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Stage-specific proteins and glycoproteins of *Plasmodium falciparum*: Identification of antigens unique to schizonts and merozoites

(malaria/synchronized cultures/erythrocyte membrane)

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ABSTRACT **Establishment of highly synchronized cultures of *Plasmodium falciparum* enabled identification of stage-specific proteins, glycoproteins, and antigens. Comparison of metabolically labeled constituents of rings, trophozoites, mature schizonts, and merozoites indicated the absence of major proteins or glycoproteins unique to rings or trophozoites. A burst of new synthetic activity occurred during schizogony when several schizont- and merozoite-specific proteins and glycoproteins became apparent. In addition to the knob protein, which was previously shown to be associated with protrusions on the host erythrocyte membrane, a major glycoprotein of parasite origin was identified on the surface membrane of schizonts. Analysis of antigens solubilized from different developmental stages indicated that immune sera, which inhibit growth of parasites *in vitro*, react mainly with merozoite- and schizont-associated antigens.**

Successful experimental vaccinations with erythrocytic stages of malaria parasites have indicated that merozoites and schizonts are the best sources of antigens that elicit a protective immune response (1–3). Identification of these stage-specific functional antigens would provide a rational basis for developing a malaria vaccine suitable for humans. Although an *in vitro* method for the cultivation of *Plasmodium falciparum*, the most virulent species of human malaria, has been available for over 3 years (4), there is no published information on stage-specific constituents or antigens. The major obstacle has been the asynchronous development of parasites in culture which has created difficulties in collection of sufficient numbers of nondegraded free merozoites. After emergence from erythrocytes, merozoites lose infectivity within about half an hour.

In this study, a combination of two published methods was used to establish highly synchronized cultures. These cultures enabled the identification of stage-specific proteins and glycoproteins. In addition, by using sera from infected humans and experimentally immunized rabbits, antigenic constituents of merozoites and schizonts were determined.

MATERIALS AND METHODS

Synchronized Cultures. *P. falciparum* (FCR-3/Gambia) was cultured in 100-mm petri dishes in a candle jar (4). To obtain highly synchronized cultures, the infection of erythrocytes by merozoites was limited to 3–4 hr as follows. Starting with asynchronous cultures, multinucleate parasites were concentrated by flotation in gelatin (5) and subcultured with fresh erythrocytes. After 7–8 hr, cultures were collected and all multinucleate parasites that had failed to develop to mature schizonts and reinfect erythrocytes were eliminated with sorbitol treatment (6). On subsequent subcultures the identical procedure was repeated but reinfection period was limited to 3–4 hr, resultant cultures were highly synchronous.

Labeling of Stage-Specific Proteins and Glycoproteins. Proteins were labeled with L-[2,3,4,5-^{3}H]proline (Amersham) at 2.5 μCi/ml (1 Ci = 3.7×10^{10} becquerels), and glycoproteins were labeled with D-[6-^{3}H]glucosamine (Amersham) at 10 μCi/ml. About 5–6 hr after infection of fresh erythrocytes, cultures were treated with gelatin (5) to ensure removal of leukocytes as well as all remnants of mature schizonts, residual bodies, and membrane fragments which do not sediment. Radioactive substrates were added when parasites were about 7–8 hr old, and different developmental stages were labeled with overlapping time intervals—i.e., rings at 8–24 hr, trophozoites at 16–34 hr, and mature schizonts and merozoites at 32–48 hr. For collection of merozoites and mature schizonts, cultures (to which labeled substrate had been added at 32 hr) were collected at 40 hr and multinucleate parasites were concentrated by flotation in gelatin. Concentrated, parasitized erythrocytes were resuspended in labeled culture medium that was supplemented with 10% erythrocyte extract. Cultures were monitored by light microscopy to determine the beginning of emergence of merozoites. Free merozoites were allowed to accumulate in the supernatant medium for 2 hr; and then cultures were transferred to centrifuge tubes. Mature schizonts and a few contaminating erythrocytes were pelleted by centrifugation (10 min, $500 \times g$) and merozoites were collected from the supernatants (15 min, $1600 \times g$).

Preparation and Analysis of Samples. Merozoite pellets were washed once with culture medium without serum. Pellets of all other developmental stages were suspended in 80 vol of ice-cold 20 mM Tris·HCl/2 mM EDTA, pH 8/0.5 mM phenylmethylsulfonyl fluoride and centrifuged (10 min, $900 \times g$). Supernatants and buffy layer of normal erythrocyte ghosts were discarded and pellets were kept at −80°C until analyzed. NaDodSO$_4$/polyacrylamide gel electrophoresis was carried out as described (7). Aprotinin (Boehringer Mannheim) was included in sample solvent at 200 μg/ml. Radioactive bands were visualized by fluorography (8).

Immune Sera. Rabbit A was immunized with merozoites prepared as described above. Rabbit B was immunized with a membrane-enriched fraction prepared from schizonts as described (9). On day 0, each antigen was emulsified with 1 ml of Freund's complete adjuvant and injected intraperitoneally. Two intravenous injections of antigen alone were given on days 21 and 45. Antisera were collected 10 days after the last injection and absorbed with normal human erythrocytes until no agglutination was apparent.

Human serum A (HA) was a gift from Irving Kagan and Henry Mathews (Center for Disease Control); it had been col-

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Abbreviations: NP-40, Nonidet P-40; HA, human serum A; HB, human serum B; RA and RB, antisera from rabbits A and B, respectively.

lected from a Nigerian and had shown high anti-*P. falciparum* titers when tested by indirect hemagglutination and indirect immunofluorescence. Human serum B (HB) was from a patient from Uganda admitted to a New York City hospital for treatment for cerebral *P. falciparum* infection. Antisera from rabbits A (RA) and B (RB) and HA were tested for *in vitro* parasite growth inhibition in tissue culutre multiwell plates (Limbro; well capacity, 3.5 ml). Routine culture conditions were used (with 10% control or immune serum) except for reduction of erythrocyte density to 2% in 0.5 ml of medium. Infection was initiated with schizonts, and growth was evaluated from stained smears 60 hr later. The same antisera were also tested for reactivity with surface antigens of merozoites by using immunoelectron microscopy essentially as described (9); incubation periods with first and second antibody were reduced to 30 min (room temperature).

Analysis of Antigens. Metabolically labeled parasites were solubilized (20 min, 37°C) in 4 vol of 1% Nonidet P-40 (NP-40) in phosphate-buffered saline, pH 7.6/0.05% sodium azide containing aprotinin at 200 μg/ml. Insoluble residue was removed by centrifugation (30 min, 17,000 × *g*). Immune or control serum (200 μl) was mixed with 150 μl of a 10% (wt/vol) suspension of protein A covalently coupled with Sepharose CL-4B (Sigma). After 30 min at 37°C the beads were pelleted and washed once with the buffer. Labeled NP-40 extract (200 μl; 400–600 μg of protein) was added to the washed beads and the samples were kept at room temperature for 2 hr. Unbound extract was removed by three washes with 0.5 M LiCl/10 mM Tris·HCl, pH 8, and one wash with 1% NP-40 buffer. Immunocomplexes were eluted with 10 M urea/6% (vol/vol) $NaDodSO_4$/0.2 M dithiothreitol, 1:1:1 (vol/vol), and analyzed by acrylamide gel electrophoresis.

Other Methods. Protein was measured by a modified Lowry method (10). For surface labeling, young schizonts were concentrated by gelatin flotation and labeled with sodium boro[^{3}H]hydride (Amersham) (11).

RESULTS AND DISCUSSION

Synchronization of Cultures and Collection of Merozoites. Highly synchronized cultures were essential for the characterization of stage-specific constituents, especially those associated with merozoites. The longevity of extracellular merozoites is quite limited; adequate numbers of nondegraded merozoites could be collected only when large numbers

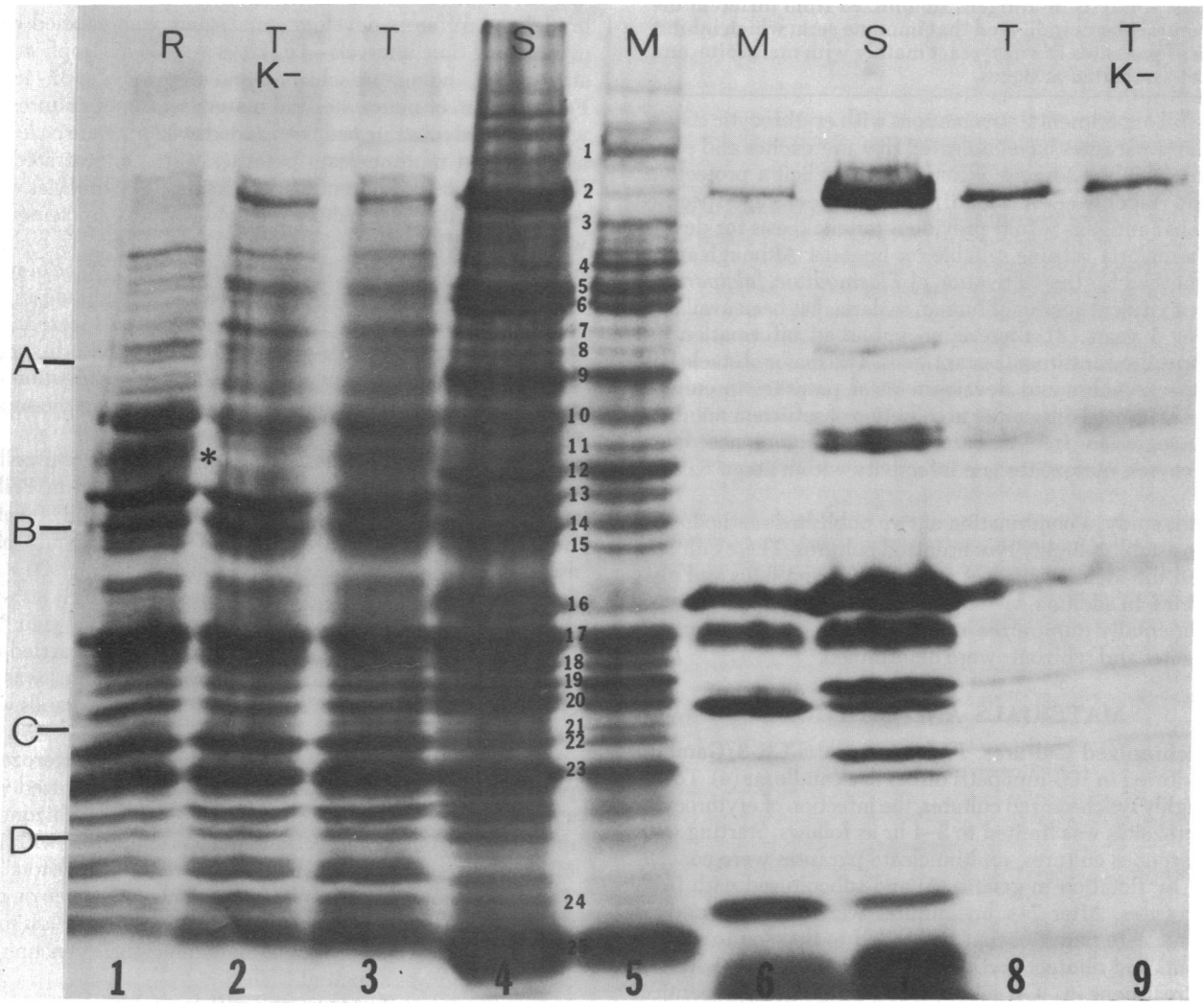

FIG. 1. Fluorogram of stage-specific proteins and glycoproteins of *P. falciparum* analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes 1–5 were labeled with [^{3}H]proline; lanes 6–9 were labeled with [^{3}H]glucosamine. R, ring; T, trophozoite; S, mature schizont; M, merozoite; K−, laboratory-derived strain which does not synthesize the knob protein. Letters at left indicate molecular weight marker proteins: A, β-galactosidase (130,000); B, bovine serum albumin (68,000); C, ovalbumin (45,000); D, chymotrypsinogen (25,000). Numbers show selected components discussed in text; asterisk marks knob protein.

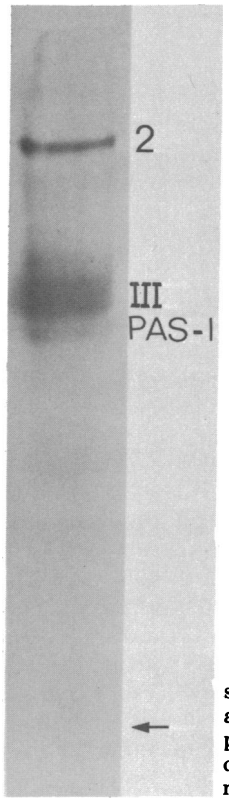

FIG. 2. Fluorogram of surface-labeled schizonts analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. 2, glycoprotein 2 shown in Fig. 1; III and PAS-1, components of normal erythrocyte membrane; arrow, gel front.

emerged from erythrocytes within a short interval of time. Two published procedures allow the separation of uninucleate and multinucleate parasites: sorbitol selectively destroys multinucleate parasites (6); treatment with gelatin (5) sediments normal erythrocytes as well as those infected with rings, leaving multinucleate parasites in the supernatant. Although each one of these procedures synchronizes cultures somewhat as judged by morphology, resulting parasites could be distributed over a developmental period of 20–22 hr. Through the combined use of these two procedures it was possible to limit the period of infection and therefore the developmental age difference could be narrowed to 3–4 hr. This procedure of synchronization is only applicable to parasites that have not undergone alteration due to maintenance in culture; flotation of infected erythrocytes in gelatin is dependent on the presence of protrusions, called knobs, on the surface membrane of the erythrocyte (12). In *in vitro* cultures, parasites eventually lose the ability to synthesize the knob protein, giving rise to infected erythrocytes with smooth membranes (K− strain) (7). Under the synchronized culture conditions, parasites appear as rings for 24–25 hr; by hr 28–30, all parasites appear as uninucleate trophozoites, and nuclear division starts soon thereafter. By hr 38–40, all parasites are multinucleate and within the last 8 hr of development, cytoplasmic division becomes apparent.

Previous studies had indicated a favorable effect of erythrocyte extract on the maintenance of structural integrity of extracellular merozoites of *Plasmodium lophurea* (unpublished data); therefore, media used for collection of merozoites were supplemented with 10% erythrocyte extract. Because most normal erythrocytes had been eliminated from schizonts during concentration by gelatin, emerging merozoites accumulated in the culture medium rather than in erythrocytes. Merozoite samples that were collected by differential centrifugation showed a few schizonts, normal erythrocyte ghosts, and membrane fragments. Even though they could be further purified by the use of antierythrocyte serum or centrifugation through Ficoll/Paque, for the purposes of this comparative study it was more important to avoid degradation through lengthy manipulations.

Stage-Specific Proteins and Glycoproteins. Although the electrophoretic pattern of stained proteins showed some distinct stage-related differences (not shown), it was not possible to distinguish parasite and host cell components. Because mature erythrocytes do not synthesize proteins, all metabolically labeled components have to originate from the parasite. The fluorogram of analyzed samples is shown in Fig. 1. Lanes 1–5 were labeled with proline, and lanes 6–9 were labeled with glucosamine. Lanes 2 and 9 were trophozoites of the laboratory derived K− strain and were included as controls for determining whether the knob protein is glycosylated. With the exception of merozoites, all other developmental stages represent equal numbers of parasites. For convenience of discussion, selected bands have been numbered; the asterisk marks the knob protein. Proteins of rings and trophozoites (lanes 1 and 3) show very few differences; the quantitative increase in some bands (e.g., bands 2 and 5) reflects the beginnings of schizont-associated components. As shown previously (7), the only apparent difference in K− strain trophozoites is the lack of the knob protein (compare lanes 2 and 3). Preliminary experiments with short intervals of labeling indicated that most of the characteristic proteins associated with merozoites were synthesized throughout schizogony; the long periods for metabolic labeling were selected to maximize merozoite-related differences even though a few other differences between younger developmental stages became masked. For example, short-interval labeling showed a decline in the synthesis of band 13 and gradual increase in band 14 during development from rings to schizonts.

Although the main developmental change between rings and trophozoites is in shape and size of the parasite, during schizogony several new morphological features that are unique to merozoites become apparent: the polar organelles, the pellicular complex, and the surface coat of merozoites. Comparison of proteins of trophozoites and mature schizonts (lanes 3 and 4) shows several obvious differences; bands 1–6, 9, 12, 16, 17, 20, and 25 appear to be augmented or unique to the latter. Gross differences would not be expected between merozoites and mature schizonts because the only additional elements that schizonts contain, besides merozoites, are remnants of the parasitophorous vacuole membrane, residual bodies, and the host erythrocyte membrane. The most obviously decreased protein in merozoite-enriched preparations, compared to mature schizonts, was band 2 (lanes 4 and 5). When a schizont-enriched sample was surface-labeled, in addition to the main band III and PAS-I components that get labeled in normal erythrocytes (11), a strongly labeled band with the mobility of metabolically labeled band 2 was apparent (Fig. 2). Therefore, this parasite constituent is exported to the surface of the host erythrocyte membrane; the possibility of its association also with the parasitophorous vacuole membrane cannot be ruled out. The small amount of band 2 in merozoites could originate from few contaminating mature schizonts or membrane fragments. With band 2 as a reference for possible contaminants, it becomes apparent that bands 1, 3–6, 9, 12, and possibly 18 and 21 are merozoite-associated proteins. Because the fluorogram in Fig. 1 has been overexposed to visualize minor components, some resolution has been lost. In better resolved autoradiograms, several broad bands apparent in Fig. 1 resolve into multiple components; for example, each of bands 9, 10, 17, and 25 represents two components.

In an initial study using [U-^{14}C]glucosamine, glycosylated

components of only schizonts and merozoites could be visualized by autoradiography. Repetition of the experiment with a 500-fold increase in the specific activity of substrate did not show detectable levels of radioactivity in rings (not included in Fig. 1); this indicates the lack of any contribution from contaminating host cell constituents to the radioactive components. Glycosylation becomes apparent in trophozoites and increases during schizogony (Fig. 1, lanes 6–9). Of 12 glycosylated bands observed in mature schizonts, only 5 appeared to be associated with merozoites: 11, 16, 17, 20, and 24 (compare lanes 6 and 7). The results clearly indicate that band 2, which was shown to be associated with the host erythrocyte surface, is a glycoprotein. Some of the bands that showed little difference in molecular weight, such as the doublets at 11, 20, and 23, could reprresent precursor and product. The low molecular weight bands (24 and at gel front) could be glycolipids rather than glycoproteins. The doublets at 11 were of interest because their mobility was similar to that of the knob protein. Because they were present in identical proportions in K− trophozoites and in the parent strain (lanes 8 and 9), they could not be associated with the knob protein. Band 11, apparent in schizonts and merozoites (lanes 4 and 5), is most likely a glycoprotein rather than the knob protein. Even though schizonts have knobs, the protein is synthesized mainly during development of young stages (lane 1 and 3); therefore, addition of labeled substrate during schizogony would not result in labeled knob protein. This has been shown previously (7).

Identification of Antigens. Despite the massive literature on immunology of malaria, several aspects of acquired immunity remain unknown. Circulating antibodies have been shown to react with surfaces of merozoites and schizonts (13–17). After identification of schizont- and merozoite-associated proteins and glycoproteins, attempts were made to specify which of these constituents were possible candidates for functional antigens. Unlike the situation in experimental models of malaria, there are no reference sera available from humans who have acquired immunity through vaccination with erythrocytic parasites. Therefore, evaluation has to be based on sera collected from humans living in endemic areas. The recent discovery that rabbit serum supports the growth of *P. falciparum in vitro** has provided an additional tool for evaluating various antigens, although it could be argued that the immune response of a rabbit could be quite different from that of humans.

In vitro inhibition of growth, as well as reactivity with surface of merozoites, was observed with a human serum (HA) from a Nigerian and two experimentally immunized rabbits (RA and RB). HA, RA, and RB showed 45%, 40%, and 18% inhibition of parasite growth *in vitro*, respectively. It was of interest that, in addition to the surface of merozoites, all three sera reacted with the surface of residual bodies and membranous fragments (not shown). HA, RA, and RB were used for identification of antigens. In addition, a serum sample (HB) from a patient with cerebral malaria was tested to determine antigens recognized by a nonimmune host. A fluorogram of the results is shown in Fig. 3; the numbers of bands are identical to those in Fig. 1. The identity of poorly resolved bands in lane RA-m is based on a duplicate experiment; substitutions were not made to enable comparison between serum samples tested with an identical extract of antigens. Lanes RA-m, RB-m, and HB-m were from a different electrophoretic run and show slight differences in mobility of various components when compared to the other lanes.

Several factors had to be taken into consideration in interpreting the data. First, were all antigens solubilized by the extraction procedure? Based on radioactivity, 80–85% of labeled

* Sax, L. J., Mrema, J. E., Campbell, G. H. & Rieckman, K. H. (1978) The American Society of Parasitologists, 53rd Annual Meeting, 95 (abstr.).

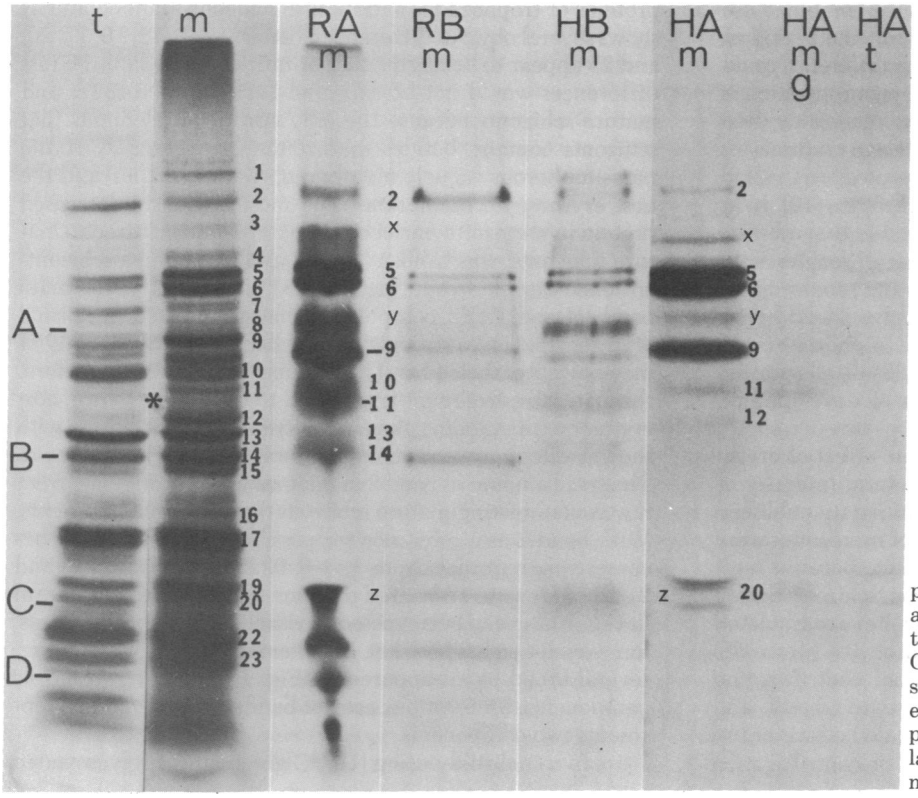

FIG. 3. Fluorogram of immunocomplexes analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. t, NP-40 extract of trophozoites; m, NP-40 extract of merozoites. Capital letters identify rabbit and human serum samples described in text. m and t extracts were from parasites labeled with proline; mg extracts were from merozoites labeled with glucosamine. Molecular weight markers are as in Fig. 1.

proteins were solubilized. Analysis of NP-40 extracts of trophozoites and a merozoite-enriched preparation (Fig. 3) indicated that most of the major proteins observed in nonextracted samples (Fig. 1) were present in extracts, with the exception of the knob protein (asterisk). This was anticipated in view of similarity of the amino acid composition of the knob protein with an unusual histidine-rich protein (unpublished data) which was characterized from *P. lophurae* (18). The histidine-rich protein of *P. lophurea* is not soluble in non-ionic detergents. A second factor for consideration is possible degradation of antigens during extraction; the absence of accumulation of small molecular weight components at the gel front indicates lack of extensive proteolysis.

Surprisingly, there were more quantitative rather than qualitative differences between the various serum samples tested. The anti-merozoite rabbit serum reacted with all antigens recognized by HA and with a few additional low molecular weight components (Fig. 3, RA-m and HA-m). This implies that antisera prepared in rabbits could be substituted for scarce immune human serum in purification of antigens. The reduction in quantity of antigens bound by RB (Fig. 3, RB-m) correlates well with the lesser extent of inhibition of growth of parasites observed with this antiserum. Rabbit B had been immunized with a crude membrane-enriched fraction, and membrane fractions have been shown to be poorer than merozoites as a source of functional antigens (19). The reactivity of RB with merozoite surface antigens, detected by immunoelectron microscopy, had not been anticipated. This could be due to possible contamination of the membrane preparation with merozoite surface constituents or to common antigens on merozoites and membranes of schizonts. Residual bodies, which were obvious contaminants of membrane preparations, could also have antigens common to surface of merozoites. Despite the crossreactivity shown by the two antisera from rabbits, some deductions could be made; band 2 and band 14 were proportionately greater in RB-m than in RA-m when compared to major antigens such as 5, 6, or 9. Similarly, when HA was tested with extracts of schizonts, a distinct relative increase in antigens 2 and 14 were observed (not shown). These results suggest that, similar to antigen 2, antigen 14 is also associated with membranes of schizonts.

Due to the limited amount of human serum available from the nonprotected patient, HB was not tested for *in vitro* inhibition of parasite growth or reactivity with merozoite surface antigens. HB-m showed mainly gross quantitative differences from HA-m; the fluorogram was exposed for twice as long as all other samples to visualize the radioactivity.

Extracts of metabolically labeled rings did not show any detectable antigens when tested with HA. Equal numbers of identical culture plates were used for preparation of NP-40 extracts from each developmental stage. It is of interest that the only detectable antigens in trophozoites were those merozoite and schizont components whose synthesis was apparent in labeled trophozoites (Fig. 3, HA-t).

The results of stage-specific constituents (Fig. 1) had indicated that proteins 1, 3–6, 9, and 12 as well as glycoproteins 11, 16, 17, and 20 are merozoite-associated. Therefore, all the numbered antigens that reacted with HA are merozoite-specific or associated with membranes of schizonts (Fig. 3, HA-m). Bands x, y, and complex z could not be identified with certainty; x ran between bands 3 and 4, and y ran between bands 7 and 8. The bands at z were always distorted because they ran just below the large peak of serum globulin apparent in stained gels. Extracts of merozoites that had been labeled with glucosamine assisted in the identification of one of the components at z as glycoprotein 20; the only other glycosylated antigen was glycoprotein 11 (Fig. 3, HA-mg).

Use of control rabbit and human sera indicated the absence of nonspecifically bound antigens. The failure to detect minor antigenic components, because of limitations of the resolution of the method used or dissociation of antigens with low affinity for the antibodies during the washing of immunocomplexes, cannot be ruled out.

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