INFECTION AND IMMUNITY, June 1994, p. 2354–2361
0019-9567/94/$04.00+0

Vol. 62, No. 6

Serum Antibody Immunoglobulin G of Mice Convalescent from *Plasmodium yoelii* Infection Inhibits Growth of *Plasmodium falciparum* In Vitro: Blood Stage Antigens of *P. falciparum* Involved in Interspecies Cross-Reactive Inhibition of Parasite Growth

PRATIMA RAY, NARESH SAHOO, BALWAN SINGH, AND FRED A. S. KIRONDE*

Malaria Group, International Center for Genetic Engineering and Biotechnology, NII Campus, New Delhi 110 067, India

Received 13 December 1993/Returned for modification 20 January 1994/Accepted 15 March 1994

We demonstrated that antibodies in the serum of BALB/c mice convalescent from *Plasmodium yoelii* infection inhibit the in vitro growth of *Plasmodium falciparum*. Blood stage *P. falciparum* antigens that cross-react with the convalescent-phase mouse serum antibodies were identified and partially characterized. Convalescent-phase mouse serum immunoglobulin G (IgG) reacted with *P. falciparum* lysates at up to a 1:15,000 dilution of the immune sera and bound to *P. falciparum*-parasitized erythrocytes at up to a 1:5,000 dilution of the sera. The cross-reactive moieties of antigens in parasite lysates were resistant to oxidation by periodate but sensitive to trypsinization. About 15 polypeptides (M_rs of 15,000 to 110,000) of *P. falciparum* blood stages were recognized by the convalescent-phase mouse anti-*P. yoelii* sera; many of these antigens were metabolically ^{35}S labeled and specifically immunoprecipitated. Also, virtually all of the cross-reactive antigens were recognized by human malaria-immune sera. The anti-*P. yoelii* serum antibodies bound, with high affinity, to at least five of the cross-reactive antigens (M_rs of 107,000, 84,000, 53,000, 36,000, and 30,000). By phase separation in Triton X-114, eight interspecies cross-reactive antigens (M_rs of 84,000, 76,000, 51,000, 31,000, 29,000, 28,000, 23,000, and 22,000) were found to be integral membrane proteins. Convalescent-phase mouse serum IgG strongly inhibited the differentiation of *P. falciparum* from schizonts to rings; 75 μg of IgG per ml caused 80% inhibition of release of merozoites and their invasion into erythrocytes. On the other hand, the anti-*P. yoelii* serum antibodies also inhibited intracellular development of *P. falciparum* from rings to schizonts; 25 μg of IgG per ml caused 50% inhibition. Inhibition of *P. falciparum* growth by anti-*P. yoelii* serum IgG suggests that some of the interspecies cross-reactive antigens contain important conserved epitopes and induce protective antibodies against *P. falciparum*.

Malaria parasites are so capable of extensive antigenic variation that it is imperative to investigate the immunogenic potential of antigens which are shared between different *Plasmodium* species and are likely to contain epitopes that have been conserved for common structure and function. Such interspecies conserved epitopes have great promise for the development of widely effective vaccines (5, 6, 31). The existence of conserved antigen analogs throughout *Plasmodium* species is well demonstrated by cross-hybridization experiments (9) and comparison of the amino acid sequences of cloned proteins from different *Plasmodium* species (8, 11, 14, 21, 30, 38). However, only a few genes encoding proteins of diverse *Plasmodium* species have been cloned and sequenced. Thus, at present, comparative analysis of the primary structure of analogous antigens from various plasmodial species is restricted to only few proteins, notably the circumsporozoite protein, the merozoite surface antigen 1 (MSA1), the apical merozoite antigen 1, and the sporozoite surface protein 2 (3, 5, 11, 14, 28, 38). On the other hand, analysis of interspecies immune response or cross-reactivity constitutes a simple and less limited approach toward identification of important conserved antigens, because, even though antigens of malaria parasites are generally species specific, antibodies raised against one *Plasmodium* species recognize several antigens from other species (17, 20, 35), confirming the presence of homologous molecules with interspecies conserved epitopes. To identify protective interspecies conserved antigens of *Plasmodium falciparum*, we have investigated the binding of proteins in *P. falciparum* lysate and on whole parasite cells by antibodies produced during repeated *P. yoelii* infection of BALB/c mice. We have also examined and demonstrated the inhibition of in vitro growth of *P. falciparum* by mouse anti-*Plasmodium yoelii* serum antibody immunoglobulin G (IgG).

MATERIALS AND METHODS

Buffers and reagents. Protein A, insolubilized on Sepharose CL-4B, alkaline phosphatase-conjugated anti-mouse IgG, and anti-human IgG antibodies were obtained from Sigma Chemical Co., St. Louis, Mo. Other reagents and chemicals were of the highest quality available from international suppliers. Extraction buffer consisted of 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 7.5), 5 mM EDTA, 5 mM ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone. Color substrate for immunoblots consisted of 0.17 mg of 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) per

* Corresponding author. Mailing address: Malaria Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, NII Campus, New Delhi 110 067, India. Phone: 91 11 6865007. Fax: 91 11 6862316.

ml, 0.33 mg of Nitro Blue Tetrazolium per ml, 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris-HCl (pH 9.5). Color substrate for enzyme-linked immunosorbent assays (ELISAs) consisted of 1 mg of nitrophenyl phosphate, 0.5 mM $MgCl_2$, and 10 mM diethanolamine (pH 9.5).

Parasites. A lethal strain of *P. yoelii nigeriensis* was obtained from G. P. Dutta of Central Drug Research Institute, Lucknow, India. The strain is resistant to chloroquine but sensitive to the combined regime of sulfadoxine and pyrimethamine (23). An Indian isolate (FCD3) of *P. falciparum* was obtained from V. K. Bhasin of University of Delhi, New Delhi, India, and maintained in human O^+ RhD^+ erythrocytes in RPMI culture medium (RPMI 1640, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 40 mg of gentamicin per ml, 10% normal human serum, 23 mM $NaHCO_3$ [pH 7.5]) according to the method of Trager and Jensen (37). Cultures of *P. falciparum* were synchronized by lysis with 5% sorbitol (25). Two successive rounds of sorbitol treatment gave more than 97% synchronized cultures.

Sera and purified serum IgG. Convalescent-phase mouse anti-*P. yoelii* serum was produced as follows. Each of a group of 15 inbred BALB/c mice was infected with *P. yoelii nigeriensis* and cured with drugs (0.5 mg of sulfadoxine and 25 μg of pyrimethamine per mouse daily for 5 days). After three infection-cure cycles, BALB/c mice become resistant to challenge doses of up to 10^8 parasitized erythrocytes per mouse (23), whereas mice given equivalent drug treatment without parasite infection (control mice) are fully susceptible. The 15 convalescent mice were bled, and the serum of each mouse was collected separately. As required, the sera were pooled (by mixing equal volumes of serum from each mouse), inactivated by heating at 56°C for 30 min, and stored in aliquots at −20°C. Mouse serum IgG was prepared by ammonium sulfate precipitation and chromatography over a column of staphylococcal protein A beads (13). The IgG was then dialyzed against RPMI culture medium and sterilized by ultrafiltration. Control mouse sera and IgG were similarly prepared from BALB/c mice that received the same drug treatment as convalescent mice but without parasite infection. Pooled human malaria-immune serum was obtained from blood samples collected from 10 residents of the Koraput district of Orissa, India. The pooled serum tested positive on smears of *P. falciparum*-infected erythrocytes, with an immunofluorescent antibody test (IFAT) titer of 1:3,000. Control (negative) human sera were obtained from six individuals reporting no previous malarial illness and, at a 1:100 dilution, were negative by IFAT.

Metabolic labeling, parasite isolation, radioimmunoprecipitation, and SDS-PAGE. Asynchronous blood stages of *P. falciparum* were metabolically labeled by incubation with [^{35}S]methionine (100 μCi/ml) in normal RPMI culture medium (containing 30 mg of methionine per liter) for 24 h. Parasites were isolated by incubation of parasitized erythrocytes with 0.02% saponin in RPMI at 37°C for 20 min, and antigens were extracted from pelleted parasites by incubation at 4°C for 1 h in extraction buffer. Insoluble material was sedimented by centrifugation (1,000 × *g* for 1 min), and the supernatant parasite lysate was stored at −70°C. *P. falciparum* lysates were incubated with anti-*P. yoelii* serum, and the immune complexes were isolated on Sepharose CL-4B–protein A beads, with stringent washing of the beads, as described previously (12, 22). Control mouse and normal human sera were included as negative controls. Antigens were eluted from the beads by heating in sample buffer (24) and were subjected to polyacrylamide gel electrophoresis (PAGE) through either gradient (5 to 15%) or 10% acrylamide gels. The gels were then processed for fluorography (16).

Selection of cross-reactive anti-*P. yoelii* IgG on *P. falciparum* antigens. Nitrocellulose membrane was blotted with *P. falciparum* antigens resolved by SDS-PAGE (0.5 mg of *P. falciparum* lysate proteins electrophoresed through slab gels [0.15 by 16 by 12 cm^3]) and incubated in blocking solution consisting of phosphate-buffered saline (PBS [145 mM NaCl, 20 mM sodium phosphate, pH 7.4]) supplemented with 3% bovine serum albumin (BSA) and 0.02% NaN_3. Antibodies were selected by probing each membrane with 50 ml of PBSAT (PBS supplemented with 1% BSA and 0.1% Tween 20) containing 1 ml of convalescent-phase mouse anti-*P. yoelii* serum for 6 h at room temperature on an orbital shaker. The nitrocellulose was washed five times, for 20 min each, with PBSAT and once with PBS. Anti-*P. yoelii* antibodies adsorbed on nitrocellulose-blotted *P. falciparum* antigens were then eluted with 0.2 M glycine buffer (pH 2.8) containing 145 mM NaCl, and the eluate was neutralized with aliquots of 4 M Na_2CO_3.

Immunoblots and ELISAs. Antibody binding of parasite lysate proteins blotted onto nitrocellulose (immunoblot assay) or applied in coats to microtiter plate wells (ELISA) was evaluated generally as described previously (23, 36). The blotted nitrocellulose or coated microtiter wells were incubated in blocking solution and probed, respectively, with primary antibody serum, alkaline phosphatase-conjugated secondary antibody, and color substrate. Convalescent-phase mouse, control mouse, normal human, and malaria-immune human sera were tested at dilutions of 1:300 (immunoblots) and 1:400 (ELISA).

Relative affinity of cross-reactive antibodies. The relative affinity of antibody was measured by using ammonium thiocyanate elution (26) of the antibody bound either to parasite polypeptides blotted on nitrocellulose or to total parasite lysate antigens applied to microtiter wells.

Inhibition of *P. falciparum* invasion of erythrocytes and intraerythrocytic development. The inhibitory effect of anti-*P. yoelii* antibody on invasion of erythrocytes by *P. falciparum* and on development of the parasites inside the erythrocytes was evaluated essentially as described in reference 39.

(i) Cross-inhibition of invasion. Thirty-four hours after the second of two sequential treatments of the culture with sorbitol (25), different amounts of IgG from pooled convalescent-phase mouse sera were added to schizont-stage parasites at 0.5% parasitemia. Twenty-four hours after the addition of purified IgG to the schizont stages, blood smears were prepared. The ring-stage parasites in the culture were then counted per 10,000 erythrocytes, and the percent parasitemia was calculated. Purified control mouse serum IgG was similarly tested at the same time.

(ii) Cross-inhibition of intraerythrocytic development. Immediately after the second of two successive synchronizations of *P. falciparum* with sorbitol, we added various amounts of purified convalescent-phase mouse serum IgG to microtiter wells containing synchronized ring-stage parasites at 3% parasitemia. After 24 h of culture in the presence of purified IgG, smears of the culture were prepared and the numbers of schizonts per 10,000 erythrocytes were determined. Negative control experiments with control mouse serum IgG were conducted at the same time. For both the invasion assay and intraerythrocytic inhibition experiments, inhibition was calculated as % inhibition = [%p (control) − %p (test)]/[%p (control)], where %p is percent parasitemia.

IFAT and Triton X-114 phase separation. Binding of convalescent-phase mouse serum IgG to *P. falciparum* proteins in smears of cultured parasitized erythrocytes was evaluated by IFAT as described previously (35). Parasites were solubilized,

and the antigens were analyzed by temperature-dependent phase separation in Triton X-114 solution as described previously (1, 33).

RESULTS

Binding of anti-*P. yoelii* convalescent-phase mouse serum IgG to *P. falciparum* antigens (titer). Serum samples from 15 mice repeatedly infected with *P. yoelii* and drug cured (convalescent-phase mouse sera) were pooled and serially diluted. Different dilutions of the sera were incubated with coats of *P. falciparum* lysates applied to microtiter wells (at 0.5 μg of lysate protein per well). The amount of mouse serum IgG specifically bound to lysate antigen was estimated by ELISA. We found that convalescent-phase mouse serum IgG could bind to coated *P. falciparum* antigens at high dilutions of the serum (titer of 1:15,000), indicating that some of the cross-reactive *P. yoelii* antigens are highly immunogenic and induce potent anti-*P. falciparum* antibodies during repeated *P. yoelii* infection. When tested individually, serum samples from all of the 15 convalescent mice showed virtually equivalent titers. For comparison, we similarly tested the convalescent-phase mouse anti-*P. yoelii* sera against coated *P. yoelii*-lysate antigens. Binding of convalescent-phase mouse serum IgG to the *P. yoelii* antigens was observed at serum dilutions of up to 1:100,000. In IFAT assays, convalescent-phase mouse serum IgG specifically bound to ring-form, trophozoite, schizont, and merozoite stages in acetone-fixed smears of *P. falciparum*-infected blood (results not shown); fluorescence was seen at serum dilutions of up to 1:5,000. These results show that *P. falciparum* antigens in parasite lysates or on smears of whole parasite cells contain interspecies cross-reactive epitopes which are recognized by potent antibodies in convalescent-phase mouse anti-*P. yoelii* sera.

Nature of the cross-reactive determinants. Several important molecules of *Plasmodium* species are glycosylated, and, in some cases, the carbohydrate moiety is believed to have a significant role in the host-parasite interaction (15, 29, 33). It has been shown that antigenic carbohydrate components of several parasite molecules are sensitive to periodate oxidation (40). In order to find out whether some of the interspecies cross-reactive epitopes of *P. falciparum* antigen are carbohydrates, we incubated 20 mM sodium periodate in microtiter wells coated with different amounts of *P. falciparum* lysate antigen. Formed carbohydrate dialdehydes were reduced with sodium borohydride, and the amount of convalescent-phase mouse serum IgG which binds to the antigen after the treatment with periodate and borohydride was estimated by ELISA. The results (Fig. 1A) show that periodate oxidation of coated *P. falciparum* lysate antigen does not alter the binding of anti-*P. yoelii* antibody. However, in control experiments with lysates of uninfected RhD^+ erythrocytes (similar to the erythrocytes used in our *P. falciparum* culture), 20 mM periodate effectively removed carbohydrate moieties from the RhD glycoprotein. On the other hand, when coats of the *P. falciparum* lysate antigens were applied to microtiter wells and incubated with various concentrations of trypsin, a dose-dependent loss of binding sites for the anti-*P. yoelii* antibody occurred, with all of the cross-reactive determinants apparently being digested in 30 min by 12.5 mg of trypsin per ml (Fig. 1B). Thus, the results from periodate oxidation and trypsinization experiments with coated parasite lysate show that the interspecies cross-reactive moieties of *P. falciparum* which are recognized by anti-*P. yoelii* serum antibody are mostly polypeptides.

Sizes and stage-specific expression of *P. falciparum* proteins recognized by anti-*P. yoelii* antibodies. During *P. falciparum* growth in erythrocytes (erythrocytic schizogony), the parasite undergoes several morphologically different stages about every 48 h. After erythrocyte invasion, the ring form appears first, feeds on the erythrocyte cytoplasm, and passes through the trophozoite stage before undergoing nuclear divisions which produce the schizont. Invasive merozoites are released when the schizont matures and the host erythrocyte membrane ruptures. These morphological changes are associated with a well-controlled pattern of polypeptide synthesis (2, 10) such that, whereas some proteins are synthesized throughout erythrocytic schizogony and are common to all blood stages, many are mainly present at a particular stage (stage specific).

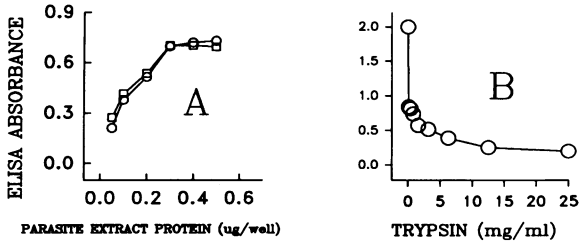

FIG. 1. Nature of the cross-reactive epitopes. (A) Microtiter wells coated with various amounts of *P. falciparum* lysate antigens (0 to 0.5 μg per well) were incubated with 20 mM sodium periodate in 50 mM sodium acetate at pH 4.5 (1 h), 50 mM sodium borohydride in PBS (30 min), and diluted (1:400) convalescent-phase mouse serum (2 h), respectively. The amount of specifically bound mouse IgG was then estimated by ELISA. □, treatment with periodate; ○, treatment with the same solutions except periodate. The ELISA readings for control wells probed with the same immune reagents without incubation with sodium acetate buffer, periodate, and borohydride (no-treatment control) showed that treatment with acetate buffer and borohydride solution alone did not affect the results. (B) Microtiter wells coated with *P. falciparum* lysate antigens (0.7 μg per well) were incubated for 30 min with the concentrations of trypsin shown and for 2 h with diluted (1:400) convalescent-phase mouse serum. The amount of bound mouse IgG was estimated by ELISA.

During in vitro culture, *P. falciparum* normally grows asynchronously (all of the blood stage forms are present in the culture medium) but can be synchronized by lysing the merozoite, trophozoite, and schizont forms with 5% sorbitol (25); rings are largely unaffected. To determine the sizes of the interspecies cross-reactive antigens and the time of their expression during erythrocytic schizogony, we prepared parasite lysates from asynchronous and synchronized cultures. Antigens in parasite lysates were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed to visualize the interspecies cross-reactive polypeptides. Figure 2 (lane A) shows that, in assays using asynchronous parasite cultures, at least 15 polypeptide bands of *P. falciparum* cross-react with mouse anti-*P. yoelii* antibody. The relative molecular masses (in kilodaltons) of the cross-reactive bands (Fig. 2, lane A) are as follows: a, 107; b, 84; c, 77; d, 53; e, 36; f, 33; g, 31; h, 30; i, 29; j, 23; k, 21; l, 19; m, 17; n, 16; and o, 15. Figure 2 (lane A) also shows that, of the cross-reactive polypeptides, at least five bands (a, b, d, e, and h) are major while the rest are minor. Some of the cross-reactive bands (Fig. 2, lane A, bands b, d, and e) are obviously double or triple bands. We found that most of the cross-reactive polypeptides are recognized by malaria-immune human sera as well (Fig. 2, lane B) and are metabolically labeled by [^{35}S]methionine and immunoprecipitated with anti-*P. yoelii* sera (results not shown). Comparison of lanes A and B (Fig. 2) shows that many *P. falciparum* antigens are not cross-reactive with anti-*P. yoelii* IgG; some of

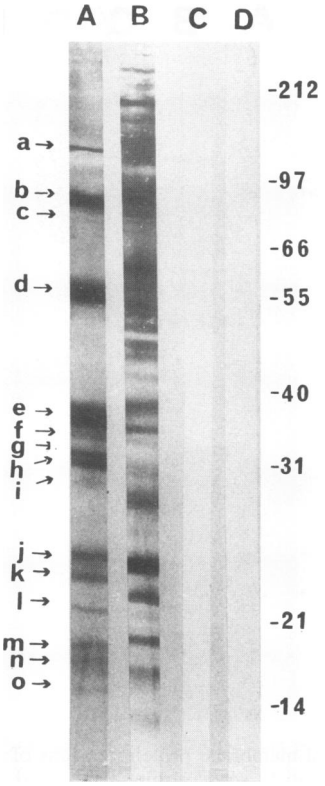

FIG. 2. Cross-reactivity of anti-*P. yoelii* sera with *P. falciparum* antigens. *P. falciparum* lysate antigens were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed with convalescent-phase mouse (lane A), human malaria-immune (lane B), control mouse (lane C), and normal human (lane D) sera. Numbers to the right are sizes (in kilodaltons) of molecular mass markers. Polypeptide bands are labeled alphabetically a to o.

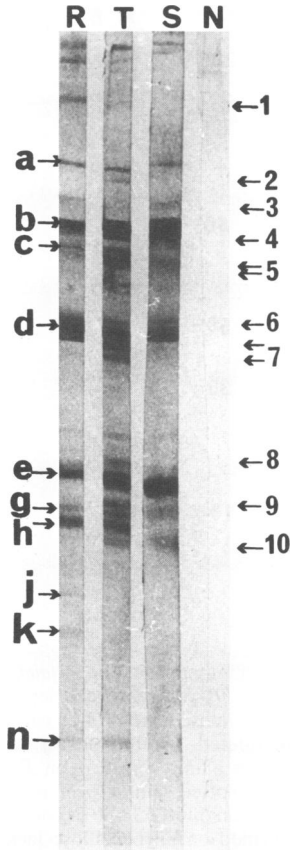

FIG. 3. Stage-specific expression of cross-reactive antigens of *P. falciparum*. Total cell lysate antigens of *P. falciparum* culture synchronized at ring-form (lane R), trophozoite (lane T), and schizont (lane S) stages were subjected to SDS-PAGE, blotted onto nitrocellulose, and probed with convalescent-phase mouse serum. Lane N was blotted with antigens from a mixture of the three synchronized cultures and probed with control mouse serum. Cross-reactive polypeptide bands are marked alphabetically on the left side of the figure. Numbers on the right side indicate polypeptides that are not expressed throughout all three of the major blood stages.

these species-specific antigens probably are associated with the unique features of each malarial parasite species, for example, host-cell specificity. Control mouse serum antibody did not bind to the *P. falciparum* proteins (Fig. 2, lane C). In Fig. 3, the expression of the interspecies cross-reactive antigens at the ring (lane R), trophozoite (lane T), and schizont (lane S) stages is shown. While most of the interspecies cross-reactive polypeptides are expressed throughout blood stage development, some are only detectable in one or two of the three major blood stages (Fig. 3, arrows numbered 1 to 10). It is also notable that few stage-specific bands appear to be poorly synthesized in asynchronous culture. For example, with anti-*P. yoelii* sera, three polypeptide bands of approximately 130, 160, and 200 kDa were consistently seen in the blots for ring stages (in lane R of Fig. 3, see the bands with molecular mass higher than that of band a) but were undetectable in the asynchronous culture (see lane A of Fig. 2). On the other hand, perhaps because of differences in the quantities of stage-specific proteins blotted to nitrocellulose, several minor polypeptides in the lower-molecular-mass ranges (see bands j to o) were consistently more detectable in asynchronous culture (lane A of Fig. 2) than in the synchronized parasites (Fig. 3).

Binding of *P. yoelii* antigens by antibodies affinity purified on *P. falciparum* lysate proteins. In order to define analogous cross-reactive antigens in *P. yoelii* blood stages, we performed two experiments. In one experiment with immunoblots and ELISA, incubation of each microliter of anti-*P. yoelii* serum with different amounts (0.1 to 50 μg) of *P. yoelii* lysate proteins showed that cross-reactive antibodies were effectively adsorbed from convalescent-phase mouse serum when each microliter of serum was incubated with 1.2 μg of *P. yoelii* lysate proteins (results not shown). In another experiment, nitrocellulose blotted with *P. yoelii* lysate proteins was probed with anti-*P. yoelii* IgG selected on membrane-bound *P. falciparum* proteins as described in Materials and Methods. Figure 4 shows that at least 10 bands (molecular weight range of 10,000 to 120,000) of *P. yoelii* lysate proteins cross-react with antibodies selected on *P. falciparum* lysate antigens.

Interspecies cross-reactive integral membrane proteins of *P. falciparum*. Integral membrane proteins of sporozoites and merozoites are major candidates for anti-malarial vaccines (33). Of these, the blood stage antigens include merozoite surface antigens MSA1 and MSA2, both of which show interspecies conserved epitopes (11, 31). Here, in order to determine which other interspecies cross-reactive antigens of *P. falciparum* blood stages are integral, we analyzed the parasite antigens by temperature-dependent phase separation in Triton X-114 solution (1, 33). We then separated the

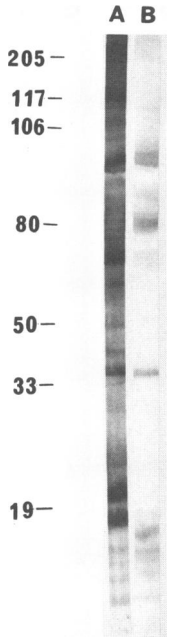

FIG. 4. Antibodies affinity purified on *P. falciparum* lysate proteins bind to *P. yoelii* antigens. *P. yoelii* antigens separated by SDS-PAGE and blotted onto nitrocellulose were probed with either convalescent-phase mouse serum diluted 1:400 (lane A) or convalescent-phase mouse serum antibodies affinity purified on *P. falciparum* antigens (lane B). Nitrocellulose blotted with *P. yoelii* antigens and probed with either normal mouse serum diluted 1:100 or convalescent-phase mouse antibodies affinity purified on nitrocellulose lacking blotted parasite antigens showed no detected polypeptide bands.

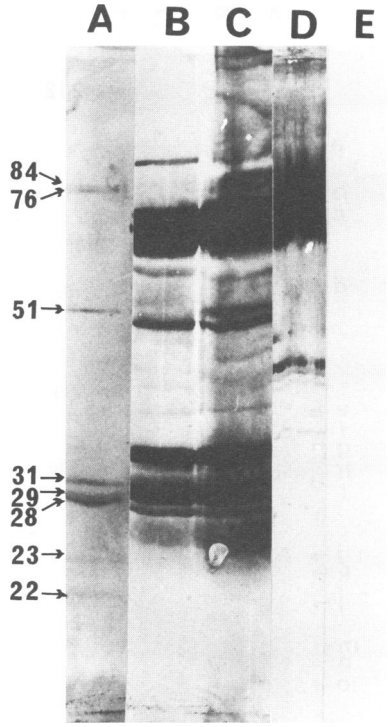

FIG. 5. Integral membrane protein antigens of *P. falciparum* that cross-react with anti-*P. yoelii* serum. Parasitized erythrocytes were solubilized and fractionated by phase separation in Triton X-114 solution. Antigens in the aqueous phase (lane B), Triton X-114 (lane A), total Triton X-114-soluble parasite proteins (lane C), and Triton X-114-insoluble proteins (lane D) were blotted onto nitrocellulose and probed with convalescent-phase mouse serum. Normal mouse or negative control mouse serum did not bind to total parasite lysate antigens (lane E). The sizes (in kilodaltons) of antigens in the Triton X-114 fraction are indicated to the left.

antigens by SDS-PAGE, blotted them onto nitrocellulose, and probed them with anti-*P. yoelii* sera. We found that at least eight integral membrane polypeptides (M_rs of 84,000, 76,000, 51,000, 31,000, 29,000, 28,000, 23,000, and 22,000) of *P. falciparum* cross-react with anti-*P. yoelii* sera (Fig. 5). When nitrocellulose blotted with Triton X-114 phase-separated *P. falciparum* antigens was probed with mouse anti-*P. yoelii* serum on the one hand and human anti-*P. falciparum* serum on the other, it was found that all of the eight cross-reactive integral proteins of *P. falciparum* were recognized by human anti-*P. falciparum* antibodies (results not shown). Thus, the cross-reactive integral molecules are targets of the immune response during natural infection. In control experiments, where albumin and lysozyme were mixed with Triton X-114 and extracted with aqueous buffer, each of these two proteins was effectively partitioned into the aqueous phase as expected.

The binding of anti-*P. yoelii* convalescent-phase mouse serum IgG to *P. falciparum* antigens (affinity). To evaluate the relative affinity of anti-*P. yoelii* IgG binding to *P. falciparum* proteins, we used the method of eluting bound antibody by different concentrations of thiocyanate (26). We found that half of the anti-*P. yoelii* antibody which binds to coats of *P. falciparum* proteins applied to microtiter wells was eluted at approximately 3.5 M NH_4SCN (Fig. 6A). This result suggested that anti-*P. yoelii* antibody binds at least some of the interspecies cross-reactive *P. falciparum* proteins with high affinity. We then conducted the following experiments to evaluate the affinity of binding of anti-*P. yoelii* antibody to individual polypeptides. Different strips of nitrocellulose blotted with separated *P. falciparum* antigens were incubated with mouse anti-*P. yoelii* serum, and each strip was then incubated individually in NH_4SCN solution. To visualize the antigen bands that retain bound anti-*P. yoelii* antibody, the strips were probed with alkaline phosphatase-conjugated anti-mouse IgG and color substrate. The results of these experiments are shown in Fig. 6B. It is clear that antibody binds to some protein bands with high affinity; in some cases, bound IgG remained uneluted by up to 8 M NH_4SCN (see bands b, d, e, and h). In contrast, other protein bands are bound by anti-*P. yoelii* antibody at such low affinity that they are well removed by 1 to 2 M NH_4SCN (see bands c, g, i, j, m, and n).

Inhibition of *P. falciparum* growth by anti-*P. yoelii* mouse serum IgG. If the interspecies cross-reactive antigens are highly conserved analogs critically associated with structure or function, we would expect antibody against such cross-reactive antigens of one *Plasmodium* species (*P. yoelii*) to inhibit the growth of another (*P. falciparum*). Figure 7 shows that mouse anti-*P. yoelii* antibody strongly inhibited the differentiation of *P. falciparum* from schizonts to rings; 75 μg of IgG per ml inhibited merozoite invasion by more than 80% (Fig. 7A). The convalescent-phase mouse IgG also inhibited intracellular development of *P. falciparum* from rings to schizonts; 25 μg of IgG per ml caused 50% inhibition (Fig. 7B).

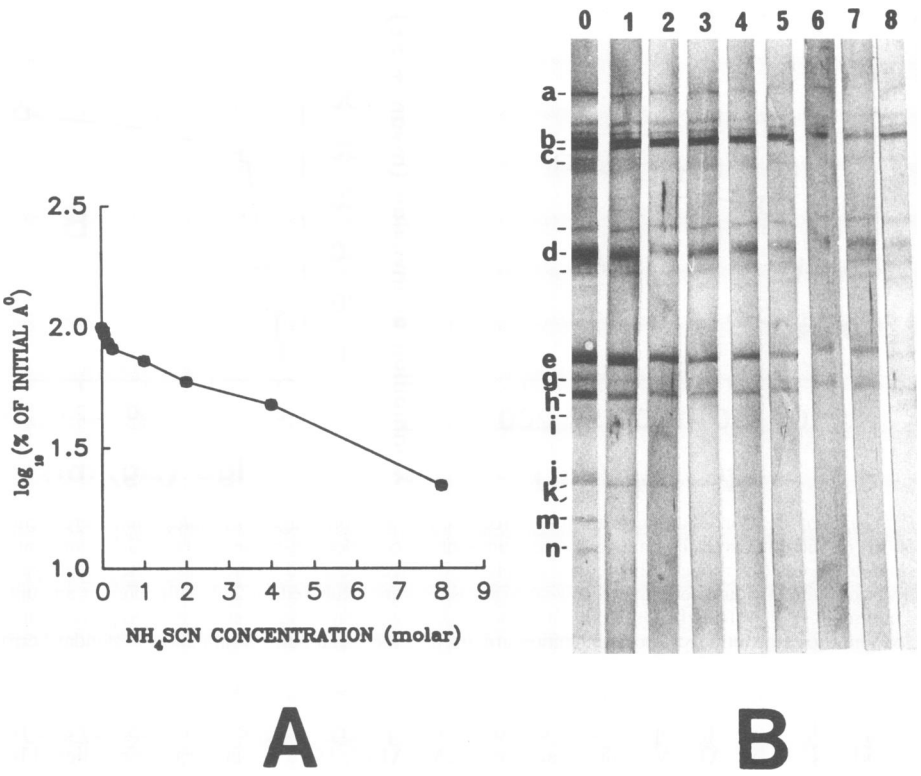

FIG. 6. Relative affinity of binding of anti-*P. yoelii* convalescent-phase mouse serum IgG to *P. falciparum* antigens. (A) Microtiter plate wells coated with *P. falciparum* lysate antigens (0.7 μg per well) were incubated for 2 h at room temperature with diluted convalescent-phase mouse serum and with the given concentrations (0 to 8 M) of NH_4SCN in 100 mM sodium phosphate (pH 6.0) for 15 min. Mouse IgG remaining bound to the wells was evaluated by ELISA. (B) Nitrocellulose blotted with *P. falciparum* antigens was probed with diluted convalescent-phase mouse serum. The membrane strips were individually incubated with the given concentration (0 to 8 M) of NH_4SCN. The strip labeled 0 was treated with buffers without thiocyanate. Polypeptides retaining bound mouse IgG were then visualized with color substrate. Cross-reactive bands are numbered alphabetically on the left-hand side.

DISCUSSION

We have demonstrated the inhibition of *P. falciparum* growth by anti-*P. yoelii* serum IgG and partially analyzed the antigenic polypeptides of *P. falciparum* recognized by the anti-*P. yoelii* antibodies. Only one or two previous reports have shown inhibition of in vitro growth of *P. falciparum* by sera from mice infected with or recovered from murine malaria. Butcher and Clark (4) recently reported inhibition of *P. falciparum* by sera from *Plasmodium chabaudi*-infected mice. However, this study implicated cytokines as the inhibitory factors in the sera. Cheng et al. (7) reported an anti-*P. yoelii* monoclonal antibody that inhibits *P. falciparum* growth and merozoite invasion. This monoclonal antibody recognizes the tetrapeptides NKND, IKND, and KKND and immunoprecipitates *P. falciparum* polypeptides of 145, 135, 102, and 76 kDa. However, human malaria-immune sera do not recognize the tetrapeptides, implying that these epitopes are poor immunogens during natural infection.

The results we have presented here show that *P. falciparum* growth (including schizont rupture, merozoite invasion, and intraerythrocytic development) is inhibited by purified anti-*P. yoelii* convalescent-phase mouse serum IgG and that this antibody binds specifically to at least 15 polypeptides (M_rs of 15,000 to 110,000) of *P. falciparum* blood stages. With asynchronous cultures, most of these polypeptides are recognized by human malaria-immune sera (Fig. 2) and can be [^{35}S]methionine labeled and immunoprecipitated by anti-*P. yoelii* antibodies. Elution of antibody by thiocyanate (Fig. 6) showed that some of the interspecies cross-reactive polypeptides are bound with high affinity by anti-*P. yoelii* antibody. The tight binding observed in the interspecies cross-reactions implies that the respective *P. yoelii* and *P. falciparum* antigen homologs have not diverged enough to render the immune complexes in the cross-reaction unstable to thiocyanate elution. Furthermore, IFAT studies showed that anti-*P. yoelii* convalescent-phase mouse serum antibodies bind to all of the blood stages of *P. falciparum*, a result in agreement with previous studies of anti-*P. yoelii* monoclonal antibodies (35). Control mouse serum IgG neither inhibited *P. falciparum* growth (Fig. 7) nor bound to whole-parasite or parasite lysate antigens (Fig. 2). Thus, we conclude that the observed inhibition of *P. falciparum* by convalescent-phase mouse serum IgG is due to interaction of anti-*P. yoelii* antibody with some of the interspecies cross-reactive antigens of *P. falciparum*. It is especially interesting (31, 33) that at least eight cross-reactive proteins in the *P. falciparum* blood stages are integral membrane molecules.

Interspecies cross-reactive antigens of *Plasmodium* species, as such, have not been much studied before, and we believe that many of the antigens of *P. falciparum* recognized by anti-*P. yoelii* serum antibodies (Fig. 2) have not been characterized. However, we recognize that on the basis of the molecular sizes of the antigens, a few of the cross-reactive molecules may be the same ones previously described by others. For example, the interspecies cross-reactive polypeptides of 107, 84, 53, 36, and

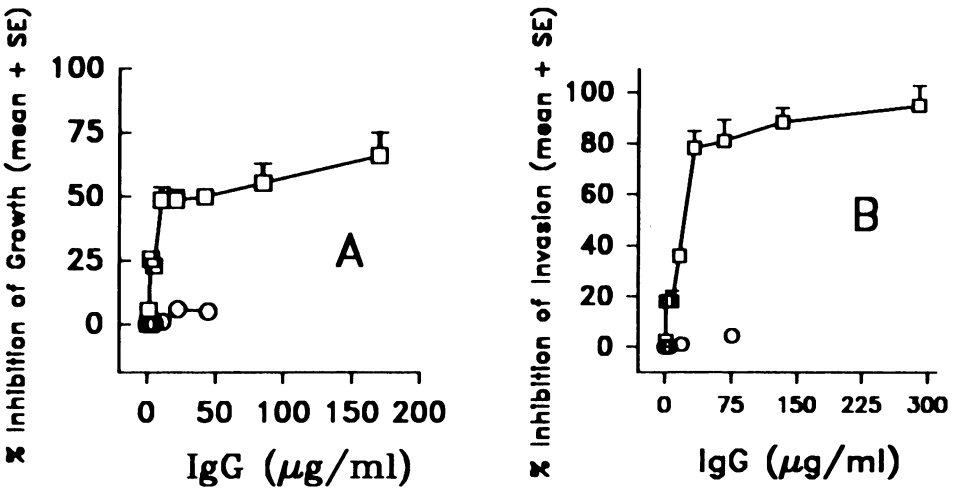

FIG. 7. Inhibition of *P. falciparum* invasion of and growth in erythrocytes. (A) Schizont stages of *P. falciparum* were incubated for 24 h in RPMI medium (total volume, 150 μl) in microtiter wells containing different amounts of convalescent-phase mouse IgG (□) or control mouse IgG (○), and the percent parasitemia (ring stages) was determined. The percent inhibition was then calculated on the basis of the percent parasitemia in control wells containing RPMI medium without added mouse serum. (B) Ring-stage parasites were incubated for 24 h with different amounts of convalescent-phase mouse IgG (□) or control mouse IgG (○) in RPMI culture medium. The percent parasitemia (schizont stages) and percent inhibition were determined. Both the results for invasion and those for intraerythrocyte inhibition are given as the mean percent inhibition + standard error (SE) (n = 12).

19 kDa (Fig. 2, bands a, c, d, e, and l, respectively) could represent processing products of MSA1. A major vaccine candidate, the MSA1 protein of *P. falciparum* is metabolically processed into several fragments (M_rs of 150,000, 110,000, 80,000 to 83,000, 45,000, 30,000 to 36,000, and 19,000) and has previously been shown to cross-react with the analogous 230-kDa molecule of *P. yoelii* (15–17). Also, the 51-kDa cross-reactive integral membrane antigen (Fig. 5) could be the MSA2 molecule (45 to 51 kDa), since recent reports (31) suggest that MSA2 (19, 34) also shows interspecies cross-reactivity. On the other hand, we believe that the interspecies cross-reactivity of most of the *P. falciparum* polypeptides recognized by convalescent-phase mouse serum IgG (Fig. 2) is not due to a common or pan-specific *Plasmodium* epitope such as the one described by Cheng et al. (7). This is because antibodies that we affinity purified on each of several cross-reactive polypeptide bands specifically bind to the same bands on nitrocellulose blotted with total parasite lysate (results not shown). In other words, many of the interspecies cross-reactive polypeptides do not appear to share a common epitope.

The convalescent-phase mouse serum IgG used in our growth inhibition assays (Fig. 7) was not affinity purified on *P. falciparum* antigen and is therefore expected to react with many immunogens in addition to *P. yoelii* antigens. Thus, the small amounts of convalescent-phase mouse serum IgG (75 μg/ml) required to inhibit parasite invasion by more than 80% (Fig. 7A) suggest high-affinity binding of potent anti-*P. yoelii* antibodies to vital antigens of *P. falciparum*. To our best knowledge, the interspecies conserved homologs associated with the cross-inhibition of *P. falciparum* blood stages (Fig. 7) remain unknown. The 230-kDa MSA molecule of *P. yoelii* (the most studied blood stage antigen of this species) has not been reported to induce antibodies that inhibit *P. falciparum* growth. It would be interesting to determine whether any of the *P. falciparum* polypeptides (bands a, b, d, e, and h, corresponding to 107, 84, 53, 36, and 30 kDa, respectively) which are bound with moderate or high affinity by anti-*P. yoelii* IgG (Fig. 6B) are involved in the cross-inhibition of invasion. To the extent that nearly all findings from human malaria vaccine experiments with preerythrocytic stages (28, 32) and from investigations of MSA1 (3, 15) were accurately predicted by the *P. yoelii* rodent malaria model, antigens of *P. falciparum* that either serologically cross-react with anti-*P. yoelii* antibodies or share sequence similarities with *P. yoelii* antigens (8, 30) are worthy of detailed investigation. We are conducting experiments designed to select a few of the interspecies cross-reactive antigens for detailed analysis.

While it is possible that a few of the interspecies cross-reactive determinants might be nonprotective conserved repeats (27), the inhibition of *P. falciparum* growth by convalescent-phase mouse serum anti-*P. yoelii* IgG (Fig. 7) suggests that some of the cross-reactive antigens of *P. falciparum* must contain protective epitopes. Such antigens are potential vaccine candidates, similar to several other cloned and sequenced molecules of *Plasmodium* species which also contain interspecies conserved sequences: for example, MSA1 (11, 15), MSA2 (31, 34), apical merozoite antigen 1 (38), sporozoite surface antigen 2 (14, 30), and the 25-kDa ookinete surface antigen (21). Whereas our results (Fig. 2) suggest that intact *P. falciparum* MSA1 (195 kDa) is not recognized by our anti-*P. yoelii* serum, Holder et al. (17) have previously reported detection of the molecule by a similar antiserum. The disparity in the results is probably due to the fact that Holder et al. used antisera raised by immunization with the purified *P. yoelii* 230-kDa merozoite surface molecule (Py230) while we probed with antisera obtained by repeated infection and cure. We feel that convalescent-phase mouse serum probably lacks adequate antibodies specific to the conserved cross-reactive epitopes in the intact molecule (3).

Finally, we have investigated a model of interspecies protective immune response by using two *Plasmodium* species: the human malarial agent *P. falciparum* and the rodent parasite *P. yoelii*. Since only a few antigens of different *Plasmodium* species have been cloned and sequenced, analysis of interspecies cross-protection constitutes a simple screening approach for the identification of important conserved antigens of *Plasmodium* species. In a recent experiment with interspecies cross-reactive immunity, mice immunized with *P. falciparum* sporozoites were protected against challenge infection with

Plasmodium berghei sporozoites and a protective noncircumsporozoite 45- to 54-kDa antigen common to *P. falciparum* and *P. berghei* was identified (18).

REFERENCES

1. **Bordier, C.** 1981. Phase separation of integral membrane proteins in triton-114 solution. J. Biol. Chem. **256:**1604–1607.
2. **Boyle, D. B., C. I. Newbold, R. J. M. Wilson, and K. N. Brown.** 1983. Intraerythrocytic development and antigenicity of *Plasmodium falciparum* and comparison with simian and rodent malaria parasites. Mol. Biochem. Parasitol. **9:**227–240.
3. **Burns, J. M., L. A. Parke, T. M. Daly, L. A. Cavacini, W. P. Weidanz, and C. A. Long.** 1989. Protective monoclonal antibody recognizes a variant-specific epitope in the precursor of the major merozoite surface antigen of the rodent malarial parasite *Plasmodium yoelii*. J. Immunol. **142:**2835–2840.
4. **Butcher, G. A., and I. A. Clark.** 1990. The inhibition of *Plasmodium falciparum* growth *in vitro* by sera from mice infected with malaria or treated with TNF. Parasitology **101:**321–326.
5. **Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M. J. Santos, and V. Nussenzweig.** 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. Cell **70:**1021–1033.
6. **Chauhan, V. S., S. Chatterjee, and P. K. Johar.** 1993. Synthetic peptides based on conserved *Plasmodium falciparum* antigens are immunogenic and protective against *Plasmodium yoelii* malaria. Parasite Immunol. **15:**239–242.
7. **Cheng, Q., G. Jones, X. E. Liu, C. Kidson, and A. Saul.** 1991. Identification of a common *Plasmodium* epitope (CPE) recognized by a pan-specific inhibitory monoclonal antibody. Mol. Biochem. Parasitol. **49:**73–82.
8. **Cowan, G., S. Krishna, A. Crisanti, and K. Robson.** 1992. Expression of thrombospondin-related anonymous protein in *Plasmodium falciparum* sporozoites. Lancet **339:**1412–1413.
9. **Da Silveira, F. J., L. Sibilli, E. Brunet, and O. Mercereau-Puijalon.** 1984. Cloning of cDNAs that code for *Plasmodium chabaudi* antigens of the erythrocytic forms and cross-hybridize with *P. falciparum*. Mol. Biochem. Parasitol. **11:**133–143.
10. **Deans, J. A., A. W. Thomas, P. M. G. Inge, and S. Cohen.** 1983. Stage-specific protein synthesis by asexual blood stage parasites of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **8:**45–51.
11. **Del Portillo, H. A., S. Longacre, E. Khouri, and P. H. David.** 1991. Primary structure of the merozoite surface antigen 1 of *Plasmodium vivax* reveals sequences conserved between different *Plasmodium* species. Proc. Natl. Acad. Sci. USA **88:**4030–4034.
12. **Firestone, G. L., and S. D. Winguth.** 1990. Immunoprecipitation of proteins. Methods Enzymol. **182:**688–700.
13. **Harlow, E., and D. Lane (ed.).** 1988. Antibodies, a laboratory manual, p. 283–318. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Hedstrom, R. C., J. R. Campbell, M. L. Leef, Y. Charoenvit, M. Carter, M. Sedegal, R. L. Beaudoin, and S. L. Hoffman.** 1990. A malaria sporozoite surface antigen distinct from the circumsporozoite protein. Bull. W.H.O. **68**(Suppl.)**:**152–157.
15. **Holder, A. A.** 1988. The precursor to the major merozoite surface antigens: structure and role in immunity. Prog. Allergy **41:**72–79.
16. **Holder, A. A., and R. R. Freeman.** 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and monoclonal antibody. J. Exp. Med. **156:**1528–1538.
17. **Holder, A. A., H. R. Freeman, and C. I. Newbold.** 1983. Serological cross-reaction between high molecular weight proteins synthesized in blood schizonts of *Plasmodium yoelii*, *Plasmodium chabaudi* and *Plasmodium falciparum*. Mol. Biochem. Parasitol. **9:**191–196.
18. **Hollingdale, M., M. Aikawa, C. Atkinson, W. R. Ballou, G. Chen, J. Li, J. Meis, B. Sina, C. Wright, and J. Zhu.** 1990. Non-CS pre-erythrocytic protective antigens. Immunol. Lett. **25:**71–76.
19. **Jones, G. L., H. M. Edundson, R. Lord, L. Spencer, R. Mollard, and A. J. Saul.** 1991. Immunological fine structure of the variable and constant regions of a polymorphic malaria surface antigen from *Plasmodium falciparum*. Mol. Biochem. Parasitol. **48:**1–10.
20. **Kamboj, K. K., J. W. Barnwell, R. S. Nussenzweig, and A. H. Cochrane.** 1988. Characterization of cross-reactive blood-stage antigens of the *Plasmodium cynomolgi* complex using anti-*Plasmodium vivax* monoclonal antibodies. J. Parasitol. **74:**403–408.
21. **Kaslow, D. C., C. Syin, T. F. McCutchan, and L. H. Miller.** 1989. Comparison of the primary structure of the 25 kDa ookinete surface antigens of *Plasmodium falciparum* and *Plasmodium gallinaceum* reveals six conserved regions. Mol. Biochem. Parasitol. **33:**283–288.
22. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a *staphylococcal* protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115:**1617–1624.
23. **Kironde, F. A. S., A. Kumar, A. R. Nayak, and J. L. Kraikov.** 1991. Antibody recognition and isoelectrofocusing of antigens of the malaria parasite *Plasmodium yoelii*. Infect. Immun. **59:**3909–3916.
24. **Laemmli, U. K.** 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
25. **Lambros, C., and J. P. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65:**418–420.
26. **Macdonald, R. A., C. S. Hosking, and C. L. Jones.** 1988. The measurement of relative antibody affinity by ELISA using thiocyanate elution. J. Immunol. Methods **106:**191–194.
27. **Moelans, I. I. M. D., and J. G. G. Schoenmakers.** 1992. Cross-reactive antigens between life cycle stages of *Plasmodium falciparum*. Parasitol. Today **8:**118–123.
28. **Nardin, E. H., and R. S. Nussenzweig.** 1993. T cell responses to pre-erythrocytic stages of malaria: role in protection and vaccine development against pre-erythrocytic stages. Annu. Rev. Immunol. **11:**687–727.
29. **Ramasamy, R., and R. T. Reese.** 1985. A role for carbohydrate moieties in the immune response to malaria. J. Immunol. **134:**1952–1955.
30. **Rogers, W. O., A. Malik, S. Mellouk, K. Nakamura, M. D. Rogers, A. Szarfman, D. M. Gordon, A. K. Nussler, M. Aikawa, and S. L. Hoffman.** 1992. Characterization of the *Plasmodium falciparum* sporozoite surface protein 2. Proc. Natl. Acad. Sci. USA **89:**9176–9180.
31. **Saul, A., R. Lord, G. L. Jones, and L. Spencer.** 1992. Protective immunization with invariant peptides of the *Plasmodium falciparum* antigen MSA2. J. Immunol. **208:**208–211.
32. **Sedegah, M., W. R. Weiss, Y. Charoenvit, and S. L. Hoffman.** 1993. Immunization of mice with irradiated *Plasmodium yoelii* sporozoites. Am. J. Trop. Med. Hyg. (Suppl.) **49:**407.
33. **Smythe, J. A., R. L. Coppel, G. V. Brown, R. Ramasamy, D. J. Kemp, and R. F. Anders.** 1988. Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **85:**5195–5199.
34. **Smythe, J. A., M. G. Peterson, R. L. Coppel, A. J. Saul, D. J. Kemp, and R. F. Anders.** 1990. Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **39:**227–234.
35. **Taylor, D. W., K. J. Kim, P. A. Munoz, C. B. Evans, and R. Asofsky.** 1981. Monoclonal antibodies to stage-specific, species-specific, and cross-reactive antigens of the rodent malaria parasite, *Plasmodium yoelii*. Infect. Immun. **32:**563–570.
36. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
37. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
38. **Waters, A. P., A. W. Thomas, J. A. Deans, G. F. Mitchell, D. E. Hudson, L. H. Miller, T. F. McCutchan, and S. Cohen.** 1990. A merozoite surface receptor protein from *Plasmodium knowlesi* is highly conserved and distributed throughout *Plasmodium*. J. Biol. Chem. **265:**17974–17979.
39. **Weiss, M. M., J. D. Oppenheim, and J. P. Vanderberg.** 1981. *Plasmodium falciparum*: assay in vitro for inhibitors of merozoite penetration of erythrocytes. Exp. Parasitol. **51:**400–407.
40. **Woodward, M. P., W. W. Young, Jr., and R. A. Bloodgood.** 1985. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods **78:**143–153.