PLASMODIUM KNOWLESI-INDUCED ANTIGENS IN PLASMA MEMBRANES OF PARASITIZED RHESUS MONKEY ERYTHROCYTES

by

R. Schmidt-Ullrich and D. F. H. Wallach

Radiobiology Division, Department of Therapeutic Radiology
Tufts University School of Medicine - New England Medical Center Hospital
Boston, Massachusetts 02111, United States of America

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3 To whom correspondence should be addressed.

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1. INTRODUCTION

Inoculation of owl or rhesus monkeys with intraerythrocytic stages of Plasmodium falciparum (Siddiqui, 1977) or P. knowlesi merozoites (Mitchell et al., 1977), respectively, using complete Freund's adjuvant produces effective antimalarial immunity. Moreover, Collins et al. (1977) have isolated a heat-stable soluble antigen from sera of P. knowlesi-infected monkeys which, when injected with complete adjuvant, will suppress parasitaemia and reduce mortality of animals challenged with homologous parasites.

The above 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. (1970), who showed that the sera of rhesus monkeys chronically infected with, or immune to infection by, a given strain of P. knowlesi possess antibodies that agglutinate erythrocytes containing schizonts of that strain. Parasite-related antigens have also been detected by immune electron microscopy of erythrocytes infected with P. falciparum (Kilejian, 1977), but it is not known whether any such antigens can elicit a protective immune response.

We have approached this area of research by biochemical and immunochemical techniques. First, a reliable method was developed for the isolation of schizonts and host-cell plasma membranes from P. knowlesi-infected rhesus erythrocytes (Wallach & Conley, 1977). These fractions have now been analysed by two-dimensional isoelectric focusing-dodecyl sulfate polyacrylamide gel electrophoresis and by immunoelectrophoretic methods utilizing high-titre rhesus monkey antisera raised against purified schizonts. Both approaches have revealed the presence in the host-cell plasma membranes of P. knowlesi-infected erythrocytes of proteins that are not present in the membranes of uninfected cells and that cannot be detected in isolated schizonts.
2. MATERIALS AND METHODS

2.1 Chemicals

The chemicals used in this study and the sources from which they were obtained were: Triton X-100, N-2-hydroxyethylpiperazine-N-2-ethanesulfonate, dithiothreitol, dextran (170 000 D) and bovine serum albumin from Sigma, St Louis, Mo., USA; dodecyl sulfate and urea from Fisher Scientific Co., Fair Lawn, N.J., USA; agarose (Lot AGS 222) from Litex, Glostrup, Denmark; acrylamide, N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate and Coomassie brilliant blue from Bio-Rad Laboratories, Richmond, Calif., USA; ampholytes (Ampholine pH 3.5-10.0) from LKB, Uppsala, Sweden; Ficoll and polyacrylamide gradient gels (4-30%) from Pharmacia Fine Chemicals, Uppsala, Sweden; complete Freund's adjuvant from Difco Laboratories, Detroit, Mich., USA; lactoperoxidase from Boehringer, Mannheim, Federal Republic of Germany; neuraminidase from Behringwerke, Marburg, Federal Republic of Germany; 125I (carrier free) from New England Nuclear, Boston, Mass., USA; sodium metrizoate (Hypaque) and chloroquine (Aralen) from Winthrop Laboratories, New York, N.Y., USA; sodium heparin from Upjohn, Kalamazoo, Mich., USA; ketamine hydrochloride veterinary anaesthetic (Ketaset) from Bristol Laboratories, Syracuse, N.Y., USA; and Dulbecco's phosphate buffered saline from Gibco, Grand Island, N.Y., USA.

Abbreviations used in this paper for some of the above substances are: DTT = dithiothreitol; DS = dodecyl sulfate; HEPEs = N-2-hydroxyethylpiperazine-N-2-ethanesulfonate; PBS = Dulbecco's phosphate buffered saline; TEMED = N,N,N',N'-tetramethylethylenediamine.

2.2 Monkeys

Rhesus monkeys (Macaca mulatta) weighing 4-8 kg,\(^1\) were first infected after a ~4-week quarantine period (Tb-negative). The infected animals were cured by an intramuscular injection of 20 mg/kg chloroquine on day 1 and two further injections of 10 mg/kg on the next two days. About three weeks after the first infection the animals were splenectomized at the Laboratory of Animal Medicine, New England Medical Center, Boston, Mass., USA. After one to two weeks convalescence, the animals could be reinfected and recured (chloroquine) for two to three times. Some very anaemic animals were supported during cure, by an intravenous infusion of 100 ml fresh blood from a healthy animal, plus 50 ml physiological, saline-dextrose.

2.3 Parasites

P. knowlesi-infected rhesus erythrocytes were obtained as described by Wallach & Conley (1977). Infection of each animal was initiated by an intravenous injection of 0.75-1.5 ml of a buffered cell suspension that had been frozen at -70°C. Frozen stocks of infected cells were prepared in the following manner: two parts of freshly drawn, infected blood (20% infected erythrocytes), anti-coagulated with sodium heparin (10 μ/ml) were mixed with one part of 3/7 glycerin/PBS (v/v) and shell frozen by swirling in liquid nitrogen. The vials were then immediately transferred to a -70°C freezer.

2.4 Evaluation of parasitaemia

To monitor the progress of parasite development in infected animals, uniform blood smears on glass slides were stained using Giemsa Azure type B stain. A solution of Glemsta stain (Fischer, Lot 74531; 1:20 dilution) was prepared in water adjusted to pH 7.2. Evaluation of parasitaemia from the methanol fixed, stained slides was as described by Wallach & Conley (1977).

\(^1\) Obtained from the Primate Import Company, Port Washington, N.Y., USA.
2.5 Preparation of membranes from uninfected erythrocytes

Freshly drawn, heparinized (10 μl/ml) venous blood was washed five times to remove leukocytes. Membranes from the erythrocytes were isolated by hypotonic lysis or by nitrogen decomposition as described by Wallach & Conley (1977).

2.6 Preparation of membranes from infected erythrocytes

When the parasitaemia in an infected monkey reached 20-30% and the parasites were in the late schizont stage, the animal was anaesthetized and 20-30 ml of infected blood were drawn by venipuncture. Schizont-infected erythrocytes were separated from uninfected erythrocytes, leukocytes and thrombocytes using Ficoll-Hypaque gradients as described by Wallach & Conley (1977). Disruption of infected cells was by nitrogen decompression (280 psi N₂ for 15 minutes). Nitrogen decomposition, as employed, disrupts the host-cell plasma membrane but not the membranes of the parasitophorous vacuole or the parasite. Isolation of the erythrocyte membranes of infected erythrocytes was as described by Wallach & Conley (1977), but using a density of 1.085 in the first gradient; for this and the following gradients, Ficoll (9% w/v) and Hypaque (25% w/v) were mixed in a ratio of 65/35 (v/v). The membrane vesicles were then washed twice in 5 mM phosphate, at pH 8.0, to release entrapped haemoglobin and cytoplasmic proteins.

2.7 Incubation of normal monkey erythrocytes with serum of parasitaemic monkeys

These experiments were designed to test whether parasite-specific proteins, released into the serum at late stages of intracellular parasite development (McColm et al., 1977), adsorb to membranes of non-parasitized erythrocytes. For this, monkey erythrocytes from freshly drawn blood were washed five times in PBS. Sera were obtained from infected monkeys at late stages of the erythrocyte cycle. At sampling, one of the monkeys had a parasitaemia of 50%, the other of 27%. About 5 × 10⁹ erythrocytes (in 0.5 ml) were incubated with 5 ml of each serum at 37°C for 3 hours and then washed three times in PBS and processed for membrane analysis.

2.8 Isolation of released parasites

The conditions described above yield parasites which, according to biochemical, immunological and electronmicroscopic criteria, are surrounded predominantly by intact parasitophorous vacuolar membranes (to be published).

2.9 Antisera

A high-titrated hyperimmune serum against purified P. knowlesi schizonts was raised in a splenectomized rhesus monkey. For this, 10⁸ parasites in 0.25 ml sucrose/HEPES (0.25M/0.01M), at pH 7.5, were mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously at multiple sites at the back. Monthly injections of the same number of parasites from different parasite preparations yielded a hyperimmune serum suitable for immunochimical analyses: The serum used for the experiments discussed in this paper was drawn ten days after the eighth booster with complete Freund's adjuvant. A splenectomized animal was used, not by choice but because of the acute shortage of rhesus monkeys.

Hyperimmune serum against normal monkey erythrocyte membranes was prepared in rabbits. The animals were initially immunized by subcutaneous injection of 0.5 mg erythrocyte membrane protein in 0.5 ml sucrose/HEPES (0.25 M/0.01M), at pH 7.5, mixed with 0.5 ml of complete Freund's adjuvant. Boosters were at monthly intervals using 1 mg of membrane proteins in complete Freund's adjuvant. All sera were processed under sterile conditions and stored at -70°C.

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¹ The serum from this second animal was kindly provided by Dr R. Gwadz, Malaria Section, Laboratory of Parasitic Diseases, National Institutes of Health, USA.
2.10 Solubilization of proteins

Membrane proteins of normal and parasitized monkey erythrocytes were solubilized as described by Schmidt-Ullrich et al. (1976) using HEPES/Triton X-100 (1 mM/1%), at pH 8.5, in two sequential extraction steps at 37°C for 15 minutes. Purified parasites were Triton-solubilized in the same manner. The protein concentration was adjusted to 5-10 mg/ml (Schmidt-Ullrich et al., 1976). For DS polyacrylamide gel electrophoresis, samples were transferred into DS/DTT (1%/40 mM) (protein concentration ~5 mg/ml) and heated for 5 minutes in boiling water.

2.11 Crossed immune electrophoresis

This method was applied as described before (Schmidt-Ullrich et al., 1976) but monkey anti-parasite antiserum was used in the second dimension.

2.12 Crossed-line immune electrophoresis

This form of crossed immune electrophoresis was used to determine cross-reactivities (Schmidt-Ullrich et al., 1976; Bjerrum & Bøgg Hansen, 1976). First, an antigen-containing sample was electrophoresed in the first dimension as in conventional crossed immune electrophoresis. An agarose strip, containing possible cross reacting antigens dispersed uniformly throughout it, was then placed parallel to the strip with the electrophoretically pre-separated proteins and the antibody-containing agarose. Upon electrophoresis in the second dimension the proteins in the second strip migrated as a single front, except in cases of cross reactivity with some of the pre-electrophoresed proteins when the origins of the immunoprecipitation arcs of the latter were displaced towards the anode.

2.13 Isoelectric focusing

Equilibrium analytical focusing was performed using 4% acrylamide gels, cross-linked with 2.5% bisacrylamide, containing 2% ampholytes, 8M urea, 1% Triton X-100 and 10% sucrose. The procedures followed were those detailed by Schmidt-Ullrich & Wallach (1977).

2.14 Bidimensional isoelectric focusing-immune electrophoresis

The procedure was as described by Schmidt-Ullrich et al. (1977) but using about 400 µg of Triton X-100 solubilized protein from normal monkey erythrocyte membranes, membranes of parasitized erythrocytes and purified intracellular parasites. In the second dimension we used 0.067 ml/ml agarose of rabbit serum against monkey erythrocyte membranes.

2.15 Bidimensional isoelectric focusing-dodecyl sulfate polyacrylamide gel electrophoresis

Isoelectric focusing was in cylindrical gels (65 mm x 3 mm; 400 µg of protein per gel), the composition of which is given in section 2.13 above, using specified pH gradients. The procedure was that detailed by Schmidt-Ullrich et al. (1977). To compare the Coomassie blue-stained slab gels obtained with different samples, these were photographed and prints prepared that matched precisely in size (e.g. Fig. 5A-C). The protein patterns were then transferred onto transparent copying paper (3M; 383). These traces were then superimposed and a third transparency used to trace components that were deleted, diminished or added in one electrophorogram versus another.

3. Results and discussion

3.1 Solubilization of membrane proteins using Triton X-100

The two-step extraction of membrane proteins from animal and parasitized monkey erythrocytes and of parasite proteins using HEPES/Triton X-100 (1 mM/1%v/v), solubilized more than 90% of the parasite protein. Only 60-70% of the membrane protein was solubilized, due to the known poor solubility of spectrin components in non-ionic detergents. However, except for the greater proportion of spectrin in DS solubilized membranes, there were no qualitative and
only minor quantitative differences in protein pattern when these were compared with Triton X-100-solubilized membranes by DS polyacrylamide gel electrophoresis.

3.2 Contamination of schizonts by erythrocyte membrane fragments

Rabbit antiserum against normal monkey erythrocyte membranes was employed in bidimensional isoelectric focusing-immune electrophoresis to detect possible contamination of purified parasites by erythrocyte membrane components. Analyses of membranes from normal and infected cells are shown in Fig. 1A, B. Normal erythrocyte membranes (Fig. 1A) reveal three major complex immune precipitates, component 1 at pI 6.0, component 2 at pI 5.8 and component 3 at pI 5.5. It is not clear why such a limited number of immune precipitates was obtained, compared with the many proteins resolved chemically by isoelectric focusing alone or combined with DS polyacrylamide gel electrophoresis. However, this fact presents no disadvantage for the purpose of this experiment.

The membranes from infected cells (Fig. 1B) showed only two components with isoelectric points very close to those of components 2 and 3. Components 2 and 3 were reduced by about 50% in height, component 3 somewhat more than component 2. Since the heights of the immune precipitates vary linearly with antigen concentration and all analyses were carried out using identical protein loading, the results suggest a deletion of component 1 and an approximately 50% loss of components 2 and 3.

It should be stressed that, in two out of four experiments, isolated schizonts treated identically gave no immune precipitates at all. The other two schizont preparations yielded hazy, trace precipitates. It can therefore be concluded that the schizonts lacked significant contamination by the membrane proteins defined by the immune precipitates. This conclusion was also supported by crossed immune electrophoresis against hyperimmune monkey serum (see section 3.4): none of the strong host-cell plasma membrane antigens (e.g. 4 and 5 in Fig. 2) could be detected in the schizont preparations over a 5-fold range of antigen/antiserum concentration ratios. Biochemical data, however, have indicated that as much as ~10% contamination of schizonts with host-cell plasma membranes can occur (Wallach & Conley, 1977).

3.3 Immunization of a rhesus monkey with purified schizonts

Tests of sera from rhesus monkeys that had acquired natural immunity to *P. knowlesi* failed to reveal sufficient concentrations of precipitating immunoglobulin for immunochemical identification of parasite specific antigens. A splenectomized animal was therefore immunized by repeated injections of purified schizonts in complete Freund's adjuvant. After immunization and three boosters, the serum revealed immune precipitates, in crossed immune electrophoresis, when run against Triton-solubilized proteins from isolated parasites or membranes of parasitized cells. Subsequent boosters in incomplete Freund's adjuvant did not maintain an adequate antibody titre. However, high titres of precipitating antibody were recovered by boosting again with antigen in complete Freund's adjuvant. This confirms previous data (Brown, 1977) demonstrating the need for complete Freund's adjuvant to obtain a high titre immune sera against intraerythrocytic plasmodial parasites.

3.4 Crossed immune electrophoresis

The immune precipitation patterns of purified membranes from parasitized erythrocytes and of isolated *P. knowlesi* schizonts electrophoresed against monkey anti-parasite serum are shown in Figs. 2 and 3, respectively. As documented in Fig. 2, infected membranes reveal seven, partially complex precipitation areas, numbered 1-7 according to electrophoretic mobility of the antigens in the first dimension. Of these antigens, only the weakly reacting components 1 and 3 and the dominant component 7 (Fig. 3) can be detected in the parasites used as antigen for the immunization.

No traces of components 2, 4, 5 and 6 were detected in any parasite preparation over a five-fold range of antigen/antibody ratios, although the heights of schizont components 1, 3 and 7 varied as expected with changing antibody/antigen ratio.
The identity between components 1, 3 and 7 of the parasites and the infected membranes, respectively, is revealed by anodal (upward) displacement of these precipitates in crossed-line immune electrophoresis with parasite protein in the intermediate strip. This is shown in Fig. 4B, where i stands for components of erythrocyte membranes from infected cells (separated electrophoretically in the first dimension), and p for parasite (in the intermediate strip). Precipitate 7 is displaced upward (anodally) to merge with the horizontal precipitation line 7p, arising from the parasite antigens. 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 in both membranes from infected cells and parasites, their interactions are not as obvious as for component 7.

There is no cross reactivity between antigens 1-7 of parasitized-cell membranes and any protein in solubilized normal monkey erythrocyte membranes included in the intermediate strip (Fig. 4A). Concordantly crossed immune electrophoresis of normal erythrocyte membranes from different monkeys against monkey anti-parasite serum yielded no immune precipitates.

It should be emphasized that no immune precipitates were revealed when membranes of normal monkey erythrocytes, incubated with serum from monkeys with 25-50% parasitaemia, were electrophoresed against monkey anti-parasite serum. This indicates that the parasite-specific components identified in membranes of parasitized cells are not adsorbed from the serum.

Bidimensional isoelectric focusing-immune electrophoresis using monkey serum and immunoglobulin purified therefrom was attempted. However, neither gave any immune precipitates with membrane proteins of parasitized erythrocytes or purified parasite. This may mean that the antigens do not tolerate present focusing conditions or that monkey antibodies are unduly sensitive to traces of urea. These questions are under study.

3.5 Isoelectric focusing

Fig. 5 illustrates the protein pattern obtained when Triton X-100 solubilized proteins of the three fractions under investigation are fractionated by isoelectric focusing. Major differences between normal membranes, membranes from infected cells and parasites are detected. Membranes from infected cells reveal components at pi 4.2, 4.3, 4.6 and 4.7, which are also found in the parasite, but not in normal membranes. On the other hand, the dominant component of normal membranes, near pi 6.0, is markedly reduced in membranes from infected cells; this probably represents the depletion of spectrin, which focuses in this pH region.

3.6 Bidimensional isoelectric focusing-dodecyl sulfate polyacrylamide gel electrophoresis

As shown in Fig. 6A-C, application of this technique reveals at least 50 protein spots in the case of schizonts, as well as in that of membranes from normal and infected erythrocytes. The patterns of given sample categories were highly reproducible and could therefore be compared by superimposing photographs of Coomassie blue-stained gel slabs. Fig. 6A-C are reproductions of the prints obtained with normal membranes, host-cell membranes and schizonts, respectively. Fig. 6D, obtained by the superimposition method, shows erythrocyte membrane components deleted or diminished upon infection. Fig. 6E shows components common to host-cell membranes and schizonts. Fig. 6F shows components that are unique to the membranes of parasitized erythrocytes, as compared to normal membranes and schizonts. The differences between samples were highly reproducible and the components apparently unique to parasitized erythrocytes were identical in three separate infections and fractionations.

No components of normal erythrocytes were detectable in purified parasites, confirming a lack of substantial contamination of parasite by host erythrocyte membranes. Furthermore, the data indicate that the surface membranes of parasitized monkey erythrocytes undergo substantial modifications: several major membrane components, i.e. spectrin and parts of the "band 3" complex, as well as some components of smaller molecular weight are deleted, or significantly diminished, as in Fig. 6D. Membranes from infected cells also contain some protein spots characteristic for the parasite (Fig. 6E). However, the relative proportions
of these components in the parasites and the membranes of infected cells suggest that their presence in membranes of infected cells is not due to simple contamination by parasite material. Crossed immune electrophoresis also leads to this conclusion.

Membranes from parasitized cells show as high a resolution as normal membranes. There is no smearing or accumulation of low molecular weight material. Any low molecular weight fragments produced by plasmodial proteases have not been retained by the host-cell membranes.

It should be stressed that the host-cell membranes consistently show three proteins (Fig. 6F) with pIs near 5.2, 4.8 and 4.5, and molecular weights near 90 000D, 65 000D and 55 000D, respectively, which have not been detected in any parasite preparation.

4. GENERAL DISCUSSION

Bidimensional isoelectric focusing DS polyacrylamide gel electrophoresis shows that the membranes of P. knowlesi-infected rhesus erythrocytes contain at least three proteins (pI 4.6-5.2; Mol wt ~55 000-90 000D), which 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 pI 4.8-5.2, 55 000D-90 000D proteins 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 labelling during parasite maturation and final analysis of separated proteins by autoradiography. This approach has been applied (Schmidt-Ullrich & Wallach unpublished observations) and labelling both with amino acids and glucosamine was obtained, but label incorporation sufficient for reliable autoradiography has not yet been achieved.

McColm et al. (1977) have demonstrated release of schizont-synthesized proteins from rhesus erythrocytes parasitized with P. knowlesi. The infected cells were first cultivated in the presence of ^3H-isoleucine and then transferred to unlabelled culture medium. Two major polypeptides (apparent molecular masses near 45 000D and 49 000D) were revealed by sodium DS polyacrylamide gel electrophoresis of the concentrated medium. Both of these proteins also occur in the schizont cytoplasm (McColm et al., 1977) and appear to be released during host cell rupture and reinvasion. These proteins are clearly separate entities from the "new" membrane we describe.

According to our immunoelectrophoresis with monkey anti-schizont serum, 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 that these represent trace erythrocyte components antigenic within the species, e.g. blood group antigens. 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 the possibility that they are protein-associated parasite-synthesized glycolipids cannot be excluded. Unfortunately no information is available as to whether P. knowlesi has the metabolic machinery for glycoconjugate synthesis. In terms of present knowledge, it appears reasonable to hypothesize that the new antigens are proteins and that they may correspond, at least in part, to the "new" proteins already discussed.

It appears most improbable that the new host-cell plasma membrane antigens represent fragments of normal membrane proteins, although conclusive proof of the alternative, that they are parasite products, would, in our system, require their metabolic labelling. However, if the antigens are parasite products, it must be asked why are they not detected in the schizont preparations used to immunize the monkeys?
Parasite and parasitophorous vacuole membranes are disrupted by complete Freund's adjuvant (immunization) as well as by Triton X-100 (immunoelectrophoresis). The following working hypothesis should be considered: (a) during intraerythrocytic maturation, *P. knowlesi* synthesizes potentially antigenic components; (b) at least four of these are either exported from the parasite immediately after synthesis and/or their antigenic sites are blocked in the schizont; (c) the exported proteins are bound to the host-cell membrane in antigenic form. If antigenicity is masked in the schizont, unmasking would have to occur after export, either by erythrocyte or plasma enzymes. The postulated parasite-induced proteins/antigens might subserve functions essential for intraerythrocytic maturation. For example, the normal erythrocyte membrane may lack the transport capabilities required for the metabolically highly-active parasite and these needs might be met by parasite-derived transport systems inserted into the erythrocyte membrane, where they would be detected as an antigen(s). The validity of this hypothesis is accessible to experimental test.

The presence of parasite-specific antigens in host-cell membranes may be relevant to the development of anti-malarial vaccines. Immunization is considered to be one of the few feasible approaches to malaria, but the search for vaccine antigens has heretofore focused on proteins associated directly with malaria parasites. However, the observation that immunized animals can produce high titre antisera 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.

The above notion is speculative. Nevertheless it can be tested in a synchronized system such as *P. knowlesi* rhesus monkeys, using either direct vaccination and/or passive serum transfer.

5. SUMMARY

Highly purified *Plasmodium knowlesi* schizonts were isolated and used to produce a hyperimmune antiparasite 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 analysed by bidimensional protein fractionation techniques.

Of seven parasite-specific antigens identified in membranes of parasitized erythrocytes, using crossed immune electrophoresis against monkey antiparasite serum, only three could be detected in the purified schizonts. Membranes of normal erythrocytes and uninfected erythrocytes which had been incubated with sera from monkeys with 25-50% parasitaemia, did not react with the monkey anti-parasite serum.

Isoelectric focusing of membranes from parasitized cells, revealed components, at pI 4.2, 4.3, 4.6 and 4.7, which are found in the parasite, but not in normal membranes. A dominant group of membrane proteins above pI 6.0, probably representing spectrin, was greatly reduced in parasitized cells. Concordant results were obtained by comparison of normal and infected membranes by bidimensional focusing-immune electrophoresis, using rabbit antiserum against normal monkey erythrocytes. Bidimensional focusing-dodecyl sulfate polyacrylamide gel electrophoresis of membranes from parasitized cells revealed three proteins, in the 55 000-90 0000 molecular weight region, with isoelectric points between pI 4.5 and pI 5.2, which could not be detected in normal membranes or purified schizonts.

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RESUME

Après avoir isolé des schizontes de Plasmodium knowlesi hautement purifiés, on s'en est servi pour produire un sérum antiparasitaire hyperimmun chez un singe rhésus.

Des protéines de membranes d'érythrocytes normaux et d'érythrocytes infectés par P. knowlesi et de schizontes purifiés ont été solubilisées dans du Triton X-100 à 1 % et analysées au moyen des techniques de fractionnement bidimensionnel des protéines.

Des sept antigènes parasito-spécifiques identifiés au moyen de l'immunoélectrophorèse croisée contre le sérum antiparasitaire de singe dans les membranes des érythrocytes parasités, trois seulement ont été trouvés dans les schizontes purifiés. Les membranes des érythrocytes normaux et des érythrocytes non infectés qui avaient été incubés avec des sérum de singes présentant une parasitémie de 25 à 50 %, n'ont pas réagi avec le sérum antiparasitaire de singe.

La focalisation isoelectrique des membranes des cellules parasitées a révélé des éléments de pH 4,2; 4,3; 4,6; et 4,7, valeurs que l'on observe chez les parasites mais non dans les membranes normales. Un groupe dominant de protéines de membranes ayant un pH supérieur à 6, et représentant probablement la spectrine, était fortement réduit dans les cellules parasitées. On a obtenu des résultats concordants en comparant des membranes normales avec des membranes infectées, cela au moyen de la focalisation bidimensionnelle/immunoélectrophorèse, effectuée avec un antisérum de lapin contre des érythrocytes normaux de singes. La focalisation bidimensionnelle/électrophorèse en gel de polyacrylamide-dodécyl sulfate des membranes des cellules parasitées a fait apparaître trois protéines de masse moléculaire se situant entre 55 000 et 90 000, avec des points isoelectriques entre 4,5 et 5,2, qui n'ont pu être décelées dans les membranes normales ou dans les schizontes purifiés.

REFERENCES


FIG. 1. BIDIMENSIONAL ISOELECTRIC FOCUSING-IMMUNE ELECTROPHORESIS OF MEMBRANE PROTEINS FROM NORMAL ERYTHROCYTES (A) AND PARASITIZED ERYTHROCYTES (B)

350 µg of protein was focused in polyacrylamide/Triton/urea/ampholytes, at pH 3.5-10.0 (4%/1%/8%/2%) and then electrophoresed into agarose containing rabbit antiserum against normal monkey erythrocyte membranes (67 µl/1 ml agarose). The pH-gradient common for plates A and B is shown in plate B. The immune precipitates are numbered starting with 1 at the alkaline end of the pH gradient. Coomassie blue staining of immunoplates.
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/l ml of agarose). Immune precipitates are numbered 1 to 7 according to their electrophoretic mobility in the first dimension. Coomassie blue staining of 50 x 50 mm immunoplates.

FIG. 3. CROSSED IMMUNE ELECTROPHORESIS OF PROTEINS FROM P. KNOWLESI SCHIZONTS, DISSOLVED IN 1% TRITON X-100

About 75 μg proteins were separated electrophoretically in the first dimension (horizontal) and then electrophoresed at right angles into agarose, containing monkey anti-parasite serum (0.18 ml/l ml of agarose). The immune precipitates are numbered 1, 3 and 7 as in Fig. 1. Coomassie blue staining of 50 x 50 mm immunoplates.
FIG. 4. BIDIMENSIONAL IMMUNE ELECTROPHORESIS OF MEMBRANES FROM PARASITIZED MONKEY ERYTHROCYTES

Proteins (150 µg) solubilized 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 monkey anti-parasite serum. Coomassie blue staining of 50 x 50 mm immunoplates.

A. Crossed-line immune electrophoresis with 400 µg of proteins from uninfected erythrocytes in the intermediate strip (I; 50 x 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). Component 1 in the parasite is too low in concentration to cause a precipitation line or to displace anodally component 1 predominant in membranes of parasitized erythrocytes.
FIG. 5. ISOELECTRIC FOCUSING OF MEMBRANES OF NORMAL AND PARASITIZED MONKEY ERYTHROCYTES AND OF SCHIZONTS

Triton X-100 solubilized proteins (120 µg) were focused in 4% acrylamide/bisacrylamide containing 1% Triton, 8M urea and 2% ampholytes, pH 3.5-10.0. The abscissa gives the pH gradients and the ordinate indicates the absorbance for Coomassie blue staining at 620 nm.

A. Membranes of normal erythrocytes.
B. Membranes of parasitized erythrocytes.
C. Purified schizonts.
FIG. 6. BIDIMENSIONAL ISEOELECTRIC FOCUSING-DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

A. Membranes of normal monkey erythrocytes.
B. Membrane proteins of cells parasitized by *P. knowlesi*.
C. Purified schizonts.

The abscissa and the ordinate give the pH gradient and the apparent molecular weights, respectively. In all cases, 350-600 µg protein, solubilized in 1% Triton X-100, was focused in 4% acrylamide-bis-acrylamide, containing 2% ampholytes (Ampholine 3.5-10.0), 8M urea, 1% Triton X-100. Prior to DS polyacrylamide gel electrophoresis in the second dimension the focused proteins were equilibrated with DS and DTT in the gel and positioned atop the acrylamide gradient gel (4-30%). One dimensional cylindrical focusing gels shown in A, B and C were laden with 100 µg of protein. Coomassie blue protein staining of cylindrical and slab gels. Results from three separate infections and fractionations.

D. Proteins in normal erythrocyte membranes deleted or diminished in membranes of parasitized cells.
E. Protein components common to membranes of parasitized erythrocytes and purified parasites.
F. Proteins unique to membranes of parasitized cells.