PROTECTIVE PLASMODIUM KNOWLESI M, 74,000
ANTIGEN IN MEMBRANES OF SCHIZONT-INFECTED
RHECUS 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, 230,000 merozoite anti-
gen 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, 230,000 component and lower M, proteins derived
therefrom in complete Freund's adjuvant (CFA) 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, 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, 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, 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
with adjuvant only and four monkeys were immunized with electrophoretically purified \( M, 74,000 \) \( P. knowlesi \) 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 \( M, 74,000 \) 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 (Aralen) 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. \(\beta\)-(tosylamide-2-phenyl)-ethyl-chloromethyl-ketone (TPCK)-trypsin was from Worthington Biochemical Corp., Freehold, NJ. RPMI 1640 with and without methionine was obtained from Gibco Laboratories, Grand Island, NY. \[^{35}\text{S}]\)methionine (>400 Ci/mmol), Na\[^{222}\text{S}]\) (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), \(\sim 4\) 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 \( M, 74,000 \) 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}\text{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 Mr 74,000 plasmodium protein, we used immune Ig from rhesus monkey 153 (21, 22).

For analytical immune precipitations, 0.1 mg of [35S]methionine-labeled, Triton X-100-solubilized plasmodium antigen (about 10⁶ 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% [35S]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 Mr 74,000 P. knowlesi Component. The Mr 74,000 protein was isolated from purified membranes of P. knowlesi-infected rhesus erythrocytes (containing 4-6 nuclei schizonts). Of the 4 × 10¹¹ cells used for subcellular fractionation (28, 29, 31), 4 × 10¹⁰ were labeled metabolically with [35S]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). [35S] Autoradiograms of the dried, unfixed gels were used to identify the Mr 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 Mr 74,000 component was assessed by reelectrophoresis and IEF (25, 30) of the [35S]methionine-labeled protein or by the chloramine T technique after [35S]methionine 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 [35S]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 Mr 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 10⁴ 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.

[^125I] labeling of Plasmodium Proteins. After DS-PAGE the proteins were localized by [35S]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% H₂O₂
(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 ¹²⁵I labeling of cross-reacting plasmodium
components with M, 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 ¹²⁵I using lactoperoxidase and H₂O₂ (22) and used for immune precipitation
with vaccination sera.

Trypsin Digestion of Plasmodium Antigens. For cleavage with TPCK-trypsin, dried gel
pieces containing ¹²⁵I-labeled plasmodium M, 74,000, 102,000, 140,000, and 230,000
proteins were hydrated with 0.05 ml of NH₄HCO₃ (0.05 M), pH 7.8. TPCK-trypsin (0.5
mg/ml) in 0.05 M NH₄HCO₃ 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 × 10⁴ cpm ¹²⁵I-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/H₂O (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/H₂O (32.5:25:5:20, vol/vol) was at right angles to the first dimen-
sion. After drying, the peptide maps were developed by ¹²⁵I autoradiography.

To prove the identity of peptides derived from the immune-precipitated plasmodium
components with M, of approximately 74,000, 102,000, 140,000, and 230,000, we
localized individual peptides by ¹²⁵I 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 ¹²⁵I radioactivity were rerun on thin layer
plates.

Results

Isolation and Purity of the M, 74,000 P. knowlesi Antigen. Electrophoretic isolation
of the M, 74,000 protein from membranes of schizont-infected erythrocytes and purifica-
tion 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, 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 metab-
olically incorporated [³⁵S]methionine or by extrinsic ¹²⁵I labeling (Fig. 1, lanes 2,
5, and 6).

As is shown in Fig. 2, the ¹²⁵I-labeled, purified M, 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, 74,000 protein showed detectable contam-
ination by other plasmodium and/or host cell polypeptides.

Vaccination. The intravenous inoculation of 10⁴ freshly drawn P. knowlesi
schizont-infected rhesus erythrocytes produced potentially fatal parasitemias in
FIGURE 1. DS-PAGE of the purified *P. knowlesi* Mr 74,000 and 230,000 proteins from membranes of schizont-infected rhesus erythrocytes. *M*, × 10⁻³. Lane 1: *M*, 230,000 protein, [³⁵S]methionine autoradiogram; lane 2: *M*, 74,000 protein, formic acid oxidation, Coomassie blue staining; lane 3: *M*, 230,000 protein, formic acid oxidation, Coomassie blue staining; lane 4: *M*, 230,000 protein, formic acid oxidation, [³⁵S]methionine autoradiogram; lane 5: *M*, 74,000 protein (10⁵ cpm), formic acid oxidation and [³⁵S]-labeling with chloramine *T*, [³⁵S]-autoradiogram; lane 6: *M*, 74,000 protein (5 × 10⁶ cpm), formic acid oxidation and [¹²⁵I]-labeling with chloramine *T*, [¹²⁵I]-autoradiogram; lane 7: *M*, 230,000 protein, formic acid oxidation and [¹²⁵I]-labeling with chloramine *T*, [¹²⁵I]-autoradiogram.

FIGURE 2. IEF of *P. knowlesi* proteins from membranes of schizont-infected rhesus erythrocytes. The membrane proteins and the purified *M*, 74,000 component were labeled with [¹²⁵I] using the chloramine *T* technique. Abscissas give the pH gradient and the gel fractions, the ordinate the [¹²⁵I] radioactivity (cpm). (--) membranes of infected erythrocytes; (-- --) purified *M*, 74,000 protein.
FIGURE 3. P. knowlesi (W-1-variant) infections in rhesus monkeys vaccinated with the P. knowlesi Mr 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 Mr 74,000 protein; (□), (■) rhesus monkeys 170 and 171 vaccinated with immunoprecipitated, electrophoretically purified P. knowlesi Mr 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 Mr 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 recrudescence 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 Mr 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 \textit{P. knowlesi} component (Fig. 4, lane 4). After the first booster immunization, only the 74,000 protein was precipitated from membranes of \textit{P. knowlesi}-infected rhesus erythrocytes (Fig. 4, lanes 1 and 7). However, Ig obtained after the second and third booster immunization deposited \textit{P. knowlesi} components with \textit{Mr} near 250,000, 140,000, and 102,000, in addition to the 74,000 protein (Fig. 4, lanes 2, 3, and 8). The apparent antigenic relatedness of these proteins was further examined by tryptic peptide mapping.

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

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

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

\textbf{FIGURE 4.} DS-PAGE of \textit{P. knowlesi} protein specifically precipitated by immune Ig from sera of vaccinated rhesus monkeys. Constant amounts of membrane protein (0.1 mg) from \textit{[35S]-methionine-labeled} \textit{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: \textit{[35S]-methionine autoradiograms}; lanes 7 and 8: \textit{[125I]} autoradiograms. Lane 1: \textit{P. knowlesi} proteins deposited by Ig after the 2nd vaccination; lane 2: \textit{P. knowlesi} proteins deposited by Ig after the 3rd vaccination; lane 3: \textit{P. knowlesi} proteins deposited by Ig after four injections of the 74,000 protein; lane 4: double immune precipitation of \textit{P. knowlesi} proteins as in 1–3, but using Ig from preimmune serum only (control); lane 5: purified 74,000 protein used for vaccination; lane 6: \textit{[35S]-methionine labeled proteins from membranes of schizont-infected rhesus erythrocytes}; lane 7: \textit{P. knowlesi} proteins, extrinsically labeled with \textit{[125I]} and chloramine T, deposited by Ig after the 2nd injection of the 74,000 protein; lane 8: \textit{P. knowlesi} proteins, extrinsically labeled with \textit{[125I]} and chloramine T, deposited by Ig after the 4th injection of the 74,000 protein.
FIGURE 5. Two-dimensional thin layer peptide maps of *P. knowlesi* Mr 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 Mr 74,000 protein, (B) tryptic peptides of the Mr 230,000 protein, (C–F) reelectrophoresis/rechromatography of peptides 1, 3, 6, and 9 isolated and combined from maps of both the Mr 74,000 and 230,000 proteins. The peptides of the Mr 230,000 protein that yields the largest number of peptides are labeled 1 through 16. The peptides numbered in the Mr 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 Mr 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 Mr near 140,000, 102,000, and 74,000 indicate that these peptides are identical to those of the Mr 230,000 protein as proven by co-migration of the isolated peptides upon reelectrophoresis-rechromatography. These results are illustrated for the Mr 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 Mr 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, 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, 102,000 protein, (B) tryptic peptides of the M, 140,000 protein, (C–F) reelectrophoresis/rechromatography of peptides 1, 3, 6, and 9 isolated and combined from maps of the components with Mr near 74,000, 102,000, 140,000, and 230,000. The peptides numbered in the maps of M, 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, 74,000 component. Further immunizations, irrespective of the antigen purification technique, induced antibodies that reacted not only with the M, 74,000 protein but also with components of M, 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, 74,000 *P. knowlesi* schizont antigen conferred protection against *P. knowlesi* similar to that described for a *P. yoelii* M, 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, 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, 74,000 component and the M, 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, 74,000 protein appeared to be contained in the M, 230,000 component. In addition, this protein could, upon oxidative denaturation, be disintegrated into polypeptides with M, near and below 74,000. Considering the peptide homology between the M, 74,000 protein and the M, 102,000 and 140,000 components, we have to conclude that the M, 230,000 protein represents a precursor that is processed by controlled proteolysis to smaller M, components as has been documented for one other plasmodium species (16). The immune reactivity of the M, 102,000, 140,000, and 230,000 components after more than two immunizations of rhesus monkeys with the M, 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, 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, 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 challenged with the heterologous W strain (24). These results confirm our previous immunochemical analyses showing that there are cross-reacting *M*, 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*, 74,000 proteins of *P. knowlesi* and *P. falciparum* is currently being investigated in more detail.

**Summary**

The immunogenic *Plasmodium knowlesi* (H strain) M, 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 \textit{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 \textit{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. Immunological analyses of the rhesus sera revealed antibody only against the \textit{Mr} 74,000 protein after the first two immunizations. Upon repeated antigen injection, antibodies reacted with components of \textit{Mr} of approximately 102,000, 140,000, and 230,000 in addition to the \textit{Mr} 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 \textit{Mr} 230,000 component represents a precursor protein that is cleaved within the infected erythrocyte into proteins with \textit{Mr} 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.

References

1. Coggeshall, L. T., and H. W. Kumm. 1937. Demonstration of passive immunity in experimental monkey malaria. \textit{J. Exp. Med.} 66:177.
2. Cohen, S., I. A. McGregor, and S. Carrington. 1961. Gamma-globulin and acquired immunity to human malaria. \textit{Nature (Lond.).} 192:733.
3. Diggs, C. L., and A. G. Osler, 1975. Humoral immunity in rodent malaria. III. Studies on the site of antibody action. \textit{J. Immunol.} 114:1243.
4. Siddiqui, W. A., D. W. Taylor, S. C. Kan, K. Kramer, and S. M. Richmond-Crum. 1978. Vaccination of experimental monkeys against \textit{Plasmodium falciparum}: a possible safe adjuvant. \textit{Science (Wash. DC).} 201:1237.
5. Reese, R. T., W. Trager, J. B. Jensen, D. A. Miller, and R. Tantravahi. Immunization against malaria with antigen from \textit{Plasmodium falciparum} cultivated in vitro. \textit{Proc. Natl. Acad. Sci. USA.} 75:5665.
6. Gwadz, R. W., and I. Green. 1978. Malaria immunization in rhesus monkeys. A vaccine effective against both the sexual and asexual stage of \textit{Plasmodium knowlesi}. \textit{J. Exp. Med.} 148:1311.
7. Mendis, K. N., and G. A. T. Targett, 1979. Immunization against gametes and asexual erythrocytic stages of a rodent malaria parasite. \textit{Nature (Lond.).} 277:389.
8. Mitchell, G. H., G. A. Butcher, J. Langhorne, and S. Cohen. 1977. A freeze-dried merozoite vaccine effective against \textit{Plasmodium knowlesi} malaria. \textit{Clin. Exp. Immunol.} 28:276.
9. Brown, G. V., R. F. Anders, G. F. Mitchell, and P. F. Heywood. 1982. Target antigens of purified human immunoglobulin which inhibit growth of *Plasmodium falciparum*. *Nature (Lond.)*. 297:591.

10. Deans, J. A., E. D. Dennis, and S. Cohen. 1978. Antigenic analysis of sequential erythrocytic stages of *Plasmodium knowlesi*. *Parasitology*. 77:333.

11. Schmidt-Ullrich, R., L. H. Miller, D. F. H. Wallach, K. Powers, and R. Gwadz. 1981. Rhesus monkeys protected against *Plasmodium knowlesi* malaria produce antibodies against a Mr 65,000 plasmodium glycoprotein on the surface of infected erythrocytes. *Infect. Immun.*. 34:519.

12. Perrin, L. H., R. Dayal, and H. Rieder. 1981. Characterization of antigens from erythrocytic stages of *Plasmodium falciparum* reacting with human immune sera. *Trans. Roy. Soc. Trop. Med. Hgy.*. 75:163.

13. Potocnjak, P., H. Yoshida, R. S. Nussenzweig, and V. Nussenzweig. 1980. Monovalent fragments (Fab) of monoclonal antibodies to sporozoite surface antigen (Pb44) protects mice against malarial infection. *J. Exp. Med.*. 151:1504.

14. Freeman, R. R., A. J. Trejdosiewicz, and G. A. M. Cross. 1980. Protective monoclonal antibodies recognizing stage-specific merozoite antigens of a rodent malaria parasite. *Nature (Lond.)*. 284:366.

15. Perrin, L. H., E. Ramirez, P. H. Lambert, and P. T. Miescher. 1981. Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies. *Nature (Lond.)*. 289:301.

16. Holder, A. A., and R. R. Freeman. 1981. Immunization against blood-stage rodent malaria using purified parasite antigens. *Nature (Lond.)*. 294:361.

17. Kilejian, A. 1978. Histidine-rich proteins as a model malaria vaccine. *Science (Wash. DC.)*. 201:922.

18. Sherman, I. W. 1981. *Plasmodium lophurae*: protective immunogenicity of the histidine-rich protein? *Exp. Parasitol.*. 52:292.

19. Kilejian, A. 1981. *Plasmodium lophurae*. Immunogenicity of a histidine-rich protein. *Exp. Parasitol.*. 52:291.

20. Perrin, L. H., and R. Dayal. 1982. Immunity to asexual erythrocytic stages of *P. falciparum*: role of defined antigens in the humoral response. *Immunol. Rev.*. 61:245.

21. Schmidt-Ullrich, R., L. H. Miller, D. F. H. Wallach, and J. Lightholder. 1982. Immunogenic antigens common to *Plasmodium knowlesi* and *Plasmodium falciparum* are expressed on the surface of infected erythrocytes. *J. Parasitol.*. 68:185.

22. Schmidt-Ullrich, R., D. F. H. Wallach, and J. Lightholder, 1979. Two *Plasmodium knowlesi* antigens on the surface of schizont-infected rhesus monkey erythrocytes induce antibody production in the immune host. *J. Exp. Med.*. 150:86.

23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)*. 227:680.

24. Butcher, G. A., and S. Cohen. 1972. Antigenic variation and protective immunity in *Plasmodium knowlesi* malaria. *Immunology*. 23:503.

25. Schmidt-Ullrich, R., S. W. Thompson, S. J. Kahn, M. T. Monroe, and D. F. H. Wallach. 1982. Simian virus 40 (SV40)-specific isoelectric point-4.7-94,000 Mr membrane glycoprotein: major peptide homology exhibited with the nuclear and membrane-associated 94,000 Mr, SV40 T-antigen in hamsters. *J. Natl. Cancer Inst.*. 69:839.

26. Brown, K. N., and I. N. Brown. 1965. Immunity to malaria: antigenic variation in chronic infections of *Plasmodium knowlesi*. *Nature (Lond.)*. 208:1286.

27. Barnwell, J., R. J. Howard, and L. H. Miller. 1982. Altered expression of *Plasmodium knowlesi* variant on the erythrocyte membrane in splenectomized rhesus monkeys. *J. Immunol.*. 128:224.

28. Schmidt-Ullrich, R., D. F. H. Wallach, and J. Lightholder. 1979. Fractionation of
Plasmodium knowlesi-induced antigens of rhesus monkey erythrocyte membranes. Bull. W. H. O. 57:115.

29. Wallach, D. F. H., and M. Conley. 1977. Altered membrane proteins of monkey erythrocytes infected with simian malaria. J. Mol. Med. 2:119.

30. Fahey, J. L. 1967. Chromatographic separation of immunoglobulins. Methods Immunol. Immunochem. 1:321.

31. Schmidt-Ullrich, R., and D. F. H. Wallach. 1978. Plasmodium knowlesi-induced antigens in membranes of parasitized rhesus monkey erythrocytes. Proc. Natl. Acad. Sci. USA. 75:4949.

32. Resch, K., W. Imm, E. Ferber, H. Fischer, and D. F. H. Wallach. 1972. Quantitative determination of soluble and membrane proteins through their native fluorescence. Naturwissenschaften. 58:220.

33. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I-131-labeled growth hormones of high specific radioactivity. Biochem. J. 89:114.

34. Elder, J. W., R. A. Rickett, II, J. Hampton, and R. A. Lerner. 1977. Radioiodination of proteins—single polyacrylamide gel slices. J. Biol. Chem. 252:6510.

35. Butcher, G. A., G. H. Mitchell, and S. Cohen. 1978. Antibody-mediated mechanisms of immunity of malaria induced by vaccination with Plasmodium knowlesi merozoites. Immunology. 77:34.

36. Chulary, J. D., M. Aikawa, C. Diggs, and J. D. Haynes. 1981. Inhibitory effects of immune monkey serum on synchronized Plasmodium falciparum cultures. Am. J. Trop. Med. Hyg. 30:12.

37. Miller, L. H., J. G. Johnson, R. Schmidt-Ullrich, J. D. Haynes, D. F. H. Wallach, and R. Carter. 1980. Determinants on surface proteins of Plasmodium knowlesi merozoites common to Plasmodium falciparum schizonts. J. Exp. Med. 151:790.