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Induction of Protective Immunity to *Plasmodium falciparum* in *Saimiri sciureus* Monkeys with Partially Purified Exoantigens

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Soluble *Plasmodium falciparum* exoantigens in crude culture supernatant fluids induced protective immunity against experimental falciparum malaria in Bolivian *Saimiri sciureus* monkeys. Susceptible squirrel monkeys were vaccinated with an aluminum hydroxide-fortified fraction purified from culture supernatants of *P. falciparum* Indochina I and Geneve/SGE-1 by cation-exchange (sulfopropyl-trisacryl) chromatography. Animals immunized with sulfopropyl-purified and corresponding control immunogens were challenged with whole blood containing monkey-adapted virulent organisms of the Indochina I strain. Hematological, serological, and parasitological profiles, including the appearance of crisis forms, served as potential indicators of protection. This immunogen conferred significant clinical protection of squirrel monkeys against needle challenge with the homologous Indochina I strain and a moderate degree of heterologous strain immunity.

Exoantigens are soluble proteinaceous moieties naturally released into the blood plasma of animals infected with *Plasmodium* and *Babesia* parasites or into the supernatant medium of in vitro cultures of these organisms. Many of these antigens have been isolated, identified, and evaluated for their immunogenic potential (19, 23). Induction of protective immunity to bovine babesiosis with culture-derived *Babesia* exoantigens has been demonstrated in cattle (22, 25), and similar efforts are presently under way in our laboratory with polypeptides produced in vitro by the human malaria parasite *Plasmodium falciparum*.

Soluble *P. falciparum* exoantigens in crude culture supernatant fluids have been shown in a series of preliminary experiments to induce protective immunity against clinical malaria in *Saimiri sciureus* squirrel monkeys (23). Subsequently, potential malarial immunogens have been purified from *P. falciparum* culture fluids by ion-exchange chromatography and have been subjected to immunochemical analysis (28). Optimal fractionation of exoantigens has been achieved with cation-exchange chromatographic procedures by using sulfopropyl (SP)-trisacryl gels (J. Thelu, unpublished data). Western immunoblot analysis indicated that the antigenic fraction, referred to as the SP fraction, contained major malarial proteins at molecular weights of 100,000 (100 K), 83K, and 70K, with minor antigens observed at molecular weights of 185 K, 43K, and 20K (L. Shamansky, H. Y. Liu, L. Hager, and M. Ristic, Mol. Biochem. Parasitol., submitted for publication).

The present study was designed to evaluate the protective immunity induced by SP-trisacryl-purified antigenic fractions from culture supernatant fluids of the Indochina I and Geneve/SGE-1 strains of *P. falciparum* in *S. sciureus* monkeys.

MATERIALS AND METHODS

Experimental animals. Healthy adult male *S. sciureus* squirrel monkeys of Bolivian origin (South American Primates, Inc., Miami, Fla.) were selected for the study. Before use in the experiments, all animals were screened for previous or current exposure to *Plasmodium* and *Trypanosoma* species and for filarial and gastrointestinal organisms and were determined to be free of parasites. Any animal found to be serologically positive for *Plasmodium* spp. was eliminated from the study. Monkeys weighing 875 ± 55 g were randomly assigned to duplicate experiments of identical design. Monkeys were housed in a controlled environment with a temperature of 21 to 29°C and 70% humidity. They were fed monkey chow (Ralston Purina Co., St. Louis, Mo.) supplemented with fresh fruits, essential minerals, and vitamins.

In vitro cultivation of *P. falciparum* strains. Parasites of the Indochina I (Vietnamese isolate; squirrel monkey adapted) and Geneve/SGE-1 (Senegalese isolate; not squirrel monkey adapted) strains of *P. falciparum* were grown in vitro by the method of Trager and Jensen (30). Cultures were initiated from cryogenically preserved stocks in 35-mm culture plates with washed infected human type A^+ erythrocytes (0.5 to 1.5% parasitized erythrocytes) at an 8 to 10% hematocrit. Cultivation was done in RPMI 1640 culture medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), antibiotics, and 10% human type A^+ serum. Parasites were subsequently propagated under similar conditions in tissue culture flasks to allow greater production of antigen-containing supernatant fluids. These cultures were incubated at 37°C in an atmosphere of 6% O_2–10% CO_2–84% N_2.

Preparation of immunogens. Asynchronous cultures of both *P. falciparum* strains were continuously maintained in human type A^+ erythrocytes at parasitemia levels of 1 to 4%. Supernatant fluids were collected daily from cultures exhibiting active parasite growth with multiple developmental forms (ring, trophozoite, and schizont) as observed on consecutive thin smears of infected erythrocytes. Culture supernatants containing potential immunogens were pooled, centrifuged at 1,000 × *g* for 15 min at 4°C, and then filtered through 0.45 and 0.22-μm-pore membrane filters (Millipore

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Corp., Bedford, Mass.). Supernatants were stored at −70°C before use in purification procedures. In addition, normal control supernatant fluids from uninfected cultures were similarly prepared.

Exoantigens were partially purified from crude culture supernatants by SP-trisacryl (LKB Instruments, Inc., Rockville, Md.) cation-exchange chromatography. Briefly, supernatant fluids were thawed, concentrated 10 times by pervaporation at 4°C, and then dialyzed against 60 volumes of 0.01 M sodium acetate buffer (pH 3.7) for 48 h at 4°C. Subsequently, this material was placed on an SP-trisacryl column (50 by 200 mm), and proteins were eluted in 0.01 M sodium acetate buffer (pH 3.7) with an increasing stepwise salt gradient to 0.3 M NaCl at a flow rate of 300 ml/h. Antigenic fractions (250 ml per fraction) were monitored by the indirect enzyme-linked immunosorbent assay (32) by using human placental immunoglobulin G from immune females living in endemic areas of Africa. The indirect fluorescent antibody (IFA) titer of pooled immunoglobulin G was 1:5,120. A corresponding control immunoglobulin G pool was prepared from placentas of malaria-free individuals. Antigenic activity was found in fractions which eluted between 0.2 M and 0.3 M NaCl concentrations (termed SP fraction). These fractions were pooled, concentrated by lyophilization, and dialyzed against 0.01 M phosphate-buffered saline (pH 7.2) for 48 h at 4°C to remove excess salts. Finally, the SP fraction was adjusted to a protein concentration of 2 mg/ml, sterilized by membrane filtration, and stored at −70°C until use as an immunogen. An SP-purified fraction from uninfected cultures was similarly prepared as a negative control immunogen.

Vaccination and challenge schedule. In total, four monkeys were vaccinated with the SP fraction derived from the Indochina I strain, eight animals were immunized with the SP immunogen derived from the Geneve/SGE-1 strain, and six animals received the control preparation. Each dose consisted of 2 mg of total protein in a 1-ml volume. These preparations were gently mixed with 127.5 μl of 1.86% aluminum hydroxide adjuvant (Superfos, Copenhagen, Denmark) for 1 h at 25°C. Two subcutaneous inoculations were administered at a 28-day interval. Challenge exposure consisted of an intravenous injection of fresh infected blood containing 10^7 virulent monkey-adapted Indochina I organisms on day 100, 10 weeks after the second vaccination. Subsequently, SP fractions derived from Indochina I and Geneve/SGE-1 culture supernatants will be referred to as homologous and heterologous immunogens, respectively.

Collection of clinicopathological data. A complete hematological profile was considered essential as an indicator of possible adverse effects caused by vaccination and of the degree of protective immunity after challenge exposure. Blood samples (200 to 300 μl) were collected by femoral venipuncture on a weekly basis beginning 2 weeks before the first vaccination and continuing until 4 weeks postchallenge. Major hematologic determinations were conducted electronically on a computerized Coulter Counter (S-Plus II model; Coulter Electronics, Inc., Hialeah, Fla.). The Coulter program analyzed each sample in triplicate and gave the mean of three observations. The parameters studied were total leukocyte count with differential analysis; erythrocyte, reticulocyte, and platelet counts; and hemoglobin and hematocrit values. Also, blood urea nitrogen (BUN) and serum glutamic pyruvic transaminase (SGPT) levels were measured as indicators of kidney and liver functions, respectively. Antibody titers specific to *P. falciparum* were monitored weekly throughout the study by IFA (26).

In addition to hematology, blood chemistry, and serology, parasitemia (percentage of parasitized erythrocytes) levels were determined every 48 h postchallenge by both thick and thin Giemsa-stained peripheral blood smears. Special emphasis was also placed on the appearance of any morphologically abnormal parasites, i.e., crisis forms (18, 27), as a potential correlate of protective immunity in vivo.

General observations, such as appetite, overall health, activity, demeanor, etc., were noted daily, and body weights were recorded every 2 weeks. Before experimentation, a decision was made to avoid chemotherapeutic treatment of clinically ill animals after the challenge exposure to allow an unbiased evaluation of protective immunity throughout the course of infection.

RESULTS

No adverse effects from immunization were noted in any of the vaccinated animals. After each inoculation, monkeys were examined for the presence of local inflammatory reactions at the injection site. None were found. Hematological values in all animals remained normal throughout the vaccination period. Specific antibody responses to the *P. falciparum* SP immunogens peaked 14 days after the second vaccination as mean IFA titers reached 1:33 (range, 1:20 to 1:80) and 1:70 (range, 1:40 to 1:160) in monkeys immunized with the homologous and heterologous immunogens, respectively. Titers in animals which received the control preparation were less than 1:10. Elevated antibody levels in vaccinated monkeys persisted for 2 months after the second vaccination.

The most pronounced hematologic abnormalities, i.e., decreases in hematocrit levels, paralleled increases in parasitemia approximately 14 days after challenge exposure (Fig. 1). The mean maximum parasitemia levels of monkeys in groups which received the SP immunogens were significantly lower (2.9% homologous, $P < 0.001$; 7.4% heterologous, $P < 0.01$) than those of the control group (14.5%). Maximum hematocrit decreases corresponded with the mean maximum parasitemia levels of each group (Table 1). Recovery to normal hematological levels was notably accelerated in the animals which received the homologous SP fraction, was moderate in monkeys immunized with the heterologous SP fraction, and was considerably delayed in control animals. No major differences in total leukocyte and reticulocyte counts were found among vaccinated and control animals. A moderate thrombocytopenia was generally evident 14 days postchallenge in monkeys of all groups; however, recovery to base-line platelet levels could be correlated with immune status and coincided with the normalization of other clinical parameters.

At 2 weeks postchallenge, control animals showed dramatic increases in SGPT (+119%) and BUN (+58%) concentrations compared with the respective levels found in monkeys vaccinated with the homologous (SGPT, +41%; BUN, −16%) and heterologous (SGPT, +43%; BUN, +12%) SP immunogens.

Antibody levels in all monkeys increased rapidly after challenge exposure, and maximal titers were observed 14 days thereafter. Titers in vaccinated animals remained stable 4 weeks postinoculation (mean IFA titers, 1:3,330 [homologous] and 1:2,945 [heterologous]), while titers in control monkeys steadily declined to 1:640. Crisis forms or damaged parasites were first observed in thin peripheral blood smears between 11 and 13 days postchallenge, with mean parasitemia levels on those days notably lower in vaccinated monkeys (Table 1). Moreover, more than 50% of the para-

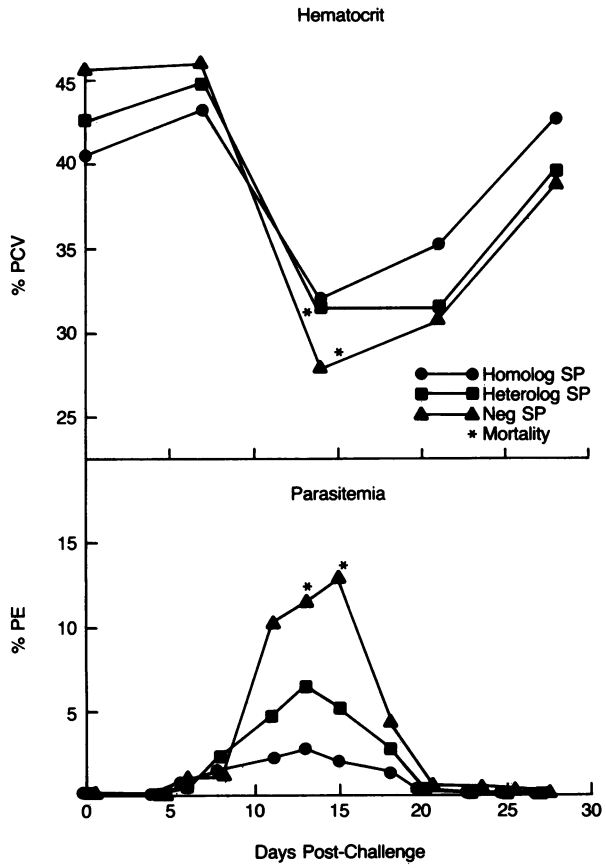

FIG. 1. Hematocrit and parasitemia levels in vaccinated (SP immunogens from the homologous Indochina I and heterologous Geneve/SGE-1 *P. falciparum* strains) and control (negative SP immunogen) monkeys after challenge exposure with 10^7 *P. falciparum* Indochina I organisms.

sitic forms seen in immunized animals at that time (day 12 postchallenge) demonstrated irregular structural features (Fig. 2). The two types of intracellular crisis forms with altered morphology observed in vivo were point or pyknotic forms and abnormal, vacuolated organisms.

On days 13 and 15 postchallenge, two of the animals of the control group died. These monkeys became lethargic and anorexic 2 days before succumbing to clinical malaria and had lost 11.1% of their prechallenge body weight. Subsequent pathological examinations revealed hepatosplenomegaly with spleens six times normal size, splenic rupture accompanied by hemoperitoneum, jaundice, and parasitic occlusions and microscopic lesions in major organs (J. Everitt, manuscript in preparation).

DISCUSSION

SP-trisacryl cation-exchange chromatography appeared to be an important first step in the purification of *P. falciparum* exoantigens from the supernatant fluid of in vitro cultures. Subsequently, this antigenic fraction, termed SP, was shown to induce protective immunity to malaria in *S. sciureus* squirrel monkeys. Shamansky et al. (submitted) have characterized the SP antigens by Western immunoblotting with a polyspecific rabbit antiserum to the SP fraction. This antiserum is primarily schizont specific, has an IFA titer of 1:1,280, and inhibits merozoite invasion of human erythrocytes in vitro (C. Fajfar-Whetstone, unpublished data). Western blots indicated that the SP fraction contained at least six parasite proteins ranging in molecular weight from 20K to 185K. The estimated yield of *P. falciparum* parasite antigens was 500 μg from 1 liter of supernatant fluid. Crossed immunoelectrophoretic analysis demonstrated that the SP fraction was free of serum immunoglobulin G and erythrocytic antigens and that the cation-exchange chromatographic procedure was effective in eliminating 80% of the proteins found in the crude supernatant fluids (M. A. James, unpublished data). Shamansky et al. (submitted) have further purified the SP fraction by high-performance liquid chromatography, isolated major parasite glycoproteins with molecular weights of 100K and 83K in pure form, and determined the N-terminal amino acid sequences.

There is good evidence indicating that soluble *P. falciparum* exoantigens are naturally released into the plasma of infected individuals (15, 17) and into the supernatant fluids of in vitro cultures (13, 14, 23, 24, 28, 33). Apparently, exoantigens are released during the schizont developmental stage or at the time of schizont rupture and merozoite invasion of new erythrocytes or both (16, 24, 33). The major 83K glycoprotein purified by Shamansky et al. (submitted) from the SP antigenic fraction is present in relatively high quantities in culture supernatant fluids and is thought to be a principal component of the merozoite surface coat (4, 7, 10). It has been suggested that the 83K antigen is a processed polypeptide derived from a high-molecular-weight precursor protein (6, 8, 9).

The protective potential of soluble malarial exoantigens was first recognized by Todorovic et al. (29) during immu-

TABLE 1. Clinicopathological responses[a] of vaccinated (homologous and heterologous *P. falciparum* SP immunogens) and control (negative SP immunogen) squirrel monkeys to challenge exposure with *P. falciparum* Indochina I

Immunogens	Maximum % decrease in:			Mean maximum parasitemia (%)	First appearance of crisis forms		Mortality
	Hematocrit	Hemoglobin	Erythrocytes		Postchallenge (days)	Mean parasitemia (%)	
Homologous SP (Indochina I)	30.5 ± 4.7[b]	31.5 ± 7.1	28.5 ± 7.4	2.9 ± 0.6[c]	12.8 ± 1.3	2.5 ± 0.9[b]	0/4
Heterologous SP (Geneve/SGE-1)	35.6 ± 5.3	39.8 ± 4.7	40.9 ± 5.8	7.4 ± 1.7[d]	12.8 ± 1.3	4.2 ± 1.8	0/8
Negative SP	47.4 ± 7.3	47.6 ± 7.5	50.0 ± 7.5	14.5 ± 3.7	11.0 ± 2.6	7.7 ± 2.4	2/6

[a] Group means plus or minus the standard error (means of two experiments).
[b] $P < 0.05$ (Student's t test).
[c] $P < 0.001$ (Student's t test).
[d] $P < 0.01$ (Student's t test).

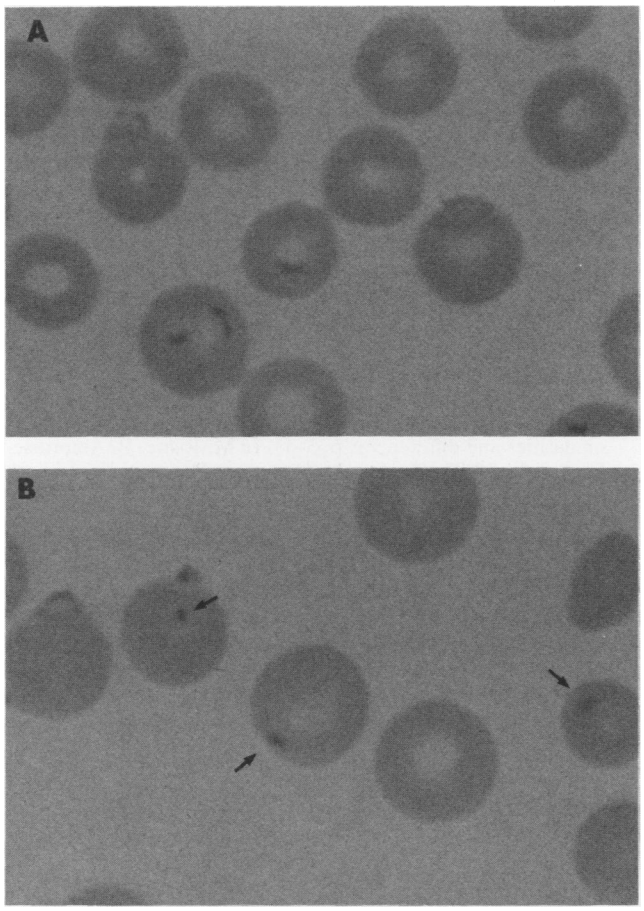

FIG. 2. Typical (more than 50% of parasites observed) morphology of parasitic forms as seen in Giemsa-stained thin peripheral blood smears from control (A) and vaccinated (B) monkeys 12 days postchallenge. A majority of normal parasitic forms (A) were seen in control animals; however, morphologically altered pyknotic parasites (B; arrows) were found in vaccinated monkeys. ×1,000.

nization studies in chickens with serum antigens of *P. gallinaceum* and subsequently by Collins et al. (1), who immunized monkeys with similar antigens of *P. knowlesi*. Recently, soluble antigens isolated from supernatants of *P. falciparum* cultures were proposed as target vaccine candidates on the basis of their identification with soluble antigens circulating in the plasma of acutely infected individuals (13). Preliminary experiments conducted in our laboratory demonstrated the protective capability of crude supernatant fluids derived from *P. falciparum* cultures (23) and served as an important prelude to our current studies with purified immunogens.

In the present study, various clinicopathological parameters were found to be important indicators of protective immunity against disease. Potential protective criteria included reduction of parasitemia levels, parasite killing as evidenced by the appearance of crisis forms, moderate hematocrit and hemoglobin decreases, and, importantly, the maintenance of normal blood SGPT and BUN levels. The latter criteria were consistent with the hepatomegaly and proliferative glomerulonephritis seen upon necropsy of control animals. Several other recent reports have described the immunization of splenectomized and intact *S. sciureus* monkeys with purified antigenic preparations chemically extracted from *P. falciparum* schizonts or merozoites or both (2, 5, 20, 21). Protection against homologous (2) and heterologous (5, 20, 21) challenge was variable and dependent on the antigenic composition of the respective immunogens. Although at least a partial degree of protective immunity was reported by other workers, the criteria used to substantiate such evidence were limited to hematocrit, parasitemia, and serological determinations. We sought to relate these parameters to biochemical and pathological changes.

The mechanism of protection induced by immunization with the purified SP fraction of *P. falciparum* culture supernatant fluids remains incompletely defined. The moderate level of humoral immunity seen after vaccination suggests that antibodies may be only a component of the total protective host immune response (2, 3, 21). Induction of parasite crisis forms (18, 27) appeared to be an important feature of these protective immune responses in vaccinated animals of the present study. Currently, sera from these immunized animals are being tested in an *S. sciureus* erythrocytic culture system (Fajfar-Whetstone, unpublished data) to determine the effect on intracellular parasite development in vitro. Jensen et al. (11, 12) and Vande Waa et al. (31) reported on the strong relationship between intraerythrocytic parasiticidal activity, i.e., crisis form induction, and protective immunity to human falciparum malaria in Sudan. Their findings substantiate the role of cell-mediated immune responses in the acquired resistance to malaria.

In conclusion, the present study demonstrated that cation-exchange-purified *P. falciparum* exoantigens induced significant homologous protection with a moderate degree of heterologous strain immunity against clinical malaria caused by a virulent challenge exposure with *P. falciparum*. These results suggest that the SP antigenic fraction of culture supernatant fluids or pure malarial protein(s) contained therein may be good vaccine candidates for immunoprophylactic regimens against falciparum malaria.

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LITERATURE CITED

1. **Collins, W. E., P. G. Contacos, A. J. Harrison, P. S. Staufill, and J. C. Skinner.** 1976. Attempts to immunize monkeys against *Plasmodium knowlesi* by using heat-stable, serum-soluble antigens. Am. J. Trop. Med. Hyg. **26**:373–376.
2. **Dubois, P., J.-P. Dedet, T. Fandeur, C. Roussihon, M. Jendoubi, S. Pauillac, O. Mercereau-Puijalon, and L. Pereira da Silva.** 1984. Protective immunization of the squirrel monkey against asexual blood stages of *Plasmodium falciparum* by use of parasite protein fractions. Proc. Natl. Acad. Sci. U.S.A. **81**: 229–232.
3. **Fandeur, T., P. Dubois, J. Gysin, J.-P. Dedet, and L. Pereira da Silva.** 1984. *In vitro* and *in vivo* studies on protective and inhibitory antibodies against *Plasmodium falciparum* in the saimiri monkey. J. Immunol. **132**:432–437.
4. **Freeman, R. R., and A. A. Holder.** 1983. Surface antigens of malaria merozoites. A high molecular weight precursor is processed to an 83,000 mol. wt. form expressed on the surface of *Plasmodium falciparum* merozoites. J. Exp. Med. **158**:1647–1653.
5. **Hall, R., J. E. Hyde, M. Goman, D. L. Simmons, I. A. Hope, M.**

Mackay, J. Scaife, B. Merkli, R. Richle, and J. Stocker. 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature (London) **311**:379–382.

6. **Hall, R., A. Osland, J. E. Hyde, D. L. Simmons, I. A. Hope, and J. G. Scaife.** 1984. Processing, polymorphism, and biological significance of P190, a major surface antigen of the erythrocytic forms of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **11**:61–80.
7. **Heidrich, H.-G., W. Strych, and J. E. K. Mrema.** 1983. Identification of surface and internal antigens from spontaneously released *Plasmodium falciparum* merozoites by radioiodination and metabolic labelling. Z. Parasitenkd. **69**:715–725.
8. **Holder, A. A., and R. R. Freeman.** 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. J. Exp. Med. **156**:1528–1538.
9. **Holder, A. A., and R. R. Freeman.** 1984. The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. J. Exp. Med. **160**:624–629.
10. **Howard, R. F., and R. T. Reese.** 1984. Synthesis of merozoite proteins and glycoproteins during the schizogony of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **10**:319–334.
11. **Jensen, J. B., M. T. Boland, J. S. Allan, J. M. Carlin, J. A. Vande Waa, A. A. Divo, and M. A. S. Akood.** 1983. Association between human serum-induced crisis forms in cultured *Plasmodium falciparum* and clinical immunity to malaria in Sudan. Infect. Immun. **41**:1302–1311.
12. **Jensen, J. B., S. L. Hoffman, M. T. Boland, M. A. S. Akood, L. W. Laughlin, L. Kurniawan, and H. A. Marwoto.** 1984. Comparison of immunity to malaria in Sudan and Indonesia: crisis-form versus merozoite-invasion inhibition. Proc. Natl. Acad. Sci. U.S.A. **81**:922–925.
13. **Jepsen, S.** 1983. Inhibition of *in vitro* growth of *Plasmodium falciparum* by purified antimalarial human IgG antibodies. Isolation of target antigens from culture supernatants. Scand. J. Immunol. **18**:567–571.
14. **Jepsen, S., and B. J. Andersen.** 1981. Immunoadsorbent isolation of antigens from the culture medium of *in vitro* cultivated *Plasmodium falciparum*. Acta Pathol. Microbiol. Scand. Sect. C. **89**:99–103.
15. **Jepsen, S., and N. H. Axelsen.** 1980. Antigens and antibodies in *Plasmodium falciparum* malaria studied by immunoelectrophoretic methods. Acta Pathol. Microbiol. Scand. Sect. C **88**:263–270.
16. **McColm, A. A., and P. I. Trigg.** 1980. Release of radioisotope labeled antigens from *Plasmodium knowlesi* during merozoite re-invasion *in vitro*. Parasitology **81**:199–209.
17. **McGregor, I. A., M. E. Turner, K. Williams, and P. Hall.** 1968. Soluble antigens in the blood of African patients with severe *Plasmodium falciparum* malaria. Lancet **i**:881–884.
18. **Ockenhouse, C. F., S. Schulman, and H. L. Shear.** 1984. Induction of crisis forms in the human malaria parasite *Plasmodium falciparum* by γ interferon-activated, monocyte-derived macrophages. J. Immunol. **133**:1601–1608.
19. **Perrin, L. H., and R. Dayal.** 1982. Immunity to asexual erythrocytic stages of *Plasmodium falciparum*: role of defined antigens in the humoral response. Immunol. Rev. **61**:245–269.
20. **Perrin, L. H., M. Loche, J.-P. Dedet, C. Roussilhon, and T. Fandeur.** 1984. Immunization against *Plasmodium falciparum* asexual blood stages using soluble antigens. Clin. Exp. Immunol. **56**:67–72.
21. **Perrin, L. H., B. Merkli, M. Loche, C. Chizzolini, J. Smart, and R. Richle.** 1984. Antimalarial immunity in *Saimiri* monkeys. Immunization with surface components of asexual blood stages. J. Exp. Med. **160**:441–451.
22. **Ristic, M.** 1984. Research on babesiosis vaccines, p. 103–122. *In* M. Ristic, P. Ambroise-Thomas, and J. P. Kreier (ed.), Malaria and babesiosis: research findings and control measures. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
23. **Ristic, M., and J. P. Kreier.** 1984. Malaria and babesiosis: similarities and differences, p. 3–33. *In* M. Ristic, P. Ambroise-Thomas, and J. P. Kreier (ed.), Malaria and babesiosis: research findings and control measures. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
24. **Rodriguez de Silva, L., M. Loche, R. Dayal, and L. H. Perrin.** 1983. *Plasmodium falciparum* polypeptides released during *in vitro* cultivation. Bull. W.H.O. **61**:105–112.
25. **Smith, R. D., M. A. James, M. Ristic, M. Aikawa, and C. A. Vega.** 1981. Bovine babesiosis: protection of cattle with culture-derived soluble *Babesia bovis* antigen. Science **212**:335–338.
26. **Sulzer, A. J., and M. Wilson.** 1971. Fluorescent antibody test for malaria. Crit. Rev. Clin. Lab. Sci. **2**:601–619.
27. **Taliaferro, W. H., and L. G. Taliaferro.** 1944. The effect of immunity on the asexual reproduction of *Plasmodium brasilianum*. J. Infect. Dis. **75**:1–32.
28. **Thelu, J., P. Ambroise-Thomas, M. Contat, and P. Kupka.** 1982. Antigènes excrétés-sécrétés par *Plasmodium falciparum* en cultures *in vitro*. Etude comparée avec les antigènes somatiques et les antigènes figurés. Bull. W.H.O. **60**:761–766.
29. **Todorovic, R., D. Ferris, and M. Ristic.** 1967. Immunogenic properties of serum antigens from chickens acutely infected with *Plasmodium gallinaceum*. Ann. Trop. Med. Parasitol. **61**:117–124.
30. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193**:673–675.
31. **Vande Waa, J. A., J. B. Jensen, M. A. S. Akood, and R. Bayoumi.** 1984. Longitudinal study on the in vitro immune response to *Plasmodium falciparum* in Sudan. Infect. Immun. **45**:505–510.
32. **Voller, A., D. Bidwell, G. Huldt, and E. Engvall.** 1974. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. W.H.O. **51**:209–211.
33. **Wilson, R. J. M., and R. K. Bartholomew.** 1975. The release of antigens by *Plasmodium falciparum*. Parasitology **71**:183–192.