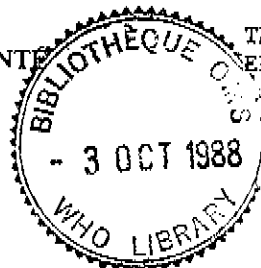




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SPOROZOITE VACCINE DEVELOPMENT AND RESEARCH
 TOWARDS DEVELOPMENT OF ASEXUAL BLOOD-STAGE VACCINES:

REPORT OF THE NINTH MEETING OF THE SCIENTIFIC WORKING GROUP
 ON THE IMMUNOLOGY OF MALARIA

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SUMMARY

The ninth meeting of the Scientific Working Group on the Immunology of Malaria of the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) reviewed progress in the development of sporozoite vaccines and in the identification and analysis of protein antigens of exoerythrocytic and asexual blood-stage parasites. Analyses of protein function and primary structure and protein-elicited immune responses are now making it possible to define the roles of specific proteins in the host-parasite interaction and to determine the potential of these molecules as candidates for malaria vaccines.

The development of sporozoite vaccines based on the circumsporozoite surface (CS) protein is continuing, using either a synthetic peptide coupled to a carrier or a recombinant protein expressed in Escherichia coli. A Phase I trial of the recombinant protein vaccine (falciparum sporozoite vaccine-1 or FSV-1) has just been completed and a trial using the synthetic peptide vaccine is about to start. The FSV-1 vaccine was well tolerated, but the antibody response of the immunized volunteers was disappointing. This may be due in part to genetic restriction in the immune-response genes, such as that described in congenic mouse strains immunized with the repeating epitope. Further analysis of the immune response to sporozoites in mice suggested that high levels of cell-mediated immunity may be required for protection, and the analysis of human sera from endemic areas revealed a greater heterogeneity in antibody specificity than that observed previously.

Studies on the exoerythrocytic (EE) stages represent an important new area of molecular analysis in malaria. An effect of gamma-interferon (γ -INF) on immunity against liver-stage parasites has been demonstrated. Although

liver-stage parasites have been shown to share some epitopes with both sporozoites and asexual blood stages, a unique liver-stage antigen was described and progress on cloning the gene for this protein was reported.

Several antigens of the asexual blood-stage parasite have now been characterized at the level of nucleotide sequence. A number of parasite-derived antigens have been examined for their ability to immunize and protect rodents and nonhuman primates. In one immunization study employing parts of the RESA/Pf155 molecule expressed from *E. coli* recombinants plus Freund's complete adjuvant, Aotus monkeys were significantly protected against *P. falciparum* challenge.

Malarial proteins on the merozoite surface, within merozoite organelles, or associated with the host erythrocyte membrane have been described and the role of these proteins in attachment and invasion of red cells is being investigated. The genes for many of these proteins contain repetitive sequences which, like those in the CS protein, are immunodominant. Repeats in different antigens may be related and as a result antibodies induced by asexual-stage antigens may crossreact with several different antigens.

Antigens of *P. vivax* blood-stage parasites have been identified, and cloned DNA for some of their genes has been isolated.

Presentation of the recombinant or synthetic antigens in a manner that will induce responses mimicking the natural protective responses observed against the parasite may be crucial to the development of malaria vaccines. The multiple mechanisms of immune responsiveness to specific antigens were outlined and different methods of antigen presentation and processing described.

A limited number of selected references to previously published studies are given in section 10. More comprehensive reference lists are given in recent reviews (Heidrich, 1986; Miller et al, 1986).

1. INTRODUCTION

The ninth meeting of the Scientific Working Group on the Immunology of Malaria reviewed the current status of sporozoite vaccine development and research towards the development of asexual blood-stage vaccines. The development of sporozoite vaccines is in an advanced stage and the results of Phase I trials of a recombinant-derived polypeptide construct were presented. Studies on the specificity of the immune response to the sporozoite, both with respect to the specificity of the immunity induced by natural infection and the possible genetic restriction of the response, were reported. Many blood-stage antigens have been described and part of the meeting was devoted to defining the relationships between antigens described by different investigators. Genes for several of these proteins have now been cloned and analysis of these, together with the specificity of the antibody response to the primary peptide sequences, have revealed a complex interaction with the immune system. The possibility of using some of these antigens as the basis for vaccines has been reinforced by immunization studies with either parasite or recombinant-derived protein. However, the potential problems of immunogenicity and genetic restriction of host responsiveness are areas where considerable further research will be required.

2. SPOROZOITE VACCINE DEVELOPMENT

The structure of the surface protein of sporozoites (the circumsporozoite [CS] protein) has been elucidated in a number of *Plasmodium* species, including *P. falciparum* (Dame et al, 1984; Enea et al, 1984), *P. vivax* (Arnot et al,

1985, McCutchan et al, 1985), *P. knowlesi*, *P. cynomolgi*, *P. berghei* and *P. yoelii*. The nucleotide sequence of each gene predicts a repetitive amino acid sequence that forms an immunodominant epitope. Although the repeat element in each species can vary in length, the overall length and amino acid composition are restricted. On each side of this structure there are charged regions with homology between the different species. In *P. falciparum*, the central repetitive region contains Asn-Ala-Asn-Pro (NANP) repeated 37-41 times, interspersed with a small number of the alternate repeat Asn-Val-Asp-Pro (NVDP). This repeat structure is found in strains from all over the world (Zavala et al, 1985; Weber & Hockmeyer, 1985). Protection against sporozoites appears, at least in part, to be mediated by antibodies directed against this immunodominant region of the CS protein.

Antisporozoite vaccines using as antigens either synthetic peptides or proteins expressed as recombinant products have been developed. Each of these approaches has a number of potential advantages and disadvantages. The synthetic peptide is a chemically defined entity, but the coupling to a larger carrier molecule introduces a number of potential variables and problems. The choice of carrier and the amount of peptide to be conjugated to it are important. Prior exposure of the individual to the carrier and antigenic competition between the peptide and the carrier may influence the performance of the immunogen. Carrier hypersensitization and rapid clearance after immunization could reduce the immune stimulation by the peptide. Also, if the carrier is providing crucial T-cell epitopes, boosting of the response by exposure to natural infection may not occur. A recombinant protein is potentially available in large amounts, although the purification to homogeneity of each type of molecule can be expensive and difficult. Such recombinant proteins may be large enough to have T-cell sites, but boosting by natural exposure may not occur. Both types of vaccine are likely to require an adjuvant.

2.1 Development of Synthetic Peptide Vaccines*

Synthetic peptides based on the repeat have been made and used in an inhibition assay for monoclonal antibody (MAB) binding to define the minimum size of the epitope. The 8-mer (NANP)₂ showed no inhibition but (NANP)₃ and larger peptides inhibited binding. In further studies the 12-mer (NANP)₃ has been used. High-titre serum from a human volunteer immunized and protected with irradiated sporozoites reacted with (NANP)₃ and the binding was 90% inhibited by MAB. In sera from an endemic area (The Gambia), the titre increased with the age of the individual. These sera recognized the sporozoite in the immunofluorescent antibody (IFA) test and the (NANP)₃ repeat in a solid-phase assay; preincubation with the synthetic peptide abolished this reaction.

* Since the time of the meeting, the following information has become available: The peptide conjugate (NANP)₃-tetanus toxoid was subsequently tested for safety and immunogenicity in healthy nonimmune human volunteers. The vaccine was well tolerated, nontoxic and immunogenic. Antibody responses correlated with vaccine dose and the antibodies recognized sporozoites. A challenge study was carried out in three volunteers who had received four monthly inoculations of vaccine and in four nonvaccinated control subjects. All controls developed patent infection within seven to ten days after challenge. One vaccinated subject was fully protected and the two others showed delay in patency until day 11.

Rabbits immunized with the synthetic peptide linked to a tetanus toxoid carrier produced antibodies reacting with the peptide, with sporozoites (by IFA) and with the CS polypeptide (on Western blots). Most of the anti-peptide activity can be absorbed by *P. falciparum* sporozoites but not by *P. berghei* sporozoites. The anti-peptide antibodies neutralized sporozoites *in vitro* and inhibited sporozoite invasion into hepatoma cells. Eight of 11 MABs raised against the peptide recognize the CS protein. Further studies have shown that the (NANP)₃-tetanus toxoid construct is immunogenic in *Aotus* monkeys. Because tetanus toxoid is a good antigen, presensitization may interfere with and reduce the response to the haptén. It has been shown that only one of several strains of inbred mice recognized the repetitive epitope on the *P. falciparum* CS protein (Good et al, 1986; Del Giudice et al, 1986). Although the synthetic peptide vaccine may overcome this restriction in immune response since the carrier can provide T-cell epitopes, the response may not be boosted in nature. Preclinical studies using several other candidate peptide-carrier conjugate vaccines are in progress.

2.2 Development of Recombinant Vaccines*

The expression of the entire CS protein in *E. coli* has proved difficult, but a number of recombinant clones expressing the repetitive epitope have been produced. Both R32tet32 and R48tet32 proved to be immunogenic in mice, but the response was increased by the addition of Freund's complete adjuvant or aluminium hydroxide (alum). R32tet32 induced high levels of antibodies in mice and rabbits, with circumsporozoite precipitation (CSP) and inhibition of sporozoite invasion (ISI) responses (Young et al, 1985). Although the response in outbred animals was good, genetic restriction of the immune response in defined mouse strains was observed (Good et al, 1986; Del Giudice et al, 1986). In rhesus monkeys, 70% of the animals responded to R32tet32. On re-immunization after six months, boosting occurred only in those monkeys that responded to the primary immunization. The duration of the response in mice showed that the antibody titre by ELISA fell in parallel to the ISI response. At 44 weeks, the response to the alum-adsorbed material was still good in both systems, but antibody levels in the animals immunized with the peptide alone were falling.

The results of Phase I safety and immunogenicity studies in human volunteers of an alum-adsorbed vaccine known as FSV-1 (falciparum sporozoite vaccine-1) were reported. This study was designed to establish in healthy nonimmune human volunteers the safety of increasing doses of FSV-1 and to evaluate whether FSV-1 induces an immune response in humans. FSV-1 consists of single-dose ampoules of sterile R32tet32 (NANP₃₂NVDF₂tet32, where tet32 refers to the first 32 amino acids encoded by a tetracycline resistance gene) and preserved with thimerosal. FSV-1 was administered intramuscularly to groups of three volunteers at selected doses (10, 30, 100, 300 and 800 µg together with alum, 0.5mg Al(3+) per dose). Volunteers received primary immunizations at week 0, boosted at the same dose at weeks 4 and 8, and were evaluated for immediate toxicity for 20 minutes after immunization and 24 and 48 hours later. Serum for antibody determination was obtained one week after the first dose and then biweekly for 16 weeks. Sera were assayed by ELISA using R32LR, a purified recombinant construct which contains only the first two amino acids of the tetracycline resistance gene as the test antigen. Pre-immunization sera for each volunteer were assayed simultaneously with

* Since the time of the meeting, six immunized volunteers subsequently received a fourth dose of FSV-1. They and two nonimmunized control subjects were challenged by bites of infected mosquitos. The controls developed patent parasitaemia on days 9 and 10, respectively. Of the immunized volunteers, one did not develop parasitaemia and the others did so on days 12 and 13. The protected volunteer had the highest antibody response to the vaccine.

post-immunization sera. Sera were also assayed for CSP and ISI reactivity and by IFA, and, in some cases, immunoglobulin class and subclass responses to R32tet32 or R32LR were measured by ELISA. Blood for studies of cellular responses was obtained prior to the first immunization and four weeks after each dose and assayed for in vitro lymphocyte proliferation in response to R32tet32.

The vaccine was generally well tolerated at all five doses. Minor local pain occurred in seven of nine volunteers receiving doses of 100 µg or greater. One volunteer who received 800 µg doses developed a generalized transient urticarial eruption within five minutes of the third dose of FSV-1. Assays of haematological, hepatic and renal function revealed no evidence of toxicity in any volunteer.

Antibody responses to FSV-1 were detected two weeks after the primary immunization and were dose-dependent. Twelve of fifteen (80%) vaccine recipients had detectable antibody when serum was assayed at a dilution of 1:50. Most were not positive beyond a 1:200 serum dilution and titres began to fall after 12 weeks. Boosting of antibody responses after primary immunization was seen in only two volunteers, both of whom received the 800 µg dose. No restriction of IgG subclass response was identified. Sera from all volunteers were screened for IgE by ELISA, using R32tet32 as antigen, but it was detected only in post-booster serum from the volunteer who developed an allergic reaction to FSV-1. CSP reactivity was detected in sera of nine of 15 volunteers, including at least one individual at each dose. In all cases, CSP reactivity developed after multiple doses of FSV-1 and was limited to 2+ reactions. No vaccine recipient developed ISI activity greater than 75% at any time during the study. Antibodies reactive with sporozoites by IFA were detected only in the sera from volunteers receiving the 800 µg dose. Lymphocyte proliferation assays showed a response in 11 of 13 volunteers. There was no apparent correlation between the magnitude of the proliferative response in vitro and either antibody production or dose received.

Vaccine examined at the conclusion of the trial showed no evidence of degradation. The possibility that lengthening the interval between booster doses may result in superior antibody response was explored in rhesus monkeys immunized with FSV-1 during preclinical studies; a delay of six months between boosters did not significantly alter the antibody responses.

The response to R32tet32 in congenic mice has been shown to be highly restricted and under the control of Ir gene products. Only a limited number of T-cell epitopes have been shown to be present on the molecule and these are distributed between both the CS repeat region and the tet32 portion of the protein. This restriction in T-cell epitopes may play a role in the observed lack of booster responses to FSV-1 in human volunteers.

FSV-1 is the first of a series of malaria vaccines which will undergo Phase I trials in humans. Several new candidate vaccines have been developed taking into account the experience with FSV-1. One approach has been to express new P. falciparum CS proteins incorporating additional T-cell epitopes to enhance immunogenicity and provide an opportunity for boosting by natural sporozoite exposure. T-cell epitopes derived from the parasite may also elicit cellular immune mechanisms that kill developing exoerythrocytic (EE) stages in the liver.

Larger parts of the CS molecule may contain additional T-cell epitopes. P. knowlesi and P. falciparum CS proteins have been expressed in vaccinia and induced antibody responses in rabbits. The P. vivax CS antigen has also been expressed in yeast and can be readily purified. Animals, including Saimiri monkeys, immunized with this recombinant antigen, using alum as adjuvant, produced antibody which inhibited invasion of sporozoites into hepatoma cells in vitro. Low primary responses were increased by boosting.

2.3 Immune Mechanisms Involved in Sporozoite Immunity

Earlier studies with P. berghei showed that irradiated sporozoites inoculated intravenously protected against challenge infection and that the CSP reaction correlated well with protection. In other studies, mu-suppressed mice could be protected, and transfer of sensitized spleen cells to recipients gave protection, the latter being lost if the cells were treated with θ -antiserum and complement (Chen et al, 1977). Nevertheless, in another study passive transfer of a monoclonal antibody conferred passive protection against homologous challenge (Potocnjak et al, 1980).

Mice were immunized with irradiated sporozoites, with synthetic peptide based on the P. berghei repetitive sequence coupled to keyhole limpet haemocyanin (KLH), or with full length P. berghei CS protein produced in E. coli, and then challenged with 1000-10 000 sporozoites. Antisporozoite antibody responses were assayed by ELISA and for CSP and ISI reactivity. Animals immunized with the recombinant protein made antibody at titres at least as high in all assays as those elicited in animals immunized with sporozoites, but on challenge with 500, 1000 or 10 000 sporozoites, they were less well protected than the latter. When challenged with 500 sporozoites, mice were protected by passive transfer of either MAB or protein A-purified IgG from mice immunized with the peptide-KLH construct, but not by serum from mice immunized with the recombinant construct or with irradiated sporozoites. Adoptive transfer of spleen cells from immune animals conferred protection against a challenge of 10 000 sporozoites. Cell populations enriched in B cells did not protect, whereas those enriched in T cells did protect on transfer. These results strongly implicate cell-mediated immunity (CMI) in protection against sporozoites.

In this rodent model the results suggest that immunization with sporozoites induces a high level of cell-mediated protection. On the other hand, immunization with peptide or recombinant protein induces a low level of protection that is antibody-mediated and high titres of ELISA, CSP and ISI antibodies. Factors including peptide length, orientation or degree of substitution of the carrier, timing of dose or route of immunization may influence the type of immune response induced. A synthetic vaccine should stimulate protective immunity that can be induced by irradiated sporozoites; however, the cellular effector mechanisms involved are not yet understood.

An antigen competition assay using IFA on wet sporozoites or dot blots was used to analyze MABs against the CS protein and synthetic (NANP)_n derivatives. The recombinant antigen R32 at different concentrations totally blocked the binding of two MABs to the sporozoite surface. Using human sera, much poorer competition was observed and the binding of some sera was not inhibited at all by the addition of R32. About half of the sera did not react with R32 in dot blots.

In the second set of assays, using antibody competition in IFA, dot blots or ELISA, it was possible to inhibit the binding of the MAB by human sera and vice versa, but although there were antibodies of the same specificity, other specificities were also present in the sera. Binding of the MAB to R32 could not be blocked by human immune sera, suggesting that these antibodies differ in their specificity for R32 epitopes.

Human lymphoblastoid cell lines specific for sporozoite or EE stages have been produced and 12 MABs have been used to screen a number of different Thai strains of P. falciparum. Considerable restriction in the numbers of sporozoites recognized in different strains was observed by IFA. For example, MAB II recognized 60% of parasites in Th I, but none in Th IV or Th VII, whereas MAB IV recognized no parasites in Th I, 60% in Th IV and 100% in Th VII. None of the other MABs recognized the Th VII strain, and the Th X strain was only recognized by MAB VII. When hyperimmune sera from individuals

living in high transmission areas were screened against the same strains, they recognized sporozoites in most strains, whereas two sera from individuals living in a low transmission area recognized only a small population of sporozoites in some isolates and many in some other isolates. These findings suggest that several antigenic specificities may be present on individual sporozoites in different isolates. It is not yet known whether these results reflect the presence of proteins other than the CS protein on the sporozoite surface, or the presence of multiple variable epitopes on the CS protein created by microheterogeneity in repeat structure.

Antibodies in the sera of mice immunized with recombinant or synthetic epitopes have a number of effects on sporozoites *in vitro*, inhibiting attachment, entry and intracellular development in liver cells (Mazier et al, 1986). There was a dose-dependent inhibitory effect of MABs using purified IgG, but total inhibition of the development of schizonts could not be achieved; at best, 85-95% inhibition was observed. A variety of antibody specificities appear to be present in human sera that vary in their reactivities with different isolates. Some sporozoites evade the antibody effects *in vitro*, even with good correspondence between the antibody and the sporozoite antigen.

Another study of ant sporozoite antibodies showed that the results of different tests in human sera do not always correlate (Hoffman et al, 1986). For example, high scores in the ELISA and ISI assays were measured in some sera that gave a poor reaction in the IFA test. Only 30% of the sera gave a positive CSP reaction, but 80% gave positive ISI and ELISA reactions, although there was no direct correlation between the two assays. Sera from different age-groups were examined: in infants (<1 year old), a positive ELISA reaction but little ISI reactivity were detected; in the 1-3-year age-group, no antibody was detected by ELISA, but these sera appeared to enhance invasion into hepatoma cells; in the 4-9-year and >20-year age-groups, increasing ISI and ELISA titres were detected.

Domains of the CS protein that bind to hepatoma cells were analysed by Scatchard plot analysis. R32tet32 bound nonspecifically; R32LR produced by trypsinization or as a recombinant construct also bound nonspecifically, and the level of binding suggested that a nonspecific binding step may be involved in invasion. The conserved region N1 from *P. falciparum* or *P. knowlesi* bound similarly and competitively inhibited each other. Antibodies to N1 from *P. falciparum* blocked sporozoite invasion, and crosslinking of N1 with HepG2-A16 cells identified two putative receptor molecules with a relative molecular mass (M_r) of 35 000 (35K) and 55K, respectively. A longer conserved peptide from the same region, N2, did not bind either specifically or nonspecifically. CS processing involving protease action at multiple lysine sites within N2 may activate N1 for binding. Invasion may thus involve nonspecific binding of the species-specific repeat region and specific binding of the conserved N1 region to hepatic receptors.

3. EXOERYTHROCYTIC-STAGE PROTECTIVE IMMUNITY

Recent studies suggest that immune mechanisms operate against the liver stages of malaria parasites. For example, following infection of Norwegian brown rats with *P. berghei* sporozoites, up to 30% of liver schizonts were shown to be invaded and destroyed by immune cells, Kupffer cells, monocytes and neutrophils (J. Macs, unpublished data).

3.1 Role of Gamma Interferon (γ -IFN)

In *P. berghei* infection in mice, interferon-inducers affect the course of sporozoite-induced blood-stage parasitaemia, for example, by abolishing the infection or lengthening the prepatent period. This effect appears to operate at the EE stage since there is no apparent effect on the sporozoites or

blood-stage parasites. The effect of recombinant interferon (IFN) on the liver stages in both rodent and chimpanzee models has been assessed using a repetitive DNA probe to enumerate the EE stages in the liver.

In Norwegian brown rats, rat γ -IFN administered either prior to or after *P. berghei* sporozoite infection inhibited the EE stage (Ferreira et al, 1986). A maximum of 90% inhibition was observed when γ -IFN was administered at five hours before sporozoite infection (-5 hours). Inhibition was achieved only when γ -IFN was given at intervals between -18 and +24 hours, but some effect was also seen if γ -IFN was given at five and 20 hours after sporozoite infection. These effects appeared to be independent of challenge size between 40 000 and 400 000 sporozoites. A dose-dependent reduction of *P. berghei* EE forms in hepatoma cells in vitro was also obtained by the addition of human γ -IFN.

Administration of human γ -IFN to chimpanzees challenged with *P. vivax* sporozoites increased the prepatent period before blood-stage parasites were seen (Ferreira et al, 1986). It is not clear whether γ -IFN caused a delay in EE development or whether there was selection of forms with reduced numbers of receptors for γ -IFN.

3.2 Antigens of Liver-Stage Parasites

Liver schizonts are antigenically distinct and yet share defined epitopes common to sporozoites and to asexual blood forms of the parasite. An understanding of the antigenic composition of liver merozoites could have important implications for malaria vaccine development, due to the pivotal role that these parasites play between the sporozoite stage and the asexual blood stage. A stage-specific antigen has been detected in immature EE forms of *P. falciparum* (Druilhe et al, 1984). Strain variation of this antigen has not been found so far. It is located at the periphery of liver schizonts, and possibly around the developing merozoites of mature forms. The antigen is heat-stable and it can be detected in infected hepatocytes in vitro. A similar antigen can be identified in *P. vivax* EE forms in Saimiri monkeys and in *P. yoelii* EE forms in vitro or in vivo.

After invasion of sporozoites and production of liver asexual blood stages, there are several modifications of protein synthesis and antigen expression. Some epitopes shared by liver stages and asexual blood stages or sporozoites could be defined by MABs. There were few common epitopes, although several epitopes showed some degree of crossreactivity. Thus, many antibodies to other stages did not react with EE parasites, but some reacted at a lower titre (for example, antibodies against the 195K blood-stage antigen) while antibodies against a 22K protein reacted with both liver and blood-stages at a similar titre.

EE stages obtained from chimpanzees infected with sporozoites have also been examined. One chimpanzee was infected with sporozoites from *P. falciparum* clone 3D7 and a second received sporozoites of clone HB3. MABs recognizing at least three epitopes of the CS protein and a polyclonal serum raised against R32tet32 were used to look for the presence of CS-like epitopes in mature liver schizonts. These antibodies reacted with late liver schizonts from both clones showing a fine granular pattern. The reaction was blocked with R32tet32 and was of lower titre than that obtained with the intact sporozoite. (None of these sera reacted with blood-stage parasites).

Some MABs that recognize different proteins in asexual blood-stage parasites also reacted by indirect immunofluorescence with liver schizonts. These MABs included: two specific for PfEMP 2, a 300K antigen located on the cytoplasmic face of the infected erythrocyte membrane; 126K and 101K proteins located in the blood-stage parasitophorous vacuole and on the merozoite surface; a series of antigens of 80, 60 and 40K that are associated with the

rhostry organelles; and two different epitopes of a 14K protein that appears to be associated with the merozoite surface. In general, the MABs reacted less strongly with the liver schizonts than with the blood-stage antigens. The MABs against PfEMP 2 and the rhostry proteins gave a fine granular pattern, whilst the other MABs produced a coarse granular pattern. Different MABs against distinct epitopes of the 195K protein reacted with late liver schizonts. The strain specificity of these MABs was identical for both the EE and blood-stage forms. All MABs to the 195K protein gave a coarse granular pattern with liver stages and had a similar titre to both liver and blood-stage parasites.

Biochemical analysis of the liver-stage proteins is difficult because of the small numbers of parasitized cells that can be obtained. No liver-stage specific MABs have been obtained, and purifying mRNA is virtually impossible. As an alternative approach, affinity-purified polyclonal antibodies were prepared from the sera of individuals who had been highly exposed to sporozoite challenge but protected against the asexual blood stages by drug prophylaxis over many years. These sera had a high titre to liver-stage antigens and sporozoites, but were negative for antibodies against the blood stage. Genomic libraries in an expression vector were screened differentially with antibody preparations. Clones identified in this way were used to affinity-select specific antibodies which were then screened against the three stages. Three clones selected antibodies which gave the same reactivity as human immune serum on early or late liver stages, and the antibodies were stage- and species-specific. The β -galactosidase fusion protein expressed by one clone, 307, had a heat-stable epitope, and DNA sequencing showed that the insert contained a 51 base-pair (bp) element (coding for 17 amino acids) repeated three and a half times. This sequence does not appear to be related to any known *P. falciparum* sequence. The gene is located on chromosome 5 in all strains, but some restriction fragment polymorphism was detected in Rsa I digests of genomic DNA from different strains. The 307 fusion protein was able to absorb antibody from human sera and totally inhibit antibody binding to the liver stages with nine out of ten sera. At the moment, the role of this antigen is unknown, but recombinant DNA methods will facilitate this analysis.

4. ASEXUAL BLOOD-STAGE ANTIGENS OF *P. FALCIPARUM*

4.1 A Comparison of Antigens and Specific Reagents from Different Laboratories

A comparison was made of antibody reagents specific for proteins in the 100-160K molecular-weight range identified in different laboratories, in order to establish the relationship between these antigenic proteins. Four distinct specific antigens (see Table 1) were defined and none of these appeared to be related to the Pf155 ring-infected erythrocyte surface antigen or the gp195 merozoite surface protein.

R140, R4 and R311 recognized antigens of different sizes when electrophoresed on parallel gel tracks. None of these antibodies gave a RESA-type IFA pattern. MAB 5E3 recognized the same antigen as R4. MAB 3D5 recognized a unique antigen.

4.2 The Major Merozoite Surface Antigen

4.2.1 The 195K glycoprotein of *P. falciparum*

The precursor to the major merozoite surface antigens is a glycoprotein synthesized throughout schizogony and transported to the surface of the intracellular parasite. Late in schizogony, possibly at the time of merozoite release, the protein appears to be processed to specific fragments, some of

TABLE 1. COMPARISON OF DATA ON REAGENTS

SOURCE OF ANTIBODY	MONOCLONAL OR SPECIFIC Ab	ESTIMATED SIZE OF ANTIGEN RECOGNIZED	SUBCELLULAR LOCATION
A.A. Holder	R140 (rabbit Ab to purified Ag) WIC 61.3 (MAB to purified Ag)	140K + 155K doublet	merozoite/ rhoptries
L.H. Perrin	R4 (rabbit Ab to purified Ag) Pilatus (serum from monkey immunized with pure Ag)	140K	schizont/ parasitophorous vacuole/ merozoite surface
J.A. Lyon	5E3 (MAB)	113K	merozoite surface
M.E. Perkins	R311 (rabbit Ab to cloned gene product)	130K	Glycophorin-binding protein (GBP)
J.A. Lyon	3D5 (MAB)	101K	schizont/ parasitophorous vacuole/ merozoite surface

Ab = antibody

Ag = antigen

MAB = monoclonal antibody

which are found on the surface of released merozoites. The precursor has a molecular weight of 180 to 205K, depending on the strain of *P. falciparum*.

Specific fragments derived from the 195K precursor in the Camp strain have been mapped to the linear gene sequence, using specific MABs and antibodies affinity-purified on recombinant clones expressing parts of the gene (Lyon et al, 1986). In another study, the fragments of this polypeptide associated with merozoites of the Wellcome strain were investigated. Specific regions of the gene were expressed in *E. coli* and antibodies raised against the products recognized species of 83, 42, 38 and 19K in extracts of surface-labelled merozoites, and additional species of 28-30 and 15-18K were detected on Western blots. Previous work had shown that the 83K fragment was derived from the N-terminus of the precursor and this was confirmed in these studies. The 42 and 19K fragments were shown to be derived from the C-terminus of the molecule and to contain epitopes constrained by cystine residues. It is proposed that the linear sequence of fragments is 83-28/30-38-42K, with the 15-18 and 19K polypeptides being subfragments of the 42K species. Epitopes associated with the 42/19K region can be detected in ring stages and this part of the molecule may be carried in on the parasite surface during invasion.

Complete DNA sequences have been obtained for the gene from three strains of *P. falciparum* and partial sequences from a number of other strains (Holder et al, 1985; Mackay et al, 1985; Weber et al, 1986). A comparison of these

sequences indicates the genetic basis of the antigenic polymorphism that has been described for the 195K protein. The gene consists of sequences distributed in variable blocks that are separated by conserved or semi-conserved sequences. Variable sequences occur not only in the regions that code for the tripeptide repeats, but also in regions without apparent repetitive structure. Variable sequences are apparently not widely polymorphic but fall into two distinct types. This suggests that the protein is encoded by dimorphic alleles capable of limited genetic exchange. Evidence at the nucleotide level indicates that intragenic recombination can generate the overall structural diversity observed within this gene from different strains.

The 195K (180-205K) antigen has been purified from extracts of cultured SGE2 parasites by affinity chromatography and electroelution of the band from SDS-PAGE gels and used to immunize groups of Saimiri sciureus monkeys, using 100 µg of antigen together with Freund's complete adjuvant (Perrin et al, 1984). All the immunized monkeys had significant specific antibody titres (measured by IFAT) at six weeks. After challenge with 2.5×10^7 asexual blood-stage parasites of FUP Palo Alto strain, the control animals had high parasitaemia by day 10 and most of them had to be treated. All the immunized monkeys developed low or moderate parasitaemia and recovered without anti-malarial treatment. The degree of protection achieved in these experiments was similar to that reported previously for immunization with whole merozoites and schizonts. All the sera from the immunized monkeys reacted with the polypeptide used for the immunization, but some of the processed products were not detectable by immunoprecipitation. The processed products, for example, the 83K fragment, were clearly detected using Western blotting against schizont extracts. The monkey sera recognized schizonts and the surface of merozoites by IFA, and no antigen was detected in ring stages. A cDNA clone containing sequences corresponding to the N-terminus of the 83K fragment was obtained and three peptides corresponding to different regions were synthesized (Cheung et al, 1986). Peptide 1 consisted of residues 1 to 43 (43 amino acids), peptide 2 consisted of residues 20 to 43 (24 amino acids) and peptide three consisted of residues 46 to 79 (34 amino acids). The polypeptides were coupled to tetanus toxoid and used to immunize rabbits. These animals produced antibodies recognizing the 180-205K protein and an 83K protein on a Western blot and reacting with the surface of merozoites by IFA. Rabbits immunized with unique sequences (peptides 1 and 2) reacted with six of six P. falciparum isolates tested, while rabbits immunized with the repeat polypeptide (SGGSVA) reacted with five of six isolates.

The 43 amino acid synthetic peptide coupled to tetanus toxoid (TT) was selected for immunization studies in squirrel monkeys. The antibody response in monkeys was much lower than that in rabbits and the protection induced by immunization was highly variable. The four control monkeys all had parasitaemias of >20% and required treatment. In the experimental group one animal had a >20% parasitaemia but with a marked delay in onset, and the other three animals recovered spontaneously after peak parasitaemias of 19, 9.2 and 3.4% on days 10, 9 and 9, respectively. Maximal parasite growth inhibition of 25% was obtained in vitro using an IgG fraction of the rabbit serum.

The identification of immunogenic regions of the molecule in which the primary structure is conserved in all isolates or which exhibit only limited antigenic variability may be important. Attention has been focused on the N-terminal end of the 195K protein. Mice were immunized with a 30K recombinant protein (Nde peptide) from the N-terminal region of gp195. The N-terminal 231 amino acids of this protein are highly conserved between the Camp and Wellcome isolates, whereas the C-terminal 47 amino acids are variable. Antisera raised against this protein reacted specifically on Western blots with gp195 and the 83K species and also reacted with the 73 and 67K fragments seen in the Camp strain. When tested by IFA with three parasite strains that have serologically different gp195 (Camp, IMTM-22 and FCR-3), the antisera reacted with all three strains, but reacted more strongly with the homologous Camp

strain. The antisera have not given convincing growth inhibition in vitro. An immune Aotus monkey serum that inhibits Camp strain parasite growth in vitro also interferes with the complete processing of gp195, in particular blocking the cleavage of p83 at the N-terminal end to the 73 and 67K species. Antibodies may act by hindering access to the protease cleavage site or by inhibiting directly the activity of the processing protease, but the precise mechanism is unknown.

4.2.2 Rodent models: the 230K protein of P. yoelii

Two murine malaria parasites, P. yoelii and P. chabaudi adami, are being used to elucidate the mechanisms of both cellular and humoral immunity which function during malaria infection. Resolution of acute infection with P. yoelii requires antibodies, whereas resolution of acute infection with P.c. adami is antibody-independent but requires T lymphocytes. This provides a model for investigating the nature of cell-mediated immune mechanisms which may aid the host in combating infection.

Mice passively immunized with MAB 302 to P. yoelii are protected against a challenge with lethal and nonlethal variants of this parasite (Majarian et al, 1984). MAB 302 immunoprecipitated a 230K molecule analogous to the gp195 molecule of P. falciparum, together with smaller polypeptides of 67 and 36K. It is unclear whether the dramatic biological activity of MAB 302 against P. yoelii results from its idiotype or its isotype (IgG3). To approach this question monoclonal and polyclonal anti-idiotypic antibodies specific for MAB 302 have been prepared. These anti-idiotypic antibodies have been used to measure the concentration of antibodies with the 302 idiotype in sera from mice infected with P. yoelii and to attempt to induce synthesis of this protective idiotype by immunization of mice with the anti-idiotypic antibodies prior to infection.

P. yoelii genomic DNA has been used to construct an expression library in λ gt11, which was screened using MAB 302. A clone has been isolated which produces a large β -galactosidase fusion protein recognized on Western blots by antiserum to the 230K antigen. The DNA insert in this clone is 4kb and, when used as a probe, recognizes an 8kb plasmodial mRNA which is operationally polyA-. A partial restriction map has been obtained for the clone, together with partial nucleic acid sequencing data on the portion of the clone containing the 1700bp open reading frame. This insert crosshybridizes slightly with P. berghei, but not with P. falciparum, DNA. High-level expression of this polypeptide will make possible the mapping of the epitope recognized by the protective antibody, as well as a variety of immunization studies. Such approaches may contribute to an evaluation of the 195K protein of P. falciparum as a protective antigen.

The role of cell-mediated immune mechanisms in resolving P.c. adami infections in mice has also been studied. It was shown previously that antigen-specific interleukin 2 (IL-2)-dependent T-cell lines could provide adoptive protection to nude mice, which lack T cells and are therefore normally extremely susceptible to this species of parasite. The protective response was dose-dependent and antigen-specific and abrogated by pre-treatment of the IL-2-propagated cells with anti-Thy 1.2 and complement. More recently, individual T-cell clones have been derived, by limiting dilution from these cell lines, and assayed for their ability to transfer protection to nude mice infected with P.c. adami. One clone, designated CTR 2.1, allowed nude mice to resolve their acute infections. This cell line displays an L3T4+, Lyt2-surface phenotype. It also secretes γ -IFN and IL-2 in response to soluble antigen and major histocompatibility complex (MHC)-compatible antigen-presenting cells in vitro. These results demonstrate that a single cloned T-cell line is capable of providing adoptive protection against this malaria infection.

4.3 The Ring-infected Erythrocyte Surface Antigen (RESA/Pf155)

The ring-infected erythrocyte surface antigen (RESA/Pf155) is present within micronemes of developing merozoites but after invasion is associated with the membrane of the newly infected red cell. The solubility characteristics of RESA/Pf155 are consistent with its being anchored at the membrane due to an interaction with components of the membrane skeleton.

The complete structure of the gene encoding RESA/Pf155 has recently been determined (Pavaloro et al, 1986). An intron in the gene separates a small exon 1 (65 amino acids) from a very much larger (1008 amino acids) exon 2. Within exon 2, there are two regions of repetitive sequences: the 3' repeat region encodes several tandem repeated of an 8-amino acid sequence (EENVEHDA) followed by a much more extensive set of tandemly repeated 4-amino acid sequences (predominantly EENV). The 5' repeat region is more degenerate, with an 11-amino acid sequence (DDEHVVEEPTVA) occurring twice and five shorter sequences derived from the 11-mer by deletions and, in some cases, by conservative substitutions.

Antibodies to this antigen are found at high levels in most sera from immune donors or from patients with repeated P. falciparum infections but are of low incidence in patients with primary infection (Perlmann et al, 1984). Studies in children living in a holoendemic area of Africa suggest that the appearance of anti-RESA/Pf155 antibodies may correlate with the development of clinical immunity. Human antibodies to RESA/Pf155 efficiently inhibit reinvasion of erythrocytes by merozoites in vitro, and the major immunogenic structures of RESA/Pf155 are highly conserved in different P. falciparum strains. This antigen may therefore be an important candidate for a vaccine against the asexual blood stages of the parasite.

RESA/Pf155 is heat-stable and present in schizonts and merozoites, as well as in supernatants of P. falciparum cultures. An antigen of similar properties was recently detected in P. chabaudi (Pchl05, 105K) (Gabriel et al, 1986). When studied by immunofluorescence, RESA/Pf155 and Pchl05 do not crossreact. However, a mouse MAB to a synthetic octapeptide, representing one of the repeated C-terminal amino acid sequences of RESA/Pf155 (see below), binds to Pchl05 as well as to RESA/Pf155 in immunoblotting. In addition, a Pchl05-specific mouse monoclonal antibody also reacts in ELISA with synthetic oligopeptides representing C-terminal repeats of RESA/Pf155, indicating the existence of crossreacting epitopes in the two antigens.

RESA/Pf155 was initially detected as a ring-like staining of the membrane of freshly invaded erythrocytes in a modified immunofluorescence assay after short glutaraldehyde fixation and air-drying. In spite of the presence of soluble RESA/Pf155 in supernatants of P. falciparum cultures, it did not bind to uninfected erythrocytes from such cultures, although it does bind selectively to human erythrocyte membranes (ghosts). Taken together, available evidence suggests that RESA/Pf155 is introduced into the RBC membrane during invasion and may have an important function in this process, perhaps by interfering with membrane and/or membrane skeleton organization. The RESA 3' repeat and the erythrocyte Band 3 N-terminus are structurally related.

RESA 3' repeat:	EENVEEYDEE
Band 3 N-terminus:	MEELQDDYEDE

A monoclonal antibody raised to Band 3 from mouse erythrocytes was found to crossreact with Pchl05 and RESA/Pf155 (immunoblotting), as well as with the synthetic peptides from the C-terminal repeat region of RESA/Pf155 (ELISA). Another MAB specific for the Pchl05 molecule, crossreacts with RESA/Pf155 and reacts with the synthetic peptides, but does not recognize Band 3.

Polyclonal antibodies were prepared in rabbits using synthetic peptides [EENVEHDA or (EENV)₂] coupled to a carrier protein (KLH). A minor fraction of the resulting peptide-specific antibodies also reacted with RESA/Pf155. Similar results were obtained with a mouse monoclonal antibody specific for EENVEHDA and with rabbit antibodies against the product of a synthetic gene expressed in *E. coli* as a fusion protein containing the sequence EENVEHDA repeated four times. All antibody reagents inhibited reinvasion of erythrocytes by merozoites *in vitro*, indicating that the corresponding amino acid sequences are accessible for immune attack on the native RESA/Pf155 molecule.

To assess the importance of the C-terminal repeat sequences of RESA/Pf155 for the human immune response, the binding of human antibodies to different synthetic peptides in ELISA was also investigated. The human response to these peptides was polyclonal. While some sera contained antibodies specific for EENVEHDA, a majority contained antibodies reacting with linear epitopes in the synthetic sequence (EENV)₂. Antibodies binding to EENVEHDA appeared to be of relatively low affinity and frequently crossreacted with related sequences from other parasite antigens. In contrast, antibodies binding to (EENV)₂ were of higher affinity and/or of more restricted specificity. The ELISA reaction with the octapeptide could be efficiently inhibited both by octapeptide and by (EENV)₂, while the reaction with (EENV)₂ could only be inhibited by (EENV)₂ and not by EENVEHDA. These latter antibodies were also the most efficient inhibitors of erythrocyte reinvasion *in vitro*, suggesting that repeats of the tetrapeptide EENV may be a suitable basis for a vaccine.

In order to study immune regulation and T-cell responses to RESA/Pf155, autologous T/B-cell cooperation systems and lymphokine assays have been set up permitting assessment of both T-cell stimulation and T-cell-dependent secretion of antimalarial antibodies *in vitro*. T-cells from RESA/Pf155 seropositive, but not those from seronegative, donors proliferated in response to RESA/Pf155, while their response to crude *P. falciparum* antigen was suppressed. Moreover, responding T cells induced autologous B cells to secrete anti-RESA/Pf155 antibodies into the culture supernatants. Both the crude antigen and RESA/Pf155 also induced secretion of γ -IFN in T-cell cultures of *P. falciparum* patients, an assay which appears to be a useful indicator of the existence of T-cell immunity in *P. falciparum* malaria.

These results indicate that the intact RESA/Pf155 molecule possesses epitopes required for the stimulation of helper T cells and perhaps also for the induction of protective cellular immunity. The intact RESA/Pf155 molecule is therefore being mapped for T-cell reactive epitopes. T cells from the peripheral blood of donors living in *P. falciparum*-endemic areas have been incubated with autologous antigen-presenting cells in the presence of various synthetic peptides from the C-terminal end of RESA/Pf155. In RESA/Pf155 seropositive donors, cell proliferation was obtained with synthetic peptides which were 16-20 amino acids long and which included the C-terminal EENV repeats also seen by antibodies. In responding donors, optimal T-cell proliferation *in vitro* was obtained with peptides predicted to have an amphipathic alpha-helical conformation. However, several of the donors responding to intact RESA/Pf155 did not respond to any of these peptides, suggesting that the anti-peptide response is genetically restricted.

Three groups of monkeys were immunized with different regions of RESA/Pf155, and a fourth group of monkeys was immunized with an irrelevant fusion polypeptide and then challenged with *P. falciparum* (Collins et al, 1986). Nine of the 14 immunized animals showed some degree of resistance to the challenge infection, whereas all the control animals were susceptible. The antibody responses of individual monkeys against peptides corresponding to the three major repetitive sequences in RESA/Pf155 were measured by ELISA. Of the five animals immunized with a fusion polypeptide largely composed of the 3' repeats of RESA/Pf155 (Group 1), the three animals that were susceptible had low antibody titres to the 8-amino acid repetitive sequence, whereas the

two resistant animals had high antibody titres to this sequence. All five animals had high antibody titres to the repetitive 4-amino acid sequence. Of nine monkeys immunized with fusion polypeptides, which included the 5' repetitive sequence (Groups II and III), seven were resistant. All seven resistant monkeys had high-titre antibody responses to the 11-amino acid repetitive sequence contained within the 5' repeat, whereas the two susceptible monkeys had very low antibody titres to this sequence. Thus, antibody responses induced by immunization, and measured prior to challenge, against two different repetitive sequences (the 5' repeat 11-mer and the 3' repeat 8-mer of RESA) predicted which animals were rendered resistant to overwhelming infection with *P. falciparum*. It is of interest that neither antibody responses against the dominant 4-mer repetitive sequences nor anti-RESA/Pf155 antibodies measured by immunofluorescence on glutaraldehyde-fixed and air-dried films predicted which monkeys would be resistant.

Although antibodies to the RESA/Pf155 11-mer and 8-mer predicted immunity in the *Aotus* vaccination trial, it is not clear that protective antibodies or other immune effector mechanisms are directed against epitopes encoded by these repetitive sequences. The possibility that RESA/Pf155 protects by priming for an enhanced response to a crossreacting antigen must be investigated.

4.4 Parasite Antigens that Bind to Erythrocytes

Heterogeneity of the receptor for *P. falciparum* merozoites has been demonstrated. Some parasites are unable to invade neuraminidase-treated erythrocytes (e.g., Camp strain). Other parasites (e.g., strains Thai-Tn and 7G8) invade neuraminidase-treated erythrocytes, albeit at a reduced rate (50% of control). Parasites that are sialic acid-dependent (Camp strain) also have a reduced capacity to invade En(a-) erythrocytes lacking glycoporphin A and MkMk erythrocytes lacking glycoporphins A and B. This suggests that the sialic acid-containing oligosaccharide, and not the peptide, is required for invasion by some *P. falciparum* merozoites. A second ligand was required for invasion by parasites of clone 7G8, which invade independently of sialic acid. This ligand is trypsin-sensitive on MkMk erythrocytes. In contrast, the sialic acid-dependent parasite does not use this trypsin-sensitive ligand on MkMk erythrocytes. The identification of parasite proteins involved in the recognition of and binding to erythrocytes is crucial to understand further the molecular bases of these interactions.

4.4.1 Glycophorin-binding protein and S-antigen

Glycophorin-binding proteins (GBPs) have been described in *P. falciparum* merozoites (Perkins, 1984). Two proteins which bind to glycophorin columns have been identified in each of 12 geographic isolates of the parasite. One of these proteins has a molecular weight of 130K (present as a triplet in culture supernatants) and it appears to be functionally and antigenically conserved. The second GBP shows size polymorphism and is antigenically complex. In some isolates (e.g., FCR-3), this second component has a molecular weight of 155K; it can be radiolabelled by incorporation of proline and glycine and it is heat-stable. In other isolates (e.g., FCQ-27), the second component has a molecular weight of 220K (depending on the isolate this molecule has a molecular weight of between 180 and 220K). In four isolates, this second component has been shown to correspond to the previously identified S-antigen.

The structure of GBP 130 has recently been determined (Ravetch et al, 1985; Kochan et al, 1986). There are 11 x 50 amino acid repeat sequences which contain conserved elements (e.g., ADPE). *E. coli*-derived recombinant protein binds to the glycophorin column, and binding is abolished by heat treatment. The antigenically invariant GBP 130 polypeptide reacts with specific antibodies raised against products from the cloned GBP 130 gene.

The S-antigen in FCQ-27 binds to the glycophorin column and can be identified using a MAB that also reacts with the product of Ag16 (an S-antigen cDNA clone) by precipitation or Western blotting. However, after binding and elution from the MAB column, the S-antigen no longer binds to the glycophorin column.

P. falciparum glycoproteins such as gp195 or gp56, labelled with glucosamine in schizont extracts, do not bind to the glycophorin column nor do specific rhoptry proteins. Some binding of the 140/120/113 antigen is observed.

The GBP 130 protein has been localized to the merozoite surface, using paraformaldehyde-fixed parasites and MAB. Fixation may be necessary to stabilize the surface coat proteins, especially those that are not anchored in the membrane. A similar treatment is not necessary to detect anti-gp195 binding to the merozoite surface.

The domain of glycophorin to which GBP 130 binds has been investigated with specific tryptic fragments. T1 (residues 1 to 39 of glycophorin) contains the majority of the oligosaccharides, and it inhibited binding of GBP to the glycophorin column. T3 (residues 40 to 61) and T5 showed some inhibition, whereas T6 (the transmembrane domain) had no effect. Sialic acid, fetuin and N-acetyl glucosamine did not inhibit binding. GBP also bound to T1 peptide coupled to a solid support, and a high concentration of fetuin on the beads also resulted in weak binding.

To investigate the contribution of the sialic acid side chains on the O-linked tetrasaccharides, T1 fragment was treated with influenza neuraminidase, which cleaves two to three sialic acid linkages, or with Vibrio cholera neuraminidase, which cleaves two to six linkages. The desialated peptide and the fully deglycosylated peptide did not compete in the GBP-binding assay. It is possible that the cluster of O-linked carbohydrate chains forms the high-affinity binding site.

In the FCQ-27 strain, the T1 fragment does not completely inhibit the binding of the 220K S-antigen, suggesting that there may be different binding specificities of these molecules. All strains dependent on sialic acid had the 155K S-antigen and showed 1-7% invasion into neuraminidase-treated cells, whereas, with one exception, those that could partially invade neuraminidase-treated cells (30-58% invasion) had the high-molecular-weight S-antigen. In isolates where the S-antigen is the 155K species, the binding of the GBPs appears to be fully dependent on the presence of sialic acid residues at the N-terminal end of glycophorin. In other isolates where the S-antigen is approximately 220K, this dependency on sialic acid is only partial. These results suggest that an S-antigen/GBP 130 complex may be involved in red-cell binding. Despite the antigenic variation shown by the repetitive sequence of the S-antigen, there may be functionally conserved regions involved in this binding. Perhaps the two different groups of S-antigen mediate binding to different receptors on the red cell.

In primary isolates, S-antigens that have the FC-27 serotype all have apparent molecular weights of 200 to 220K, whereas those that have the NF7 serotype vary widely in size. Although the repeats are different, so too is the N-terminal sequence (70-75% homology between different strains). At the C-terminal end of the NF7 repeat, there are 2 x 15 amino acid repeats, which have now been found in some other S-antigens (including KI from Thailand), and antibodies to this repeat are relatively common.

4.4.2 Erythrocyte-binding antigen

To identify parasite molecules that may be involved in initial recognition, intact erythrocytes have been used as affinity substrates. This

approach has the advantage of maintaining erythrocyte ligands in their natural environment with their native configuration. Erythrocyte-binding antigens (EBAs) were investigated by incubation of supernatants from parasite cultures (labelled with ^3H -isoleucine) with erythrocytes (Camus & Hadley, 1985). Four antigens of 175, 120, 65 and 46K were identified, which bound to human erythrocytes. No direct relationship between EBAs and GBPs could be established. The EBAs were investigated using erythrocytes from animals susceptible or resistant to invasion and human erythrocytes naturally resistant to invasion (Cad or Tn cells) or resistant to invasion after enzymatic treatment (trypsin, neuraminidase). A correlation was observed between the binding of the 175K antigen to erythrocytes and the susceptibility of cells to invasion by merozoites. Such a correlation was not observed for the 120, 65 and 46K antigens. The 175K EBA was also able to bind to merozoites, which suggested that this antigen could be used by the parasite to bind to erythrocytes.

Studies of the susceptibility of EBA-coated erythrocytes to parasite invasion suggested that there is a strain specificity for the EBA and that merozoites need the EBA of the homologous strain to invade erythrocytes.

When the 175K antigen was eluted from EBA-coated cells, it was able to rebind to erythrocytes, suggesting that its binding is specific for a membrane ligand. When human immune sera containing anti-EBA antibodies were incubated with EBA-coated erythrocytes, the EBA-antibody complexes were shed from the erythrocyte surface. If a similar mechanism occurs *in vivo*, the antibodies could eliminate the EBA bound to the erythrocyte, leaving the surface ligand free and accessible for another EBA.

4.5 Antigens of the Parasitophorous Vacuole/Merozoite Surface

Comparison of antibody reagents revealed that schizont polypeptides estimated at either 113, 120, 126 or 140K in different laboratories were identical (see Table 1). This protein is associated with schizonts, probably in the parasitophorous vacuole, and is released in large amounts at the end of schizogony. The protein can be isolated on the merozoite surface in immune complexes or by glutaraldehyde fixation. A second unique protein, p101, appears to have a similar location.

The 140K protein has been purified and used to immunize squirrel monkeys which were challenged with parasites from a heterologous strain (Perrin et al, 1984). All control monkeys had high parasitaemias by day 10 and most had to be treated, whereas the immunized animals showed only transient, low-level parasitaemias.

When *P. falciparum* parasites are cultured with certain immune sera, merozoites are agglutinated by antibodies to form clusters within which some normally soluble antigens accumulate in relatively insoluble immune complexes. MABs reacting with several of these antigens have been produced, including MAB 3D5, which recognizes a 101K antigen (p101), and MAB 5E3, which recognizes a 113K antigen (p113). Both MABs gave a grape-like pattern of rimmed fluorescence around merozoites contained within mature schizonts, and the antigen recognized by MAB 3D5 was localized to the surface of individual merozoites. Both MABs precipitated antigens that were synthesized by mature trophozoites and young schizonts and that appeared in the culture medium when schizont rupture occurred.

MAB 3D5 reacted in immunoblots with a 101K antigen from three *P. falciparum* strains tested. The electrophoretic mobility of p101 was similar whether SDS-PAGE was performed under reducing or non-reducing conditions. With some antigen preparations, an additional 92K parasite antigen was recognized.

MAB 5E3 reacted in immunoblots with a 113K antigen from three P. falciparum strains tested under both reducing and non-reducing conditions; an additional 100K antigen was detected when antigens were electrophoresed under non-reducing conditions. Prolonged exposure of immunoprecipitation gels showed that, in addition, small amounts of 100, 70 and 50K parasite antigens were also precipitated by MAB 5E3.

A P. falciparum genomic DNA expression library was prepared in λ gt11 and screened with immune monkey serum. Antibodies affinity-purified from the immune serum with the protein expressed by one of the clones reacted with p101. This clone contained a 3100 bp insert, beginning with an open reading frame of approximately 1200 bp that was in-frame with β -galactosidase.

Three clones expressing p113 were identified with immune rabbit serum RSB10. Antibodies selected from these expression proteins reacted strongly with p113 and weakly with 80 and 50K P. falciparum antigens. Each of these three clones contained a 400 bp insert. One clone was sequenced and had an open reading frame that was in-frame with β -galactosidase and contained 27 consecutive codons for serine.

Logical candidates to study in the search for a vaccine against the erythrocytic stages of malaria include antigens such as these, which are accessible at the parasite surface and which react with antibodies in immune serum that inhibit parasite invasion.

4.6 Rhoptry Antigens

A number of polypeptides of differing size are associated with the rhoptry organelles of merozoites. These proteins are synthesized by schizonts, but may be detectable in ring-stage parasites. Protection of squirrel monkeys with one rhoptry protein preparation has been described.

4.6.1 A 230K antigen

A 230K protein was identified by a MAB that was produced by immunization of mice with antigen from culture supernatants. The 230K molecule is synthesized during schizogony and within 30 minutes processed to a 215K species. This antigen was identified at the neck of the rhoptry organelles by immunoelectron microscopy (IEM) using this MAB (Holder et al, 1985).

4.6.2 A 140K antigen

A 140K protein has been located in the rhoptries by immunogold electron microscopy. A 155K doublet (not related to RESA/Pf155, as judged by lack of reaction with a Pf155-specific MAB) coprecipitates with this protein and may be noncovalently associated within the parasite; no structural homology was found between these proteins. On Western blots of non-reduced gels, the 140K protein migrated as a 130K species, and a ladder of lower mobility species was observed with the purified protein preparation, implicating the presence of cystine residues in the structure of the protein.

4.6.3 A 77-82K antigen

This protein has been identified by MABs, together with 76 and 66/63K fragments that may be proteolytic cleavage products. A polypeptide of 37 to 42K appears to coprecipitate with this molecule from detergent extracts, but there is no evidence to support a primary structural relationship. MABs against this protein recognize conserved and variable epitopes, and in vitro inhibition with two of these MABs has been reported.

4.6.4 40-42K antigens

It is possible that there may be two polypeptides of this size localized in the rhoptries and synthesized at the end of schizogony. MABs either react with a 41K polypeptide alone or with a 41K polypeptide and a coprecipitated 82K antigen.

Two preparations of the 41K protein have been used to immunize squirrel monkeys (Perrin et al, 1985). One was purified by affinity chromatography from an NP40 extract of SGE2 schizonts (native antigen), and a second was purified by affinity chromatography followed by electroelution from SDS-PAGE gels (denatured antigen containing at least 0.1% SDS). Using micro-ELISA assays, it was found that both groups of monkeys had similar antibody titres against the native antigen, but the monkeys immunized with the denatured antigen reacted 20 times better than monkeys immunized with the native antigen. On the other hand, the degree of protection against challenge infection observed in monkeys immunized with the native antigen was much higher than that observed in monkeys immunized with the denatured antigen, indicating that the native structure of the immunizing molecule is an important factor in the induction of a protective antimalaria immune response.

4.7 Cytoadherence and Proteins Interacting with the Infected Erythrocyte Membrane

4.7.1 P. falciparum erythrocyte membrane protein 1 (PfEMP 1)

The properties of this protein are fully consistent with its role as the cytoadherent moiety for P. falciparum-infected cells. PfEMP 1 is a malarial protein ranging in apparent molecular weight from 250 to 300K in different isolates (Leech et al, 1984). It is a cell surface protein which has been identified on the surface of K+B+ infected erythrocytes and not on K- cells (Aley et al, 1984). These properties are consistent with the anticipated expression of the cytoadherent moiety. PfEMP 1 has been demonstrated on the surface of Aotus erythrocytes, human erythrocytes containing in vitro culture-adapted parasites, and human erythrocytes taken from Gambian patients and cultured for about 24 hours in vitro (Aley et al, 1986).

PfEMP 1 is not solubilized from Aotus-infected erythrocytes by non-ionic detergents in isotonic solution. It is solubilized by treatment with 1-2% SDS, which also disrupts the host erythrocyte membrane skeleton. It has been suggested that PfEMP 1 is attached to the submembrane skeleton of the host cell. It has been inferred that PfEMP 1 is expressed at the cell surface only at knobs. Polyspecific human or monkey antisera from hyperimmune hosts only react at the cell surface at knobs when tested by IEM.

When ¹²⁵I-labelled intact infected cells are treated with low levels of trypsin, cleavage of ¹²⁵I-PfEMP 1 occurs in parallel with loss of cytoadherent capacity, indicating that PfEMP 1 is either the cytoadherent moiety or intimately associated with the functional moiety. Under the conditions of trypsin treatment used, there is no apparent cleavage of other ¹²⁵I-labelled proteins.

PfEMP 1 is antigenically diverse. The antigenically diverse epitope(s) on PfEMP 1 is expressed on the external surface of intact infected cells. Antisera from Aotus monkeys infected with a single isolate react with PfEMP 1 only from the homologous isolate. Complexes of specific Aotus antibody and PfEMP 1 can be extracted from intact cells with 1% Triton X-100, whereas this detergent does not extract PfEMP 1 alone, suggesting that binding of antibody to PfEMP 1 may perturb its anchoring to the membrane skeleton.

The capacity of Aotus antisera to react with PfEMP 1 on intact cells coincides with its capacity to block or reverse cytoadherence. Thus, antiserum

which blocks cytoadherence of FVO strain alone will react with PfEMP 1 of FVO but not with that of other isolates. Antisera which blocked cytoadherence of multiple strains (from hyperimmune animals) reacted with the PfEMP 1 of multiple strains. Antisera from infected humans in diverse geographic regions specifically blocked cytoadherence of a single isolate or a limited subset of isolates. It was concluded that antibody binding to the antigenically diverse epitope(s) on PfEMP 1 blocked cytoadherence. However, the possibility that a separate molecule close to PfEMP 1 has the cytoadherent function and that it is blocked by strain-specific antibody against PfEMP 1 cannot be excluded.

4.7.2 P. falciparum erythrocyte membrane protein 2 (PfEMP 2)

PfEMP 2 is a malarial protein of about 300K as measured on an SDS-PAGE gel under reducing conditions. It is size variant, ranging in apparent molecular weight from 280 to 400K in different isolates, migrating as a sharp band. Although these properties are very similar to those of PfEMP 1, PfEMP 2 is uniquely distinguished by reaction with two mouse monoclonal antibodies (MAB 8B7.4 and 4H9.1).

Like PfEMP 1, PfEMP 2 is not extracted by non-ionic detergents in isotonic solution. PfEMP 2 is only extracted under conditions which solubilize the host erythrocyte membrane skeleton, suggesting that it is attached to the membrane skeleton.

To date, all isolates of P. falciparum tested, whether from Aotus or Gambian patients, react strongly with MAB 8B7.4, whereas not all isolates react with MAB 4H9.1. These differences are seen by both IFA and immunoprecipitation of biosynthetically radiolabelled malarial proteins. Thus, PfEMP 2 bears an antigenically conserved structure (defined by MAB 8B7.4) and may be either antigenically diverse or show deletion of some epitope(s) in various parasites (defined by MAB 4H9.1). These MABs give the same IFA pattern, reacting with the parasite cytoplasm, the parasitophorous vacuole membrane and/or parasite plasma membrane and with spherical granules within the erythrocyte cytoplasm, and showing reactivity over the host erythrocyte membrane.

PfEMP 2 is not expressed on the cell surface. It was identified by IEM as a submembrane component of the electron-dense material at knobs; it was found in electron-dense spheres and membrane whorls in the host erythrocyte cytoplasm and in association with the parasitophorous vacuole membrane and parasite cytoplasm.

PfEMP 2 is a major product of parasite protein synthesis. It is first synthesized by early trophozoites and accumulates rapidly thereafter. It is expressed under the erythrocyte membrane up to the stage of infected-cell rupture and merozoite release. The size of PfEMP 2 does not change during the intracellular growth of the asexual parasite.

The function of PfEMP 2 is unknown. Its submembrane location precludes a direct role in the cytoadherence property. Since it is associated with the electron-dense material under knobs, it may play a role in knob structure and/or function. Knobs may exert functions other than cytoadherence: P. malariae-infected cells bear knobs and associated electron-dense material but do not cytoadhere.

4.7.3 P. falciparum knob-associated histidine-rich protein (KAHRP/PfHRP 1)

This histidine-rich protein (HRP) has also been called PfHRP 1 to distinguish it from two other HRPs also expressed by asexual P. falciparum parasites, PfHRP 2 and 3 (Kilejian, 1979; Hadley et al, 1983; Vernot-Hernandez & Heidrich, 1984, 1985). KAHRP/PfHRP 1 constitutes part of the electron-dense material under knobs (Leech et al, 1984). With K+ parasites, KAHRP/PfHRP 1 is labelled strongly by uptake of radioactive-histidine, but

only poorly or not at all by other amino acids. Rabbit antisera and MABs to the P. lophurae HRP, 72% of which is histidine, crossreact with KAHRP/PfHRP 1 (Kilejian, 1983; Kilejian & Rosenbaum, 1985).

KAHRP/PfHRP 1 migrates as a broad band with an apparent molecular weight of 80 to 120K in different K+ isolates. It reacts specifically with a mouse IgG MAB (MAB 89) by both immunoprecipitation and Western blotting. A rabbit antiserum raised against KAHRP/PfHRP 1 cut from a preparative gel reacted specifically with this protein on Western blots, but also with PfHRP 2 on immunoprecipitation. MAB 89 reacted only with K+ parasites by IFA on methanol or acetone-fixed cells, giving a granular pattern of reactivity with spherical granules both in the host cell cytoplasm and in association with the erythrocyte membrane. MAB 89 did not react by IFA with intact, non-fixed K+ erythrocytes, nor did it react with the cell surface by IEM. MAB 89 and rabbit anti-KAHRP/PfHRP 1 had no effect on cytoadherence.

KAHRP/PfHRP 1 was identified by IEM as a submembrane component at knobs and as a constituent of electron-dense spheres in the host erythrocyte cytoplasm, and shown to be attached to both the parasitophorous vacuole membrane and parasite plasma membrane. IEM of saponin-treated K+ cells showed KAHRP/PfHRP 1 to constitute part of the electron-dense material under knobs.

KAHRP/PfHRP 1 is expressed by all K+ P. falciparum isolates or clones examined to date, regardless of cytoadherence phenotype. Since K+B- parasites also express KAHRP/PfHRP 1, and the immunofluorescence pattern was identical to that of K+B+ parasites, it can be inferred that this protein alone does not confer cytoadherence. Although KAHRP/PfHRP 1 is a component of knobs, like PfEMP 2 it appears not to be accessible to the cell surface and its expression at the erythrocyte membrane does not correlate with expression of the cytoadherence function. It may play an indirect role in mediating cytoadherence.

4.8 Transferrin Receptors

The Plasmodium parasite has a requirement for iron. Lethal P. vinckei infection is suppressed by desferrioximine in vivo, and P. falciparum is inhibited in vitro by addition of this compound (Pollack & Fleming, 1984). In addition, certain individuals in endemic areas undergoing iron replacement therapy become parasitaemic. Two recent studies suggest that the parasite can synthesize its own transferrin receptor to supply its iron needs. However, the size estimates for the molecule differ and it will be important to confirm that the same species has been identified.

In one study, fluorescein isothiocyanate (FITC)-labelled ferrotransferrin was added to cultures of P. falciparum and its uptake by parasitized cells was examined at five-minute intervals by fluorescence microscopy (Rodriguez & Jungery, 1986). Initially, parasitized cells had a faint peripheral staining and with time this appeared to be localized in large vesicles, possibly moving towards the parasite, finally accumulating around the parasite. Transferrin, but not apotransferrin, was specifically bound, and unlabelled transferrin could compete with the FITC-labelled transferrin. Using cells fixed in 10% methanol in acetone and Rhodamine-conjugated rabbit anti-human transferrin, a pattern of vesicles and accumulation in the parasite were observed.

To identify the parasite encoded molecules involved in this process, extracts of ³⁵S-methionine-labelled parasites were mixed with ¹²⁵I-labelled transferrin, electrophoresed on an SDS-PAGE gel and compared to similar extracts in the absence of added transferrin. A band with an apparent molecular weight of 200K was observed, which was probably a complex of parasite protein and transferrin that was not denatured by SDS. The same band could be detected by Western blotting with anti-transferrin antibody, and it was not present in extracts of uninfected erythrocytes. Using transferrin covalently coupled to Sepharose 4B, a 93K parasite protein was still bound to

the column after washing. On blots of total parasite proteins fractionated on SDS-PAGE gels and probed with transferrin, binding in the region of 200, 93 and 46K was observed.

Results of a second study (Halदार et al, 1986) indicate that a protein of 102K, synthesized by the intracellular parasite and inserted into the erythrocyte membrane of mature infected cells, is a receptor for serum ferrotransferrin. Schizont-infected erythrocyte membranes of the Gambian clone FCR-3/A2 isolated on Affigel 731 beads contain a parasite protein of 102K. Polyclonal antisera to the SDS-PAGE-purified 102K protein were raised in rabbits. At physiological pH, antibody-purified protein bound human ferrotransferrin but not apotransferrin. Conversely, antibody to human transferrin was used to purify the ferrotransferrin receptor complex from infected cells. The isolated receptor was specifically recognized by polyclonal rabbit antisera to the 102K molecule. A smaller radiolabelled fragment of 45K was sometimes detected in antibody-purified preparations of the receptor and is probably due to limited proteolysis of the 102K polypeptide. The plasmodial receptor has approximately the same molecular weight as its human counterpart when analysed by SDS-PAGE under reducing conditions. However, under non-reducing conditions the human receptor appears to be a dimer of 200K, whilst the mobility of the parasite receptor is unchanged. Preliminary studies indicate 60 000-100 000 parasite transferrin receptors per cell, with a single high-affinity binding site for human ferrotransferrin. The membrane-bound receptor is acylated, which may be important for its association with the erythrocyte membrane and its regulation in the infected cell.

4.9 Antigens Identified with Immune Sera

In Saimiri monkeys, protective antibody can be demonstrated by passive transfer of IgG from immune donors to naive animals which are then protected against a challenge with 1×10^8 infected red blood cells. There was no correlation between protective effects in vivo and inhibition in vitro, and therefore these antibodies are not neutralizing. The intact antibody was required for protection (Fab fragments were not effective), and perhaps phagocytosis or antibody-dependent cytotoxicity mechanisms are involved. Comparative studies using protective and nonprotective antibody preparations suggested that the recognition of proteins of 41, 71/72, 76/82, 96/100 and (possibly) 140K correlated with protection, whereas no such correlation was established with recognition of 195, 125 or 110K proteins. When used to immunize squirrel monkeys, the 71/72 and 90K polypeptides were found to be immunogenic, but little reaction to the 76K species could be detected (Dubois et al, 1984). Four to seven days after challenge, the titre increased four-fold and all the animals were protected.

More recently, the humoral response of humans from hyperendemic areas of West Africa has been analysed. A correlation was observed between levels of antibodies reacting with p72 and p96 and the time of exposure to malaria infection. The 96K protein, which may be that identified previously, is resistant to boiling and is recognized by protected monkey sera and by more than 90% of human sera from endemic areas of Africa.

Using MABs and monospecific sera, polypeptides have been characterized with regard to their electrophoretic mobilities, isoelectric points, time of synthesis in the parasite life cycle and cellular location. A 72K protein, two 90K proteins and many 96K proteins were recognized. One 90K protein ($pI=6.1$) and a 72K protein were recognized by vaccinated monkeys. Only the 72K protein appears to be present in ring stages, and the 72 and 90K antigens are major products in trophozoites. The 42, 72, 76 and 96K antigens are present in early schizonts, and the 41, 71, 76 and 96K species are detected in late schizonts.

Clones coding for parts of the 71 and 72K proteins were detected in a λ gt11 library and confirmed by affinity purification of antibodies on the fusion proteins. No clones for the 90K protein could be isolated from the genomic library, but a cDNA clone in pUC9 was obtained for this protein.

A DNA clone, 11.1, appears to code for a 260K protein and this may be the precursor to the 96K protein. This protein contains the sequence (EEVVVEEVVP)₄₀ (Koenen et al, 1984). Antibodies to a synthetic peptide based on this sequence reacted with 260, 96 and 48K polypeptides on immunoblots. This fusion protein has been used to immunize monkeys, but no protection was observed. Although the animals had good antibody titres by ELISA against the synthetic (EEVVVEEVVP)₂ peptide, the IFA titres were very low. After challenge, the IFA titre increased (fivefold), but these results suggest that the antibodies did not recognize the native protein, and it will be necessary to identify the important epitopes.

5. CROSSREACTIONS AMONG ANTIGENS OF P. FALCIPARUM

Many protein antigens of P. falciparum contain short sequences that are repeated in tandem arrays. Recently, it has become clear that the naturally immunogenic epitopes encoded by these repeats are involved in extensive networks of crossreactivities. These crossreactions, which are both intramolecular and intermolecular, involve a large number of the antigens of P. falciparum that have been characterized to date, including RESA/Pf155, the major merozoite surface glycoprotein and the CS protein -- all antigens shown to have potential as vaccine components. The various levels of crossreactivity found with some of these antigens are listed in Table 2.

TABLE 2. LEVELS OF CROSSREACTIVITY AMONG P. FALCIPARUM ANTIGENS

LEVELS OF CROSSREACTIVITY	ANTIGENS INVOLVED*
Between different epitopes within one block of repeats	CS proteins, S-antigens RESA/Pf155, FIRA
Between epitopes within different blocks of repeats in the one antigen	RESA/Pf155, FIRA
Between repeats in different asexual antigens	RESA and FIRA, different HRPs, different asparagine-rich proteins
Between repeats in antigens of different life-cycle stages	CS protein and circumsporozoite protein-related antigen (CRA)
Between repeats in the equivalent antigen in different <u>P. falciparum</u> strains	S-antigens, the major merozoite surface antigen
Between repeats in antigens of different species of <u>Plasmodium</u> or other species	RESA/Pf155, hsp70

*A single epitope may be involved in crossreactivities at several levels.

The most extensive network of crossreactions that has been defined involves the RESA/Pf155 molecule. Intramolecular crossreactions within the 3' repeats or between 3' and 5' repeat sequences have been defined with monoclonal antibodies and with antibodies raised against, or affinity-purified on, RESA/Pf155 fusion polypeptides. For example, some MABs raised against the Ag28 protein (which includes RESA/Pf155 3' repeat sequences) only react with the 4 x 4-mer (EENV)₄, others also react with the 3' 8-mer (EENVEHDA) and yet others react with the 5' 11-mer. Intermolecular crossreactions between RESA/Pf155 and several other antigens have been reported. One of these crossreacting antigens is the falciparum interspersed repeat antigen (FIRA), which has a very complex repeat structure based on many related hexameric sequences. Another molecule of 210K which crossreacts with RESA/Pf155 was identified on immunoblots with antisera to RESA/Pf155-fusion polypeptides. Interestingly, although no variation in RESA/Pf155 epitopes has been observed amongst different isolates of *P. falciparum*, a crossreacting epitope is found in the 210K antigen of isolate FCQ-27/PNG but not in the Vietnamese isolate V1. The RESA/Pf155 network of crossreacting antigens includes RESA/Pf155, FIRA, CARP, Pf11.1 and 332. A histidine-rich protein network includes the small histidine and alanine-rich protein (SHARP, HRP 3) and HRP 2 (some cloned parasites do not express HRP 2); anti-SHARP antibodies react with HRP 2 but not with KAHRP/HRP 1. Another set of crossreactions involves asparagine-rich proteins. Affinity-purified antibodies to the repetitive sequence in gp195 from FCQ-27 recognize ABRA (acidic-basic repeat antigen), a schizont protein that is conserved in at least four different strains, but these same antibodies do not crossreact with the alternative repeats in gp195 from other strains.

The crossreactions are not restricted to *Plasmodium* antigens. One antigen-expressing λ gt11-Amp3 clone corresponds to a protein having sequence homology with heat-shock protein (hsp) 70. Antibodies to this protein cross-react with hsp70 of other protozoa.

A recombinant clone, clone 332, encoding a *P. falciparum* antigen which seems to be associated with membranes of infected red blood cells, has been described. Antibodies from a pool of human immune sera affinity-purified on the 332 fusion protein reacted in immunoblots with a series of parasite polypeptides of 260, 215, 205, 155, 96/92 and 48K. The same polypeptides were recognized by antibodies immunopurified on the fusion proteins produced by two other recombinant clones coding for antigens 11.1 and RESA/Pf155. The presence of common epitopes in these antigens was confirmed by the use of two MABs, MAB 33G2 and MAB 9 B11/15. Both reacted with the recombinant antigens produced by clones 11.1, 332 or RESA/Pf155, and MAB 33G2 reacted with synthetic peptides based on the repetitive sequences. Comparison of the amino acid sequences (Table 3) suggests a possible structural basis of the cross-reactivity: the antigens of this family are rich in acidic residues and contain several regularly spaced Glu-Glu dipeptides.

TABLE 3. PARTIAL AMINO ACID SEQUENCES OF FOUR RECOMBINANT CLONES

CLONE	PARTIAL SEQUENCE
333	ESVTEEIAEDK SVIEEAVEKQG SVTEELVEEEE
332	ESVTEEIAEDK
RESA	EENVEHDA EENVEENV
11.1	EEVVEEVVP L I

Two other parasite genes were identified from a second P. falciparum genomic DNA library constructed in λ gt11. Of 200 clones examined, 60 immunoselected antibodies that recognized a series of polypeptides. Serological and DNA hybridization classification suggested that many of these 60 were of the 11.1 type; eight clones were of the RESA/Pf155 type, while 332 and H102 appeared to be unique. At least five genes contribute to this family; partners for 18 other genes have not been identified so far.

The biological significance of the repeat structures and the cross-reacting antigenic epitopes they encode, is not understood. The vaccine trial of RESA/Pf155 in Aotus indicates (as do studies on the CS protein) that repeat epitopes may be targets of protective immune response. However, the prevalence of antibodies to many repeat epitopes is inconsistent with all repeat epitopes being targets of protective responses, and it appears probable that the network of crossreactivities underlies an evasion mechanism. It has been suggested that this may occur as a result of failure of the normal maturation of high-affinity antibody responses, a process that depends upon antigen selection of B lymphocytes sustaining somatic mutations in their antigen receptors. The presence of many crossreacting epitopes (many structural analogues) may cause the persistence of an abnormally high percentage of mutated B cells, resulting in large amounts of low-affinity antibody and delayed emergence of high-affinity antibodies.

MAB 5.1 is a crossreacting antibody that identifies a blood-stage antigen (CRA) and reacts with glutaraldehyde-fixed sporozoites (Hope et al, 1984). Since many immune sera from endemic areas (e.g., The Gambia) react with sporozoites, it is important to determine whether the crossreacting blood-stage antigen induces antibodies to sporozoites. Anti-CS protein antibody levels may reflect the rate of transmission in endemic areas, but such analysis would be complicated if blood stages also induced these antibodies. MAB 5.1 reacts with the sporozoite CS protein and with a 23K blood-stage antigen that contains one element of the CS repeat (NANP) and an adjacent region rich in asparagine, proline and alanine. MAB 5.1 binds to a 22-residue peptide based on the 23K protein sequence and also binds to (NANP)₃. The binding of hyperimmune sera (obtained in The Gambia) to these sequences was investigated. 98% of sera contained antibodies against blood-stage parasites, 80% had antibodies against the CS protein and reacted with (NANP)₃, but only 5% of these sera reacted with the 23K 22-mer. Preabsorption studies with the 22-mer had no effect, whereas (NANP)₃ abolished binding to the CS peptide. In a study of four Gambian sera, (NANP)₃ did not remove reactivity with the 22-mer, nor did the 22-mer remove reactivity with (NANP)₃. Serum from a volunteer immunized with irradiated sporozoites only reacted with (NANP)₃ and these antibodies could be almost totally absorbed by this peptide, but not at all by the 22-mer.

6. ASEXUAL-STAGE ANTIGENS OF P. VIVAX

P. vivax-infected cells from patients' blood have been used to prepare monospecific immune reagents against antigens of the asexual blood stage (K.N. Mendis and P.H. David, unpublished results). A collection of 34 MABs reacting on Western blots with parasite components of different molecular weight were grouped into six categories according to their pattern of IFA reactivity with air-dried intraerythrocytic parasites, as follows:

Type I - fine dots distributed evenly over the entire infected erythrocyte, giving a speckled appearance. Six of the seven MABs in this group reacted with a series of antigens of different size. They did not react with gametocytes or with P. falciparum or P. cynomolgi.

Type II - coarse dots superimposed on a fine speckled background. This could be a derivative of Type I because some MABs produce either a Type I

or Type II staining, depending on the isolate. There were eight MABs in this group.

Type III - a coarse irregular fragmented pattern or a uniform staining of the parasite. Two of the five MABs in this group reacted with gametocytes, and one of these also reacted with P. falciparum and P. cynomolgi.

Type IV - generalized low-intensity staining of the parasite. Two of the ten MABs in this group also reacted with P. falciparum and P. cynomolgi.

Type V - an intense staining around the perimeter of individual merozoites within developing schizonts. One of the three MABs in this group recognized a 200K protein.

Type VI - distinct dots, similar to the staining pattern which in P. falciparum is attributed to rhoptry antigens. There was only one MAB in this category.

Twenty of these MABs were used to screen by IFA 50 different parasite isolates from various geographic regions in Sri Lanka. Although six MABs recognized antigens conserved in all isolates, many of the other MABs revealed a high degree of antigenic polymorphism.

Antigen(s) located on the surface of schizont-infected erythrocytes of P. vivax were detected with human immune serum in an IFA assay using unfixed infected erythrocytes in suspension. Thirteen isolates from patients with acute infections were screened with sera from acute infections or rabbit polyclonal antiserum. Some, but not all, isolates showed an antigen detectable on the schizont surface. The reactivity of heterologous human immune sera indicated that these surface antigens are also polymorphic.

A P. vivax genomic DNA expression library was constructed in λ gt11 using DNA isolated from the Belem strain of parasites in the squirrel monkey. Immune screening was performed with monkey immune serum and MABs. This led to the isolation of P. vivax antigen-expressing clones on which specific antibodies could be purified from hyperimmune monkey serum. Reaction of these antibodies with Western blots of different strains of P. vivax confirmed the existence of antigenic polymorphism.

Screening of the library was performed with a MAB that recognized a 200K polypeptide of P. vivax. This antibody produced a grape-like pattern of immunofluorescence with segmenters, evocative of the pattern obtained with the 195K P. falciparum schizont/merozoite antigen. One strongly reactive clone produced a large fusion protein on which monospecific anti-200K antibody could be immunopurified from hyperimmune anti-P. vivax monkey serum.

7. OPTIMIZATION OF IMMUNOGENICITY

7.1 T-cell Responses in the Context of Malaria Vaccines

While B lymphocytes secrete immunoglobulins which are the principal effector molecules of humoral immunity, T lymphocytes have major effector functions in antibody-independent cell-mediated immunity (CMI). T cells also have an important regulatory role, which includes positive or negative regulation of antibody production by B cells. Thus, the induction of an efficient and long-lasting antibody response against malaria-derived polypeptides is a T-cell-dependent process.

To achieve efficient T-cell activation, the use of an intact plasmodial polypeptide would appear desirable, as it may be expected to contain a sufficient number of T-cell sites to circumvent genetic MHC-restriction.

However, the intact proteins may also induce suppression or give rise to nonprotective antibodies. These negative effects may be avoided by using synthetic oligopeptides of known B-cell reactivity and coupled to a carrier protein (e.g., tetanus toxoid) or, alternatively, by using a fusion protein containing relevant plasmodial epitopes. However, in cases where the T-cell reactive structures are part of the foreign protein, boosting of the vaccine-induced immune response by subsequent natural infection may not take place.

What are the structural constraints required to confer T-cell reactivity to an oligopeptide? Available evidence indicates that there are no particular constraints with regard to length. The minimal size for efficient T-cell activation seems to be approximately ten amino acids, similar to the requirement for efficient peptide-antibody interaction. Likewise, the chemical properties required for specific interaction of a peptide with the T-cell receptor (e.g., hydrophilicity, charge) appear to be the same as those of the B-cell epitopes. Studies of T-cell reactivity of soluble globular proteins have shown that a T-cell reactive site should contain both hydrophilic and hydrophobic structures, preferentially in an amphipathic alpha-helical conformation. It was suggested that the hydrophilic face of such a peptide was necessary for interaction with the T-cell receptor, while the hydrophobic face would be required for interaction with the lipid membrane of the antigen-presenting cell and/or the presenting MHC-class II molecule. There is evidence that certain amphipathic alpha-helical sequences contained in the C-terminal repeat region of RESA/Pf155 from *P. falciparum* merozoites may be good stimulators of T cells from some (but not all) RESA/Pf155 seropositive human donors. However, it should be emphasized that structural properties other than amphipathicity may also confer T-cell reactivity to an oligopeptide (e.g., sequential arrangement of hydrophobic and hydrophilic regions, strong positive charge as in polylysine, etc.).

For the design of efficient malaria vaccines, the various candidate polypeptides should be screened for both B-cell and T-cell reactive epitopes. Several groups have recently produced human T-cell clones which specifically respond to various *P. falciparum* antigens in an MHC-class II restricted manner. Such clones and the corresponding T-cell hybridomas may soon become as important for characterization and screening of plasmodial antigens as are MABs at the present time.

7.2 Genetic Control of the Immune Response to FSV-1 and the Circumsporozoite (CS) Protein

Different H-2 congenic mice were immunized with synthetic peptide or the recombinant *P. falciparum* sporozoite vaccine currently being used in human vaccination trials (Good et al, 1986). Only mice carrying the I-A^b gene responded to the 24-mer (NANP)₆. When using the whole vaccine molecule, R32tet32, two of seven other mouse strains responded with antibody formation and T-cell proliferation in vitro, suggesting that this molecule contains additional T-cell sites. Vaccination of mice with a recombinant vaccinia virus containing the entire *P. falciparum* CS gene gave an anti-CS response in several other mouse strains, suggesting the presence of additional T-cell activating sites in the CS molecule outside the repeat region. It was concluded that a vaccine should contain, in addition to B-cell epitopes, several T-cell epitopes to ensure a response in the majority of vaccinated subjects. Moreover, to give a proper anamnestic response, these structures should originate from the same natural parasite protein and be covalently linked.

8. EXPRESSION OF MALARIA ANTIGEN GENES IN VACCINIA VIRUS

The recent development of techniques for the insertion of foreign genes into the genome of vaccinia under the control of vaccinia promoter sequences

has facilitated the expression of a number of malarial antigens in cells infected with the virus in vitro and in animals vaccinated with the recombinant virus. Antigens which have been expressed include two different S-antigens, RESA/Pf155, SHARP and CRA (the CS protein crossreacting antigen).

The S-antigen was found to be secreted from cultured mammalian cells infected with the appropriate recombinant vaccinia virus; however, immunization with the virus induced a very poor antibody response to the S-antigen. Subsequently, the S-antigen was targeted to the surface of virus-infected cells by adding sequences encoding the hydrophobic and intracellular domains of a mouse immunoglobulin gene to the 3' end of the malaria coding sequence. Much greater antibody responses were elicited in rabbits and mice immunized with virus expressing the anchored form of the S-antigen.

The repeats in the gene encoding the membrane-targeted S-antigen have been deleted, leaving a Bam HI site to facilitate replacement with 16, 32 or 48 copies of the 4-amino acid repeating sequence of the P. falciparum CS protein. In this way it should be possible to obtain expression of the CS protein repeats or epitopes of other important antigens on the surface of the infected cells.

The RESA/Pf155 gene cloned into vaccinia has been altered by in vitro mutagenesis, first, to remove its intron, thereby allowing expression in recombinant vaccinia, and, second, to delete naturally immunogenic epitopes from the intact molecule. If the intron was not removed, a number of small polypeptides could be detected on Western blots of cell lysates; deletion of the intron allowed the expression and detection of a protein slightly larger than the P. falciparum product, together with a number of fragments detected in cell lysates. The contribution each part of the molecule makes to the anti-RESA/Pf155 immune response can now be assessed.

The entire CRA gene has been expressed in recombinant vaccinia as one approach to examining the biological significance of the crossreaction between this asexual blood-stage antigen and the CS protein. The vaccinia product was slightly larger than the P. falciparum product on SDS-PAGE gels. Rabbits immunized with the CRA recombinant vaccinia virus produced an antibody response to CRA and also to (NANP)₃. Thus, an "antisporeozoite" antibody response can be induced by immunization with recombinant vaccinia virus encoding the crossreacting asexual blood-stage antigen.

9. GENERAL DISCUSSION

It was suggested that a coordination meeting be held to bring together groups, including manufacturers, involved in malaria vaccine development to address a number of technical and regulatory issues, including the definition of a common standard for manufacture (such as the purity of antigen preparations) and negotiations with regulatory authorities, in which WHO might play a coordinating role. It is clear that malaria vaccine development is breaking new ground and generating the need for much more basic research in areas which have not been fully addressed to date, such as the phenomenon of genetic restriction in response to specific peptides and the relationship between the immunogenicity of native proteins and that of synthetic derivatives.

There are several P. falciparum antigens which may have functional equivalents in other human malarial species, such as P. vivax, and which include current vaccine candidate antigens. It will be extremely difficult to evaluate all current candidates, including specific derivatives of them, since much of the analysis required is empirical. At present, it cannot be assumed that all candidate antigens have been identified, and there is a continuing need for basic research. There are too few nonhuman primates available for

extensive testing of antigens in vivo, but there is no in vitro test for predicting protection. Immunological studies in rodent models using analogous molecules from rodent malaria species will be essential. It will therefore be necessary to be selective in the development of candidate vaccine antigens and to base their selection on rigorous scientific grounds. Progress in this area will also be highly dependent on collaboration with laboratories in endemic areas to maximize the use of field research results in the development of vaccines.

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