MALARIA IS ONE of the major parasitic diseases affecting humans today, contributing greatly to the high mortality rates in the developing world. The original epidemiologic concept of "malaria eradication" for large endemic areas, introduced by the World Health Organization (WHO) in the 1960s, has now been replaced by the concept of "control," given the increase in the morbidity and mortality rates. The resurgence of malaria can be attributed to the appearance of parasite strains and insect vectors resistant to the eradication measures taken. This situation has favored emphasis on basic malaria research oriented toward a better understanding of the host's immune response and the parasite's molecular biology and biochemical characteristics, with the aim of obtaining immunoprophylactic methods capable of preventing the further spread of this disease. In this report we will review some of the attempts carried out by the different research groups around the world, focusing toward the end on the work conducted at the Instituto de Inmunologia, Hospital San Juan de Dios, Bogota, Colombia.

IMMUNE RESPONSE IN MALARIA

Natural immunity. The existence of a natural immune mechanism, or innate resistance, in endemic areas has received several partial explanations such as (1) genetic disorders that alter the development of the asexual cycle of the parasite in the host's RBC, as is the case of the several hemoglobinopathies and the glucose 6-phosphate dehydrogenase deficiency; (2) alterations in the merozoite penetration mechanisms into the erythrocyte, eg, the lack of the Duffy blood group (Fya- and Fyb), which prevents invasion by Plasmodium vivax; (3) the absence of glycophorin A in the red cell membrane, as in En(a-); mutants that can confer resistance to the Plasmodium falciparum merozoite invasion process; and (4) differences in the isozyme patterns of the plasma enzymatic systems, as well as many other factors.

Acquired immunity. The mechanistic basis of acquired immunity to malaria is more controversial. We do not know why patients who live in endemic areas are slow to develop antimalarial immunity. Children from immune women are protected during the first 3 months of life; after this period they become susceptible and can present as many as two or three episodes of malaria annually. To partially explain this phenomenon we should consider some of the mechanisms that alter immune reactivity. One of these is the parasite's capacity of avoiding the immune response.

Parasite evasion of immunity. P. falciparum is essentially an intracellular parasite. It is only free from the cell at the sporozoite and merozoite stages. This extracellular period is probably too short to ensure adequate antigen presentation by accessory cells of the immune system. Conversely, although several soluble and exoantigens are released into the blood stream by the infected cells, they have not yet been shown to elicit a protective immune response.

It has also been found that most P. falciparum antigens expressed at the mature sporozoite and schizont stages contain large regions of repeated sequences, which might induce T-cell-independent B-cell polyclonal activation. This in turn could explain the hypergammaglobulinemia in patients with malaria. In general, repeated sequences have been implicated as "immundistractor" mechanisms used by plasmodia, trypanosomes, and other parasites as a means of evading the protective immune response. If this interpretation is correct, it could mean that a large number of antigens remain hidden and are never recognized. This aspect of the immune response will be reviewed later in a report by Dr R. Anders.

Another probable evasion mechanism involves the attachment of the infected erythrocytes to the vascular endothelium, preventing the passage of the mature stages of the parasite through the endoplasmatic reticulum of the spleen, allowing it to avoid being cleared from the blood stream. This issue will be addressed in a future review article by Dr R. Howard.

The development of P. falciparum gametocytes from asexual blood-stage parasites is a complex phenomenon that involves poorly understood processes. Multiple antigenic and morphologic modifications characterize this differentiation process. Interestingly, the host's immune response against the parasite's asexual stages does not appear to influence maturation of the gametocytes, suggesting an immune evasion mechanism that allows transmission of the disease. Immune evasion by erythrocytes infected with sexual stage parasites is also suggested by the observation that immature gametocyte-infected cells are sequestered from the peripheral circulation. The time of appearance of mature gametocytes in the blood stream actually coincides with the evening biting habits of the Anophelines, thereby favoring disease transmission.

The presence of immunomodulator mechanisms in acute malarial infections has been described. Measuring the lymphoproliferative response of peripheral blood mononuclear cells of patients in acute stages of the disease has shown that an immunosuppression status exists in the presence of merozoite antigens. The absence of in vitro reactivity does not correlate with either the degree of the humoral immune response or the polyclonal activation of the CD8+ cellular...
subpopulation. Nevertheless, an additional unknown functional alteration leads to a modification in the immune reactivity against the asexual blood forms, permitting the parasite to remain alive.

PARASITE ANTIGENS AND DEVELOPMENT OF THE VACCINE

There are several conceptual problems to deal with when formulating a strategy for development of a malaria vaccine. Among them are (1) acquired immunity which is stage- and species-specific; (2) the permanence of the parasite for long periods of time within the host's cell; and (3) the induction of immunosuppression and the complex relationship between host and parasite. All of these factors lead us to suggest that strategies used in the development of other types of vaccines may not be applicable to malaria.

During the last 40 years there have been repeated attempts to obtain an effective vaccine against malaria. A conventional approach was used in the first trials, where antigens were obtained by precipitation with alumina and experimental animals were immunized with the use of adjuvants. Clyde et al. performed the first human trials using sporozoites from irradiated mosquitoes as immunogens. These experiments produced the first clear indication that induced immunity in susceptible hosts was a potential method of controlling the disease. Nevertheless, the use of this type of immunogen as a vaccine is highly impractical.

Basic research permitted the development of techniques to isolate and characterize various antigenically relevant proteins of the parasite. The continuous culture method for \textit{P. falciparum}, developed by the seminal work of Trager and Jensen, permitted the preparation of large quantities of antigen without depending exclusively on isolates, either from patients or experimental animals. This, together with advances in immunologic and biotechnologic tools such as monoclonal antibodies and genetic engineering, has permitted the antigenic characterization of many isolates, as well as the cloning and expression of a large number of plasmodial genes and their products.

It is clear that for final protection trials, very pure antigens are needed. These must be safe, innocuous, and free from any contaminant material that could induce toxic or secondary damage, allergic reactions, or otherwise be able to induce partial or total immunosuppression. Contaminant material could also elicit cross-reactive antibodies against "self" antigens, or be the cause of any other anti-"self" reactivities. The purity needed can only be achieved by using purified antigens, obtained either by gene cloning in high expression vectors or by chemical synthesis of peptides corresponding to \textit{P. falciparum} immunogenic epitopes.

There are five stages in which the parasite is accessible to the immune system of the host: sporozoite, merozoite, mature infected erythrocyte, exoerythrocyte, and gametocyte. The hepatic phase of the parasite's development, known as the exoerythrocytic stage, has been poorly characterized. Attempts to identify the antigens of these exoerythrocytic forms have largely been directed towards identification of shared epitopes with antigens in the sporozoite and intraerythrocytic stages. For example, it has been shown that monoclonal antibodies directed against the glycoprotein Pf 195-200 of the asexual stage are capable of recognizing antigens of the exoerythrocyte. More recently, Guerin-Marchand et al. have reported the cloning of a specific gene of the exoerythrocytic stage that encodes an immunogenic \textit{P. falciparum} protein containing several 17-amino acid repeats. Immunology studies in a murine model by Schofield et al. demonstrated very interesting gamma-interferon inhibitory activity acting on the exoerythrocyte stage in vivo. This effect was suppressed with animals depleted of CD8 + cells. This result clearly demonstrates that the cellular immune response plays an active role in the control of the development of the hepatic forms. These two recent findings open new horizons for the development of an effective vaccine against this stage and rapid progress is anticipated in this area.

Merozoite proteins used in vaccine trials. The production of new merozoites in the infected erythrocyte is a complex process that leads to the expression of a wide variety of antigens in different \textit{P. falciparum} isolates. Most of the described antigens have been identified either intracellularly or on the surface of mature schizonts and free merozoites. Different assays with some of these surface antigens have demonstrated the capacity of antibodies directed against these molecules to confer passive immunity in vivo or to block the reinvasion by merozoites in vitro. Some of these antibodies induced a variable protective immune response. Since the surface proteins of the merozoite have been implicated in the mechanism of host erythrocyte invasion, they may be excellent vaccine candidates.

The first immunization and experimental challenge trials against the asexual blood forms of \textit{P. falciparum} were performed with merozoites obtained from continuous cultures. The monkeys immunized with these immunogens were partially protected, although it should be pointed out that the usefulness of these antigens is very limited, due to their possible contamination with immunogenic proteins of the erythrocytes.

The major surface antigen identified in the mature schizonts and merozoites of the \textit{P. falciparum} parasite is the protein with relative molecular mass (Mr) 195,000 d (195 Kd). This molecule, one of the best-studied proteins of the plasmodia, is the precursor of several smaller proteins (83, 42, and 19 Kd), some of which are expressed on the merozoite's surface. The complete structure of the 195-Kd protein has been deduced by several different groups. It was determined that the N-terminal fragment corresponds to the 83-Kd molecule, and the C-terminal fragment corresponds to the 42-Kd molecule. The 83-Kd protein and its processed products are lost during the reinvasion process. It is not expressed in the ring stage and can be recovered after reinvasion from supernatants of continuous synchronized cultures. Monoclonal antibodies directed against this protein are capable of inhibiting the in vitro development of asexual blood forms. Saimiri monkeys have been used in protective trials using the purified 83-Kd protein as immunogen; synthetic peptides, constructed from the N-terminal sequence of this protein, coupled to tetanus toxoid, have also been used for the same purpose. These trials showed...
that these immunogens were capable of inducing specific antibodies, as detected by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent assays (IIFA), as well as eliciting partial protection in animals experimentally challenged with different *P falciparum* strains.

An Mr 155,000 d parasite protein can be found in the newly invaded ring-infected erythrocyte membrane, suggesting that the protein is transferred from the merozoite to the host cell during the invasion process. The gene encoding for this 155-Kd protein, also known as ring erythrocyte surface antigen (RESA), has been cloned; it encodes two blocks of separate oligopeptide repeats. Antibodies directed against the dimeric tetrapeptide from the carboxy terminal region of this protein have proven capable of inhibiting the in vitro reinvasion of erythrocytes. RESA contains T epitopes recognized by mononuclear cells of peripheral blood from patients with a previous history of malaria. Recombinant proteins containing elements from the Pf155/RESA molecule have been tested to see whether they induce protective immunity in susceptible monkeys. From the 14 *Aotus* monkeys immunized with these proteins, nine were partially protected. There was a direct correlation between protection and induction of antibodies against repetitive fragments of the RESA molecule.

The S antigen was perhaps the first protein found in sera from patients with high parasitemia, and was later found in continuous cultures of *P falciparum*. This antigen shows great thermostability, heterogeneity, and serologic diversification. The sequence of several isolates has been reported to comprise a block of tandem repeats that are not homologous to each other. Contradictory results have been found regarding the participation of the S antigen in the induction of the protective immune response. Saul et al showed that a monoclonal antibody directed against the S antigen, present in the isolate FCQ-27/PNG, inhibits the in vitro reinvasion of erythrocytes by *P falciparum* merozoites. Nevertheless, levels of antibodies directed against the S antigen present in sera from patients living in endemic areas do not correlate with the in vitro inhibitory capacity. In addition, its great heterogeneity is more likely associated with “immunodistractive” functions.

Miettinen-Baumann et al have characterized a 46-Kd glycoprotein from the surface of merozoites. The protein is not related to the 195-Kd molecule, but is also synthesized during the late stages of the intraerythrocytic cycle. Polyclonal antibodies directed against the 46-Kd molecule partially inhibit the in vitro reinvasion of erythrocytes by merozoites. Further biologic characterization of this protein will follow gene cloning and knowledge of its amino acid sequence.

In addition to merozoite surface molecules that could be receptors for erythrocyte invasion, another class of merozoite molecules are of great interest. The paired rhoptry organelles at the merozoite apex are involved in formation of a “tight junction” between the merozoite membrane and erythrocyte membrane. Rhoptry proteins may be transferred to the host cell membrane at the time of junction formation. Rhoptry proteins of 143, 132, and 102 Kd have been isolated by immunoaffinity methods and used for immunization of *Aotus* monkeys. They have been shown to be capable of inducing partial protective immunity. To date, insufficient information is available to evaluate their value as vaccine molecules.

Dubois et al have characterized *P falciparum* proteins immunoprecipitated by polyclonal antibodies from experimentally infected *Saimiri* monkeys. They compared the proteins that reacted with antibodies from protected and immunized, nonprotected animals. 100-, 96- and 76-Kd malarial proteins were immunoprecipitated only by antibodies from protected animals. These proteins were purified, and when used to immunize *Saimiri* monkeys, induced partial protective immunity. Immune sera from endemic-area patients also precipitate the 96-Kd protein, showing that it might be related to the naturally acquired protective immunity. Characterization of the 96-Kd antigen by isoelectric focusing identified three proteins with different isoelectric points (pl). One of them, pl 5.25, is thermostable and can be recovered from *P falciparum* culture supernatants.

Bonnefoy et al have reported the cloning and expression, in *Escherichia coli*, of the gene that encodes a 96-Kd protein. This protein is composed of 50-amino acid repeats similar to those found in two other malarial proteins (glycophorin binding protein 130 and Ag 78).

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**Fig 1.** Isolated proteins of *P falciparum* merozoites, analyzed by Western blotting. (A and B) 195 Kd; (C through E) 145 Kd + 155 Kd; (H) 112 Kd; (I) 97 Kd; (J) 96 Kd; (K) 96 Kd; (M) 83 Kd; (N through P) 83 Kd; (Q) 59 Kd; (R) 84 Kd; (S) 52 Kd; (Y) 50 Kd; (T) 35 Kd; L, merozoite lysate control.
Antigenic evaluation of Colombian isolates. Our research has focused on the isolation and chemical characterization of *P. falciparum* merozoite proteins in order to test their ability to induce a protective immune response in the *Aotus* monkey experimental model. Proteins of molecular weights 195, 155, 115, 112, 105, 97, 96, 95, 90, 83, 60, 55, 54, 52, 50, 40, 35, 30, and 23 Kd have been studied because they are among the most prominent proteins in this stage of the parasite's life cycle.2,3 Some of these proteins are present in the merozoite membrane. We have isolated 200 to 400 μg of each in a highly purified form (Fig 1).

To induce a protective immune response against the parasite, we immunized *Aotus* monkeys three times with the purified preparations of proteins 155, 115, 105, 90, 83, 60, 55, 40, 35, 30, and 23 Kd. These monkeys are highly susceptible to laboratory strains of *P. falciparum* and require drug treatment to survive plasmodial infections. Serologic analysis performed five days before challenge showed that the *Aotus* had variable antibody titers against blood stages of the parasite, as demonstrated by IIFA and ELISA. After challenge, animals immunized with the 155-Kd and 55-Kd proteins showed delayed onset of parasitemias by five to seven days, suggesting a partial protective immunity induced by the vaccination. Monkeys immunized with the 84-Kd fragment of the 195-Kd glycoprotein were completely protected, showing either no detectable blood infection or very low parasitemia with spontaneous recovery. A similar behavior was found in monkeys immunized with the 35-Kd molecule. Immunization with the other proteins conferred no evident protection, since challenge produced infections similar to the controls, requiring drug treatment (Table 1).

Simultaneous to the demonstration that specific merozoite proteins could confer protective antimalarial immunity in *Aotus* monkeys,46 we determined the N-terminal amino acid sequences of the following proteins: 105, 100, 87, 82, 58, 55, 47, 40, 38, 37, 35, 26, 18, and 16 Kd (unpublished data, July 1984). Oligonucleotide probes were also synthesized in order to identify the clones of the 55-Kd and 35-Kd proteins in a *P. falciparum* genomic library that we had constructed. Other groups had already cloned and sequenced the genes encoding for several other merozoite proteins. The sequence of Pf155/RESA protein identified by Perlmann et al54 was obtained by Coppell et al12 and Cowman et al13 using cDNA cloning techniques. The amino acid and nucleotide sequences of the 195-Kd molecule were reported by Holder et al48 and subsequently by Mackay et al.15 On the basis of the amino acid sequences reported by these authors and our own data, we chemically synthesized several peptides corresponding to

### Table 1. Postchallenge Parasitemia in Monkeys Immunized With Purified Proteins

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Time course of parasitemia in *Aotus* monkeys immunized with purified proteins from *P. falciparum* merozoites. Control monkeys were immunized with saline solution emulsified with Freund's adjuvant. Challenge was performed by intravenous inoculation of 5 × 10⁷ live ring-forms of *P. falciparum* FVO strain maintained through passage in naive *Aotus* monkeys. Parasitemias were monitored daily by examination of peripheral blood smears stained with acridine orange.

*Q* indicates the beginning of chloroquine therapy.
different fragments of the 83-Kd protein, representing 47% of the entire protein sequence. Twelve more peptides, corresponding to the amino acid sequence of the molecules 35, 42, 55, and 155 Kd were also synthesized.70

These synthesized peptides were used to immunize another group of Aotus monkeys five times, using Freund's adjuvant and bovine serum albumine (BSA) as a carrier. Most of the animals developed low antibody titers against P falciparum schizonts before the challenge. On day 90, animals were challenged by intravenous injection of five million infected RBCs of P falciparum FVO strain.

Several peptides did not elicit a protective immune response against the experimental infection, regardless of high antibody titers detected by both IIFA and ELISA.71 Nevertheless, immunization with particular peptides (83.1, 55.1, 35.1, 83.2, 83.26, 83.18, 83.30, and 83.23) delayed the onset of the disease in some of the vaccinated animals, suggesting that these peptides were able to elicit partial protective immunity.71

Based on this information, a new immunization scheme was developed, using a combination of two or three of the partially protective peptides.71 The immunized monkeys developed a moderate level of antibodies, as detected by the techniques mentioned above. When the challenge on this new group of Aotus was carried out, four of eight animals immunized with a mixture of two peptides (SPf 31.1 and SPf 55.1) developed a disease similar to the controls. The remaining four animals developed moderate parasitemia and spontaneously recovered.

Of six monkeys immunized with a mixture of three peptides (SPf 35.1, SPf 55.1, and SPf 83.1), three developed low parasitemia levels that peaked between days 10 and 15, significantly later than in the control groups. These six monkeys recovered spontaneously. The remaining three animals never developed parasitemia (Fig 2). This experiment was repeated with another group of 25 animals. Eighteen monkeys were vaccinated using the mixture of the three synthetic peptides as mentioned before; seven more served as controls. While all the controls rapidly developed high parasitemias that required treatment, nine of this new vaccinated group acquired complete protection, five developed low parasitemias that spontaneously regressed, and the remaining four behaved as the controls. This experiment has been repeated several times with similar results (unpublished data, August 1986).

Based on this information, and in order to overcome
carrier problems, we designed two hybrid protein polymers. As seen in Fig 3, these hybrid polymers contain epitopes known to induce partial or complete protection in experimentally infected models; therefore they were good candidates for a malaria vaccine.60,64,71 The safety, immunogenicity, and protective efficacy of SPF(66)30 as well as its individual peptides were first assayed in monkeys. No histopathologic, behavioral, or clinical laboratory abnormalities were observed in any of the immunized and killed animals. The immunogenicity and protective efficacy of some of the individual peptides of SPF(105)20 had been previously assayed by us71 and by others55 in monkeys.

To examine the safety, immunogenicity, and protection offered by these synthetic proteins against malaria in humans and, taking into account all the national and international ethical considerations including WHO recommendations for human trials,40,72 13 male volunteers were selected to be vaccinated from a total of 109 healthy high school graduate volunteer soldiers from the Colombian Military Forces.73

Slight pain, local erythema, and induration at the inoculation site were noted in all of the subjects after immunization. None of the volunteers presented fever or showed significant changes in blood cell count, blood chemistry, or urinalysis. All the autoimmunity tests were negative.

Antibody titers were determined by peptide-antipeptide ELISA using the synthetic protein molecules as antigens. IIFA showed that all sera contained antibodies to merozoites-schizonts in titers between 1:20 and 1:160. All immunized sera and naive individuals were negative or had titers below 1:20 by IIFA tests. No correlation was found between antibody levels and antimalaria protection. Proliferation assays of peripheral mononuclear cells (PBMC) using either the hybrid protein SPF(66)30 or sonicates of purified schizonts as antigens showed stimulation indexes (SI) below 3.0 before the first vaccination. After each vaccination, and before the challenge, the stimulation indexes varied from 0.61 to 35.10, but still did not correlate with either antibody titers or antimalarial protection.

After the seventh day of the challenge, the volunteers who received saline solution had parasitemias that rose in 12 hours from very low levels to more than 0.5%. Drug therapy was administered immediately, with rapid clinical response and without residual effects or sequelae. Two of four volunteers vaccinated with SPF(105)20 showed partial control of the infection with low parasite counts during days 13 and 14, followed by development of parasitemias greater than 0.5%. These subjects received drug therapy and recovered with no clinical problems, complications, or sequela. The other two behaved as the controls and were treated similarly. Three of five volunteers vaccinated with SPF(66)30 had mild infections with steady decrease in parasite counts and total recovery by day 21. The fourth volunteer had parasitemias below 0.41%, and on day 10, with a very low parasitemia, decided to leave the study and receive prompt chemotherapy. The fifth developed parasitemia similar to the control group (Table 2).

Several points of this study deserve emphasis. Synthetic hybrid molecules were used containing epitopes from different infectious stages of the parasite. This strategy could be used for future design of vaccines with epitopes from multiple pathogens. The molecules were polymerized to form large synthetic proteins containing several epitopes; thus they were highly immunogenic without recourse to foreign carriers. The synthetic proteins were used without special immunopotentiators, although addition of such potentiators may further increase their protective capacities. However, surprisingly there was no correlation between the different humoral and cellular immunologic parameters measured and the antimalarial protection observed. A considerable delay of the infectious process in half of the individuals vaccinated with the SPF(105)20 molecule suggests this immunogen may also be effective against sporozoite challenge. SPF(66)30 gave strong protection in most of the inoculated volunteers, even those who received only two doses, suggesting that with more potent adjuvants or slightly improved formulation, complete protection may be achieved.

The synthetic hybrid polymer SPF(66)30 is the first synthetic vaccine for human use against the asexual blood stages

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Fig 3. Amino acid sequences of the synthetic hybrid molecules SPF(66)30 and SPF(105)20.
### Table 2. Development of Postchallenge Parasitemia in the Vaccinated Volunteers

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Time course of parasitemia in human volunteers immunized with the polymeric synthetic hybrid proteins SPF(66)30 and SPF(105)20. Control volunteers were immunized with alumina. Challenge was performed by intravenous inoculation of 1 × 10⁷ live ring-infected erythrocytes of *P. falciparum* (a wild Colombian strain) derived from a naive volunteer previously infected by transfusion. Parasitemias were monitored every 12 hours after the third day, by thick and thin blood smears stained with Giemsa, field stain, and acidine orange. Results of acidine orange staining are shown.

*Beginning of chloroquine therapy.
of *P. falciparum* malaria. It is necessary to evaluate the epidemiologic impact after introduction of this new immunogen in communities living in endemic zones. Given that the size of the infecting inoculum during natural exposure is much smaller than the one experimentally used, it is possible that the protection would be more substantial with naturally acquired infections. Since this is a merozoitic vaccine, the long-term objective is to confer protection against asexual blood stage infection equivalent to that naturally acquired by many exposures. It is also necessary to study the molecular mechanisms involved in the immune response induced by synthetic polymeric molecules.

**ACKNOWLEDGMENT**

The Military Forces of Colombia, especially the volunteers and medical personnel of the Military Hospital, deserve a special gratitude for their courage and desire to cooperate in the development of this vaccine. The suggestions and the critical reading of this manuscript by Dr. Russell Howard and the staff of the Instituto de Inmunologia are greatly appreciated. The authors thank Fanny Calvo de Simon for her help with the manuscript.

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Development of an asexual blood stage malaria vaccine

A Moreno and ME Patarroyo