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Plasmodium knowlesi-induced antigens in membranes of parasitized rhesus monkey erythrocytes

(two-dimensional isoelectric focusing/immunity against malaria)

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ABSTRACT **Highly purified *Plasmodium knowlesi* schizonts were used to produce a hyperimmune anti-parasite serum in a rhesus monkey. Proteins of membranes from normal and *P. knowlesi*-infected erythrocytes, as well as purified schizonts, were solubilized in 1% Triton X-100 and analyzed by bidimensional electrophoretic techniques. Of seven parasite-specific antigens identified in membranes of parasitized erythrocytes by crossed immune electrophoresis against monkey anti-parasite serum, only three could be detected in the purified schizonts. Bidimensional focusing-dodecyl sulfate/polyacrylamide gel electrophoresis of membranes from parasitized cells revealed three proteins, in the 55,000–90,000 molecular weight region, with isoelectric points between pH 4.5 and pH 5.2, that could not be detected in normal membranes or purified schizonts. Membranes of normal erythrocytes and uninfected erythrocytes that had been incubated with sera from monkeys with 25–50% parasitemia did not react with the monkey anti-parasite serum.**

Inoculation of owl or rhesus monkeys with intraerythrocytic stages of *Plasmodium falciparum* (1) or *P. knowlesi* merozoites (2), with complete Freund's adjuvant, produces effective antimalarial immunity. Moreover, Collins *et al.* (3) have isolated a heat-stable soluble antigen from sera of *P. knowlesi*-infected monkeys which when injected with complete adjuvant, will suppress parasitemia and reduce mortality of animals challenged with homologous parasites. These experiments show that antimalarial immunity can be achieved experimentally by vaccination with parasite antigens. However, they do not provide information about parasite-induced host-cell antigens. These were first recognized by Brown *et al.* (4), who showed that the sera of rhesus monkeys chronically infected with, or immune to infection by, a given strain of *P. knowlesi* contained antibodies that agglutinate erythrocytes containing schizonts of that strain of *P. knowlesi*. Possibly related antigens have also been detected by immune electron microscopy of erythrocytes infected with *P. falciparum* (5), but it is not known whether any such antigens can elicit a protective immune response.

We have approached this area by biochemical and immunochemical techniques, first developing a reliable method for the isolation of schizonts and host-cell plasma membranes from *P. knowlesi*-infected rhesus erythrocytes (6) and now analyzing these fractions, by two-dimensional isoelectric focusing-dodecyl sulfate ($DodSO_4^-$)/polyacrylamide gel electrophoresis and by immunoelectrophoretic methods using high-titer monkey antisera raised against purified schizonts.

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MATERIALS AND METHODS

Chemicals. Reagents for electrophoretic separations were as in refs. 6–8; other chemicals were as in ref. 6.

Monkeys. Rhesus monkeys (*Macaca mulatta*), weighing 4–8 kg, obtained from the Primate Import Company (Port Washington, NY), were first infected after about a 4-week quarantine period (Tb-negative). Cure was by an initial intramuscular injection of 20 mg of chloroquine per kg, followed by two daily injections of 10 mg/kg. About 3 weeks after the first infection the animals were splenectomized; after 1–2 weeks of convalescence, the animals could be reinfected two to three times.

Parasites. *P. knowlesi*-infected rhesus erythrocytes were obtained as described (6). Infection of each animal was initiated by an intravenous injection of 0.75–1.5 ml of a buffered cell suspension (20% parasitemia) that had been stored at −70°. Microscopic evaluation of parasitemia was as in ref. 6.

Membrane Isolation. Freshly drawn, heparinized (10 units/ml) venous blood was washed five times to remove leukocytes. Membranes from uninfected erythrocytes were isolated as in ref. 6. Membranes were prepared from infected erythrocytes as follows: At 20–30% parasitemia (late schizont stage), 20–30 ml of blood was drawn (10 units of heparin per ml) and schizont-infected erythrocytes were separated from leukocytes, thrombocytes, and uninfected erythrocytes by Ficoll/Hypaque gradients (6). Infected cells were disrupted by nitrogen decompression and the membranes of infected erythrocytes were isolated as in ref. 6, but a density of 1.085 was used in the first gradient. For this, Ficoll (9% wt/vol) and Hypaque (25% wt/vol) were mixed in a ratio of 65/35 (vol/vol). The membrane vesicles were then washed twice in 5 mM phosphate (pH 8.0) to release soluble proteins.

Incubation of Normal Monkey Erythrocytes with Serum of Parasitemic Monkeys. To test whether parasite proteins, released into the serum, adsorb to erythrocyte membranes, we washed fresly drawn normal cells five times in phosphate-buffered saline. Sera were obtained from infected monkeys at late stages of the erythrocyte cycle. At sampling, one of the monkeys had a parasitemia of 50%, and the other (serum provided by R. Gwadz, Malaria Section, National Institute of Health) of 27%. About $5 \cdot 10^9$ erythrocytes (in 0.5 ml) were incubated with 5 ml of each serum at 37° for 3 hr, washed three times, and processed for membrane analysis.

Isolation of Released Parasites. We purified the parasites from erythrocyte lysates by Ficoll/Hypaque gradients and differential centrifugation (6).

Antisera. A hyperimmune serum against purified *P. knowlesi* schizonts was raised in a splenectomized rhesus

Abbreviations: $DodSO_4^-$, dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

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monkey: 10^8 parasites in 0.25 ml of 0.25 M sucrose/10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes), pH 7.5, were mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously at multiple sites on the back. Injections of the same number of parasites from different parasite preparations were repeated monthly. The serum used for the experiments illustrated here was drawn 10 days after the eighth booster with complete adjuvant. We used a splenectomized animal not by choice, but because of shortage of monkeys.

Immune serum against normal monkey erythrocyte membranes was prepared in rabbits. The animals were initially immunized by subcutaneous injection of 0.5 mg of erythrocyte membrane protein mixed with 0.5 ml of complete Freund's adjuvant. All sera were processed under sterile conditions and stored at −70°.

Solubilization of Proteins. Membrane proteins of normal and parasitized monkey erythrocytes were solubilized as in ref. 7, with 1 mM Hepes/1% Triton X-100, pH 8.5, in two sequential extraction steps at 37° for 15 min. Purified parasites were solubilized in the same manner. The protein concentration was adjusted to 5–10 mg/ml (8).

Electrophoretic Procedures. Crossed immune electrophoresis and crossed-line immune electrophoresis, to determine crossreactivities (9), were as described (7, 8). Isoelectric focusing was performed as detailed (10). Bidimensional isoelectric focusing-immune electrophoresis and $DodSO_4^-$/polyacrylamide electrophoresis, were as described (8). To compare the Coomassie blue-stained slab gels obtained by the last procedure with different samples, we photographed the gels and prepared prints that matched precisely in size (e.g., Fig. 5 *A–C*). The protein patterns were transferred onto transparent copying paper (3M; 383). These traces were then superimposed and a third transparency was used to trace components that were deleted, altered in intensity, or added.

RESULTS

Solubilization of Membrane Proteins. The two-step Triton extraction solubilized more than 90% of the parasite protein and 60–70% of the membrane protein, due to the poor solubility of spectrin in Triton. Except for the greater proportion of spectrin in $DodSO_4^-$-solubilized membranes, there were only minor quantitative differences when these were compared with Triton X-100-solubilized membranes by $DodSO_4^-$/polyacrylamide gel electrophoresis.

Contamination of Schizonts by Erythrocyte Membrane Fragments. Bidimensional isoelectric focusing-immune electrophoresis, using antiserum against normal monkey erythrocyte membranes, of normal erythrocyte membranes (Fig. 1*A*) revealed three major immune precipitates, at pI 6.0 (*1*), pI 5.8 (*2*), and 5.5 (*3*). The membranes from infected cells (Fig. 1*B*) gave no precipitate at pI 6.0, and those at pI 5.8 and 5.5 were reduced about 50% in height. Isolated schizonts gave no, or hazy, trace immune precipitates. These results indicate that the schizonts lack significant contamination by those membrane proteins giving the immune precipitates, as is also suggested by crossed immune electrophoresis against hyperimmune monkey serum. Biochemical data, however, indicate as much as ~10% contamination of schizonts with host-cell membranes (6).

Immunization of Rhesus Monkey with Purified Schizonts. Sera from monkeys with immunity to *P. knowlesi* fail to give satisfactory immune precipitates with parasite antigens. However, after immunization of a splenectomized monkey and three to eight boosters in complete adjuvant, the serum gave immune precipitates with Triton-solubilized proteins from isolated parasites or membranes of parasitized cells. High titers of precipitating antibody required boosters with antigen in complete Freund's adjuvant (see ref. 11).

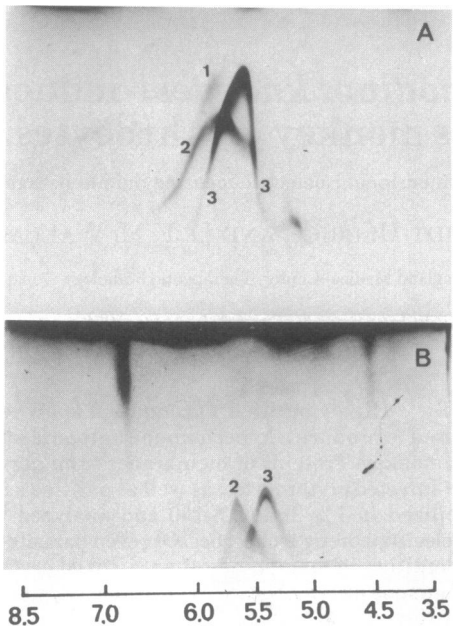

FIG. 1. Bidimensional isoelectric focusing-immune electrophoresis of membrane proteins from normal (*A*) and parasitized (*B*) erythrocytes. Protein (350 μg) was focused in 4% polyacrylamide/1% Triton/8 M urea/2% ampholytes, pH 3.5–10.0, and then electrophoresed into agarose containing rabbit antiserum to normal rhesus erythrocyte membranes (67 μl/ml). pH gradient is shown below *B*. Immune precipitates are numbered starting with *1* at alkaline end of pH gradient. Coomassie blue staining.

Crossed Immune Electrophoresis. The precipitation patterns of membranes from parasitized erythrocytes and isolated schizonts, electrophoresed against monkey anti-parasite serum, are shown in Figs. 2 and 3, respectively. Infected membranes reveal seven partially complex precipitation arcs. Of these antigens *only* the weakly reacting components *1* and *3* and the dominant component *7* (Fig. 3) can be detected in the parasites. No traces of components *2*, *4*, *5*, and *6* were detected in any parasite preparation over a 5-fold range of antigen/antibody ratios, although the heights of schizont components *1*, *2*, and *7* varied as expected with changing antibody/antigen ratio.

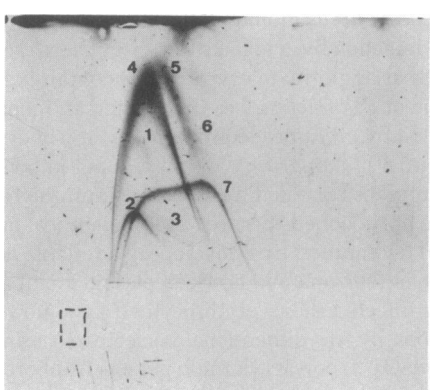

FIG. 2. Crossed immune electrophoresis of membrane proteins from monkey erythrocytes infected with *P. knowlesi*. Membrane proteins (150 μg; solubilized in 1% Triton X-100) were first electrophoretically separated in antibody-free agarose and then electrophoresed at right angles into agarose containing monkey anti-parasite serum (0.12 ml/ml of agarose). Immune precipitates are numbered *1–7* according to their electrophoretic mobility in the first dimension. Coomassie blue staining.

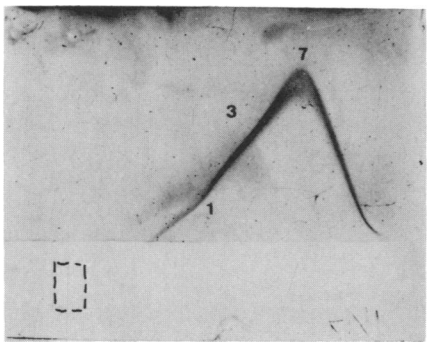

FIG. 3. Crossed immune electrophoresis of proteins from *P. knowlesi* schizonts, dissolved in 1% Triton X-100. About 75 μg of proteins were separated electrophoretically in the first dimension (horizontal) and electrophoresed at right angles into agarose containing monkey anti-parasite serum (0.18 ml/ml of agarose). Immune precipitates are numbered as in Fig. 1. Coomassie blue staining.

Identity of components *1*, *3*, and *7* of parasites and the infected membranes was documented by anodal (upward) displacement of these precipitates in crossed line immune electrophoresis with parasite protein in the intermediate strip (Fig. 4*B*). Precipitate *7* is displaced upward to merge with the horizontal precipitation line *7p*, arising from the parasite antigens.

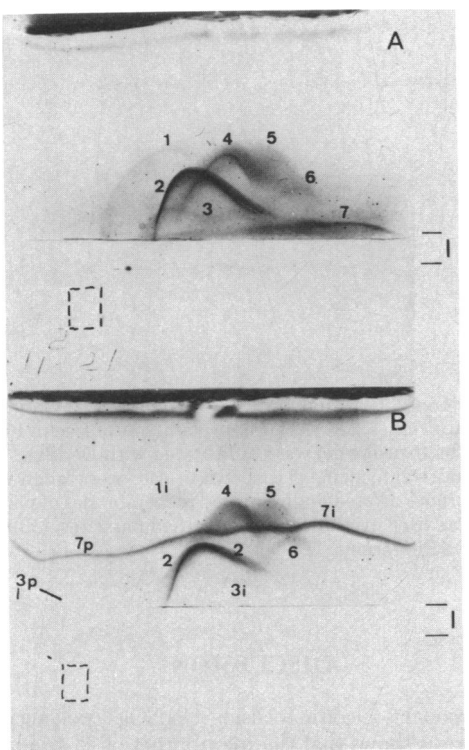

FIG. 4. Bidimensional immune electrophoresis of membranes from parasitized monkey erythrocytes. Proteins (150 μg; in Triton X-100) were separated in the first dimension (horizontal) and then electrophoresed in the second dimension (vertical) into agarose containing 0.12 ml of monkey anti-parasite serum. Coomassie blue staining. (*A*) Crossed-line immune electrophoresis with 400 μg of proteins from uninfected erythrocytes in the intermediate strip (*I*; 50 × 6 mm). The immune precipitates *1–7* are identical to those in Fig. 1; however, longer electrophoresis in the first dimension resulted in a better horizontal resolution, advantageous for this analysis. (*B*) Crossed-line immune electrophoresis with 400 μg of parasite protein in the intermediate strip (*I*). Precipitation lines *3p* and *7p* originate from proteins in the intermediate strip and fuse with the identical antigens in membranes from infected cells (*3i* and *7i*).

Similarly, precipitates *1i* and *3i* originate from precipitation lines *1p* (not marked) and *3p* arising from the intermediate strip. Due to the low concentration of components *1* and *3*, their interactions are not as obvious as for component *7*.

There is no crossreactivity between antigens *1–7* and any protein of normal monkey erythrocyte membranes included in the intermediate strip (Fig. 4*A*). Concordantly, crossed immune electrophoresis of normal erythrocyte membranes from different monkeys against monkey anti-parasite serum yielded no immune precipitates. Also, no precipitates developed when membranes of normal erythrocytes, incubated with sera from infected monkeys, were run against monkey anti-parasite serum. This indicates that the parasite-specific components in membranes of parasitized cells are not adsorbed from the serum.

Isoelectric Focusing. Fig. 5 illustrates the protein pattern obtained after Triton X-100-solubilized proteins of the three fractions under investigation were fractionated by isoelectric focusing. The membranes from infected cells gave bands at pI 4.2, 4.3, 4.6, and 4.7. These were also found in the parasite, but not in normal membranes. The ~pI 6.0 component of normal membranes is markedly reduced in membranes from infected cells, probably representing spectrin depletion.

Bidimensional Isoelectric Focusing-DodSO_4^-/Polyacrylamide Gel Electrophoresis. As shown in Fig. 6 *A–C*, this

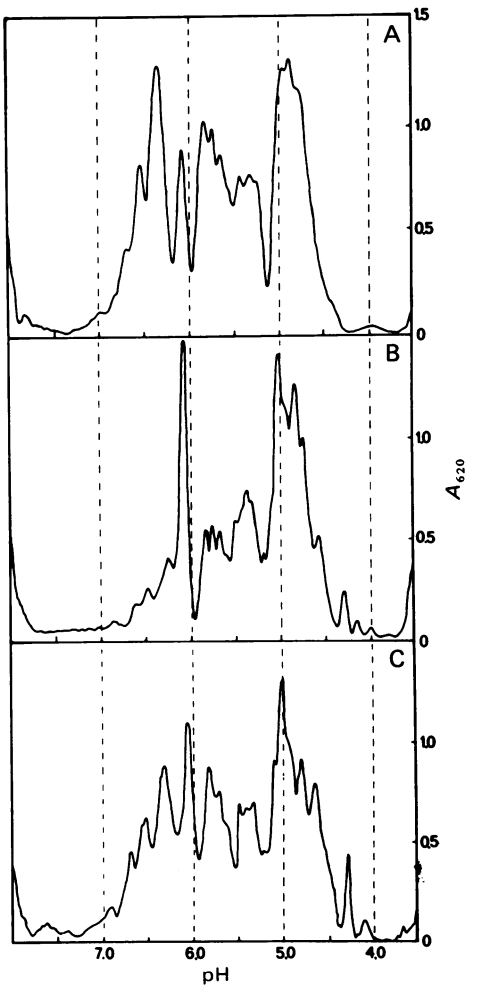

FIG. 5. Isoelectric focusing of membranes of normal and parasitized monkey erythrocytes and of schizonts (120 μg of Triton X-100-solubilized proteins). Absorbance for Coomassie blue staining is given. (*A*) Membranes of normal erythrocytes; (*B*) membranes of parasitized erythrocytes; (*C*) purified schizonts.

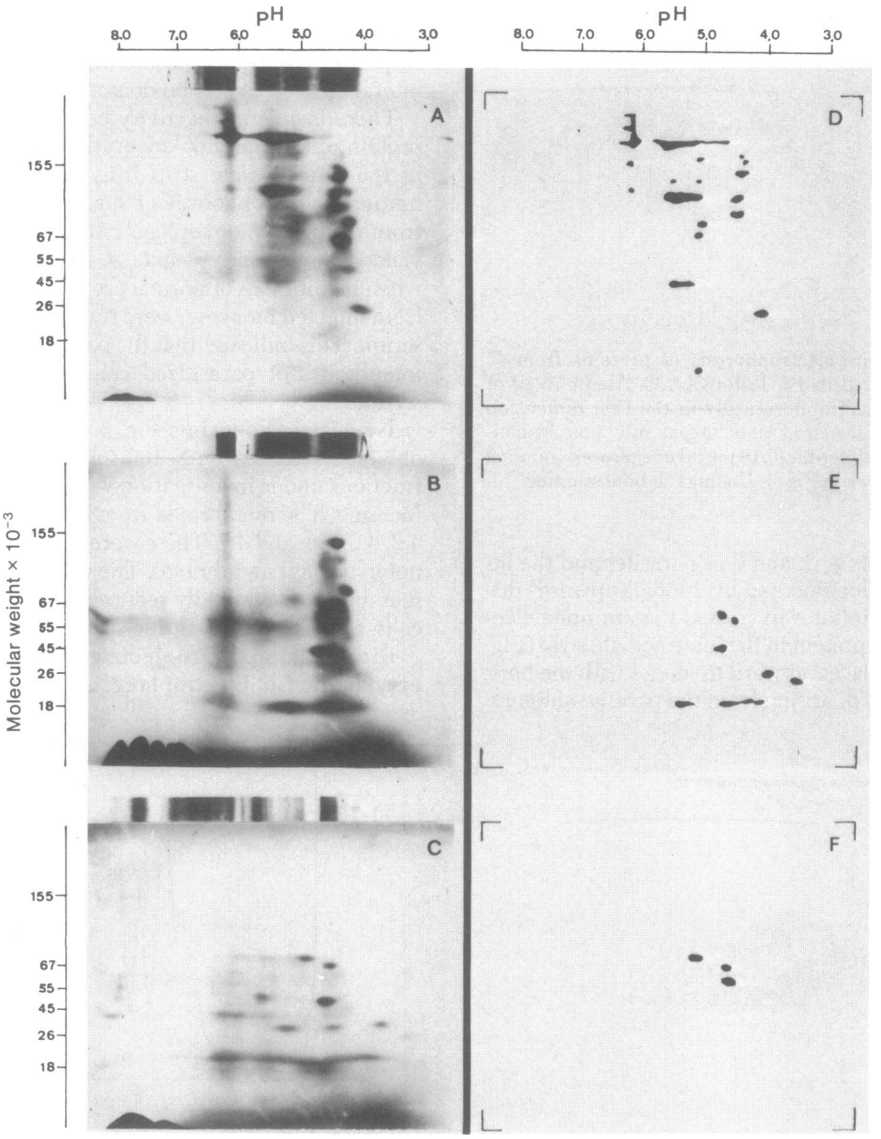

FIG. 6. Bidimensional isoelectric focusing-DodSO_4^-/polyacrylamide gel electrophoresis. (*A*) Membranes of normal monkey erythrocyte cells; (*B*) membrane proteins of cells parasitized by *P. knowlesi;* (*C*) purified schizonts. In all cases, 350–400 μg of protein, solubilized in 1% Triton X-100, was analyzed. Prior to DodSO_4^- gel electrophoresis in the second dimension, the focusing gel was equilibrated with DodSO_4^- and dithiothreitol and positioned atop an acrylamide gradient gel (8). One-dimensional cylindrical focusing gels shown in *A–C* were laden with 100 μg of protein. Coomassie blue protein staining. (*D*) Proteins in normal erythrocyte membranes deleted or diminished in membranes of parasitized cells (superimposition method). (*E*) Protein components common to membranes of parasitized erythrocyte and purified parasites. (*F*) Proteins unique to membranes of parasitized cells. Results from three separate infections and fractionations.

technique reveals at least 50 protein spots in schizonts, as well as in membranes from normal and infected erythrocytes. Differences between samples were highly reproducible and components apparently unique to parasitized erythrocytes were identical in three separate infections and fractionations. The data show that the membranes of parasitized erythrocytes undergo substantial modifications. Several major membrane components, i.e., spectrin and parts of the "band 3" complex, as well as some smaller molecular weight components, are diminished or deleted. Membranes from infected cells also contain trace parasite components (Fig. 6*E*). Most importantly, however, they consistently show *three* proteins (Fig. 6*F*) with pIs near 5.2, 4.8, and 4.5 and molecular weights near 90,000, 65,000, and 55,000 that have not been detected in any parasite preparation.

DISCUSSION

Bidimensional isoelectric focusing-DodSO_4^-/polyacrylamide electrophoresis shows that the membranes of *P. knowlesi*-infected rhesus erythrocytes contain at least *three* proteins (pI 4.6–5.2; molecular weight ~55,000–90,000) that cannot be detected in normal membranes or purified schizonts. These proteins might be membrane-associated cleavage products due to the action of parasite proteases or glycosidases of normal membrane proteins, e.g., the components shown depleted by isoelectric focusing or focusing-immune electrophoresis. The proteins of pI 4.8–5.2 and molecular weight 55,000–90,000 could also be parasite products, possibly the new antigens detected by crossed immune electrophoresis. A clear distinction between these possibilities will require *in vitro* metabolic labeling during parasite maturation and final analysis of sepa-

rated proteins by autoradiography. However, the "new" proteins we detect are not identical to the excreted proteins described by McColm *et al.* (12), since these are also found in the parasite cytoplasm.

Our immunoelectrophoretic data with monkey antischizont serum show that membranes from parasitized erythrocytes contain at least *four* antigens that are not detectable in schizont preparations identical to those used to produce the antiserum. Because parasites from different monkeys were used for immunization and boosting, it is unlikely these represent trace erythrocyte components. This interpretation is supported by the fact that no immune precipitates were obtained with normal erythrocyte membranes from five different monkeys. According to their electrophoretic mobilities these antigens are proteins, but we cannot exclude the possibility that they are protein-associated, parasite-synthesized glycolipids. Unfortunately no information is available as to whether *P. knowlesi* has the metabolic machinery for glycoconjugate synthesis.

It appears most improbable that the new host-cell membrane antigens represent fragments of normal membrane proteins. However, if the antigens are parasite products one must inquire why they are not detected in the schizont preparations used to immunize the monkey. We suggest the following working hypothesis. During intraerythrocytic maturation, *P. knowlesi* synthesizes potentially antigenic components. At least four of these are either exported from the parasite immediately after synthesis or their antigenic sites are blocked in the schizont. The exported proteins associate with the host-cell membrane in antigenic form. If antigenicity is masked in the schizont, unmasking would need to occur after export, either by erythrocyte or plasma enzymes.

The presence of parasite-specific antigens in host-cell membranes may be relevant to the development of antimalarial vaccines. Immunization is considered to be one of the few feasible approaches to malaria control, but the search for vaccine antigens has heretofore focused on proteins associated directly with malaria parasites. However, the observation that immunized animals can produce antibodies against parasite-induced antigens located in host-cell membranes suggests a strategy directed against those antigens. Suitable antigen preparations may lead to long-term immunity that causes destruction of parasitized cells, and therewith parasites, early *after* infection.

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1. Siddiqui, W. A.(1977) *Science* **197**, 388–389.
2. Mitchell, G. H., Butcher, G. A., Langhorne, J. & Cohen, S. (1977) *Clin. Exp. Immunol.* **28**, 276–279.
3. Collins, W. E., Contacos, P. G., Harrison, A. J., Stanfill, P. S. & Skinner, J. C. (1977) *Am. J. Trop. Med. Hyg.* **26**, 373–376.
4. Brown, K. N., Brown, I. N. & Hills, C. A. (1970) *Exp. Parasitol.* **28**, 304–317.
5. Kilejian, A., Abati, A. & Trager, W. (1977) *Exp. Parasitol.* **42**, 75–164.
6. Wallach, D. F. H. & Conley, M. (1977) *J. Mol. Med.* **2**, 119–136.
7. Schmidt-Ullrich, R., Wallach, D. F. H. & Davis, F. D. G., II (1976) *J. Natl. Cancer Inst.* **57**, 1117–1126.
8. Schmidt-Ullrich, R., Thompson, W. S., Lin, P.-S. & Wallach, D. F. H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5069–5072.
9. Bjerrum, O. J. & Bøg Hansen, T. C. (1976) in *Biochemical Analyses of Membranes*, ed. Maddy, A. T. (Chapman and Hall, London, England), pp. 378–426.
10. Schmidt-Ullrich, R. & Wallach, D. F. H. (1977) in *Biological and Biomedical Applications of Isoelectric Focusing*, eds., Catsimpoulas, N. & Drysdale, J. (Plenum, New York), pp. 191–209.
11. Schmidt-Ullrich, R., Thompson, W. S. & Wallach, D. F. H. (1977) in *Proc. Natl. Acad. Sci. USA* **74**, 643–647.
12. McColm, A. A., Shakespeare, P. G. & Trigg, P. I. (1977) *Bull. WHO* **55**, 277–283.