ANTIBODIES IN MALARIAL SERA TO PARASITE ANTIGENS IN THE MEMBRANE OF ERYTHROCYTES INFECTED WITH EARLY ASEXUAL STAGES OF *PLASMODIUM FALCIPARUM*

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Malaria is one of the most widely spread human diseases today and constitutes a major public health problem for a large part of the world's population. The dramatic rise of the disease since the mid-1960's has resulted in renewed attempts to find ways of controlling the disease by immunological measures. Although the immune response to the malaria parasites is strictly regulated by the T cell system and may at least in part be cell mediated (1–3), there is good evidence that antibodies play an important role in providing protection (4, 5). Therefore, major efforts are now being made to characterize plasmodial antigens that may elicit protective humoral immune responses and thus may be suitable candidates for malaria vaccines (6). In the past few years, conspicuous advances in this direction have been made with regard to the plasmodial sporozoites, i.e., the life cycle forms of the parasite which the mosquito transmits to the mammalian host (7, 8). However, since mortality and morbidity of the disease are caused by the asexual blood stages of the parasite, it is generally agreed that development of vaccines against these forms is of equally high priority in malaria research (9). Here, some of the antigens of special interest are those which give rise to antibodies interfering with reinvasion of noninfected erythrocytes (E)[1] and with intraerythrocytic growth (10–14) or with the sequestration of infected E, thereby preventing their destruction in the spleen (15, 16). Such antigens appear to be derived from the mature blood forms (schizonts, merozoites) of the parasite and/

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[1] *Abbreviations used in this paper:* ALP, alkaline phosphatase; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; E_i, erythrocytes infected with *P. falciparum*; E_0, noninfected erythrocytes; GA, glutaraldehyde; IF, immunofluorescence; IFA, immunofluorescence assay; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBH, Tris-buffered Hanks' solution.

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or may also be located on the surface of infected E. However, because of the complexity of the immune response of the *Plasmodium*-infected host, the characterization of these antigens poses some major logistic problems, including the existence of an antigenic variability both between and within strains of a given plasmodial species (17–19).

By means of immunofluorescence (IF) and an enzyme-linked immunosorbent assay (ELISA) we have identified a new antigenic modification of the surface of human E infected with *Plasmodium falciparum*, the most lethal of the human malaria parasites. These antigens are recognized by antibodies from patients or immune individuals and are present on the surface of E containing early stages of the parasite already shortly after invasion. By eluting antibodies from the surface of infected E, we have been able to show that the major antigen involved in this reaction is a parasite-derived polypeptide of 155,000 mol wt, probably deposited on the E by the invading merozoite.[2]

Materials and Methods

Parasites

The main source of parasites was a Tanzanian strain of *Plasmodium falciparum* (F32) isolated in 1978 (21) and cultured in vitro in blood group O^+ E according to Trager and Jensen (22). Parasites were also obtained from the blood of patients with acute *P. falciparum* infection.

Immune Sera

Immune sera were from Swedish or South American (Colombian) patients with acute *P. falciparum* malaria or from Africans living in a holoendemic area of Liberia with high transmission of *P. falciparum*. For details see (23).

E Monolayers

Infected E (E_i) from *P. falciparum* cultures (parasitemia, 5–10%) or freshly drawn from infected patients (parasitemia, usually 3–5%) were sedimented by centrifugation, washed three to four times with Tris-buffered Hanks' solution (TBH) (10 ml 0.15 M Tris buffer, pH 7.2, 90 ml 0.9% NaCl, 100 ml Hanks' solution). They were then diluted to give 1% suspensions. E from noninfected cultures (E_0) were used for control. Eight-well multitest slides (Flow Laboratories, Rockville, MD) were treated for 30 min with one drop per well (~20 μl) of a coating buffer, pH 9.6 (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 200 mg NaN_3/liter). Immediately after aspiration of the coating buffer, one drop of the 1% E suspensions was added to each well and the cells were left to settle for 30 min at room temperature. Unbound E were rinsed off by gentle shaking of the slides, immersed upside down in a petri dish filled with TBH. The wet E monolayers were quickly but gently covered (on top) with 1–2 ml of 1% glutaraldehyde (GA) in phosphate-buffered saline (PBS). After ~10 s, the GA solution was decanted and fixation was repeated once. The slides were then washed with distilled water and air-dried. They could be stored at −20°C for many months with fully retained reactivity.

IF of GA-fixed and Air-dried Monolayers

The slides were treated sequentially for 30 min with 20 μl of (*a*) various dilutions of immune or normal human serum; (*b*) affinity-purified and biotinylated goat antibodies to human IgG (reacting with both heavy and light chains, 30 μg/ml; Vector Laboratories, Inc., Burlingame, CA); and (*c*) avidin conjugated with fluorescein isothiocyanate (50 μg/

[2] These results have been presented in a preliminary form at recent meetings: see *Scand. J. Immunol.* (1983) 18:71 (Abstr.) and Proc. 5th Int. Congr. Immunol., Kyoto, Japan, August 21–27, 1983, p. 226.

ml; Vector Laboratories, Inc.). Between the incubations, the slides were washed with TBH supplemented with 50 μl Tween 20 per 100 ml. All incubations were performed at room temperature in a humid chamber. To visualize parasitized E, the monolayers were counterstained after the IF staining by adding one drop per well of ethidium bromide (10 μg/ml; Sigma Chemical Co., St. Louis, MO). After a few seconds, the slides were washed with distilled water, mounted, and scored with a 100× oil immersion lens in incident ultraviolet light in a Zeiss Universal research microscope (Carl Zeiss Co., Stockholm, Sweden) equipped for simultaneous observation of IF (green) and nuclear staining (orange) (band filter 450–490, beam splitter FT 510, and barrier filter LP520). Extra barrier filters LP560 or LP590 were used for exclusive observation of IF or ethidium bromide fluorescence, respectively.

Inhibition of IF

Lysates of E_i or E_0. Monolayers of E_i (~10% parasitemia) or E_0 were prepared in 35-mm plastic petri dishes (No. 1008, 35 × 10 mm; Falcon Labware, Oxnard, CA) and were treated with 1 ml coating buffer followed by 1 ml of the 1% E suspensions. Nonadsorbed E were aspirated with a Pasteur pipette and the monolayers were washed once with 1 ml TBH. One confluent monolayer corresponded to ~22×10^6 E. The E monolayers were air-dried but not GA fixed. Lysates were prepared by sequential incubation with 0.5 ml TBH for 30 min at room temperature of three dishes containing either E_i or E_0. Under these conditions, most of the E membranes and parasites remained bound to the plastic. Before use the lysates were centrifuged at 1,000 *g* for 10 min. A strongly positive Liberian serum, diluted 1:500, was then mixed with either TBH or lysate to a final serum dilution of 1:5,000 and used in the IF assay (IFA) as described.

Culture supernatants. Medium (containing 10% normal human serum) in which the parasites had been growing for ~24 h was collected from *P. falciparum* cultures of 5–10% parasitemia. The culture was centrifuged at 750 *g* for 10 min to sediment E_i and E_0 and the supernatant was then centrifuged once more at 70,000 *g* for 30 min. 90 μl of different dilutions of the supernatant were mixed with 10 μl of a hyperimmune Liberian serum and were used in the IFA. The final dilution of the immune serum in these tests was 1:1,000. In some experiments, the culture supernatants were processed further by passage over an immunoadsorbent prepared from another *P. falciparum* hyperimmune serum (see below). For inhibition of IF, both the passed and the eluted fractions of the supernatant were used. Elution of the material adsorbed to the immunoadsorbent was performed essentially as described by Jepsen and Andersen (21). From one liter culture supernatant applied to the column, 14 mg adsorbed protein were eluted with 3 M potassium thiocyanate. The material was dialyzed against PBS and concentrated to 3 mg protein/ml. For IF inhibition a series of four antigen dilutions was mixed with the hyperimmune serum. The final dilutions in the tests were 1:1,000 or 1:2,000 for the serum and 0.01–10 μg antigen protein/ml. In additional experiments the eluates were similarly tested after incubation in boiling water for 5 min, followed by centrifugation to remove insoluble material.

Parasite extracts. The pelleted material obtained from the centrifuged culture supernatants (see above) was used. It was enriched in free merozoites but also contained pigment and some cell debris from both parasites and E. The pellet was washed twice in PBS and then sonicated on ice for two 30-s periods at 50 W (Bronson sonifier B-12; Bronson Sonic Power Co., Danbury, CT). The sonicate was freed from insoluble material by centrifugation at 100,000 *g* for 30 min. Sonicates of ghosts made from normal blood group O^+ E as described (24) were prepared in the same way. Stock solutions of the sonicates containing 6 mg protein/ml were kept frozen until use. For the inhibition assay, 90 μl of four different dilutions of the sonicates were diluted to 100 μl with a hyperimmune Liberian serum, giving final serum dilutions of 1:1,000 and 0.06–60 μg/ml of the sonicates.

Elution of Antibody from E Monolayers

E monolayers were prepared in plastic petri dishes (No. 1008; Falcon Labware), GA fixed, and air-dried. Dishes containing either E_i (22×10^6 E/dish; parasitemia, 5–10%) or

E_0 were incubated with 1 ml/dish of a hyperimmune Liberian serum diluted 1:100 with TBH. After incubation for 30 min at 37°C and in the refrigerator overnight, the serum was aspirated and each dish was washed three times with 1–2 ml TBH containing 0.4% bovine serum albumin (BSA). Four dishes with either E_i or E_0 were then treated sequentially with 1 ml 0.2 M glycine buffer, pH 2.8, 2 min/dish. After the last dish, the buffer was neutralized by transfer to a tube containing 0.1 ml 2 M Tris-base. 0.1 ml of the eluate was used for IF and the rest for antigen analysis.

Enzyme Treatment of E Monolayers

Monolayers of either E_i or E_0 on multitest slides were treated with enzymes either before or after GA fixation and drying. The enzymes used were trypsin (bovine pancreas, type III, twice crystallized, 9,440 U/mg; Sigma Chemical Co.), pronase (nonspecific protease, *Streptomyces griseus*, type XIV, 5.8 U/mg; Sigma Chemical Co.), or neuraminidase (*Clostridium perfringens*; Sigma Chemical Co.). One drop of enzyme solution at the concentration indicated below was added to each well for 30 min at 37°C. Enzyme treatment was interrupted by washing with TBH containing 15% fetal calf serum. The slides treated with enzyme before fixation were then fixed with 1% GA and air-dried.

Immunoadsorbent

IgG from a *P. falciparum* hyperimmune Liberian serum (Kinon) was prepared by ammonium sulfate precipitation and DEAE Sephadex ion exchange chromatography as described (25). The IgG was coupled to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) at a concentration of 5 mg per ml of gel (65 ml gel total) and the coupled gel was packed in a column (K26/40; Pharmacia Fine Chemicals).

Immunoblotting

Immunoblotting was performed essentially as described previously (26). One ml IgG of immune serum (1 mg IgG/ml) or of the antibody preparation eluted from E monolayers (E_i or E_0) was diluted with 2 ml incubation buffer (PBS, 1% BSA, 0.1% Triton X-100, 0.02% sodium dodecyl sulfate [SDS], and 0.02% NaN_3) and incubated with nitrocellulose strips containing electrophoretically transferred polypeptides from SDS-polyacrylamide gel electrophoresis (PAGE) separations of either merozoite-enriched parasite preparations or soluble culture supernatant antigens eluted from an immunoadsorbent (see above). Antibodies bound to the blotted polypeptides were detected by staining for alkaline phosphatase (ALP) after reaction of the strips with rabbit anti-human IgG (polyvalent) conjugated with ALP.

In some experiments, metabolically radiolabeled parasite material was used. *P. falciparum* cultures (5% haematocrit, 5–10% parasitemia) were labeled for 15 h with L-(^{75}Se)-selenomethionine (25 μCi/ml medium, 20–50 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom) in methionine-free medium (Parker 199; State Bacteriological Laboratories, Stockholm). A merozoite-enriched fraction was prepared as described above and was used as antigen in immunoblotting experiments (~5×10^5 cpm per nitrocellulose strip). After development of the immunoblottings, the nitrocellulose strips were subjected to autoradiography for 3–5 d, using Kodak X-Omat RP film and an intensifying screen.

ELISA

The content of antibodies to *P. falciparum* or E in different sera was determined as described elsewhere. The parasite antigen was a sonicate of trophozoite/schizont-enriched fractions obtained by Percoll gradient centrifugation (Pharmacia Fine Chemicals) of the laboratory cultures (23).

Cell ELISA. To determine the antibodies binding to the surface of GA-fixed E_i, a cell ELISA was developed. The wells of micro-ELISA plates were treated with bicarbonate coating buffer, pH 9.6. Fifty μl/well of a 1% suspension of E_i (~10% parasitemia) or E_0 were added and incubated for 30 min at room temperature. Nonattached cells were removed by gentle flooding of the plates with TBH and aspiration. Washing was repeated three times, leaving the bottom layer wet every time. 100 μl of 1% GA was then added

to each well and removed immediately by shaking of the plates upside down. Fixation was repeated once. The plates were then washed with distilled water, air dried, and stored at −20°C until use. In both ELISA systems the antibody content of different sera was determined with an ALP-conjugated rabbit anti-human immunoglobulin serum as described (23).

Results

When monolayers of GA-fixed and air-dried E were incubated with *P. falciparum*-immune serum and then tested in the indirect IFA, a distinct surface staining of E_i was observed. No staining was obtained with sera from controls never exposed to malaria. Fresh E or E which had not been air dried after fixation were not stained. Immunofluorescence was restricted to the surface of parasitized E, as seen by counterstaining of the parasite nuclei with ethidium bromide (Fig. 1 *a–c*). By using this double staining procedure, positive IF could easily be related to the maturity of the parasite. Whereas E containing rings and trophozoites displayed strong IF (Fig. 2 *a*), it was usually absent or very weak on E containing schizonts. IF was an early event, already comprising large parts of the E surface when the merozoite penetrated the E (Fig. 2 *b*). Very rarely, staining of noninfected E around a bursting schizont was observed (Fig. 2 *c*). IF of intracellular parasites was not seen.

The IF shown above was obtained by exposing E infected by in vitro culture with our laboratory strain (Tanzania) to sera of acutely infected patients from Colombia or Sweden. Exactly the same results were obtained when parasitized E from acutely infected patients were exposed to the patient's own serum (Fig. 2 *d*). To check for possible strain specificity, *P. falciparum*-infected E from four Colombian, two Tanzanian, and two Kenyan patients were tested with E surface-positive sera of either Colombian or African origin. IF was similar throughout. Sera from five patients acutely infected with *P. vivax*, three infected with *P. ovale*, and one with *P. malariae* were also tested with *P. falciparum*-infected E. Two *P. vivax*, one *P. ovale*, and the *P. malariae* serum gave a positive E surface IF. The rest were negative. However, the positive sera were from Indian or African donors and had positive *P. falciparum* titers in ELISA, suggesting that the donors had previously also been exposed to *P. falciparum*. In contrast, the sera that were negative in this group were from Swedish patients with primary infections. *P. vivax*-infected E from seven acutely infected patients were not stained by either *P. vivax* sera or by some of the most reactive *P. falciparum* sera available on our panel.

Sera from 60 *P. falciparum* patients or immune individuals were screened for reactivity in the E surface IFA and in ELISA with total *P. falciparum* antigen as described by Wahlgren et al. (23). The serum dilutions used for screening were 1:10 for IFA and 1:1,000 for ELISA. In general, Swedish or Colombian sera (acute infections) giving an OD_{405} of <0.8 in ELISA were also negative in the E surface IFA. Sera giving ELISA values ≥ 0.8 at the 1:1,000 dilution (39 sera) were then subjected to endpoint titration in IFA (Fig. 3), While the sera from four ELISA-positive Colombians and three Swedes were negative, all others were positive in the E surface IFA. However, there was no correlation between IFA titers and the strength of ELISA against total *P. falciparum* antigen. To design an ELISA permitting a more restricted identification of antigens on the surface

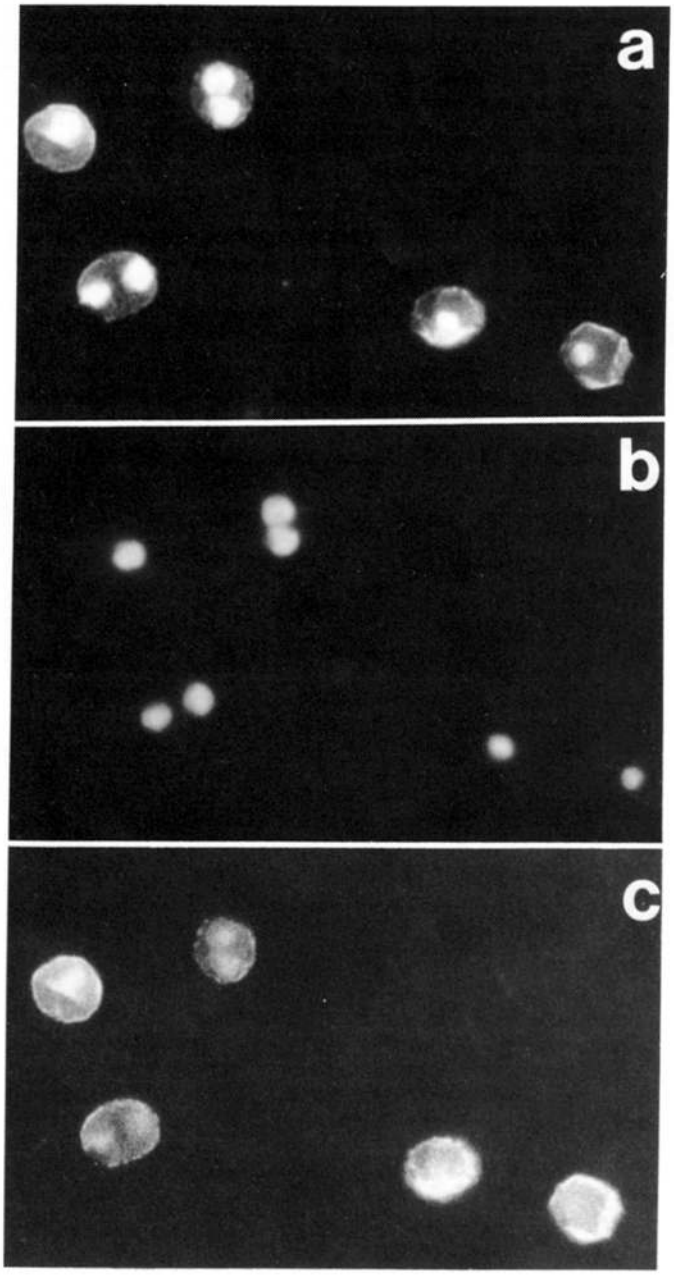

FIGURE 1. Indirect IF of GA-fixed and air-dried monolayers of *P. falciparum*-infected erythrocytes (laboratory strain) exposed to malarial sera from a Colombian donor. The parasite nuclei were counterstained with ethidium bromide, parasitemia ~10%. (*a*) IF (green) of surface of trophozoite containing E and intracellular fluorescence of parasite nuclei (orange); (*b*) the same field but only showing parasite nuclei, or (*c*) E surface IF.

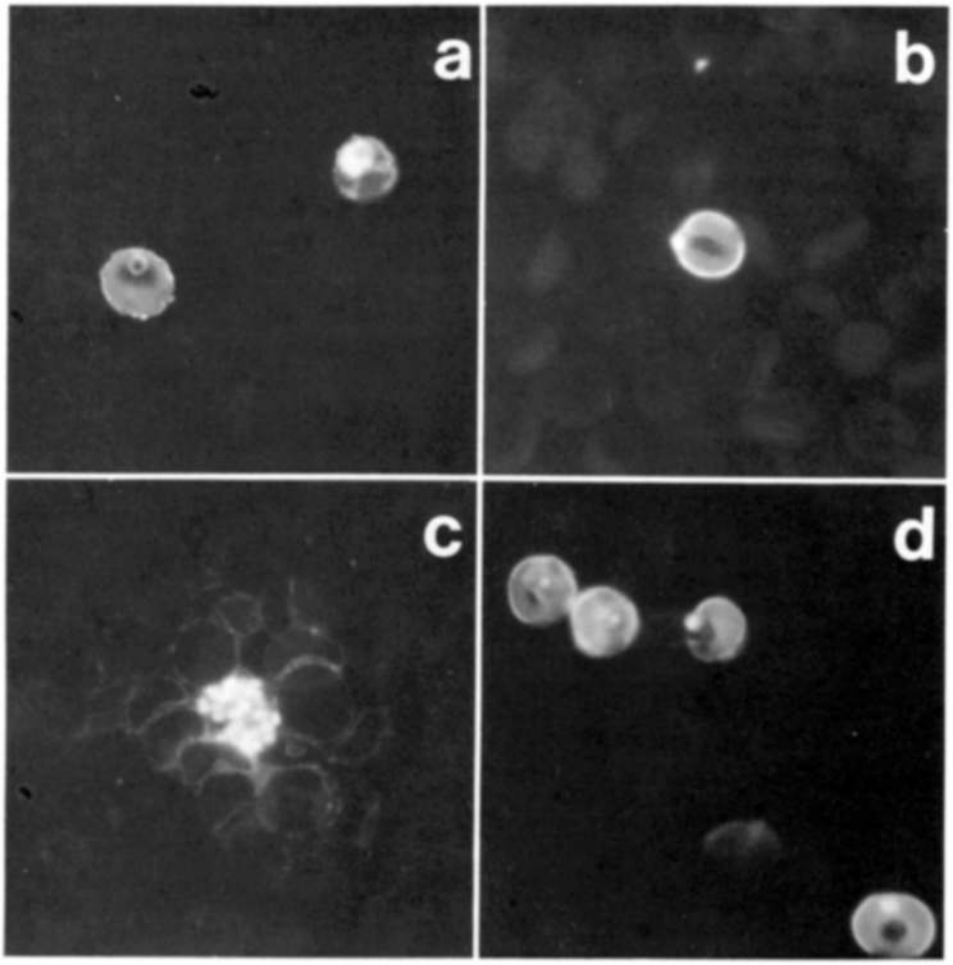

FIGURE 2. Indirect IF of GA-fixed and air-dried monolayers of *P. falciparum*-infected erythrocytes (for further explanations see Fig. 1). (*a*, *b*) Double-stained E containing young trophozoites or penetrating merozoites, respectively; (*c*) surface IF of noninfected E around bursting schizont; merozoite nuclei counterstained; (*d*) IF of parasitized E freshly drawn from a Colombian patient and stained with the patient's own serum; parasite nuclei counterstained.

of infected E, we developed a cell ELISA in which monolayers of GA-fixed and dried E were used as antigen. Fig. 4 shows the results of a representative experiment with a *P. falciparum*-immune Liberian serum and a normal control serum tested with either E_i or E_0. As seen, the immune serum gave a strong and dose-dependent reaction with E_i, significantly different from the three controls. In Table I, three sera with IFA titers $\geq$1:625 were compared with three sera having IFA titers $\leq$1:125. For the cell ELISA, the sera had to be used at relatively high dilutions to eliminate background binding to E_0. This was probably the main reason for the absence of specific reactions with the low-titered sera. On the other hand, sera with elevated IFA titers always gave strong and highly specific reactions in the cell ELISA.

To elucidate the nature of the antigens responsible for the binding of antibod-

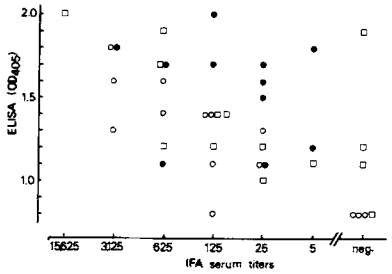

FIGURE 3. Comparison of ELISA (total *P. falciparum* antigen) and E surface IFA. (Abscissa) Reciprocals of highest serum dilutions giving positive surface IF of E infected with *P. falciparum* laboratory strain, ~10% parasitemia; (ordinate) ELISA values (OD_{405}) at serum dilution 1:1,000 (○, ●, □) Swedish, Liberian, or Colombian sera, respectively. Sera giving ELISA values ≤0.8 not included.

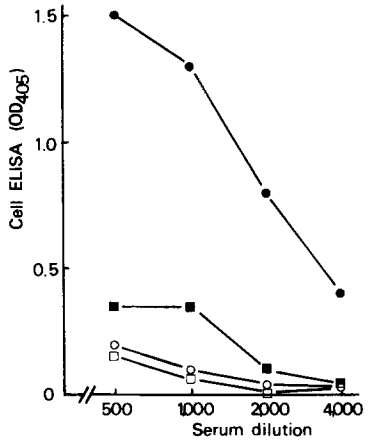

FIGURE 4. Cell ELISA. (Abscissa) Serum dilutions; (ordinate) OD_{405}; (○, ●) *P. falciparum*-immune serum (Liberian); (□, ■) normal human serum (Swedish); (●, ■) *P. falciparum*-infected E, 10% parasitemia; (○, □) noninfected E.

ies to the surface of E_i, the cells were treated with proteolytic enzymes or neuraminidase. Some E_i monolayers in multitest slides were treated with enzyme before fixation with GA. They were then washed, fixed, air-dried, and stained as described. As seen from Table II, under these conditions neither trypsin nor neuraminidase abolished IF which was indistinguishable from that of untreated cells. Pronase was also without effect except when added at very high concentrations. However, when enzyme treatment was performed after GA fixation and air drying, pronase completely abolished staining at both concentrations used. In contrast neither trypsin nor neuraminidase affected antibody binding.

To establish the origin of the antigens reacting in the E surface IFA, inhibition experiments were performed. The representative results of three out of a total of eight independent experiments are shown in Table III. The soluble fraction of E_i lysates (~10% parasitemia) completely abolished surface IF given by a strongly positive immune serum (Exp. 1) whereas the soluble fraction of E_0 lysates had no effect. Similarly, the soluble fractions of sonicated, merozoite-enriched parasite preparations were fully inhibitory at a concentration of 6 μg protein/

TABLE I
Comparison of E Surface IFA and Cell ELISA

Sera*	IFA titers‡	Cell ELISA (OD_{405})‡	
		E_i	E_o
Immune, group 1			
Lib 11	12,500	1.27	0.09
AC	3,125	1.26	0.31
Lib 6	625	0.57	0.17
Normal§	0	0.06	0.05
Immune, group 2			
HM	125	0.37	0.29
JC	25	0.18	0.29
X27	5	0.15	0.19
Normal§	0	0.20	0.12

* *P. falciparum*-immune sera with IFA titers ≥1:625, dilution in cell ELISA 1:4,000 (group 1) or ≤1:125 and 1:1,000 (group 2).
‡ Antigen: E infected with *P. falciparum* laboratory strain, parasitemia ~10%. IFA titers are reciprocals of highest serum dilution showing positive immunofluorescence.
§ Mean values obtained with five normal sera; these sera had no IFA titers at lowest dilution tested (1:5).

TABLE II
*Surface IF of Enzyme-treated E**

Enzymes	Enzyme treatment‡	
	Before fixation and drying	After fixation and drying
Trypsin		
400 μg/ml	+	(+)
100 μg/ml	+	+
Pronase		
400 μg/ml	(+)	−
100 μg/ml	+	−
Neuraminidase		
0.02 U/ml	+	+

* IFA of E_i (~10% parasitemia) was done with a hyperimmune Liberian serum (Kinon), diluted 1:1,000.
‡ 30 min at 37°C of E monolayers on multitest slides either before or after GA fixation and drying. +, strong IF; (+), weak IF; −, no IF.

ml, while no inhibition was seen at 0.6 μg/ml (Exp. 2). In contrast, similar extracts made from normal E ghosts were not inhibitory at concentrations >25 times higher. Centrifuged supernatants from cultures of the laboratory *P. falciparum* strain were inhibitory at a dilution of 1:8. When passaged over an immunoadsorbent made from the IgG fraction of the serum of a healthy but *P. falciparum*-hyperimmune Liberian donor, most of its inhibitory activity disappeared (Exp. 2). This activity was fully recovered in the material eluted from the immunoad-

TABLE III
*Inhibition of E surface IF**

Exp.	Inhibitor‡	Lowest concentration needed for total inhibition‡
1	E_i lysate	14×10^7 E/ml, 9% parasitemia
	E_o lysate	—§
	Merozoite extract	6.5 μg protein/ml
	E ghost extract	—§
2	Culture supernatant	
	Before IS passage	1:8 dilution
	After IS passage	1:2 dilution
	IS eluate	1 μg protein/ml
3	Culture supernatant	
	IS eluate	1 μg protein/ml
	IS eluate (100°C, 5 min, centrifuged)	1 μg protein/ml

* *P. falciparum* laboratory culture tested with hyperimmune Liberian serum Gbor, final dilution 1:5,000 (Exp. 1) or Kinon, final dilution 1:1,000 (Exps. 2, 3).
‡ See Material and Methods; IS, immunoadsorbent prepared from Kinon IgG.
§ No inhibition with highest concentrations tested (14×10^7 E/ml and 160 μg ghost protein/ml, respectively).

sorbent (Exps. 2 and 3). Incubation in boiling water for 5 min (27) did not noticeably affect the inhibitory capacity of these eluates.

For closer studies of the antigens exposed on the surface of E_i, the antibodies binding to GA-fixed and air-dried E were isolated by acid elution. Antibodies eluted from E_i monolayers were fully active in the E surface IFA when added to fresh E_i monolayers. No IF was seen with similarly prepared eluates from E_0 monolayers. These antibody preparations were used for antigen analysis. Soluble antigens from culture supernatants were first adsorbed to a *P. falciparum*-reactive immunoadsorbent, eluted, and subjected to SDS-PAGE on 5–15% gradient slab gels (26). The separated antigens were then electrophoretically transferred to strips of nitrocellulose and probed either with the total IgG fraction of a *P. falciparum*-hyperimmune serum or with antibody eluates prepared as described above. In these experiments, the same Liberian serum (Kinon) was used both for preparing the antibody eluates, the immunoadsorbent, and for developing the immunoblots. The results of a typical experiment are shown in Fig. 5. Upon the addition of the ALP anti-human Ig conjugate and zymogram staining, all strips displayed a strong but diffuse staining in the molecular weight regions typical for human Ig chains (μ, γ, light). The stained zones appeared also on the control strips not treated with Kinon antibodies, indicating that the antigen preparation contained substantial amounts of human Ig. This was derived from the human serum present in the culture supernatant and was retained on the immunoadsorbent, which was rich in anti-Ig activity (Wahlgren, unpublished results). When the antigen mixture was probed with the total Kinon IgG, at least 25 distinct

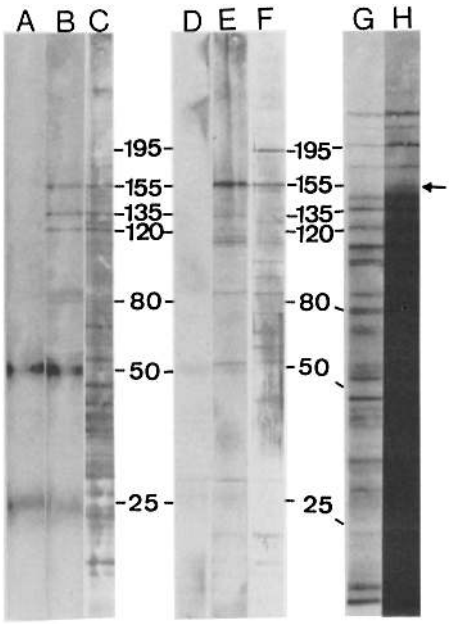

FIGURE 5. Reactivity of antibodies to immunoadsorbent-purified *P. falciparum* culture supernatant antigens (*A*, *B*, and *C*) and antigens of a merozoite-enriched fraction of *P. falciparum* (*D*, *E*, and *F*) after electrophoretic transfer of polypeptides from SDS-PAGE to nitrocellulose membranes. (*A*, *D*) Polypeptides recognized by antibodies eluted from monolayers of normal human E; (*B*, *E*) polypeptides recognized by antibodies eluted from monolayers containing *P. falciparum*-infected E; (*C*, *F*) polypeptides recognized by whole IgG from *P. falciparum*-hyperimmune serum (Kinon); (*G*, *H*) autoradiograms of nitrocellulose strips to which the polypeptides of a ^{75}Se-methionine-labeled merozoite-enriched fraction of *P. falciparum* were transferred after SDS-PAGE. In *H*, the autoradiogram has been overexposed in order to visualize the weakly labeled polypeptide at 155 K (arrow). Numbers indicate approximate molecular weights × 10^{-3}.

bands of widely differing molecular weights were observed. In contrast, the antibodies eluted from E_i monolayers reacted with only a few of the transferred polypeptides, primarily a doublet of M_r 155,000 (155 K) and two faster migrating bands of M_r 135 K and 120 K, respectively. Some weakly stained bands in the 70–80 K mol wt region were seen in some but not all experiments. The three slower migrating bands also appeared when the antigen mixtures were heated to 100°C for 5 min before SDS-PAGE. However, none of these antigens was seen when the strips were probed with eluates from E_0 monolayers, indicating the absence of the corresponding antibodies in these preparations.

To directly prove the origin of the antigens responsible for antibody binding to the surface of E_i, similar experiments were performed with extracts of parasites metabolically labeled with ^{75}Se-methionine. The antigen source was soluble extracts of merozoite-enriched preparations, prepared as described above and subjected to SDS-PAGE. Probing of the nitrocellulose strips with Kinon serum showed that >20 antigenic polypeptides had been transferred, including a strongly stained band (doublet) of M_r 155 K. Autoradiography of the SDS-polyacrylamide gels indicated the presence of at least 20 radioactive bands and most of these polypeptides were also detected on the nitrocellulose strips,

regardless of whether or not these were probed with antibodies. Comparison of the zymogram and the autoradiographs suggested that a majority but not all of the antigens made visible with the Kinon serum were of parasite origin. These included a 155 K M_r polypeptide that was weakly but distinctly labeled (Fig. 5.) and was the predominating antigen when the strips were probed with antibodies eluted from E_i monolayers. In the zymograms, these strips also showed two weak bands of M_r 135 K and 120 K which were labeled in the autoradiographs. These antibodies brought down three additional bands (110 K doublet, 90 K, and 52 K) which had no counterparts in the autoradiographs and at least one of which (52 K) appeared to be of erythrocyte origin. There were some fast-migrating components which, however, were also stained when the strips were probed with the eluate from E_0 monolayers. The nature of these components is under investigation. Parallel tests with E ghost polypeptides as antigen showed that the Kinon-IgG reacted with E polypeptides of M_r 220, 70, 65, 52, and 48 K but had no reactivity in the 155–120 K region (data not shown) (see 26).

Discussion

By means of an indirect IFA, we have shown that sera from patients with *P. falciparum* malaria or from immune donors contain antibodies that bind to the surface of E_i. With some very rare exceptions (Fig. 2*c*), antibody binding was restricted to the surface of E_i. It was most pronounced when the parasites were in early phases of their developmental cycle (ring stages, trophozoites) and was already seen when the merozoites penetrated uninfected host cells. In some sera the titers of the antibodies for this reaction were very high (>1:15,000).

To demonstrate this antibody binding to E_i, we have developed a new indirect IFA in which E monolayers were very briefly fixed with dilute GA and air-dried before they were exposed to malarial sera and anti-Ig conjugate. Under these conditions, the antibodies did not penetrate the E membrane and intracellular parasites were not stained. The presence of parasites in the antibody-binding E was demonstrated by counterstaining with a fluorescent nuclear dye similar to what has been described by others (28).

As demonstrated in this assay, antibody binding was the same regardless of whether the E_i were obtained from cultures of a *P. falciparum* strain kept in the laboratory for many years or were freshly drawn from the blood of patients with acute infection. However, surface staining was never seen unless the E were air-dried. The reasons for this are not clear. Obviously, antibody staining reflects a change in the surface structure of the E occurring early in connection with invasion, but the corresponding antigenic structures are not exposed before dehydration. This conclusion was also supported by experiments in which the E were treated with pronase. When E_i were treated before fixation and drying, subsequent IF staining was not affected. In contrast, enzyme treatment after fixation and drying abolished antibody binding.

The E surface IF appeared to be species specific since strongly positive *P. falciparum* sera did not react with *P. vivax*-infected E. However, as we have not as yet seen any positive reactions when testing *P. vivax*-infected E with sera from patients with *P. vivax* malaria, we do not know whether the results reflect species specificity of the antibodies in *P. falciparum* sera or differences in antigen

expression on E infected with either one of these two plasmodial species. In a rodent malaria system we have observed a similar antibody binding to the surface of *P. chabaudi*-infected mouse E exposed to sera from mice immunized with this parasite. These sera did not react with mouse E infected with *P. yoelii*. Moreover, there were no cross-reactions between *P. chabaudi* and *P. falciparum* when E and sera were tested in reciprocal combinations (unpublished results).

Screening of many malarial sera suggested that repeated or prolonged exposure to *P. falciparum* is a prerequisite for the appearance of antibodies giving the E surface IF described in this study. Thus, sera from residents of a holoendemic area of Africa (Liberia) were all positive. In contrast, a higher frequency of negative reactions was found among Colombian and Swedish patients with acute malaria, particularly those suffering from their first infections. For the latter two groups, sera that displayed relatively low reactivity with total *P. falciparum* antigen in ELISA (23) were usually also negative in the E surface IFA. With this exception, there was no obvious correlation between IF and ELISA titers and some sera which gave strong ELISA reactions were negative in E surface IF. On the other hand, when GA-fixed and air-dried E were used in ELISA (cell ELISA) the correlation to the IFA was good, indicating that the two test systems detect the same antibodies. If confirmed by further experiments, the cell ELISA should be well-suited for screening of sera or B cell supernatants (29) for the occurrence of antibodies reacting with the surface of infected E. In particular, it should be suitable for studies of the specificity of the antibodies in quantitative inhibition experiments. Its sensitivity can probably be greatly increased by using higher parasitemias and synchronized parasite cultures as antigen.

The fact that certain sera were negative in the E surface IFA may mean that the relevant antibodies had not been raised or were not available for reaction because of the formation of complexes with antigen present in the blood during the acute phase of infection. Alternatively, antibodies may recognize antigens that vary for different *P. falciparum* strains. The existence of inter- and intrastrain variability of plasmodial antigens on the E surface has been reported for both *P. knowlesi* (17, 30, 31) and *P. falciparum* (16, 20, 32). Thus far, we have not seen this variability, since the sera that gave positive IF with our laboratory *P. falciparum* culture and which were from different parts of the world reacted equally well with freshly isolated E_i from African or South American patients, including both heterologous and autologous serum/parasite combinations. However, as our positive sera may have been polyspecific, the occurrence of a strain variation in the E surface IF is not excluded (20). In an attempt to study this problem we have recently also tested surface IF-positive antibodies from monoclonal B cell cultures, obtained by Epstein-Barr virus transformation of the lymphocytes of a hyperimmune Liberian donor, with E infected either with Tanzanian or Colombian parasites. In the experiments performed up to now, no differences in E surface IF between these parasites were observed. Further experiments, using both human monoclonal antibodies (29) and genetically defined parasite isolates, will be used to answer this important question.

Treatment of E_i after fixation and drying with neuraminidase had no effect on antibody binding, indicating that terminal sialic acid residues were not involved in these reactions. Although treatment of the cells with trypsin was also

without effect, treatment with pronase completely abrogated antibody binding, suggesting that the antigens were polypeptides. Treatment of the monolayers with chloroform/methanol (2:1) did not change antibody binding, arguing against the involvement of lipids (unpublished results).

In accordance with the finding that E_o were not stained by immune sera was the fact that lysates of normal E or extracts of normal E membranes did not inhibit E surface IF even when added at very high concentrations. In contrast, antigens extracted from merozoite-enriched parasite preparations or present in centrifuged supernatants of *P. falciparum* cultures gave an efficient and dose-dependent inhibition. The soluble supernatant antigens eluted from insolubilized antibodies of a hyperimmune Liberian serum were the most effective inhibitor of all those tested. Moreover, this inhibition was heat resistant, suggesting that the inhibitory antigens may be related to the S antigens, a serologically complex family of soluble, parasite-derived antigens of different molecular weights present both in patient sera and in supernatants of *P. falciparum* cultures (27, 33, 34).

Plasmodial infection is known to cause substantial changes in the surface structure and composition of parasitized E (35). Although our results strongly favored the conclusion that the antigens responsible for E surface IF were parasite derived, they did not exclude the involvement of altered host components, recognized by antibodies present in malarial sera. Therefore, to further elucidate the nature and origin of these antigens, we analyzed them with antibodies eluted from the surface of E_i pretreated with a hyperimmune serum under conditions similar to those of the E surface IFA. Immunoblotting after SDS-PAGE of soluble and immunoadsorbent enriched antigens from *P. falciparum* cultures gave many bands when the blots were probed with the IgG fraction of the same hyperimmune serum as was used to prepare the immunoadsorbent. However when probed with eluates made up of the E surface-binding antibodies from the same serum, a very restricted number of antigens was detected. The consistent finding was that of a distinct, strongly stained doublet of M_r 155,000 and of two faster-migrating bands of lower apparent molecular weights (135 K, 120 K). The immunological relationship of these polypeptides to each other is not known. Similar results were obtained when the antigens were heat treated before SDS-PAGE, thus confirming the heat stability noted in the inhibition experiments.

Further evidence for the origin of the antigens was sought by using extracts of metabolically labeled parasites (mainly merozoites) as the antigen source. Here, immunoblotting suggested that only one dominating antigen, also of M_r 155 K, and some faster-migrating minor polypeptides were recognized by the antibodies eluted from monolayers of E_i, as contrasted to >20 polypeptides recognized by immune serum. Three of these polypeptides, including the 155 K M_r band, were also among the ~20 bands which were radioactive according to the autoradiographs. Labeling with ^{75}Se-methionine of the 155 K M_r polypeptide was weak but significant. The results further support the conclusion that the E surface IF seen by us was due to parasite-derived antigens recognized by antibodies from patients or individuals with acquired *P. falciparum* immunity.

The occurrence of parasite antigens on the surface of infected E in malaria is well established (16, 17, 30–32, 36–39). In most instances, these antigens appear to be synthesized during relatively late phases of parasite maturation and are

assumed to be inserted into the E surface by the parasites from within the infected host cell (35). They have only been found on the surface of E containing mature trophozoites or schizonts. This is different from the findings reported here where the antigens were readily detected on E_i already at invasion and in the early phases of parasite development whereas they appeared to be absent from schizont-containing E. Taken together, our results suggest that these antigens are released from bursting schizonts or merozoites and are deposited in the E membrane in connection with invasion. That antigens are released during schizont development and rupture or merozoite invasion has been reported (12, 40, 41). The antigens responsible for the E surface IF described by us may be part of the merozoite coat that is peeled off at invasion or may be released from the merozoite rhoptries (14, 42–44). Our findings further imply that they have affinity for receptor structures in the E membrane (45–47). It has recently been reported (48) that the major 195 K M_r glycoprotein found on the surface of *P. falciparum* schizonts is processed into fragments corresponding in size to those of the antigens described by us. Fragmentation appears to occur when the schizonts mature and merozoites are released. However, although the immune serum used in our antigen analysis contained antibodies that recognized the 195 K M_r glycoprotein (Fig. 5 and Berzins, unpublished results), the antibody fraction eluted from the surface of E_i did not. Further investigations are required to establish the relationship of the antigens involved in the E surface interactions studied by us to that of other known parasite antigens described in the current literature.

In recent experiments we have found that the antibodies eluted from fixed and air-dried monolayers of E_i strongly inhibit parasite reinvasion in vitro (Wåhlin, manuscript in preparation). This suggests that the corresponding antigens may have an important role in the invasion process. Whether this involves the schizont burst, a recognition function necessary for merozoite attachment (45–47), or an alteration of the E membrane to facilitate merozoite penetration (49) is presently unknown. In any event, the experiments also suggest that the antibodies against these antigens may have a host protective function in malaria infection. If so, protection would probably not operate at the level of the E_i (e.g., by opsonization) since the antigens appear to be buried in the surface and are not readily accessible for antibody binding. For these reasons, the antibodies are probably also different from those believed to protect by blocking or reversal of sequestration of E_i (16). Rather, a possible protective action of the antibodies would be expected to take place at the level of the bursting schizont or free merozoites. Experiments intended to answer these questions are in progress.

Summary

Monolayers of human erythrocytes (E) infected with *Plasmodium falciparum* were briefly fixed with 1% glutaraldehyde and air dried. They were then exposed to sera from patients with *P. falciparum* malaria or from donors immune to this parasite and tested in an indirect immunofluorescence assay (IFA). Parasites in infected E were made visible by counterstaining with ethidium bromide. Immunofluorescence (IF) was restricted to the surface of infected E. No antibody binding was detected unless the E were dried, suggesting that the relevant

antigens were not available on the outer layers of the E surface. Staining over large parts of the E surface was seen already when the merozoite penetrated noninfected cells and was strong in E containing early stages of the parasite (rings, trophozoites). It was weak or absent from E containing schizonts. Antibodies in sera from different parts of Africa, Colombia, or Sweden reacted similarly with E infected with a Tanzanian *P. falciparum* strain kept in culture for many years and with parasitized E freshly drawn from African, Swedish, or Colombian patients. All sera from residents of a holoendemic area (Liberia) were IFA positive. In contrast, some sera from Colombian or Swedish patients with primary infection gave negative results. The results of the IFA and of an enzyme-linked immunosorbent assay in which fixed and dried E were the targets were well-correlated, suggesting that the same antibodies were detected by these assays. The antigens involved in the IFA were susceptible to pronase but not to trypsin or neuraminidase. E surface IF was inhibited by lysates of infected E, merozoite extracts, or soluble antigens present in *P. falciparum* culture supernatants but not by lysates of normal E or ghost extracts. The inhibitory antigens were heat stable (100°C, 5 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting of either antigen-enriched preparations from culture supernatants or merozoite extracts showed that antibodies eluted from monolayers of infected E reacted consistently with a predominant polypeptide of M_r 155,000 and two to four minor polypeptides of lower molecular weights. Metabolic labeling of the parasites with ^{75}Se-methionine indicated that these antigens were parasite derived. We conclude that the antigens involved in these reactions are released from bursting schizonts or merozoites and are deposited in the E membrane in the course of invasion. A possible protective significance of the immune response to these antigens is suggested by preliminary experiments which showed that the antibodies eluted from infected E very efficiently inhibit E reinvasion by merozoites in vitro.

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