PROTECTIVE *PLASMODIUM KNOWLESI* M_r 74,000 ANTIGEN IN MEMBRANES OF SCHIZONT-INFECTED RHESUS ERYTHROCYTES

BY R. SCHMIDT-ULLRICH, J. LIGHTHOLDER, AND M. T. M. MONROE

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

The protective properties of antiplasmodial antibodies in malaria have been well established (1–3). It has also been shown that primate hosts can be protected against potentially lethal plasmodium infections by vaccination with purified whole blood-stage parasites and adjuvants (4–8). More recently, sera of hosts protected against malarial infections (9–12) and monoclonal antibodies against defined plasmodium antigens (13–15) have been used to identify plasmodium polypeptides that might be used for vaccination against malaria.

Monoclonal antibodies against a *Plasmodium yoelii* M_r 230,000 merozoite antigen have been shown to confer passive protection when injected into BALB/c mice infected with *P. yoelii* malaria (14). A subsequent study showed that repeated inoculation with the M_r 230,000 component and lower M_r proteins derived therefrom in complete Freund's adjuvant (CFA)[1] protected against otherwise fatal blood-induced challenges with *P. yoelii* (16). Passive protection of BALB/c mice by monoclonal antibodies against the *P. berghei* M_r 44,000 sporozoite surface protein (13) suggests that this antigen can confer protection against sporozoite-induced infections in mice. The protective nature of a *P. lophurae* histidine-rich protein (17) is under debate (18, 19). Evidence about antigens that might protect man against *P. falciparum* is indirect and derives from demonstration of antibody-mediated inhibition of parasite growth in vitro (9, 15). Two candidate antigens, of approximately 82,000 and 96,000 M_r, have been identified in schizont-infected human erythrocytes by monoclonal antibody (20) and patient sera (9, 12), respectively.

We have identified an immunogenic *P. knowlesi* glycoprotein (11, 21) that is expressed on the surface of *P. knowlesi*-infected rhesus monkey erythrocytes (22), and migrates with an M_r of 74,000 in a discontinuous electrophoresis system (23). The presence of antibodies against this protein correlates positively with protection against *P. knowlesi* malaria (11) and we have now demonstrated that immunization with this protein can protect rhesus monkeys against potentially lethal infections with *P. knowlesi*.

In a limited vaccination trial of six rhesus monkeys, two animals were injected

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DS-PAGE, dodecyl sulfate-polyacrylamide gel electrophoresis; FCS, fetal calf serum; IEF, isoelectric focusing; IFA, incomplete Freund's adjuvant; PMSF, phenylmethylsulfonyl fluoride; TPCK, L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/83/07/0146/13 $1.00
Volume 158 July 1983 146–158

with adjuvant only and four monkeys were immunized with electrophoretically purified M_r 74,000 *P. knowlesi* antigen isolated from membranes of schizont-infected erythrocytes. Upon challenge with the W-1 variant of *P. knowlesi* the control monkeys developed fatal parasitemias within 7 d (24). The vaccinated monkeys, in contrast, limited the infections to peak parasitemias of between 7 and 11% 14–16 d after challenge. Immunochemical analyses of the sera during vaccination showed that antibodies reacted initially only with the M_r 74,000 antigen but, after the second and third booster, reacted also with related antigens of approximately 102,000, 140,000, and 230,000 M_r.

Materials and Methods

Reagents. Reagents for dodecyl sulfate-polyacrylamide gel electrophoresis (DS-PAGE) and isoelectric focusing (IEF) in polyacrylamide were as described before (11, 22–25). Bovine serum albumin (BSA), Triton X-100, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co., St. Louis, MO. Hypaque (Na-diatrizoate) and chloroquine (Aralene) were purchased from Winthrop Laboratories, New York; Ficoll and protein A-Sepharose 4B from Pharmacia Fine Chemicals, Piscataway, NJ. Lactoperoxidase, from Boehringer Mannheim Biochemicals, Indianapolis, IN, in 3.2 M $(NH_4)_2SO_4$ was freed of ammonium sulfate by dialysis against the buffer used for labeling. L(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone (TPCK)-trypsin was from Worthington Biochemical Corp., Freehold, NJ. RPMI 1640 with and without methionine was obtained from the Tufts Cancer Center, Boston, MA, and dialyzed fetal calf serum (FCS) from Gibco Laboratories, Grand Island, NY. [^{35}S]methionine (>400 Ci/mmol), Na^{125}I (carrier free in 0.1 M NaOH, 17 Ci/mg), and Enhance were purchased from New England Nuclear, Boston, MA. CFA and incomplete Freund's adjuvant (IFA) were obtained from Difco Laboratories, Detroit, MI. Chloramine T and X-Omat AR X-ray film were from Eastman Kodak Co., Rochester, NY, and cellulose thin layer plates (Polygram Cell 300) were from Brinkmann Instruments, Inc., Westbury, NY.

Monkeys and Plasmodium Strains. Male U. S.-bred rhesus monkeys (Macaca mulatta), ~4 kg body weight, were obtained from Thorsen Breeding Laboratories, Enosburg Falls, VT. Two strains, the H strain of Malaysian origin and the W-1 variant of the W strain (24, 26) of *P. knowlesi*, were used. Both strains were passed in nonsplenectomized rhesus monkeys only. The H strain (maintained at the National Institutes of Health [NIH]) and the W-1 variant were kindly provided by Dr. J. W. Barnwell (27) and Dr. S. Cohen (24), respectively. The NIH isolate was used to purify the M_r 74,000 antigen and the vaccinated rhesus monkeys were challenged with parasites of the W-1 variant. The parasitemia was monitored using methanol-fixed blood smears stained with Giemsa blood stain.

Metabolic Labeling and Solubilization of Plasmodium Antigens. Purified schizont-infected erythrocytes (22, 28) were adjusted to a hematocrit of 10% using methionine-free RPMI 1640 fortified with 10% dialyzed, heat-inactivated FCS. The cells were incubated at a [^{35}S]methionine concentration of 0.2 mCi/ml for 3 h. The incubator was maintained at 37°C and equilibrated with humidified $O_2/CO_2/N_2$ (5.5%:5%:89.5%, vol/vol). After labeling, the parasitized erythrocytes were washed three times in RPMI 1640, 0.01 M, in unlabeled methionine and then disrupted by nitrogen decompression (22, 28). Subcellular fractionation into parasites and host cell membranes was as described elsewhere (29).

For immune precipitation the plasmodium proteins were solubilized by two sequential extractions in 0.01 M Hepes, 1% Triton X-100 (vol/vol), 0.002 M PMSF, pH 8.7 (21). The solubilization buffer for proteins to be fractionated by DS-PAGE was 2% (wt/vol) 0.08 M DS, dithiothreitol (DTT), 0.02 M Tris-HCl, 10% glycerol (vol/vol), pH 6.8 ("sample buffer").

Immunochemical Reagents and Immunoprecipitation. Sera were drawn from the vaccinated rhesus monkeys before immunization, and 7 to 10 d after each of the second and third immunizations and after intravenous challenge with antigen. Immunoglobulin (Ig) was purified (30) from all sera and adjusted to 2.5 times the serum concentration. For the

immune precipitation of the M_r 74,000 plasmodium protein, we used immune Ig from rhesus monkey 153 (21, 22).

For analytical immune precipitations, 0.1 mg of [^{35}S]methionine-labeled, Triton X-100-solubilized plasmodium antigen (about 10^6 cpm) was first reacted with 0.02 ml of nonimmune Ig and 0.08 ml of 50% (vol/vol) hydrated protein A-Sepharose 4B for 60 min. The preabsorbed antigen was then equilibrated with 0.02 ml of immune Ig for 90 min, at 4°C and 37°C, followed by an incubation with 0.08 ml of protein A-Sepharose at 4°C for 60 min. Proteins bound nonspecifically were eluted from the protein A-Sepharose by three washes in 0.13 M NaCl, 0.02 M Tris-HCl, 1% Triton X-100 (vol/vol); 0.01 M methionine, 0.02% BSA (wt/vol), pH 8.0 ("washing buffer"), one wash in washing buffer containing LiCl (0.5 M), and one in washing buffer without BSA. In control experiments the antigen was incubated with preimmune Ig for both reactions.

For the large scale immune precipitation, five 10-mg aliquots of Triton X-100-extracted membrane antigens (90% unlabeled and 10% [^{35}S]methionine-labeled protein; see below) were preadsorbed as described above and then reacted with 2 ml of immune Ig for 90 min at 37 and 4°C. The antigen-antibody complexes were deposited using 5 ml of a 50% (vol/vol) protein A-Sepharose. The protein A-Sepharose beads were washed extensively as described above. Specifically bound antigens were eluted with sample buffer and fractionated on analytical or preparative scale DS-polyacrylamide slab gels (23). After electrophoresis the gels were dried without fixation and the protein was localized by autoradiography.

Isolation of the M_r 74,000 P. knowlesi Component. The M_r 74,000 protein was isolated from purified membranes of *P. knowlesi*-infected rhesus erythrocytes (containing 4–6 nuclei schizonts). Of the 4×10^{11} cells used for subcellular fractionation (28, 29, 31), 4×10^{10} were labeled metabolically with [^{35}S]methionine as described above. One half of the host cell membrane proteins were solubilized by two sequential extractions in Triton X-100 and immune precipitated with rhesus immune Ig (monkey 153) (11); the other half was equilibrated in sample buffer. The proteins of both samples were fractionated on multiple slab gels by DS-PAGE (23). ^{35}S autoradiograms of the dried, unfixed gels were used to identify the M_r 74,000 plasmodium protein that was isolated in two separate batches; one from gels in which the DS-solubilized host cell membrane proteins were fractionated, the other from gels containing the immune-precipitated parasite protein. The gel strips containing the protein were recovered, rehydrated in siliconized glass tubes using Na-phosphate (0.005 M), pH 8.0. The extraction was terminated when 70–80% of the protein was extracted by three sequential equilibrations of the gel pieces in Na-phosphate/DS (0.005 M:0.1%,wt/vol), pH 8.0. The protein was quantitated fluorometrically (32).

The purity of the M_r 74,000 component was assessed by reelectrophoresis and IEF (25, 30) of the [^{35}S]methionine-labeled protein or by the chloramine T technique after ^{125}I labeling. The latter procedure was chosen as a most sensitive tool to search for possible protein contaminants that were derived from the host cell membrane and were not metabolically labeled with [^{35}S]methionine.

Vaccination of Rhesus Monkeys. Six male rhesus monkeys (Nos. 163–166, 170, and 171) previously unexposed to malaria were selected for the vaccination trial with the M_r 74,000 protein. The extracted proteins, electrophoretically purified protein (0.6 mg) and immune-precipitated sample (0.5 mg), were each split into eight aliquots for immunization of rhesus monkeys Nos. 165 and 166, and Nos. 170 and 171, respectively. For each immunization, 0.1 ml of antigen (0.07 mg) was mixed with 0.15 ml CFA and injected half intramuscularly and half subcutaneously at multiple sites. The injections were repeated with IFA for the first and second booster immunization given at 3-wk intervals. The third booster immunization was given intravenously without IFA 3 wk later, 10 d before challenge of the monkeys with 10^4 freshly drawn schizont-infected erythrocytes (W-1 variant of *P. knowlesi*). Rhesus monkeys 163 and 164 were injected according to the same schedule with CFA and IFA only and challenged with the same number of viable parasites.

^{125}I-labeling of Plasmodium Proteins. After DS-PAGE the proteins were localized by [^{35}S]methionine autoradiography and isolated in narrowly cut gel strips. They were

subjected to formic acid oxidation before they were iodinated and digested by TPCK-trypsin. Performic acid was produced by co-incubation of formic acid and 30% H_2O_2 (19:1, vol/vol) for 150 min at 0°C. The protein-containing gel pieces were then incubated in performic acid/methanol (8:2, vol/vol) for 30 min at 4°C. The chloramine T technique (33) was used to achieve high specific activity ^{125}I labeling of cross-reacting plasmodium components with M_r of approximately 74,000, 102,000, 140,000, and 230,000. The iodination was performed in polyacrylamide (34) with modifications as described (25). Triton X-100-solubilized proteins from membranes of schizont-infected erythrocytes were labeled with ^{125}I using lactoperoxidase and H_2O_2 (22) and used for immune precipitation with vaccination sera.

Trypsin Digestion of Plasmodium Antigens. For cleavage with TPCK-trypsin, dried gel pieces containing ^{125}I-labeled plasmodium M_r 74,000, 102,000, 140,000, and 230,000 proteins were hydrated with 0.05 ml of NH_4HCO_3 (0.05 M), pH 7.8. TPCK-trypsin (0.5 mg/ml) in 0.05 M NH_4HCO_3 was added in the same volume that was required for complete hydration of gel pieces. Trypsinization was for 40 h at 37°C with a second addition of 0.05 mg TPCK-trypsin after 16 h. Controls were run as described (25).

Peptide Mapping. Two-dimensional thin layer peptide mapping was used to define the positions of peptides in a two-dimensional grid. About 5×10^5 cpm ^{125}I-labeled peptides were applied to a 10 × 10-cm thin layer chromatography plate near the cathode. The plate was then moistened with acetic acid/formic acid/H_2O (15%:5%:80%, vol/vol) and a dye mixture of orange G/acid fuchsin (2%:1%, wt/vol) spotted near the anode. Electrophoresis in the first dimension, at 400 V, was terminated when the dye reached the sample spot. The plates were dried under cold air. Chromatography in butanol/pyridine/acetic acid/H_2O (32.5:25:5:20, vol/vol) was at right angles to the first dimension. After drying, the peptide maps were developed by ^{125}I autoradiography.

To prove the identity of peptides derived from the immune-precipitated plasmodium components with M_r of approximately 74,000, 102,000, 140,000, and 230,000, we localized individual peptides by ^{125}I autoradiography. The peptides were scraped off the cellulose thin layer plates and eluted from the cellulose with acetic acid/formic acid/water (15%:5%:80%, vol/vol). In all reelectrophoresis-rechromatography experiments, identical amounts of peptides, as determined by ^{125}I radioactivity were rerun on thin layer plates.

Results

Isolation and Purity of the M_r 74,000 P. knowlesi Antigen. Electrophoretic isolation of the M_r 74,000 protein from membranes of schizont-infected erythrocytes and purification by immune precipitation followed by DS-PAGE both yielded ~0.5 mg from 50 mg of membrane protein. This indicates that the protein represents ~1% of the total membrane protein. The M_r 74,000 component could be purified electrophoretically (with or without immune precipitation) from most gels as a homogeneous component yielding a single band upon reelectrophoresis. This was true whether the protein was monitored by metabolically incorporated [^{35}S]methionine or by extrinsic ^{125}I labeling (Fig. 1, lanes 2, 5, and 6).

As is shown in Fig. 2, the ^{125}I-labeled, purified M_r 74,000 protein focused in polyacrylamide near an isoelectric point (pI) of 5.2. Identical results were obtained whether the protein was isolated by DS-PAGE only or by immune precipitation and DS-PAGE. Neither DS-PAGE nor IEF of the metabolically labeled and extrinsically iodinated M_r 74,000 protein showed detectable contamination by other plasmodium and/or host cell polypeptides.

Vaccination. The intravenous inoculation of 10^4 freshly drawn *P. knowlesi* schizont-infected rhesus erythrocytes produced potentially fatal parasitemias in

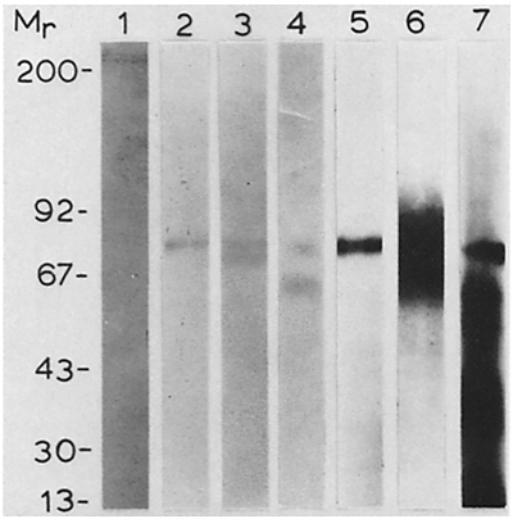

FIGURE 1. DS-PAGE of the purified *P. knowlesi* M_r 74,000 and 230,000 proteins from membranes of schizont-infected rhesus erythrocytes. M_r, $\times 10^{-3}$. Lane 1: M_r 230,000 protein, [^{35}S]methionine autoradiogram; lane 2: M_r 74,000 protein, formic acid oxidation, Coomassie blue staining; lane 3: M_r 230,000 protein, formic acid oxidation, Coomassie blue staining; lane 4: M_r 230,000 protein, formic acid oxidation, [^{35}S]methionine autoradiogram; lane 5: M_r 74,000 protein (10^5 cpm), formic acid oxidation and ^{125}I-labeling with chloramine T, ^{125}I autoradiogram; lane 6: M_r 74,000 protein (5×10^6 cpm), formic acid oxidation and ^{125}I-labeling with chloramine T, ^{125}I autoradiogram; lane 7: M_r 230,000 protein, formic acid oxidation and ^{125}I-labeling with chloramine T, ^{125}I autoradiogram.

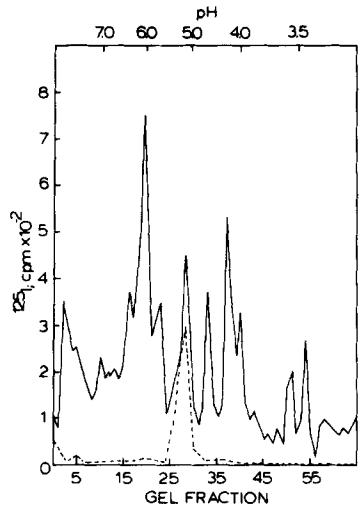

FIGURE 2. IEF of *P. knowlesi* proteins from membranes of schizont-infected rhesus erythrocytes. The membrane proteins and the purified M_r 74,000 component were labeled with ^{125}I using the chloramine T technique. Abscissas give the pH gradient and the gel fractions, the ordinate the ^{125}I radioactivity (cpm). (—) membranes of infected erythrocytes; (– – –) purified M_r 74,000 protein.

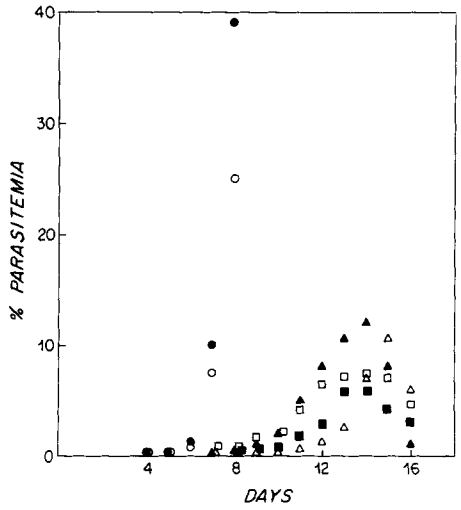

FIGURE 3. *P. knowlesi* (W-1-variant) infections in rhesus monkeys vaccinated with the *P. knowlesi* M_r 74,000 protein (see text for details). Abscissa gives the days after challenge of the monkeys with 10^4 viable *P. knowlesi* schizonts. Ordinate indicates the percent parasitemias. (○), (●) rhesus monkeys 163 and 164 injected with complete adjuvant only; (△), (▲) rhesus monkeys 165 and 166 vaccinated with electrophoretically purified *P. knowlesi* M_r 74,000 protein; (□), (■) rhesus monkeys 170 and 171 vaccinated with immunoprecipitated, electrophoretically purified *P. knowlesi* M_r 74,000 protein.

rhesus monkeys 163 and 164, injected with adjuvants only. In these two monkeys a patent parasitemia of ~0.1% was first detected on days 4 or 5 (Fig. 3). Within 7 d, the parasitemia rose to lethal levels of 25 and 39% for rhesus monkeys 163 and 164, respectively. At this time, the animals were rescued by chloroquine injections and transfusion of ~40 ml of packed, washed rhesus erythrocytes.

After inoculation with 10^4 viable schizont-infected erythrocytes, the four rhesus monkeys (Nos. 165, 166, 170, and 171) that were immunized with the *P. knowlesi* M_r 74,000 protein exhibited a delayed onset of patent parasitemia (~0.1%) on days 8 or 9 postinoculation. After an additional 6–7 d, the parasitemias peaked between 7 and 11% (Fig. 3). Thereafter, all four monkeys cleared the parasites spontaneously and recovered without drug treatment. Only monkey 166 developed an anemia on day 16 postinoculation (hematocrit of 22%), at a time of decreasing parasitemia. On day 17, he required a transfusion of 20 ml washed, packed erythrocytes, but no chloroquine treatment. Monkeys 165, 170, and 171 underwent self-cure with anemias at expected levels. Weekly monitoring of all monkeys for four more months did not reveal recrudescent plasmodium infections.

With the initiation of self-cure of monkeys 165, 166, 170, and 171, no schizonts were seen in the peripheral blood. However, a low level of reinfection was suggested by the persistence of (decreasing) concentrations of predominantly normal ring and trophozoite stages. The more mature trophozoites appeared pycnotic.

Antigen Reactivity of the Vaccination Sera. No appreciable differences in qualitative antibody responses to the M_r 74,000 antigen could be observed in the four vaccinated rhesus monkeys, 165, 166, 170, and 171. The results shown were

obtained with pooled sera. None of the preimmune Ig nor Ig from rhesus monkeys 163 and 164 specifically deposited any *P. knowlesi* component (Fig. 4, lane 4). After the first booster immunization, only the M_r 74,000 protein was precipitated from membranes of *P. knowlesi*-infected rhesus erythrocytes (Fig. 4, lanes 1 and 7). However, Ig obtained after the second and third booster immunization deposited *P. knowlesi* components with M_r near 230,000, 140,000, and 102,000, in addition to the M_r 74,000 protein (Fig. 4, lanes 2, 3, and 8). The apparent antigenic relatedness of these proteins was further examined by tryptic peptide mapping.

Peptide Homology Between the M_r 74,000 Proteins and Immune Precipitated Higher M_r Components. Performic acid oxidation before extrinsic iodination and tryptic peptide mapping resulted in a reproducible degradation of the M_r 230,000 protein into a M_r 74,000 and into components with M_r between 70,000 and 65,000 (Fig. 1, lanes 1, 3, 4, and 7).

Oxidation and chloramine T radioiodination degraded the M_r 230,000 component into a M_r 74,000 protein and components of smaller M_r. In contrast, the M_r 74,000, 102,000, and 140,000 components were not degraded by oxidative denaturation.

The structural relatedness between the M_r 230,000, 140,000, 102,000, and 74,000 components was demonstrated by the high degree of homology in their

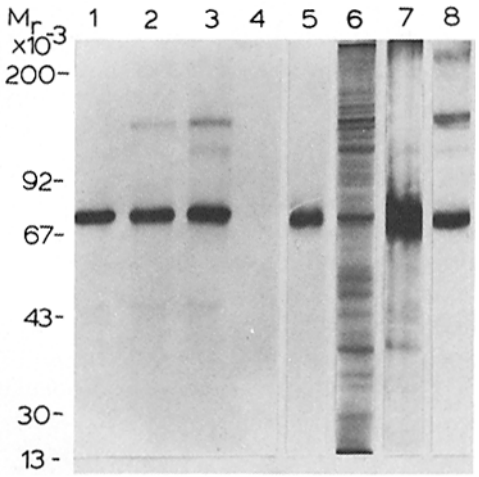

FIGURE 4. DS-PAGE of *P. knowlesi* protein specifically precipitated by immune Ig from sera of vaccinated rhesus monkeys. Constant amounts of membrane protein (0.1 mg) from [^{35}S]-methionine-labeled *P. knowlesi*-infected rhesus erythrocytes were preincubated with Ig from preimmune serum and protein A-Sepharose followed by specific deposition of the antigen by immune Ig and protein A-Sepharose. Lanes 1–6: [^{35}S]methionine autoradiograms; lanes 7 and 8: ^{125}I autoradiograms. Lane 1: *P. knowlesi* proteins deposited by Ig after the 2nd vaccination; lane 2: *P. knowlesi* proteins deposited by Ig after the 3rd vaccination; lane 3: *P. knowlesi* proteins deposited by Ig after four injections of the M_r 74,000 protein; lane 4: double immune precipitaton of *P. knowlesi* proteins as in 1–3, but using Ig from preimmune serum only (control); lane 5: purified M_r 74,000 protein used for vaccination; lane 6: [^{35}S]methionine labeled proteins from membranes of schizont-infected rhesus erythrocytes; lane 7: *P. knowlesi* proteins, extrinsically labeled with ^{125}I and chloramine T, deposited by Ig after the 2nd injection of the M_r 74,000 protein; lane 8: *P. knowlesi* proteins, extrinsically labeled with ^{125}I and chloramine T, deposited by Ig after the 4th injection of the M_r 74,000 protein.

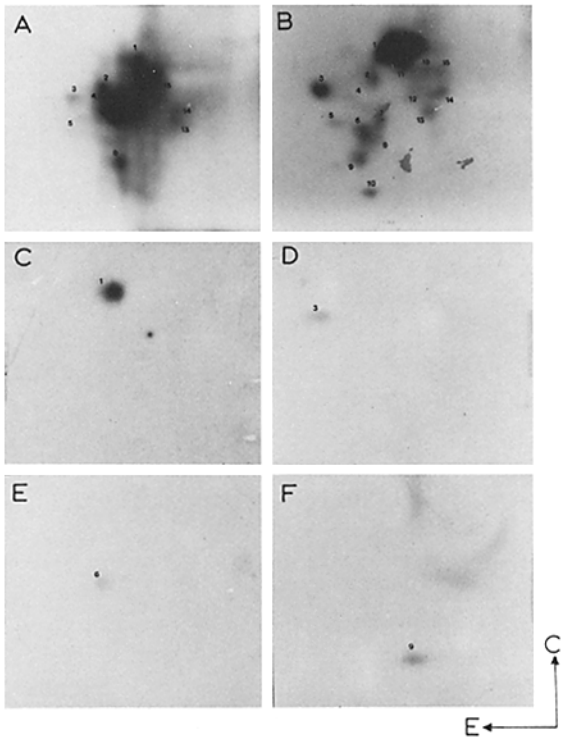

FIGURE 5. Two-dimensional thin layer peptide maps of *P. knowlesi* M_r 74,000 and 230,000 proteins from membranes of schizont-infected rhesus erythrocytes. The proteins were denatured by formic acid oxidation, labeled with ^{125}I using the chloramine T technique, and digested with TPCK-trypsin. ^{125}I autoradiograms of (*A*) tryptic peptides of the M_r 74,000 protein, (*B*) tryptic peptides of the M_r 230,000 protein, (*C–F*) reelectrophoresis/rechromatography of peptides 1,3, 6, and 9 isolated and combined from maps of both the M_r 74,000 and 230,000 proteins. The peptides of the M_r 230,000 protein that yields the largest number of peptides are labeled 1 through 16. The peptides numbered in the M_r 74,000 protein map are identical to those of the 230,000 protein, as exemplified for peptides 1, 3, 6, and 9.

^{125}I-tryptic peptide maps (Figs. 5 and 6). As the M_r 230,000 yielded the largest number of peptides, the map of this protein was used to assign a number to each peptide. Identical numbers in maps of components with M_r near 140,000, 102,000, and 74,000 indicate that these peptides are identical to those of the M_r 230,000 protein as proven by co-migration of the isolated peptides upon reelectrophoresis-rechromatography. These results are illustrated for the M_r 230,000 and 74,000 antigens in Fig. 5, *C–F* on peptides 1, 3, 6, and 9, and on the same peptides for all four antigens in Fig. 6, *C–F*.

Discussion

Immunization of four rhesus monkeys with a M_r 74,000 *P. knowlesi* protein purified from membranes of schizont-infected rhesus erythrocytes conferred protection against otherwise fatal blood-induced infections with the W-1 variant of *P. knowlesi* (24). While 10^4 viable W-1 schizonts produced fatal infections within 7–8 d in two rhesus monkeys that were injected with adjuvant alone,

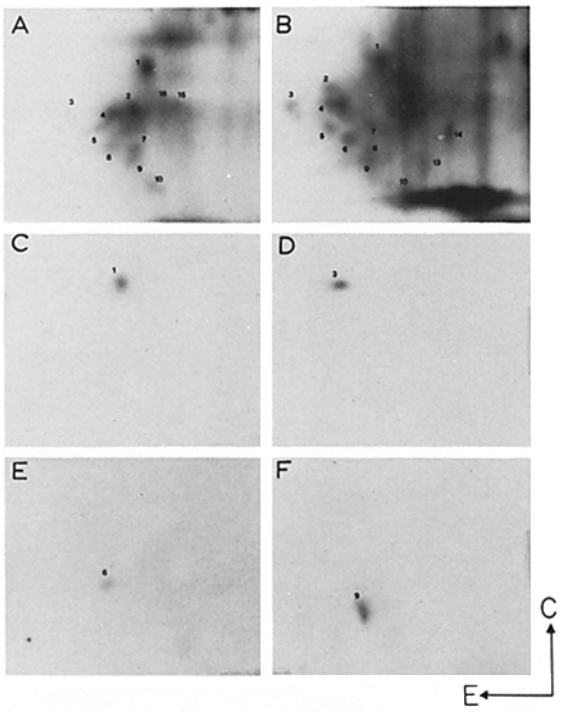

FIGURE 6. Two-dimensional thin layer peptide maps of *P. knowlesi* M_r 102,000 and 140,000 proteins from membranes of schizont-infected rhesus erythrocytes. The proteins were denatured by formic acid oxidation, labeled with ^{125}I using the chloramine T technique, and digested with TPCK-trypsin. ^{125}I autoradiograms of (*A*) tryptic peptides of the M_r 102,000 protein, (*B*) tryptic peptides of the M_r 140,000 protein, (*C–F*) reelectrophoresis/rechromatography of peptides 1, 3, 6, and 9 isolated and combined from maps of the components with M_r near 74,000, 102,000, 140,000, and 230,000. The peptides numbered in the maps of M_r 102,000 and 140,000 proteins are identical to those of the 230,000 protein (see Fig. 5) as exemplified for peptides 1, 3, 6, and 9.

protection of the vaccinated monkeys was indicated by delayed onset of patent parasitemia and maximum parasitemias between only 7 and 11% on days 14 and 15 post-challenge. During the period of self-cure, limited reinfection was indicated by the presence of normal ring and trophozoite stages, but no schizont-infected erythrocytes were seen in the peripheral blood. Immunochemical evaluation of the vaccination sera revealed that during the first two immunizations antibody production was confined to the M_r 74,000 component. Further immunizations, irrespective of the antigen purification technique, induced antibodies that reacted not only with the M_r 74,000 protein but also with components of M_r near 102,000, 140,000, and 230,000. Tryptic peptide homology between these proteins is compatible with the immunological cross-reactivity.

Vaccination of rhesus monkeys with the M_r 74,000 *P. knowlesi* schizont antigen conferred protection against *P. knowlesi* similar to that described for a *P. yoelii* M_r 230,000 merozoite antigen in this murine malaria (16). Immunizations with purified antigens (15) and with various preparations of whole-blood-stage parasites (4–8) required transient parasitemias of a maximum of 15% before the

previously induced immunity led to self-control of the infections. The mechanism of protection contrasted to immunity with very low transient parasitemia that has been observed in some rhesus monkeys after vaccination with *P. knowlesi* merozoites (24, 35) and has led to the proposal that the complete interruption of plasmodium infections occurred at the merozoite stage (35). However, persistent low level parasitemias in our vaccinated rhesus monkeys during the period of self-cure indicated that the parasites were affected not only during merozoite release or reinvasion but also during schizont maturation. The specific action of anti-M_r 74,000 antibodies on *P. knowlesi* schizonts has been confirmed in in vitro experiments in which these antibodies significantly inhibited the maturation and biosynthetic activity of parasites (R. Schmidt-Ullrich, unpublished results). These data fit observations showing growth retardation of *P. falciparum* schizonts and inhibition of merozoite release when grown in vitro in the presence of sera from immune Aotus monkeys (36). Our own and others' results (36) suggest that antibodies against plasmodium antigens of schizont-infected erythrocytes could interrupt the erythrocyte schizogony at that stage.

In the absence of detectable contamination by other proteins, the most likely reason for immunochemical cross-reactivity between the M_r 74,000 component and the M_r 102,000, 140,000, and 230,000 proteins represented antigenic homology. Tryptic peptide analyses of these proteins indicated that a significant portion of the M_r 74,000 protein appeared to be contained in the M_r 230,000 component. In addition, this protein could, upon oxidative denaturation, be disintegrated into polypeptides with M_r near and below 74,000. Considering the peptide homology between the M_r 74,000 protein and the M_r 102,000 and 140,000 components, we have to conclude that the M_r 230,000 protein represents a precursor that is processed by controlled proteolysis to smaller M_r components as has been documented for one other plasmodium species (16). The immune reactivity of the M_r 102,000, 140,000, and 230,000 components after more than two immunizations of rhesus monkeys with the M_r 74,000 protein is most likely due to an increased avidity and/or a broader specificity of the antibodies and to some structural heterogeneity of the reacting proteins.

We have demonstrated that the M_r 74,000 *P. knowlesi* protein in membranes of schizont-infected rhesus erythrocytes represents a protective antigen that induces antibodies active against the schizont-stage. We have also shown that the M_r 74,000 antigen is common to at least two different *P. knowlesi* strains (22), because the antigen for immunization was isolated from rhesus erythrocytes infected with the H strain while the animals were challanged with the heterologous W strain (24). These results confirm our previous immunochemical analyses showing that there are cross-reacting M_r 74,000 antigens in membranes of rhesus and human erythrocytes infected with different strains of *P. knowlesi* and *P. falciparum*, respectively (21, 22, 37). The homology between the M_r 74,000 proteins of *P. knowlesi* and *P. falciparum* is currently being investigated in more detail.

Summary

The immunogenic *Plasmodium knowlesi* (H strain) M_r 74,000 protein in membranes of schizont-infected rhesus erythrocytes was purified on a large scale, free

of other polypeptides as monitored by dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing. In a limited vaccination trial, four rhesus monkeys were immunized four consecutive times with the M_r 74,000 protein and Freund's complete and incomplete adjuvants. Two monkeys were injected with adjuvant only. Upon challenge with 10^4 viable *P. knowlesi* schizonts of the heterologous W strain, the control monkeys developed fatal parasitemias after 7 d. In contrast, the vaccinated monkeys exhibited a delayed onset of patent parasitemias and underwent self-cure on days 14 to 16 after peak parasitemias of between 7 and 11%. The protective immunity that was induced crossed different strains of *P. knowlesi*. Blood smears at the time of cure demonstrated limited reinfection, as indicated by the presence of normally appearing ring and trophozoite stages. The absence of schizont stages in the peripheral blood suggested a specific interruption of the erythrocytic schizogony at that stage. Immunochemical analyses of the rhesus sera revealed antibody only against the M_r 74,000 protein after the first two immunizations. Upon repeated antigen injection, antibodies reacted with components of M_r of approximately 102,000, 140,000, and 230,000 in addition to the M_r 74,000 protein. Besides immunological cross-reactivity, relatedness between all four immune-precipitated proteins was indicated by a >50% tryptic peptide homology, suggesting that the M_r 230,000 component represents a precursor protein that is cleaved within the infected erythrocyte into proteins with M_r of approximately 140,000, 102,000, and 74,000.

We thank Dr. D. F. H. Wallach for continuous interest in the progress of this work and for critical review of the manuscript.

Received for publication 11 February 1983.

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