

## Expression of *Plasmodium falciparum* surface antigens in *Escherichia coli*

(human malaria/cDNA clone/schizont-merozoite antigen)

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**ABSTRACT** The asexual blood stages of the human malarial parasite *Plasmodium falciparum* produce many antigens, only some of which are important for protective immunity. Most of the putative protective antigens are believed to be expressed in schizonts and merozoites, the late stages of the asexual cycle. With the aim of cloning and characterizing genes for important parasite antigens, we used late-stage *P. falciparum* mRNA to construct a library of cDNA sequences inserted in the *Escherichia coli* expression vector pUC8. Nine thousand clones from the expression library were immunologically screened *in situ* with serum from *Aotus* monkeys immune to *P. falciparum*, and 95 clones expressing parasite antigens were identified. Mice were immunized with lysates from 49 of the bacterial clones that reacted with *Aotus* sera, and the mouse sera were tested for their reactivity with parasite antigens by indirect immunofluorescence, immunoprecipitation, and immunoblotting assays. Several different *P. falciparum* antigens were identified by these assays. Indirect immunofluorescence studies of extracellular merozoites showed that three of these antigens appear to be located on the merozoite surface. Thus, we have identified cDNA clones to three different *P. falciparum* antigens that may be important in protective immunity.

The worldwide resurgence of human malaria, despite attempts to control this disease by vector eradication and drug treatment, makes the development of an anti-malaria vaccine a very desirable goal. A protective vaccine against *Plasmodium falciparum*, the species that causes the most severe form of human malaria, will probably have to include antigens from at least two developmental stages of the parasite: the sporozoites, which are injected into the blood by the mosquito and which appear to contain one immunodominant antigen (1), and the asexual blood stages, which contain many immunogens (2, 3), only some of which are candidates for protective antigens.

Data from several investigations (2, 4-6) indicate that most of the putative protective antigens of asexual stage parasites are present in schizonts and merozoites, the more mature developmental forms of the asexual cycle. However, large-scale production of pure antigens from parasites grown in culture is impractical and expensive because of the requirement for human blood products. One approach toward identifying, characterizing, and, perhaps, producing specific plasmodial antigens is the cloning and expression of parasite genes in bacteria. An important development was the cloning and characterization of the gene for a major sporozoite surface antigen (7, 8). Two groups have reported the construction of expression libraries containing genes that are

transcribed during the asexual blood cycle of *P. falciparum* (9, 10). A major problem for all investigators has been the identification of those clones corresponding to protective antigens. Kemp *et al.* (9) have made a cDNA library in the expression vector  $\lambda$ gt11-Amp 3 and screened the library with immune human sera. They analyzed two antigens expressed by clones from this library: the heat-stable S antigen, which varies greatly among parasite isolates (11), and a  $M_r$  155,000 protein that they believe is located on the surface of parasitized erythrocytes (12) and therefore may be a potential candidate for a vaccine antigen. McGarvey *et al.* (10) constructed a cDNA library with mRNA from asynchronous cultures of *P. falciparum*. They used differential screening with radiolabeled cDNA from either early- or late-stage parasites to identify cDNA clones corresponding to 12 genes expressed only during schizogony. They also obtained expression in *Escherichia coli* of some of these schizont-specific genes inserted in the inducible expression vector pPL31A.

In this paper, we describe the construction of a cDNA expression library made from late-stage *P. falciparum* mRNA and the isolation of bacterial clones encoding schizont and merozoite antigens. The library was constructed in the vector pUC8 (13, 14), and clones expressing plasmodial antigens were identified by immunological screening with sera from immune *Aotus* monkeys. Immune *Aotus* sera inhibit growth of the parasite in culture (15), provide passive immunity to naive animals upon transfer (unpublished data), and contain antibodies that bind to the surface of merozoites and parasitized erythrocytes (16). Therefore, cDNA clones encoding protective antigens were expected to be included among those that reacted with *Aotus* sera. Lysates of bacterial clones identified by the *Aotus* sera were used to immunize mice. The mouse sera were tested for reactivity to plasmodial antigens by immunoprecipitation, by protein electrophoresis and immunoblotting, and by indirect immunofluorescence on both fixed and intact cells. These assays allowed us to identify the blood-stage antigens encoded by several cDNA clones. Indirect immunofluorescence studies of intact extracellular merozoites showed that at least three different antigens appear to be located on the merozoite surface, making them strong candidates for host-protective antigens.

### MATERIALS AND METHODS

**Parasite Culture.** The Honduras I isolate of *P. falciparum* (Center for Disease Control, Atlanta) was cultured using standard methods (17). Cultures were routinely started from cryogenically preserved stocks every 3 months to minimize any changes that might occur in the parasites during long-

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Abbreviation: kbp, kilobase pairs.

term cultivation (18). Mature parasites were separated from ring-stage parasites with Physiogel (19) and then stored under liquid nitrogen.

**mRNA Preparation.** Frozen cell pellets were rapidly lysed in 4 M guanidinium thiocyanate/0.1 M 2-mercaptoethanol/25 mM sodium citrate/0.5% *N*-lauroylsarcosine (20). DNA in the lysate was sheared by several passages through a 25-gauge needle, and the RNA was pelleted through a cushion of 5.7 M cesium chloride by centrifugation at 35,000 rpm in a Beckman SW 50.1 rotor overnight at 15°C. Poly(A)<sup>+</sup> RNA was selected by two cycles of oligo(dT)-cellulose chromatography (21). To test the integrity of each poly(A)<sup>+</sup> RNA preparation, it was translated in a rabbit reticulocyte cell-free extract (22) and the pattern of total translated products (23, 24) was examined by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (25).

**Construction of cDNA Clones in pUC8.** pUC8 plasmid DNA was digested with *Eco*RI and *Sal* I endonucleases and the 2.7-kilobase pair (kbp) fragment was separated from the 18-bp fragment by agarose gel electrophoresis. The large fragment was recovered by electrophoresis onto a DEAE-membrane (NA45, Schleicher & Schuell) followed by elution with 1 M NaCl/50 mM arginine base at 70°C for 90 min, phenol extraction, and ethanol precipitation. Beginning with 12.5 µg of poly(A)<sup>+</sup> RNA, double-stranded cDNA was prepared by standard procedures (26). *Sal* I octanucleotide linkers (New England Biolabs) were first ligated to the cDNA at the ends equivalent to the 3' termini of the mRNA. After treatment with nuclease S1 and filling-in with the Klenow fragment of polymerase I (New England Biolabs), *Eco*RI decanucleotide linkers (New England Biolabs) were ligated to the cDNA. The cDNA was then digested with excess *Eco*RI/*Sal* I and fractionated on a 1.5% agarose gel. Two size classes of cDNA molecules (200 bp to 900 bp and 900 bp to 4.3 kbp) were purified as described above by electrophoresis onto DEAE-membrane filters. About 350 ng of cDNA was obtained in each size class.

cDNA was ligated to vector at a weight ratio of 1:20, then used to transform *E. coli* strain DH1 by the high-efficiency procedure of Hanahan (27). Transformants were plated on LB-ampicillin agar and harvested by scraping the plates, using LB medium to resuspend the bacteria. These suspensions were stored in 7% dimethyl sulfoxide at -80°C in 15 pools containing 200 to 3000 independent transformants each.

**Immunological Screening of Bacterial Colonies.** Sera from two *Aotus* monkeys, M33 and M39, were pooled in equal volumes. Monkey 33 (karyotype III) had been immunized as described by Reese and Motyl (15) with *P. falciparum* strains FCR-3 (17) and Honduras I; monkey 39 (karyotype VI) was only exposed to FCR-3. Normal serum from monkey 202 (karyotype VI) was used as a control. Sera were preadsorbed with equal volumes of lysates of DH1 containing pUC8. The lysates were prepared by concentrating a stationary culture of DH1 100-fold in 50 mM Tris·HCl, pH 7.6/150 mM NaCl/0.5% Nonidet P-40 and boiling the cells for 20 min. Preadsorption was for 1 hr at 4°C, and it was repeated four times. Bacterial colonies were plated on Millipore Triton-free HATF filters and two replicas were made from each master filter. Colonies on the two replicas were lysed with CHCl<sub>3</sub> vapor and subsequently treated as described by Helfman *et al.* (14), except that 1% casein replaced bovine serum albumin in all incubations. The preadsorbed *Aotus* serum was used at a final dilution of 1:300. The second antibody was affinity-purified rabbit anti-*Aotus* IgG, labeled with <sup>125</sup>I to a specific activity of 10<sup>7</sup> cpm/µg by the chloramine-T procedure (28).

**Immunization of Mice with Bacterial Lysates.** Exponential growing cultures of cDNA clones were concentrated 10-fold in 0.05% Triton X-100/50 mM Tris·HCl, pH 8.0/12.5

mM EDTA/2 mM phenylmethylsulfonyl fluoride containing lysozyme at 200 µg/ml, leupeptin at 10 µg/ml, and pepstatin at 10 µg/ml. MgCl<sub>2</sub> was added to 20 mM, and DNase I, to 10 µg/ml. Lysates were stored frozen at -70°C. Two mice (C3H or Balb/c) were injected i.p. per lysate; each received 200 µl of lysate emulsified with 300 µl of Freund's complete adjuvant. Mice received two booster shots; on day 21, by s.c. injection of 150 µl of lysate emulsified in Freund's incomplete adjuvant, and on day 36, by i.p. injection of 150 µl of aqueous lysate. Mice were bled 12 days after the first booster shot and every 2 weeks thereafter. Antiserum against *E. coli* strain DH1 containing pUC8 was used as the negative control in all experiments with the mouse sera.

**Transfer of Proteins to Nitrocellulose and Detection of Antigens.** Late-stage *P. falciparum* cultures were concentrated to 70–80% parasitemia with Physiogel, suspended in 10 vol of 10 mM sodium phosphate (pH 7.2) to release soluble erythrocyte proteins, and centrifuged. Proteins in the pellet were solubilized by boiling in sample buffer (25), resolved by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis on 8% gels (25) (50 µg of total protein per lane), and then electrophoretically transferred to nitrocellulose (29).

The nitrocellulose sheets were incubated with 1% casein in 50 mM Tris·HCl, pH 7.6/150 mM NaCl for 1–16 hr and then probed with individual mouse sera at a 1:50 dilution in the same buffer. The nitrocellulose was then washed three times in Tris/NaCl with 0.05% Tween 20, treated with <sup>125</sup>I-labeled affinity-purified rabbit anti-mouse IgG (specific activity, 10<sup>7</sup> cpm/µg), washed four times, and autoradiographed as described (30).

**Biosynthetic Labeling and Immunoprecipitation.** Trophozoites were concentrated by Physiogel treatment and 2 × 10<sup>8</sup> infected cells were grown for 12–18 hr in 10 ml of methionine-free RPMI medium (Irvine Scientific)/15% human serum containing 1 mCi of [<sup>35</sup>S]methionine (Amersham; 1400 Ci/mmol; 1 Ci = 37 GBq). Infected erythrocytes and free merozoites were pelleted, suspended at 10<sup>8</sup> cpm/ml in 50 mM Tris·HCl, pH 7.2/0.5 M NaCl/20 mM EDTA/0.5% Triton X-100/0.1% NaDodSO<sub>4</sub> containing leupeptin and pepstatin at 10 µg/ml each (wash buffer), and centrifuged for 45 min at 35,000 rpm in an SW 50.1 rotor.

Lysates (2 × 10<sup>6</sup> cpm per sample) were preadsorbed at 4°C for 1 hr with 0.25 vol of normal mouse serum, treated for another hour with 0.5 vol of packed protein A-Sepharose beads (Pharmacia), and centrifuged at 15,000 × g. The supernatants were incubated with 20 µl of immune mouse serum and 40 µl of 50 mM Tris·HCl, pH 7.2/0.5 M NaCl/20 mM EDTA/0.5% Triton X-100/1% casein at 4°C for 1.5 hr and then the mixture was adsorbed with 25 µl packed swollen protein A-Sepharose beads for another 1.5 hr at 4°C. The beads were washed five times, using 1 ml of wash buffer each time, suspended in 2× sample buffer (25), and boiled for 3 min. Proteins were resolved on 8% NaDodSO<sub>4</sub>/polyacrylamide gels, treated with Amplify (Amersham), and fluorographed.

**Immunofluorescence Studies of Fixed Cells.** Smears were made from a 50% suspension of concentrated late-stage parasites in phosphate-buffered saline (P<sub>i</sub>/NaCl) 50% horse serum. Slides carrying the air-dried smears were stored dessicated at -20°C. Immediately before antibody treatment, the smears were fixed for 1 min in acetone at -20°C, then washed four times with distilled water. Smears were incubated for 1 hr at room temperature with drops of a 1:10 dilution of the specific mouse serum, washed three times with P<sub>i</sub>/NaCl, treated with drops of a 1:50 dilution of fluorescein-conjugated goat anti-mouse immunoglobulin (Tago, Burlingame, CA), washed three times with P<sub>i</sub>/NaCl, mounted in glycerol, and photographed under UV light through a Zeiss microscope, using Kodacolor 400 ASA film or Ektachrome P800/1600 film push-processed to 3200 ASA.

**Immunofluorescence Studies of Intact Merozoites.** Parasite cultures were synchronized and concentrated at the trophozoite stage with Physiogel, then returned to culture without addition of fresh erythrocytes until mature schizonts developed. The schizonts were pelleted, suspended in RPMI medium/20% human serum, and incubated at 37°C until merozoites were released (<1 hr). Erythrocytes were then pelleted at 400 × *g* for 10 min, and merozoites were harvested from the supernatant by centrifugation at 1300 × *g* for 10 min. Merozoites were washed once with RPMI medium, briefly fixed with 0.08% glutaraldehyde (electron microscopy grade), washed three times with RPMI medium, and pelleted in Microfuge tubes. Such glutaraldehyde treatment stabilizes merozoites but does not permeabilize them or destroy their antigenicity (31). The lightly fixed merozoites were treated with mouse serum and fluorescein-conjugated goat anti-mouse immunoglobulin exactly as described for the fixed-parasite smears and then were examined by fluorescence microscopy.

## RESULTS

RNA from mature trophozoites and schizonts of *P. falciparum* was used to construct a cDNA library in the expression vector pUC8. cDNA inserted between the *EcoRI* and *Sal I* sites of pUC8 can be expressed from the transcriptional and translational signals of the *lacZ* gene, which are located upstream of the *EcoRI* site (13). Sequential addition of linkers to the cDNA, as described by Helfman *et al.* (14), was used to ensure that a high percentage of cDNA molecules would be inserted in the correct orientation for expression. The yield of clones from the two size classes of fractionated cDNA was markedly different. We obtained only 6000 clones from 270 ng of the larger size class (900 bp to 4.3 kbp, class A) whereas the smaller size class (200 bp to 900 bp, class B) yielded 26,000 clones from 180 ng of cDNA.

Plasmid DNA was prepared from a few cDNA clones of both size classes, and the insert sizes were examined by agarose gel electrophoresis following digestion of the DNA with *EcoRI/Sal I*. Seventy percent of the clones in class B and 50% of those in class A contained inserts of the expected sizes; the rest apparently contained no cDNA and probably resulted from head-to-tail ligation of vector molecules. The cDNA inserts from a few clones were purified and used as hybridization probes on blots (32) of restriction digests of *P. falciparum* DNA to confirm that the cloned fragments did indeed correspond to plasmodial sequences (data not shown).

When the clones were screened with immune *Aotus* sera for expression of plasmodial antigens, about 2.5% of the class B clones were positive while only 0.5% of the class A clones reacted. A total of 8000 colonies from class B and 1000 from class A were screened. Clones reacting with *Aotus* serum were picked, replated on filters at a density of about 100 colonies per plate of 10-cm diameter, and rescreened in duplicate to confirm their antigenicity and also for single colony purification. Ninety-five positive clones were accumulated, 91 from class B and 4 from class A. These clones may not all be independent since the library was amplified after the initial transformation.

Most of the cDNA clones that reacted positively with the *Aotus* sera contained small inserts (250–900 bp) and so were unlikely to be expressing full-length parasite proteins. To identify the plasmodial proteins corresponding to the antigens expressed from the cloned cDNA fragments, we immunized mice with extracts of several clones and then used three different immunological techniques to screen these mouse sera for reactivity with parasite antigens. Before they were injected into mice, the bacterial lysates were spotted on nitrocellulose and tested for reactivity with monkey sera.

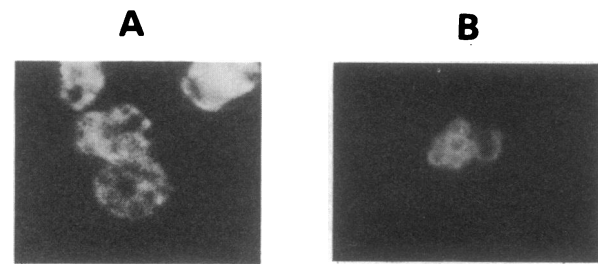


FIG. 1. Fluorescence micrographs of indirect immunofluorescence staining patterns produced by anti-C7 serum with fixed smears of infected erythrocytes containing schizonts and trophozoites (upper right corner) of *P. falciparum* (A) and a cluster of extracellular merozoites (B).

About one-third of the clones lost their antigenicity during preparation of the lysates, perhaps because of degradation of the expressed peptide, and these were not used as immunogens.

Mouse sera were obtained against 49 bacterial clones. Of these sera, 24 precipitated labeled plasmodial proteins in an immunoprecipitation assay, and 21 of the same 24, as well as 9 others, a total of 30, detected specific antigens on nitrocellulose blots of electrophoretically resolved proteins from parasitized cells. In addition, 32 of the 49 sera reacted with antigens of parasitized erythrocytes when tested by indirect immunofluorescence on slides of fixed parasites. Three of the parasite antigens identified by these methods appear to be located on the merozoite surface and are described here in more detail.

All mouse sera raised against cDNA clones B10, C1, and C7 gave a similar pattern of strong surface fluorescence with intact extracellular merozoites (Fig. 1B). The antigen recognized by these sera was also present in trophozoites and

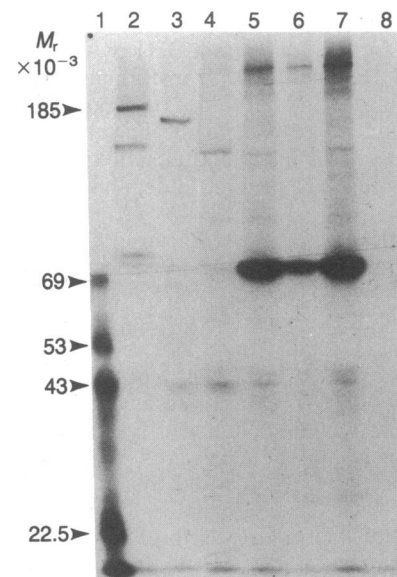


FIG. 2. Autoradiogram of a NaDodSO<sub>4</sub>/polyacrylamide gel of immunoprecipitates of <sup>35</sup>S-labeled *P. falciparum* proteins. Lanes: 1, <sup>14</sup>C-labeled molecular weight protein markers (New England Nuclear) [bovine serum albumin, *M<sub>r</sub>*, 69,000; gamma globulin heavy chain, *M<sub>r</sub>*, 53,000; ovalbumin, *M<sub>r</sub>*, 43,000; gamma globulin light chain, *M<sub>r</sub>*, 22,500]; 2, immunoprecipitate of *P. falciparum* protein p185 (30) with a mouse monoclonal antibody 4-13-4B (H. A. Stanley and R. F. Howard, personal communication), p185 was used as a molecular weight marker; 3–7, proteins immunoprecipitated by mouse sera to cDNA clones E4, C5, C1, B10, and C7; 8, control immunoprecipitation with serum to the parental *E. coli* strain DH1 containing pUC8.

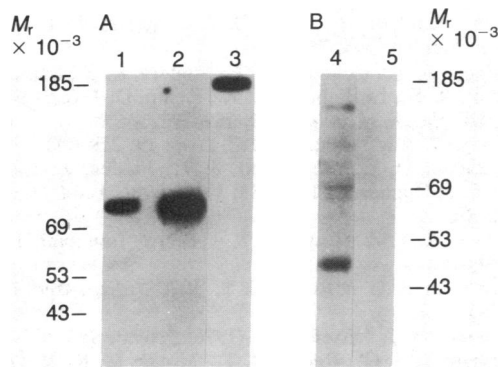


FIG. 3. Detection by immunoblotting of the antigens from *P. falciparum*-infected erythrocytes that react with mouse sera directed against bacterial clones. Mouse sera were preadsorbed twice with intact human erythrocytes and twice with erythrocyte ghosts. A and B represent independent experiments. Nitrocellulose strips were treated as follows: lane 1, mouse serum to clone B10; lane 2, serum to clone C1; lane 3, culture supernatant of hybridoma 4-13-4B, which reacts with *P. falciparum* protein p185 (H. A. Stanley and R. F. Howard, personal communication); lane 4, serum to clone C5; lane 5, serum to *E. coli* DH1 containing pUC8 (negative control).

schizonts, as determined by immunofluorescence on fixed parasitized cells (Fig. 1A). All three sera precipitated a *P. falciparum* protein of  $M_r$  75,000, as well as a minor protein species at  $M_r$  225,000 (Fig. 2, lanes 5–7). Immunoblotting experiments with any of these three sera resulted in the same protein patterns as produced by immunoprecipitation (Fig. 3, lanes 1 and 2), although the  $M_r$  225,000 protein was not always detected.

C5 is another cDNA clone which appeared to encode a merozoite surface antigen, as determined by immunofluorescence of intact parasites. The fluorescence pattern was essentially the same as that of C7, albeit somewhat weaker. Mouse antiserum to C5 immunoprecipitated a protein of  $M_r$  140,000 (Fig. 2, lane 4). However, in immunoblotting studies of proteins of parasitized erythrocytes, anti-C5 serum reacted with proteins having approximate  $M_r$  values of 140,000, 68,000, and 45,000 (Fig. 3, lane 4). The relative proportions of these three protein species varied in different antigen preparations.

A protein with an approximate  $M_r$  of 180,000 was precipitated from extracts of  $^{35}\text{S}$ -labeled parasite proteins by mouse serum raised against clone E4 (Fig. 2, lane 3). In addition, in some experiments, anti-E4 serum precipitated a protein of  $M_r$  59,000. Anti-E4 serum did not react convincingly with any parasite protein in immunoblotting experiments, suggesting that the epitopes recognized by this serum are destroyed by boiling in 2% NaDodSO<sub>4</sub> under reducing conditions. The antigen corresponding to clone E4 also shared the fluorescence pattern of the C7 antigen and thus appears to be localized to the merozoite surface. The high molecular weight plasmodial protein corresponding to the clone E4 did not co-migrate on NaDodSO<sub>4</sub>/polyacrylamide gels with gp185, the schizont glycoprotein that has been extensively described in the literature and is believed to be a precursor to some of the major merozoite proteins (33).

## DISCUSSION

The results of the colony immunoassay of the *P. falciparum* cDNA library allow us to estimate that the library should contain 600–700 clones expressing antigens recognized by immune *Aotus* sera, although each positive clone will not be the product of an independent cloning event since the library was amplified. McGarvey *et al.* (10) have suggested that only 12 major gene families are expressed specifically in the schizont and merozoite stages. Since it appears that antigens

expressed in schizonts and merozoites play a major part in eliciting protective immunity against malaria (2, 4–6), this collection of several hundred clones expressing late-stage *P. falciparum* antigens should include clones for many of the important protective antigens from the asexual cycle.

It is not clear why the fraction of clones reacting with *Aotus* serum was lower in the clone pools with larger cDNA inserts (class A). One possible explanation is that large foreign polypeptides expressed constitutively in *E. coli* may be more susceptible to degradation than smaller ones, making the parasite antigens expressed in class A clones relatively unstable and thus difficult to detect.

The strategy of immunizing animals with lysates of cDNA clones as a way of identifying the polypeptides expressed by inserted cDNA has been used before for hepatitis B antigen (34) and for a  $M_r$  120,000 malaria antigen (35). In the latter case, the clones were expressing levels of peptide too low for detection by colony immunoassays, and only a small fraction (3/50) of immunized mice produced positive sera. In contrast, we selectively immunized mice with lysates that were antigenically positive with serum from monkeys immune to *P. falciparum*. Consequently, most (32/49) of the mouse sera we obtained reacted with parasite antigens. However, the positive anti-parasite responses were only detected 2 to 3 months after immunization of the mice and were weak in many cases, perhaps due to low concentrations of the expressed plasmodial protein in the bacterial lysates.

Polyclonal, monospecific sera against particular plasmodial proteins are scarce because of technical difficulties in purifying individual parasite proteins. Therefore, the mouse sera we obtained are valuable reagents, as each one is specific for the single plasmodial protein encoded by the cDNA clone used for immunization.

Each of the cDNA clones used to raise antibodies against these three different antigens was genetically purified and contained only one cDNA fragment. Yet each of the three antisera against clones C7, C5, and E4 recognized more than one protein species, either in immunoblotting or immunoprecipitation experiments. These proteins have been ascribed  $M_r$  values of 75,000 and 225,000 (C7); 140,000, 68,000, and 45,000 (C5); and 180,000 and 59,000 (E4). It remains to be elucidated whether the multiple protein bands observed for each cloned antigen are immunologically cross-reactive products of different genes, whether there is a precursor-product relationship between the protein species, or whether the multiple bands are an artifactual result of proteolysis during sample preparation. Our results show that epitopes related to those encoded by each of the cloned gene fragments are located on the merozoite surface. However, we still do not know which specific protein in each group carries the surface epitope.

In this context, Jungery *et al.* (36) discovered that erythrocyte glycoporphins, thought to serve as receptors for invading merozoites, bind to *P. falciparum* proteins of  $M_r$  140,000 and 70,000 that are synthesized during schizogony and are inferred to be located on the merozoite surface. Also, proteins of  $M_r$  202,000, 142,000, and 46,000, among others, have been identified by Howard and Reese (30) as merozoite proteins, based on the results of biosynthetic labeling experiments.

The results of immunoprecipitation experiments with immune human sera from different parts of the world have defined several *P. falciparum* proteins that may be important in protective immunity and have  $M_r$  values comparable with those of the three antigens we have described (37–39). Monoclonal antibodies to *P. falciparum* antigens have also been used to identify possible protective antigens. A  $M_r$  140,000 protein is among those recognized by monoclonal antibodies inhibiting the *in vitro* growth of asexual stages (2). Inhibitory monoclonal antibodies have also been characterized that bind to an antigen of  $M_r$  >220,000 (40), but the

fluorescence pattern described does not resemble that produced by the anti-C7 mouse serum. The relationship between these antigens and the three described here will require much further investigation.

Evidence for the protective role of proteins of  $M_r > 200,000$ , 140,000, and 75,000 has also been obtained from vaccination trials. Dubois *et al.* (41) have successfully vaccinated squirrel monkeys with a schizont protein fraction eluted from the  $M_r$  75,000 region of a gel, and Perrin *et al.* (42) have immunized two groups of squirrel monkeys with partially purified proteins of  $M_r > 200,000$  and 140,000, respectively, that they believe to be located on the surface of either schizonts or merozoites.

For malarial antigens to play a protective role in immunity, they should be exposed to the immune system on the surface of either the infected erythrocyte or the merozoite. The results of indirect immunofluorescence assays indicate that all three antigens discussed here appear to be located on the merozoite surface. If their surface localization is confirmed by immunoelectron microscopy, these molecules may be useful in the development of a general *P. falciparum* vaccine, especially since preliminary experiments show that they are not strain-specific for the Honduras I isolate (unpublished data).

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