of the target antigen.

I will finish my comments by a brief discussion on the sporozoite vaccine. It is by far the most advanced project in the area, thanks to the extraordinary work developed by the Nussenzweig's for many years. It is entirely unnecessary to summarize the excellent research work developed until now which was described today in our section by Ruth. I will only remark concerning the checking of the box, that the target antigen in this case is unique and perfectly well identified, cloned, sequenced and characterized. No variants of the CSP protein have been observed until now in natural isolates of P.falciparum all over the world. I'll still add that the Nussenzweig's accumulated a large

amount of evidence to show that the basic mechanism of protective immunity is dependent on neutralizing antibodies: Fab fragments of monoclonal antibodies are able to inactivate sporozoites in vitro and low amounts of monoclonal antibodies injected in mice are able to protect the animals against infective bites. These arguments are important for the interpretation of the results obtained in the in vitro neutralizing tests. Such neutralizing antibodies have been raised in primate model for P.knowlesi by injection of synthetic peptides reproducing the epitopes representing the repeats of amino acids present in the CSP. However, the crucial experience is missing: the induction of protective immunity by injection of the antigen. In the absence of a convenient primate model, the proposed solution is the use of human volunteers to be immunized with a synthetic peptides or DNA recomitant molecules. Confirming the rumors we have been hearing abroad, we had in this meeting the anouncement that the experiment is projected for the near future by two different groups: the Nussenzweig group and the USA Department of Defense in collaboration with NIH. It is not necessary to comment on the suspense which is now created and to the enormous expectations arising in the scientific, medical and sanitary world about the results of such experiments. All of us deeply wish that the experiments succeed.

However, not to finish my comments with all 5 out of 5 for the Nussenzweig's, I will raise a last point. I am personally convinced that a positive result in the experiment with volunteers will be not enough to justify the immediate development of clinical trials in the field and I think that additional experiments must then be developed using animal models. In the absence of a good model for P. falciparum, primate malaria like P. knowlesi or P. cynomolgi could be employed. The reason for such precautions arises from our ignorance on the effect of the immuno selection pressure in the evolution of the CSP protein. If one assumes that genomic rearrangements or mutations can give rise to variants of the CSP protein the introduction of selective pressure upon the introduction of mass vaccination, in the absence of of control measures for the development of the blood cycle, could rapidly originate and spread vaccine resistent strains. This possibility can not be analysed in experiments with humen volunteers since the blood infection, if occuring, must be immediately treated.

WORKSHOP SESSION II CANDIDATE ANTIGENS

Rapporteurs: Graham Mitchell and Leslie Tam

The candidacy of blood stage antigens stands in sharp contrast to that of gamete and sporozoite antigens. Whereas, in the sporozoite/sexual stages, a restricted number of surface protein antigens have been identified and are obvious candidates to be tested as vaccines preventing sporozoite invasion of hepatocytes or blocking transmission, the nature or priority rating of numerous blood-stage antigens is less clear. A major task confronting participants in the workshop session was to clarify the relationships between antigens identified through conventional, monoclonal antibody or gene cloning approaches in numerous laboratories. This was felt necessary in order to facilitate discussion on the vaccination efficacy of different antigen preparations. The following table represents a concensus opinion on the major blood stage antigens reported to date:

Asexual Blood Stage Antigens of P. falciparum

Designation	Identifying Characteristic	Cloned	Reference
I Merozoite/schizont membrane-associated antigens:			
Pf 185/200 83	Merozoite surface	+	1 - 8
Pf 185/200 .... 40/46 (42)	Glycosylated, product carried into rings	+	9
N-acetylglucosamine binding protein	140/70/35	+	10
Glycophorin binding protein	low methionine, 130K	+	11,12,13
Pf 96/100 doublet	-	-	14,15
Pf 75	major surface protein of merozoite	+	16
Pf 76	-	-	17
II High M_r rhoptry proteins:			
RESA	rhoptry + ring stage	+	18,19,20
Pf 155/140	punctate fluorescence of merozoite	-	5,21,22

III Low M_r rhoptry proteins:

Pf 82 (76,75) 41	punctate fluorescence of merozoite	-	21,23,24

IV Membrane-associated antigens of infected erythrocytes:

A knob asociated protein	80-95K		25,26,27
SICA-like protein	sequestration protein? variable (± knob associated)		28

V Antigens in the parasitophorous vacuole:

S-antigens	variable, heat stable	+	29-31
90K protein	-	-	32
CRA	23K	+	33,34
FIRA	300K, highly immunogenic	+	35

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*As yet it is unclear whether the 83/100K purified exoantigen of Ristic et al (this conference) is identical to the 83K major merozoite protein, or in fact, is the PF 83 (76,65) 41K antigen (low M_r rhoptry proteins. N-terminal sequence data will resolve this shortly.

Brief References:

1 Holder and Freeman 1983 JEM 160, 624.
2 Odink et al 1984 FEBS 173, 108.
3a Hall et al 1983 MBP 7, 247.
3b Hall et al 1984 MBP 11, 61.
3c Hall et al 1984 Nature 311, 379.
3d McBride et al 1985 JEM 161, 160.
4 Perrin et al 1982 IR 61,245.
5 Kan et al 1985 this conference.
6 Howard RJ 1984 MBP 11, 349.
7 Pirson/Perkins 1985 JI 134, 1946.
8 Ristic et al 1985 this conference.
9 Howard RF/Reese 1984 MBP 11, 349.
10 Jungery et al 1983 PNAS USA 80,1018.
11 Perkins 1984 MBP 10,67.
12 Ravetch et al 1985 Science 227, 1593.
13 Coppel et al 1983 EMBO.
14 Dubois et al 1985 in press.
15 Brown GV et al 1982 Nature 297,591.
16 Andershir et al 1985 in press.
17 Gysin et al 1982 PI
18 Perlmann et al 1984 JEM 159, 1686.
19 Wahlin et al 1984 PNAS USA 81, 7912

20 Coppel et al 1984 Nature 310, 789.
21 Campbell et al 1984 AJTMH 33, 319.
22 Freeman et al 1985. MBP in press.
23 Ferrin et al 1982 IR 61,245.
24 Howard RF et al 1984 AMTMH 33,1055.
25 Kilejian et al 1980 PNAS.
26 Leech et al 1984 JCB 98,1256.
27 Wallach et al 1984 MBP 12, 85.
28 Leech et al 1984 JEM 159,1567.
29 Wilson 1980 Nature 284,451.
30 Coppel et al 1983 Nature 306, 751.
31 Saul et al 1984 PI 4,39.
32 Jendoubi et al 1985 JI 134, 1941.
33 Hope et al 1985 NAR 10, 369.
34 Coppel et al 1985 PNAS in press.
35 Stahl et al 1985 PNAS in press.

MBP = Molecular Biochemical Parasitology
PI = Parasite Immunology
NAR = Nucleic Acid Research
AJTMH = American Journal Tropical Medicine Hygeine
PNAS = Proceedings National Academy Science
FEBS = Federation of European Biochemical Societies
JCB = Journal Cell Biology
IR = Immunological Reviews

CURRENT STATUS OF THE DEVELOPMENT OF MALARIA VACCINES
EVIDENCE OF PROTECTION

Theodore Green and Kenton Kramer, Rapporteurs

Antigen candidates for the development of vaccines against sporozoite, schizont/merozoite and gametocyte stages were discussed. These antigens were characterized by their fluorescent staining characteristics, reactivity with monoclonal antibodies (McAb) and/or relative molecular weights. For a working classification of the asexual blood stage refer to the preceding section on "Asexual Blood Stage Antigens of *P. falciparum*".

Dr. Meuuwissen reported that gametocytes were produced in synchronized cultures in the presence of N-acetylglucosamine and matured for 7 days. Monoclonal antibodies specific for gametocyte antigens were described.

Monoclonal antibodies against a 48/45 kd gametocyte surface antigen and monoclonals against a 25 kd gametocyte surface protein were discussed. These antigens are expressed on the gametocytes and on the surface of gametes/zygotes and ookinetes. The monoclonals specific for the 48/45 kd antigens were found to be of 2 types: "transmission blocking" and "non-blocking". It was shown that transmission blocking and non-blocking monoclonals recognized two different epitopes on each protein. Combination of blocking and non-blocking McAb resulted in potentiation of the blocking effect. The 48/45 kd antigens were shown to consist of no more than one polypepetide chain.

The 25 kd antigens is present in small quantities in early stage gametocytes and becomes expressed on the surface of gamete/zygotes. Attachment of McAb against the 25 kd antigen did not significantly influence the number of ookinetes formed, yet almost no oocysts resulted. Prehaps penetration of the gut wall was hampered. In the strains tested, there was no evidence of antigen diversity, as the McAb bound equally well to all isolates.

Sporozoite surface antigens, designated CSP antigens, were described by Dr. Ruth Nussenzweig for *Plasmodium berghei*, *P. knowlesi*, *P. cynomolgi* and *P. falciparum*. The *P. falciparum* CSP antigen was designated 58(CS). Its Mr was 58 kd and it was derived from precursors of Mr 65-67 kd. Its isoelectric point was 5.3 (5.0-6.0). All of the described CSP antigens were found to be immunodominant and protective. The McAb to CSP's are species specific (except that recognition of *P. malariae* and *P. brasilianum* were identical). The McAb were found to block the binding of specific immune sera to sporozoites. Six different geographic strains of sporozoites were found to react with the same McAb, showing only slight difference in molecular weights.

This maybe indicative of different numbers of amino acid repeats.

To summarize, the CSP antigens were found to cover the surface of the sporozoite, to be shed, to be of Mr of 40-60 kd depending on the species, and to be derived from 2 precursors. The antigens had a single immunodomoinant epitope and were synthesized by mature sporozoites . The immunodominant area of P. knowlesi is composed of 12 tandem repeats of 12 amino acids. Relevant findings included: 1) Fab McAb fragments inhibited sporozoites as well as complete antibody in-vivo and in-vitro, 2) CSP covers the entire surface of mature sporozoite, 3) CSP is stage specific, and 4) CSP remains in association with parasite membrane after penetration of hepatocytes. A repetitive DNA hybridization probe has been developed which is exquisitety sensitive in detecting blood stage parasites.

Dr. Perrin described the induction of protection against asexual blood stage polypeptides. These were 1) Pf185-200 (merozoite/schizont membrane associated protiens), 2) Pf41 (low molecular weight rhoptry proteins) and 3) Pf140 (high molecular weight rhoptry proteins). These polypeptides were selected by their preferential recognition by Saimiri monkey immune sera or the basis of McAb inhibition of asexual blood stage growth. The polypeptides were purified by affintiy chromatography and/or electroelution from SDS PAGE gels. Challenge of all monkeys was by a heterologous parasite strain. Native Pf41 polypeptide was used as an immunogen in Saimiri monkeys and gave evidence of good protection upon challenge. The conformation of the Pf41 polypeptide is important in conferring protective immunity since the level of protection was diminished in the animals receiving SDS denatured Pf41. In another experiment, a 43 amino acid synthetic polypeptide corresponding to a portion of the Pf200 protein was coupled to tetanus toxoid and used in a Saimiri monkey vaccination trial, which resulted in evdience of protection.

Several conclusions were reached concerning this work. They are: 1) polypeptides can equal merozoites as protective antigens, 2) partial protection was found to be independent of isolate specificity and 3) structural configuration of blood stage immunogens may play a role in conferring protection.

Siddiqui et al., reported on the use of Aotus polyclonal defined antigens and a combination of two McAb defined antigens as immunogens in Aotus trivirgatus azure (karyotype VI). The McAb defined antigens were from 1) merozoite/schizont membrane associated proteins and 2) high molecular weight rhoptry proteins. There was one animal in each group. Each animal received 3 injections with FCA. By solid phase RIA, the 14 day tertiary antibody titers were identical, that is >1/31,250, in the animals receiving either McAb defined antigens or polyclonal defined antigens. By SDS PAGE, three immunizations were necessary to induce antibodies to all sets of antigens recognized by the monoclonal or Aotus polyclonal antibodies. When challenged with FUP K+ parasites, neither animal developed

a patent parasitemia.

Dr. Ristic reported the immunization of Saimiri monkeys with partially purified SP antigen containing an 83 kd protein from P. falciparum culture supernatants. SP antigen was prepared by cation-exchange chromatography. The 4 monkeys immunized with 2 mg of alum precipitated SP protein and challenged with the homologous Indochina I isolate gave evidence of protection. The 8 animals immunized with 2 mg of alum precipitated SP protein and challenged with the heterologous Geneve isolate showed less protection. No mortality was observed in the immunized groups, while 2 of the control animals died.

An 83 kd protein has been purified from the SP fraction by the use of conventional column chromatography, high performance size exclusion liquid chromatography and high performance anion exchange liquid chromatography. The 83 kd protein exists as a dimer in the native state and has been identified as a parasite protein by western immunoblotting and ELISA. The N-terminal sequence of the first 35 amino acids residues has been determined. A similar 100 kd antigen was also reported. As yet it is unclear whether the 83/100 kd purified exoantigens of Ristic et al., is identical to any of the previously described antigens. N-terminus sequence data will resolve this issue.

Dr. Collins reported on the use of monkey malarias and human malarias in primate models for the testing of malaria vaccines. Two species of monkey malaria, Plasmodium knowlesi and P. cynomolgi are likely candidates for testing sporozoite vaccines in Macaca mulatta monkeys. The advantages of this model is the predictable time frame for detecting patent parasitemias and the readily availability of sporozoites for challenge. For testing erythrocytic stage vaccines, P. fragile appears to be an appropriate choice in Rhesus monkeys. Infection results in fulminating infections and if the animals survive, the infection is characterized by its long-term persistence.

Sporozoite vaccines against human malarias using monkeys is more difficult. Only Aotus monkeys of karotypes II, III, and IV are sufficiently susceptible to sporozoite inoculation to be used in vaccine trials. In addition, only the Santa Lucia strain of P. falciparum has been transmitted with enough frequency to establish transmission rates. In this model system 25% of intact and 85% of splenectomized monkeys inoculated with relatively large numbers of sporozoites have subsequently developed patent parasitemias.

Erythrocytic stage vaccines have been tested in Aotus and Saimiri monkeys. The limited availability of Columbian Aotus monkeys has slowed the development of vaccines against P. falicparum. However limited numbers of Aotus monkeys from Peru and Bolivia are available. In addition, Saimiri monkeys from Guyana, Peru and Bolivia have been used in immunization trials with the FUP and Indochina I strains of P. falciparum. Only Indochina I has been adapted for growth in all monkeys examined.

However, additional strains are soon expected.

Dr. Pereira da Silva discussed the human condition with regard to protection against malaria. He recalled that immune adults frequently have blood stage asexual parasites and gametocytes circulating in their blood, but are considered protected. However, these individuals represent a source of infection for unprotected children and migrants. A reduction of transmission is also required. Partial immunity may not be a rigorous enough criterion for a vaccine. Five questions were posed for application to the candidate vaccines:

1. Can the "ideal" vaccine be developed without being polyvalent?

2. Do we have a list of good candidate proteins?

3. Do we currently possess the method to rigorously evaluate immune status?

4. Do we already understand the parameters needed for the practical use of successful vaccines?

5. Have we presented enough information to set up clinical trials?

It was argued that malaria vaccines will be "second generation" vaccines, that is genetically engineered vaccines. It was felt that in regard to blood stage vaccines the above criteria could not be answered in the affirmative at this time, although great strides are being made and relevant antigens and epitopes are being studied in many laboratories. However, the limitations of the existing animal models must be considered and more research is thought to be necessary. A primate model which could reproduce the entire life cycle of the parasite would be ideal.

A gametocyte vaccine is needed for the production of a polyvalent vaccine. Gamete stability of the target antigens has been demonstrated, and genetic engineering should be able to provide enough antigens for testing.

Thanks to the Nussenzweigs, the sporozoite vaccine is by far the most advanced. All of the 5 questions posed earlier can be answered in the affirmative. Only production of CSP for immunization is still needed. With regard to early testing in human volunteers, Dr. Pereira da Silva felt that not all the needed answers could be obtained. He felt that the question of parasite mutation under vaccine pressure needs to be seriously addressed.

In the discussion that followed it was stated that history has shown that in vaccines such as polio, measles, tetanus, etc., that vaccine pressure induced mutations have been very rare. Dr. Pereira da Silva insisted that the malaria vaccine

can not be compared to viral vaccines in this regard. The feasibility of allowing breakthrough and subsequent gametocyte production to occur in human subjects was discussed and discarded as not feasible. Dr. Victor Nussenzweig stated that preliminary studies in rats with P. berghei had been performed to address this question of vaccine pressure induced mutations without evidence of its occurence being noted.