TWO *PLASMODIUM KNOWLESI*-SPECIFIC ANTIGENS ON THE SURFACE OF SCHIZONT-INFECTED RHESUS MONKEY ERYTHROCYTES INDUCE ANTIBODY PRODUCTION IN IMMUNE HOSTS*

By RUPERT SCHMIDT-ULLRICH, DONALD F. H. WALLACH, and JOHN LIGHTHOLDER

From Tufts-New England Medical Center, Department of Therapeutic Radiology, Radiobiology Division, Boston, Massachusetts 02111

The work of several investigators has established the fact that intraerythrocytic maturation of several types of *Plasmodia* causes the appearance of new antigens on the surfaces of infected erythrocytes. Thus, Brown et al. (1, 2) have demonstrated that rhesus monkey erythrocytes infected by *Plasmodium knowlesi,* are agglutinated by serum from immune animals; this agglutinability appears to be specific for the strains and variants of *P. knowlesi* involved, but does not necessarily correlate with protective immunity of the host (3–5).

Immunoelectron microscopy has also demonstrated the appearance of parasite-specific antigens on the surfaces of erythrocytes infected with *P. falciparum* or *P. coatneyi* (6) and these studies, as well as work on erythrocytes infected with *P. knowlesi* (7) or other plasmodial species (8, 9), suggest that the antigenic modifications involve localized ultrastructural changes in the host cell membrane. These observations, together with the information that at least partial protective immunity can be obtained by immunizing susceptible animals with various asexual forms of plasmodia (4, 5), have generated explorations into the molecular properties of parasite-induced host-cell antigens (7, 9–12).

In previous publications we have described methods for the efficient separation of host-cell membranes from *P. knowlesi*-infected rhesus erythrocytes (10, 12) and biochemical anomalies in the membrane proteins of the host erythrocytes (10, 12). Further, using a hyperimmune monkey serum that was produced in response to purified schizonts injected with complete Freund's adjuvant, we showed that *P. knowlesi*-infected erythrocytes bear at least three parasite-induced plasma membrane antigens, as defined by crossed immune electrophoresis, corresponding to membrane neoproteins in the 55,000–90,000 daltons, pI 4.5–5.2 region (12).

In another study, *P. knowlesi*-infected rhesus monkey erythrocytes were enriched for different blood stages (11) and analyzed by crossed immune electrophoresis using

* Dedicated to Professor Herbert Fischer, Max-Planck Institute for Immunology, Freiburg, Germany, F. R., on the occasion of his 60th birthday. Supported by the Malaria component of the United Nations Development Programme/World bank/World Health Organization Special Programme for Research and training in Tropical Diseases; MAL-T16/181/MZ/9 to R. Schmidt-Ullrich; and from the U. S. Army contract 17-74-C-4118 to D. F. H. Wallach.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/79/07/0086/14$1.00
Volume 150 July 1979 86–99

pooled, monkey anti-*P. knowlesi* merozoite serum. Of 11 immune precipitates, two appeared stage specific and four were considered to be expressed on the surfaces of infected rhesus monkey erythrocytes, by the criterion of lactoperoxidase-catalyzed radioiodination of intact erythrocytes (13). The techniques used in (11, 13) suggested that all antigens detected on the surfaces of intact erythrocytes were also expressed in the merozoites used to produce the reagent antisera.

The approaches used in (4, 5, 11, 12) indicate that immunization with purified schizonts or isolated merozoites can yield protective immunity, but they do not provide information whether one or more of the *P. knowlesi*-induced erythrocyte proteins can generate this protective immunity. We have therefore used crossed immune electrophoresis to compare antigens detected by antisera raised against purified schizonts injected in complete Freund's adjuvant, vs. antigens detected by sera from monkeys rendered immune by infection with *P. knowlesi* and three subsequent challenges with infected erythrocytes. In this we have further utilized surface radioiodination of parasitized erythrocytes and absorption of sera by such cells. We find that two out of >20 antigens are immunogenic during the course of natural infection and that these two antigens are prominently expressed on the host-cell membrane.

Materials and Methods

Chemicals. Reagents used were of highest purity available. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N'N'*-tetramethylethylene diamine, ammonium persulfate, and Coomassie Brilliant Blue were purchased from Bio-Rad Laboratories (Richmond, Calif.), dithiothreitol (DTT),[1] Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dextran (170,000 daltons, molecular mass) from Sigma Chemical Co. (St. Louis, Mo.), and dodecylsulfate from Fisher Chemical Corp. (Fair Lawn, N. J.). Ficoll from Pharmacia (Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) was extensively dialyzed against deionized water, lyophilized, and used as a 9% (wt/vol) solution in water. Lactoperoxidase from Boehringer (Mannheim Biochemicals, Indianapolis, Ind.) in 3.2 M $(NH_4)_2SO_4$ was freed of ammonium sulfate by dialysis against the buffer used for labeling. Hypaque (sodium diatrizoate, 50%) and chloroquine (Aralene) hydrochloride were obtained from Winthrop Laboratories (N. Y.), agarose (lot AGS 222) from Litex (2600 Glostrup, Denmark), rabbit IgG against rhesus monkey IgG from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.), Freund's complete adjuvant from Difco Laboratories (Detroit, Mich.) and Cowan I strain of *Staphylococcus aureus* (10% suspension) was from the Tufts-Enzyme Center (Boston, Mass.). Carrier-free $Na^{125}I$ (17 Ci/mg) in 0.1 M NaOH from New England Nuclear (Boston, Mass.), was neutralized with an equimolar quantity of 0.1 M HCl before use.

Monkeys. Rhesus monkeys (*Macaca mulatta*), weighing 4–8 kg, obtained from the Primate Imports Company (Port Washington, N. Y.), were first infected after a 4-wk quarantine period (Tb negative). Cure was by an initial intramuscular injection of 20 mg of chloroquine/kg, followed by two daily injections of 10 mg/kg. About 3 wk after the first infection the animals were splenectomized; after 1–2 wk of convalescence, the animals could be reinfected at 6-wk intervals for production of schizont-infected erythrocytes.

Parasites. *P. knowlesi*-infected rhesus erythrocytes were obtained as described (10, 12). Infection of each animal was initiated by an intravenous injection of 0.75–1.5 ml of a buffered erythrocyte suspension (20% parasitemia) that had been stored at −70°C. Microscopic evaluation of parasitemia was as in references 10 and 12.

Two strains of *P. knowlesi*, the Malaysian and Philippine strain (kindly provided by R.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CIE, crossed immune electrophoresis; DS, dodecyl sulfate; DTT, dithiothreitol; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

Gwadz and L. H. Miller, Malaria Section, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) were employed for experiments detailed below.

Subcellular Fractionation of Schizont-Infected Erythrocytes. At 15–30% parasitemia (schizonts with 6–10 nuclei) 20–30 ml of blood was drawn (10 U of heparin/ml). Infected erythrocytes were purified by two sequential Ficoll-Hypaque gradients of densities 1.085 and 1.080 to remove uninfected erythrocytes, leukocytes, and thrombocytes (10, 12). Disruption of purified parasitized erythrocytes by nitrogen decompression and isolation of schizonts and erythrocyte membranes were as described in references 10 and 12.

Antisera. For immunochemical analyses, two types of sera were raised in rhesus monkeys. For the anti-schizont serum two monkeys were immunized with 10^8 schizonts (*P. knowlesi*, Malaysian strain) in Freund's complete adjuvant (0.5-ml total volume) and at least three sequential boosters of 10^8 schizonts in Freund's complete adjuvant at monthly intervals. The antigen was injected subcutaneously at the back at multiple sites to prevent formation of skin ulcers. Sera were obtained 10 d after the third and any sequential booster.

The second immunochemical reagent employed was serum from two rhesus monkeys rendered naturally immune during the first infection. The animals were challenged thrice at 3-week intervals using erythrocytes infected with the Malaysian strain of *P. knowlesi*. This antiserum is referred to as a natural hyperimmune serum.

For immunochemical analyses immunoglobulin (Ig) fractions were obtained from all the sera by precipitation with $(NH_4)_2SO_4$ (30% wt/vol; 14). The Ig fractions were concentrated fivefold relative to the serum.

Absorption of Antisera. Ig from anti-schizont antisera was absorbed with purified intact schizont-infected erythrocytes. For this, 10^9 erythrocytes were incubated with 0.2 ml of the concentrated Ig fraction at 20 and 0°C for 30 min each time. Less than 5% of the infected erythrocytes lysed during absorption, as determined by hemoglobin content of the serum in comparison to total hemoglobin released from the cells after lysis in H_2O.

Iodination of Schizont-Infected Erythrocytes. Purified parasitized erythrocytes were suspended to a cell concentration of 10^9/ml in Dulbecco's phosphate-buffered saline (PBS), 10^{-6} M NaI, and 0.001 mg/ml butylated hydroxytoluene. Iodination was in 1-ml aliquots at 20°C (10, 15). 30 μCi of $Na^{125}I$ and 0.2 mg of lactoperoxidase (1 mg/ml) was added to each tube. The iodination was initiated by addition of 40 μl of 0.002% H_2O_2 followed by four 20-μl aliquots at 60-s intervals. The iodination was quenched by two washes in ice-cold PBS, containing BSA, NaI, and glucose (1%, wt/vol, 5 mM, 10 mM) and one wash in PBS, containing 10 mM glucose.

Iodination of the proteins of isolated membranes from infected erythrocytes was performed on unsealed ghosts, prepared as in reference 10, at a protein concentration of 2 mg/ml. The buffer throughout the system was 5 mM PO_4, pH 8.0. The concentrations of the other reagents and the labeling conditions were as for infected erythrocytes, as was the washing except that the membrane fragments were pelleted at 10^7 g × min.

Coprecipitation of ^{125}I-Labeled Membrane Proteins of Parasitized Erythrocytes. Membranes from iodinated, infected erythrocytes and ^{125}I-labeled isolated membranes were used as antigens for coprecipitation. 200 μg of each antigen were first incubated with 20 μl of concentrated Ig from monkey natural hyperimmune serum in a total volume of 0.1 ml PBS, 2 mM in PMSF. As a control reagent, we used concentrated Ig from rhesus monkeys never exposed to *P. knowlesi* parasites. The reaction was at 20°C for 90 min. In the secondary reaction, the antigen-antibody complexes were precipitated by addition of 100 μl of rabbit Ig against monkey immunoglobulin or 50 μl of a 10% *S. aureus* suspension. After incubation at 20°C for 90 min, the immune aggregates were pelleted at 10^5 g × min, washed twice in PBS, and dissolved in dodecyl sulfate (DS) and DTT (1%/40 mM). *S. aureus* was removed by centrifugation at 10^5 g × min and the supernate subjected to DS-polyacrylamide gel electrophoresis (PAGE) (16). The ^{125}I-radioactivity was monitored after fractionation of gels into 1-mm slices.

Protein Fractionation. Proteins/glycoproteins of purified schizonts and membranes of infected erythrocytes were dissolved in DS/DTT or Triton X-100 as in (10, 12). The antigen was quantified by protein estimation using the ninhydrin method (17) with BSA as standard.

Crossed immune electrophoresis (CIE) was performed as described before (12, 18). For all immunochemical assays, constant amounts of schizont protein (0.15 mg) and membrane protein of infected erythrocytes (0.25 mg) were used. The identity of the antibodies of the two types of

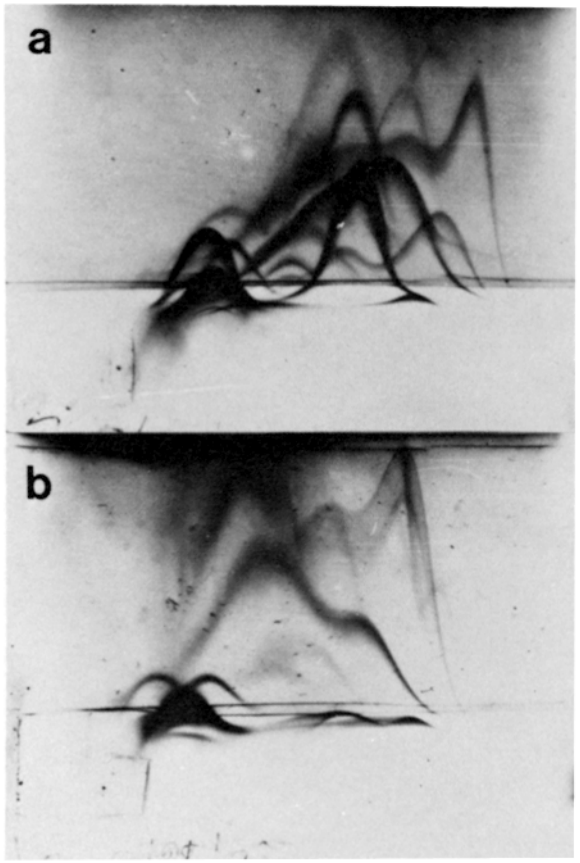

FIG. 1. Crossed immune electrophoresis of proteins from (a) purified *P. knowlesi* schizonts and (b) membranes of schizont-infected rhesus monkey erythrocytes. The Triton X-100-solubilized antigens (a) 0.15 mg of schizont protein; (b) 0.25 mg of membrane protein from infected erythrocytes were electrophoretically separated in the first dimension and then electrophoresed at right angles into agarose containing 0.025 ml monkey anti-schizont Ig per milliliter. Throughout the system, 1% agarose (wt/vol) and 1% Triton X-100 (vol/vol) were used. Coomassie Blue protein staining. The results are representative of four independent experiments.

sera employed was defined by mixing Ig fractions of both sera, and the quantitative evaluation of the heights of the precipitation arcs in comparison to control plates that contained identical amounts of antigen but only one of the sera (18).

DS-PAGE was in 7.5% polyacrylamide, cross-linked with 2.5% bisacrylamide as in (16).

Proteins/glycoproteins labeled with ^{125}I were monitored by autoradiography (Kodak Safety film NS, Eastman Kodak Co., Rochester, N. Y.) or counting of gel slices in the Autogamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) for CIE or DS-PAGE, respectively.

Results

Immune Status of the Rhesus Monkeys. Immunization and repeated boosters of rhesus monkeys with 10^8 purified schizonts in Freund's complete adjuvant resulted in a relative immunity, i.e., upon challenge with schizont-infected erythrocytes, the parasitemia rose to 1% after 8–10 days, followed by a gradual clearance of infected

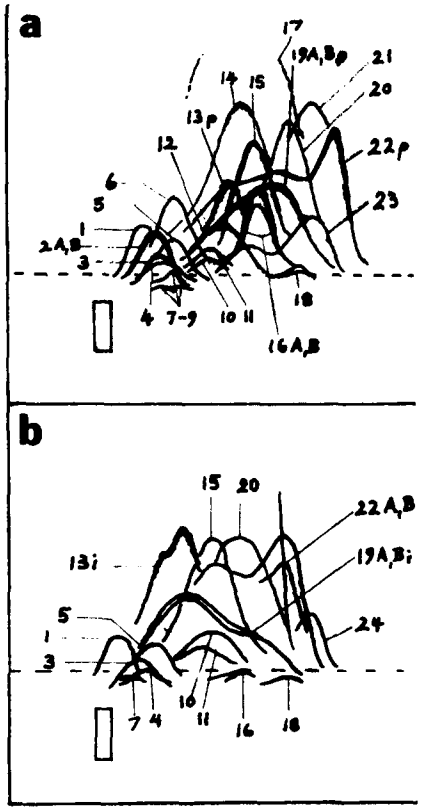

FIG. 2. Crossed immune electrophoresis of proteins from (a) purified *P. knowlesi*-schizonts and (b) membranes of infected rhesus monkey erythrocytes. A schematic depiction of results shown in Fig. 1. The precipitation arcs are numbered according to increasing relative mobility of the antigens during the first dimension electrophoresis.

erythrocytes from the blood. In two monkeys, an effective immune defense developed during the first infection after a parasitemia of 5–10% indicated by a spontaneous decrease of parasitemia to <1%. The monkeys were nevertheless treated with chloroquine after the first infection and after each challenge, which lead to a transient parasitemia never exceeding 1%.

Immunochemical Comparison between Purified Schizonts and Membranes of Schizont-Infected Erythrocytes. Anti-schizont sera of immune competent rhesus monkeys identify 25 well-defined precipitation arcs when isolated schizonts (6–10 nuclei) and membranes of infected erythrocytes are analyzed by CIE (Fig. 1a,b). The precipitates are numbered according to the increasing relative mobility of the antigens during the first dimension electrophoresis (Fig. 2a,b). As reported previously (7), there are components common to the intracellular parasites and the membranes of infected erythrocytes (components 1, 3, 4, 5, 7, 10, 11, 16, and 20), and some that are found predominantly in the parasite (components 2A,B, 6, 8, 12, 13p, 14, 16A,B, 19A,Bp, 21, 22p, and 23). However, components 13i, 19A,Bi, 22A,B, and 24 are unique to the membrane of parasitized erythrocytes. Components 2, 16, 19, and 22 represent duplicate precipitates, indicating antigenic microheterogeneity. The proportions of

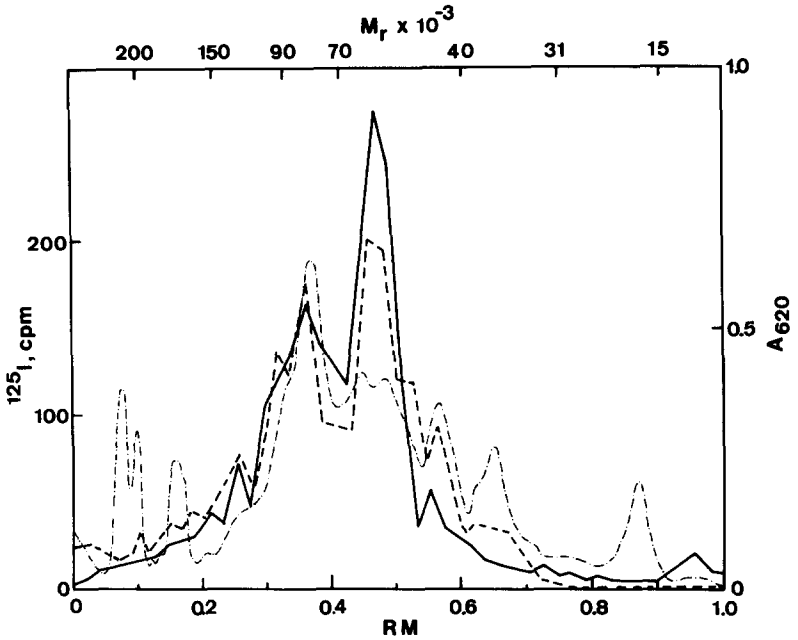

FIG. 3. DS-PAGE of membrane proteins from parasitized erythrocytes after lactoperoxidase-catalyzed surface radioiodination of intact cells. The abscissas give the relative mobility (RM) and molecular size (M_r) and the ordinates the distribution of ^{125}I (cpm) and the absorbance (A) of Coomassie Blue stained proteins at 620 nm (-•-). The ^{125}I-profiles are derived from 60 1-mm slices. The solid line gives the ^{125}I-distribution when 0.05 mg of membrane protein is fractionated; the broken line represents the proteins from 1.0 mg of iodinated membranes specifically reacted with Ig from natural hyperimmune serum and secondarily precipitated by rabbit anti-monkey IgG or *S. aureus*. The results are representative of three independent experiments.

parasite-specific antigens in the schizonts and membranes of infected erythrocytes differ markedly when the relative heights of precipitates (e.g. 1 *vs.* 3 and 4 or 10 and 11 *vs.* 15) are compared; this excludes simple cross-contamination. Component 13 is present in both the schizonts and the membranes of infected erythrocytes, but appears as a two-peak precipitate in the infected membrane (13i, Fig. 2b) with consistently lower relative mobility than in the parasite (13p, Fig. 2a).

Parasite-Specific Components Exposed on the Surface of Infected Erythrocytes. Lactoperoxidase-catalyzed radioiodination of intact infected erythrocytes yields 90–95% of the total ^{125}I covalently associated with the isolated erythrocyte membranes and <2% bound to purified schizonts. This represents an at least 1,000-fold higher specific ^{125}I activity in the infected membrane. DS-PAGE shows a localization of label predominantly of two major proteins/glycoproteins with molecular masses ≅90,000 and 65,000 daltons and two minor components in the 130,000 and 50,000 daltons molecular mass regions (Fig. 3). Autoradiograms of immunoplates show that only three components (1–4, 13, and 19) in the infected membranes are accessible to iodination (Fig. 4a,b). The autoradiograms suggest that the highest specific ^{125}I-radioactivity is in component 13, which is characteristic for the membranes of parasitized erythrocytes.

As an alternative technique to define parasite-induced antigens on the surface of infected erythrocytes, anti-schizont Ig is absorbed with intact schizont-infected eryth-

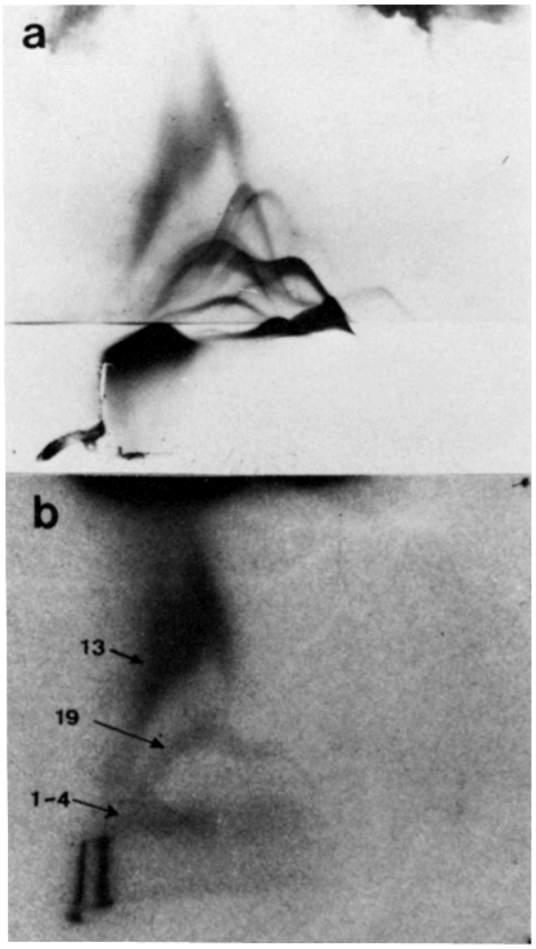

FIG. 4. Crossed immune electrophoresis of membranes from schizont-infected erythrocytes surface-labeled by lactoperoxidase-catalyzed radioiodination. About 0.25 mg of membrane protein is electrophoretically separated in the first dimension and then electrophoresed at right angles into agarose containing 0.025 ml of anti-schizont Ig/ml. Throughout the system, 1% agarose (wt/vol) and 1% Triton (vol/vol) were used. (a) Coomassie Blue protein staining; (b) autoradiograms after exposure for 6 d. Components 1–4, 13, and 19 are assigned in reference to Figs. 1 b and 2 b. The results are representative of two independent experiments.

rocytes. The immune precipitation patterns obtained with absorbed sera is demonstrated in Fig. 5 a,b. When tested against schizont proteins (for control see Fig. 1 a), there is an overall twofold increase of the height of the precipitates as a result of nonspecific adsorption of Ig to erythrocytes (components 2, 3, 4, 7, 10, 12, 16, 18, and 22, Fig. 5 a). Importantly, antibodies against components 1, 5, 13, and 19 are eliminated (Fig. 5 a,b). The elimination of antigen 1 and 13 in membrane of infected erythrocytes (for control, see Fig. 1 b) is compatible with the surface iodination data and also suggests that these components are exposed on the erythrocyte surface.

Parasite-Specific Components That Are Immunogenic during the Natural Course of Infection. The CIE patterns for schizonts and membranes of infected erythrocytes

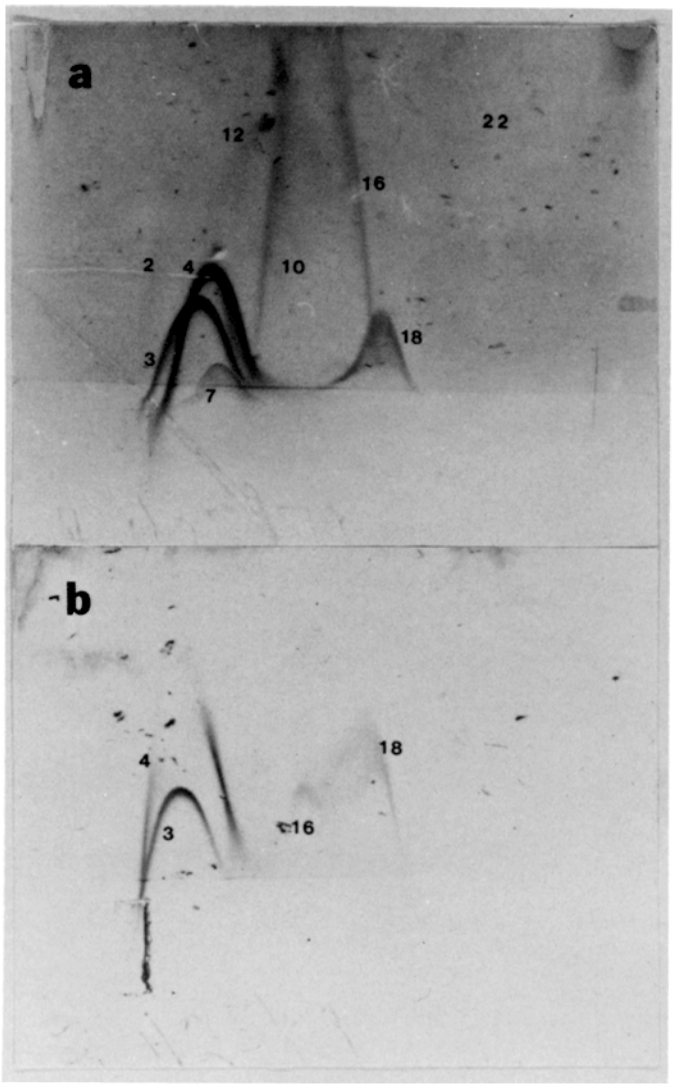

FIG. 5. Crossed immune electrophoresis of proteins from purified *P. knowlesi* schizonts and membranes of parasitized rhesus monkey erythrocytes. Triton X-100-solubilized antigens (a) 0.15 mg of schizont protein and (b) 0.25 mg of membrane protein from infected erythrocytes were electrophoretically separated in the first dimension and then electrophoresed at right angles in agarose containing 0.05 ml of absorbed monkey anti-schizont Ig per milliliter (more details are given in Materials and Methods). Throughout the system, 1% agarose (wt/vol) and 1% Triton X-100 (vol/vol) were used. Coomassie Blue protein staining. The results are representative of three independent experiments.

using natural hyperimmune serum are presented in Fig. 6a,b. These sera recognize two major and one minor component in Triton-solubilized schizonts (Fig. 6a) and two components common to infected membrane and schizonts are identical to components 1 and 13, as identified by the anti-schizont serum. This is documented in

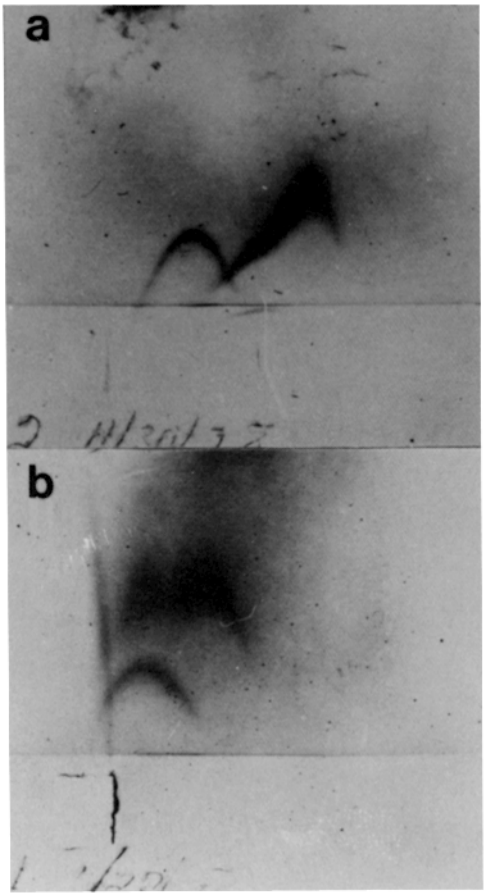

FIG. 6. Crossed immune electrophoresis of proteins from purified *P. knowlesi* schizonts and membranes of parasitized erythrocytes. Triton X-100-solubilized antigens (a) 0.15 mg of schizont protein and (b) 0.25 mg of membrane protein from infected erythrocytes were electrophoretically separated in the first dimension and then electrophoresed at right angles into agarose containing 0.025 ml of Ig from natural hyperimmune serum per milliliter. Throughout the system, 1% agarose (wt/vol) and 1% Triton X-100 (wt/vol) were used. Coomassie Blue protein staining. The results are representative of three independent experiments.

Fig. 7a,b, showing that mixing the anti-schizont and the natural immune serum produces a selective decrease in the height of precipitate 1 by some 40% and of component 13 by 70% (in comparison to the anti-schizont serum alone). The degree of suppression is expressed as the ratio of the precipitates 1 and 13 in relation to component 16, which is the most constant antigen. The natural hyperimmune serum also reveals the distinction between component 13 in schizonts vs. the membranes of infected erythrocytes. In comparison to schizonts, the component in the infected membrane has a lower electrophoretic mobility in the first dimension and appears as a two-peak component (Figs. 1 b and 4).

The molecular weights of two antigens recognized by the natural hyperimmune serum were obtained by double immune precipitation techniques. After reaction of

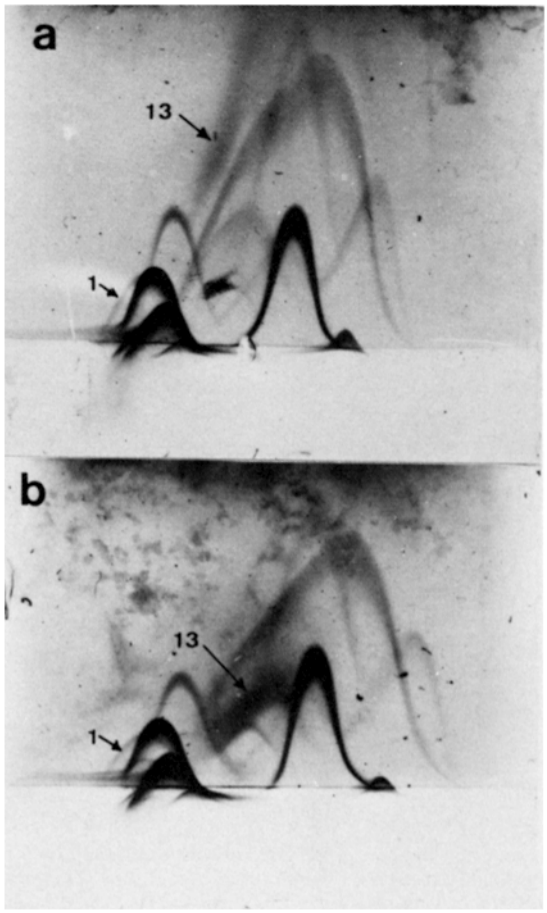

FIG. 7. Crossed immune electrophoresis of purified *P. knowlesi* schizonts. Triton X-100-solubilized antigen (0.15 mg) was electrophoretically separated in the first dimension and then at right angles electrophoresed into agarose containing (a) 0.025 ml anti-schizont Ig per milliliter and (b) 0.025 ml anti-schizont and 0.025 ml Ig from natural hyperimmune serum per milliliter. Throughout the system, 1% agarose (wt/vol) and 1% Triton X-100 (vol/vol) were used. Coomassie Blue protein staining. The results are representative of three independent experiments.

both antigen preparations with the primary natural hyperimmune Ig, 2–4% of the ^{125}I-labeled membrane protein is specifically precipitated using the secondary IgG or *S. aureus*. All precipitates analyzed by DS-PAGE (Fig. 3) yield two major peaks in the 90,000 and 65,000 D molecular mass region (although all the membrane proteins are iodinated when isolated membranes are labeled; data not shown). In comparison to the hyperimmune sera, <5% of the ^{125}I-radioactivity is precipitated when the Ig of normal monkey serum is reacted with membrane proteins of parasitized erythrocytes.

Antigenic Differences Between Different Strains of P. knowlesi. To obtain some information on antigenic differences of different strains of *P. knowlesi*, purified schizonts and membranes of parasitized erythrocytes of both the Malaysian and the Philippine strain are analyzed using the anti-schizont antiserum and the natural hyperimmune

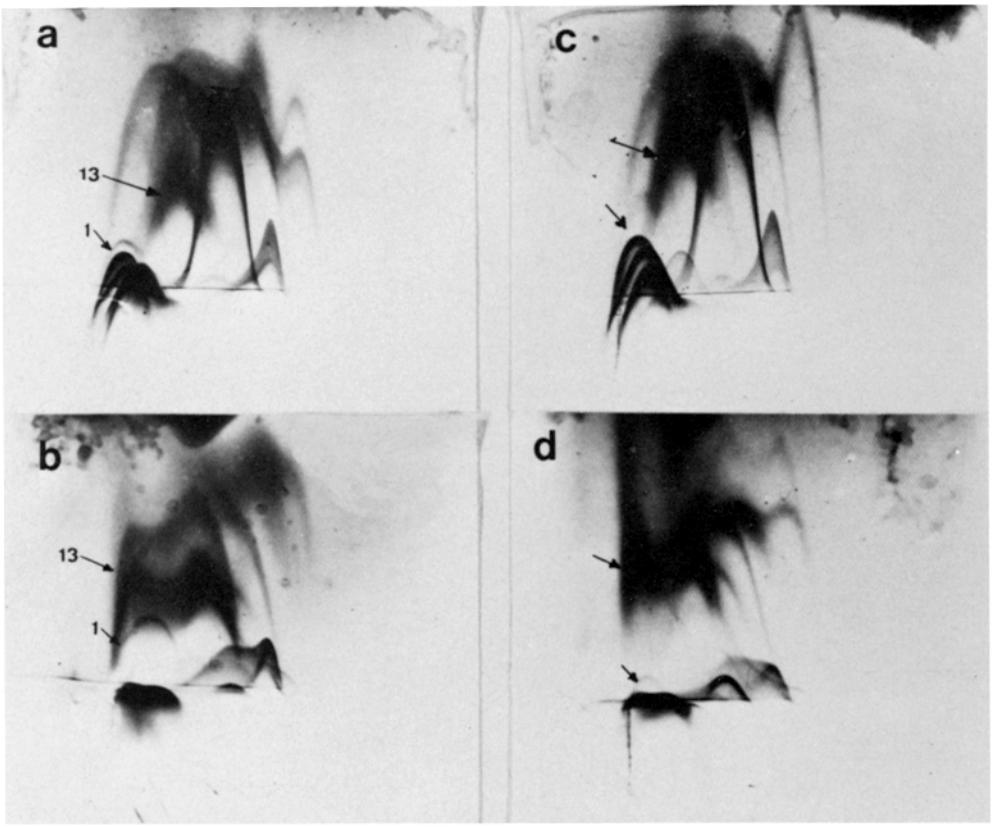

FIG. 8. Crossed immune electrophoresis of purified schizonts and membranes of parasitized erythrocytes from the Malaysian and Phillippine strains of *P. knowlesi*. Triton X-100-solubilized antigens (a and c) 0.15 mg of schizont protein and (b and d) 0.25 mg of membrane protein from infected erythrocytes were electrophoretically separated in the first dimension and then electrophoresed against anti-schizont Ig (Malaysian strain; 0.025 ml/ml agarose). Throughout the system, 1% agarose (wt/vol) and 1% Triton X-100 (vol/vol) were used. Arrows point to components 1 and 13. Coomassie Blue protein staining. The results are representative of three independent experiments.

sera (Figs. 8 and 9). Despite some minor quantitative differences in components 19 and 22, components 1 and 13 are qualitatively and quantitatively identical in both parasites and infected membranes, irrespective of the plasmodium strain analyzed (Fig. 8a–d). The same conclusions have to be drawn from the results shown in Fig. 9a,b, in which schizont proteins of the Malaysian (Fig. 9a) and the Philippine strain (Fig. 9b) are electrophoresed against natural immune serum of rhesus monkeys immune against the Malaysian strain of *P. knowlesi*. Component 1 is prominent immediately after the last challenge, subsequently declines relative to component 13 and is not detectable after 6–8 wk.

Discussion

Crossed immune electrophoresis using antisera raised in rhesus monkeys by immunization with purified *P. knowlesi* schizonts, reveals >20 immune precipitates in

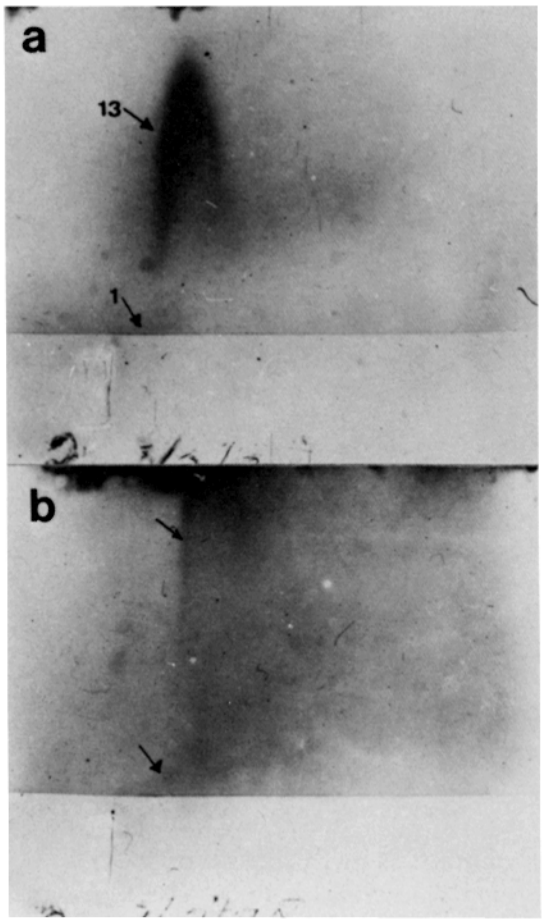

FIG. 9. Crossed immune electrophoresis of purified schizonts from the Malaysian and Philippine strain of *P. knowlesi.* Triton X-100-solubilized antigens (0.15 mg of schizont protein) were electrophoretically separated in the first dimension and then electrophoresed at right angles into agarose containing 0.025 ml of Ig/ml from natural hyperimmune serum. Throughout the system, 1% agarose and 1% Triton X-100 were used. Coomassie Blue protein staining. The results are representative of two independent components.

Triton-solubilized schizonts and at least seven parasite-specific antigens in the membranes of infected erythrocytes. In contrast, the sera of monkeys rendered naturally immune by infection with *P. knowlesi,* yield only two immune precipitates—against components 1 and 13—identified by the anti-schizont sera. Importantly, after infected cells are surface-labeled by lactoperoxidase-catalyzed radioiodination before membrane immunochemical analysis, only components 1, 13, and 19 are found to contain label, indicating that these components represent parasite-specific host cell membrane proteins that are exposed at the surface of the infected cells.

In our limited experience with two naturally immune monkeys, precipitating antibodies against these two components coincide with protective immunity of the host when challenged with the same (Malaysian) strain of *P. knowlesi.* However, the

titer against component 1 declines after antigenic challenge, whereas that against component 13 does not, (2 mo after the last challenge component 1 is not precipitated) and the animals remain immune. We therefore suggest that component 13, a 65,000 or 90,000 D protein may be the antigen responsible for the observed protective immunity. In the same molecular mass regions we find parasite-specific proteins in the membranes of infected erythrocytes (12) and metabolic incorporation of both ^{14}C-amino acids and [^{14}C]glucosamine indicating the parasite origin of these proteins/glycoproteins (Schmidt-Ullrich et al., unpublished observations).

Schizonts and host-cell membranes from rhesus erythrocytes infected with the Philippine strain of *P. knowlesi* give the same immune precipitates, on crossed immune electrophoresis against anti-schizont serum or natural immune serum, against the Malaysian strain of *P. knowlesi.* In particular, no qualitative or quantitative differences are observed in components 1 or 13. Thus, these membrane proteins do not appear to be strain specific. Moreover, because only component 13 appears to correlate with protective immunity, the possibility exists that this protein might induce protective immunity against all strains of *P. knowlesi.*

Earlier experiments by Miller et al. (5) and contradictory data (1–4) on immunity vs. agglutination titer for parasitized erythrocytes early during the course of infection suggest that antibodies circulating in immune monkeys differ in antigen specificity from those responsible for agglutination of parasitized erythrocytes. The latter exhibit a high strain and even variant specificity, while antibodies yielding a protective immunity may have different specificities and may belong to a different group of immunoglobulins.

Summary

Purified schizonts (6–10 nuclei) and membranes of schizont-infected erythrocytes from the Malaysian and Philippine strain of *Plasmodium knowlesi* are analyzed immunochemically using immunoglobulin of rhesus monkey hyperimmune sera against schizonts and of sera from naturally immune monkeys. The anti-schizont Ig identifies >20 immune components in Triton X-100-solubilized schizonts and membranes of infected cells. Of these antigens, 9 (component 1, 3, 4, 5, 6, 10, 11, 18, and 20) are common to parasites and membranes of infected erythrocytes, and 12 (2A,B, 6, 8, 9, 12, 13p, 14, 16A,B, 19 A,Bp, 21, 22p, and 23) are predominantly found in the parasite; 4 components (13i, 19A,Bi, 22A, B, and 24) are unique to the membrane of infected erythrocytes. Only three parasite-specific components (1, 13, and 19) are exposed on the surface of parasitized erythrocytes as revealed by both lactoperoxidase-catalyzed radioiodination and extensive absorption of anti-schizont Ig using intact infected erythrocytes. Two plasmodium-specific antigens (1 and 13) on the surface of infected erythrocytes are recognized by sera of rhesus monkeys rendered naturally immune against *P. knowlesi* infections and, therefore, represent antigens in vivo.

Analyses of schizonts and membranes of parasitized erythrocytes of the two different strains of *P. knowlesi* yields only some minor quantitative, but no qualitative differences when analyzed with both types of antisera. Importantly, components 1 and 13 appear identical in both strains.

Received for publication 22 February 1979

References

1. Brown, K. N., and I. N. Brown. 1965. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature (Lond.)*. **208:**1286.
2. Brown, I. N., K. N. Brown, and L. A. Hills. 1968. Immunity to malaria: the antibody response to antigenic variation by *Plasmodium knowlesi*. *Immunology*. **4:**127.
3. Butcher, G. A., and S. Cohen. 1972. Antigenic variation and protective immunity in *Plasmodium knowlesi* Malaria. *Immunology*. **23:**503.
4. Mitchell, G. H., G. A. Butcher, and S. Cohen. 1975. Merozoite vaccination against *Plasmodium knowlesi* Malaria. *Immunology*. **29:**397.
5. Miller, L. H., K. G. Powers, and T. Shiroishi. 1977. *Plasmodium knowlesi*: functional immunity and antimerozoite antibodies in rhesus monkeys after repeated infection. *Exp. Parasitol.* **41:**105.
6. Kilejian, A., A. Abati, and W. Trager. 1977. *Plasmodium falciparum* and *Plasmodium coatneyi*: immunogenicity of "knob-like protrusions" on infected erythrocyte membranes. *Exp. Parasitol.* **42:**157.
7. Miller, L. H., H. N. Fermount, and S. A. Luse. 1971. Deep vascular schizogony of *Plasmodium knowlesi* in *Macacca mulatta*. Distribution in organs and ultrastructure of parasitized red cells. *Am. J. Trop. Med. Hyg.* **20:**816.
8. Luse, S. A., and L. H. Miller. 1971. *Plasmodium falciparum* malaria. Ultrastructure of parasitized erythrocytes in cardiac vessels. *Am. J. Trop. Med. Hyg.* **20:**655.
9. Aikawa, M., C-L. Hsieh, and L. H. Miller. 1977. Ultrastructural changes of the erythrocyte membrane in Ovale-type malarial parasites. *J. Parasitol.* **63:**152.
10. Wallach, D. F. H., and M. Conley. 1977. Altered membrane proteins of monkey erythrocytes infected with simian malaria. *J. Mol. Med.* **2:**119.
11. Deans, J. A., E. D. Dennis, and S. Cohen. 1978. Antigenic analysis of sequential erythrocytic stages of *Plasmodium knowlesi*. *Parasitology*. **77:**333.
12. Schmidt-Ullrich, R., and D. F. H. Wallach. 1978. *Plasmodium knowlesi*-induced antigens in membranes of parasitized rhesus monkey erythrocytes. *Proc. Natl. Acad. Sci. U.S.A.* **75:**4949.
13. Deans, J. A., and S. Cohen. 1979. Localization and chemical characterization of *Plasmodium knowlesi*. *Bull. W. H. O.* **59**.
14. J. L. Fahey. 1967. Chromatographic separation of immunoglobulins. *Methods Immunol. Immunochem.* **1:**321.
15. Phillips, D. R., and M. Morrison. 1971. Exposed proteins on the intact human erythrocyte. *Biochemistry*. **10:**1766.
16. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10:**2606.
17. Moore, S., and W. G. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* **176:**367.
18. Bjerrum, O. J., and T. C. Bog-Hansen. 1976. Immunochemical gel precipitation techniques for analysis of membrane proteins. *In* Biochemical Analyses of Membranes. A. H. Maddy, editor. London, Chapman, and Hall, John Wiley & Sons, Inc., New York. 378.