

BIOSYNTHESIS OF Pb44, THE PROTECTIVE ANTIGEN OF SPOROZOITES OF *PLASMODIUM BERGHEI*

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Injection of rodents, primates, and human volunteers with x-irradiated sporozoites, the infective stage of malaria parasites, leads to protective immunity (1). In a rodent malaria system *Plasmodium berghei* (2, 3), and most recently in primate malaria *P. knowlesi* (4), it has been found that monoclonal antibodies against a surface protein of mature sporozoites neutralize the infectivity of the parasite. The protective antigen of *P. berghei* is a protein of 44,000 mol wt (Pb44)¹ covering the entire surface of mature sporozoites (5).

These findings raise the possibility of developing vaccines against human malaria containing purified sporozoite surface proteins. However, to date the only source of mature sporozoites is the salivary gland of the infected mosquito, which places severe constraints on the quantity of purified material that can be obtained.

Application of recombinant DNA technology to this system may provide an alternative for obtaining large amounts of the protective antigen. The present investigation was undertaken as an initial step in this direction. Our aim was to select the stage of the parasite's life cycle containing mRNA for the protective antigen, which could be used for cloning the relevant gene. For this purpose the biosynthesis of the protective antigen of sporozoites of *P. berghei* by its various developmental forms was studied.

Materials and Methods

Media and Reagents. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and medium 199 were obtained from Grand Island Biological Co., Grand Island, N. Y. Bovine serum albumin (BSA) was obtained from Miles Laboratories, Inc., Research Products Div., Elkhart, Ind., gentamicin from Schering Corp., Kenilworth, N. J. Saponin, trypsin (type III), soybean trypsin inhibitor, aprotinin, leupeptin, antipain, diisopropylfluorophosphate, and neuraminidase type VI were purchased from Sigma Chemical Co., St. Louis, Mo. Nonidet P-40 (NP-40) was purchased from Particle Data Laboratories, Ltd., Elmhurst, Ill., and *Staphylococcus aureus*, Cowan I (Pansorbin), from Calbiochem-Behring Corp., La Jolla, Calif. Orosomucoid was obtained from the American Red Cross, Washington, D. C.

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; IEF, isoelectric focusing; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; Pb44, 44,000 mol wt protective antigen of sporozoites; *Pb44, 44,000 mol wt polypeptide; *Pb52, 52,000 mol wt polypeptide; *Pb54, 54,000 mol wt polypeptide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Staph A, *Staphylococcus aureus*, Cowan 1 strain; TCA, trichloroacetic acid.

The chemicals for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) were obtained from the following sources: *N,N'*-methylenebisacrylamide, acrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, SDS, urea, 2 β -mercaptoethanol from Bio-Rad Laboratories, Richmond, Calif., ultrapure urea from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., and ampholines, pH 3.5-5.0 and 5-7 from LKB Instruments, Inc., Rockville, Md.

The radiolabeled compound L-[³⁵S]methionine, specific activity > 400 Ci/mmol, and ¹²⁵I, carrier free, 17 Ci/mg, were obtained from New England Nuclear, Boston, Mass.

The P3U1 cell line, used to produce the 3D11 hybridoma, was a gift from Dr. J. Unkeless, The Rockefeller University, New York.

Source of Sporozoites. Sporozoites of *P. berghei* (NK65) were harvested from *Anopheles stephensi* salivary glands 18-20 d after the infective blood meal (6). Sporozoites from the mosquito midgut were recovered 13-14 d after infection. The purification procedure was carried out as previously described (2).

Radiolabeling of Sporozoites. Surface iodination was carried out by a lactoperoxidase-catalyzed reaction (7). Metabolic labeling was carried out as follows. Partially purified sporozoites were incubated in DMEM without methionine, and containing 0.2% BSA, 0.01% gentamicin, 400 μ Ci/ml L-[³⁵S]methionine, in a total volume of 0.5 ml. After incubation at room temperature for varying periods of time, medium 199 containing 10 mM cold methionine was added, and the parasites were washed three to four times with the same medium.

The extent of incorporation of L-[³⁵S]methionine into protein was determined by treating 50 μ l of a suspension of labeled sporozoites for 40 min at 4°C with trichloroacetic acid (TCA) at a final concentration of 20%. The TCA precipitate was filtered through wet GF/A filter paper (Whatman, Ltd., England), washed three times with 10 ml TCA and once with methanol. The filter paper was then transferred to a glass vial containing 5 ml of hydrofluor (National Diagnostics, Inc., Advanced Applications Institute Inc., Somerville, N. J.) and the radioactivity measured in a liquid scintillation counter.

Preparation of Soluble Extract of Labeled Parasites. Labeled parasites were washed by centrifugation three to four times in medium 199 containing 0.2% BSA, and then treated with NP-40 at a final concentration of 0.5% in the presence of the following protease inhibitors: aprotinin (2 trypsin inhibiting units/ml), antipain and leupeptin (25 μ g/ml), diisopropylfluorophosphate (4 mM). After incubation for 15 min at room temperature, the extract was centrifuged at 8,000 *g*, and the pellet was discarded. In most experiments, between 40 and 60% of the counts were recovered in the supernate.

Incorporation of [³⁵S]Methionine by Blood Forms. 2×10^9 parasitized erythrocytes were collected from Swiss mice infected with *P. berghei* and washed with DMEM without methionine. The infected erythrocytes were then incubated in the presence of 200 μ Ci of [³⁵S]methionine in a candle jar at room temperature for 15-17 h (8). After several washings with medium 199 to eliminate extracellular [³⁵S]methionine, the erythrocytes were lysed with 30 ml of 0.1% saponin (9) containing the protease inhibitors as above, and then centrifuged at 7,000 *g* for 20 min. The pellet was resuspended in 1 ml of PBS and centrifuged at 150 *g* for 10 min to sediment some contaminating leukocytes. The supernatant fluid containing the free parasites was extracted with NP-40 as described previously. As a control, noninfected erythrocytes were subjected to the same procedure.

Treatment of ³⁵S-labeled P. berghei Sporozoites with Trypsin. Partially purified sporozoites were incubated with 200 μ Ci of [³⁵S]methionine for 2 h at room temperature as described above. The parasites were washed with PBS and then incubated with 0.5 ml of PBS containing 100 μ g/ml of trypsin for 10 min at 37°C. The reaction was stopped by adding 0.5 ml of 100 μ g/ml of soybean trypsin inhibitor, and the parasites were then washed several times with medium 199 containing 1 mg/ml of BSA. Controls consisted of labeled sporozoites incubated in a mixture of trypsin and soybean inhibitor.

Immunoprecipitation. Immunoprecipitation of the labeled extracts was performed as described by Kessler (10) using a 10% suspension of formaldehyde-treated *S. aureus*, Cowan 1 strain (Staph A), to bind immune complexes. Three washings of the Staph A-bearing immune complexes were carried out by centrifugation in a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% NP-40, and 0.1% BSA, pH 8.6. A final washing was performed with 50 mM Tris

buffer, pH 8.6, containing 0.1% SDS, 0.05% NP-40, and 300 mM NaCl. The immune complexes were eluted from the Staph A with a solution containing 2% SDS, 10% glycerol, 10% β -mercaptoethanol, and 6 M urea.

In most instances the immunoprecipitation was carried out with the monoclonal antibodies 3D11 that recognize the protective antigen of *P. berghei* (2). The antibodies were obtained either from supernates of cell cultures of the hybridoma, or from the serum or ascites of hybridoma-bearing mice. Controls were immunoprecipitated either with the supernates from cultures of plasmacytoma P3U1 or with normal mouse serum.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE (11) was carried out in slab gels using 3% acrylamide for the stacking gel and 10% for the separation gel. After fixation in a mixture of methanol (25%), water (63%), and acetic acid (12%), the gel was fluorographed by impregnating with Enhance (New England Nuclear), dried, and exposed to X-omat R film XR-2 (Eastman Kodak Co., Rochester, N. Y.) at -70° C. Proteins with mol wt ranging from 14,000–94,000 daltons were used as markers (kit from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.).

Two-dimensional Analytical Slab Gels. These were performed by a modification (12) of the O'Farrell technique (13). Immunoprecipitates bound to Staph A were eluted in a buffer containing 1% ampholine (pH 3.5–5 and 5–7), 5% β -2 mercaptoethanol, 2.5% NP-40, and 9 M urea and subjected to IEF in triplicate in a vertical 4.5% acrylamide slab gel. After the run, one of the gel tracks was fixed, impregnated with Enhance, dried, and subjected to radioautography. A second track of the gel slab was used for pH gradient determinations by cutting 1-cm segments and eluting them in degassed distilled water. A third track was equilibrated for 1 h in 10 ml of SDS-PAGE sample buffer containing 2% SDS, 10% glycerol, 0.005% bromophenol blue, 5% β -mercaptoethanol in 80 mM Tris buffer, pH 6.8. This track was then placed on the top of an SDS-PAGE slab gel consisting of 3.5% stacking gel and 7% running gel. After electrophoresis, the slab was processed for radioautography.

Elution of the Labeled Proteins from SDS-PAGE Gels. The procedure described by Beemon and Hunter (14) was followed. In brief, after the electrophoretic separation, the SDS-PAGE gel was soaked for 1 h in 200 ml of water containing 2% of ion exchange resin (Bio Rex AG-501-X8 from Bio-Rad Laboratories), then dried and radioautographed. The portions of the gel corresponding to the sporozoite-specific bands were cut out and rehydrated by incubation with a small volume of a buffer containing 50 mM ammonium bicarbonate, 0.1% SDS, and 5% β -mercaptoethanol. The swollen gel was cut into small fragments and homogenized further in a glass tissue grinder. Proteins were eluted by incubating the gel fragments overnight at 37° C with 0.5 ml of the same buffer. The samples were centrifuged at 10,000 *g* for 30 min and NP-40 was added to the supernates to a final concentration of 0.1%. The supernates were then dialyzed for 2 d against Tris-NaCl buffer, pH 8.6, containing 0.1% NP-40.

Treatment of Extracts of Sporozoites with Neuraminidase. Attempts were made to remove sialic acid residues from glycoproteins present in the extracts of sporozoites by treatment with neuraminidase. The labeled extracts in medium 199, pH 6.5, were mixed with orosomucoid (final concentration 500 μ g/ml) and incubated with neuraminidase (1 U/ml) at 37° C. At different time intervals, samples were taken and analyzed by high voltage agarose electrophoresis. Incubation was terminated at a time when the results of the electrophoresis showed that all orosomucoid had been converted to asialorosomucoid.

Results

*Incorporation of [35 S]Methionine into Protein by *P. berghei* Salivary Gland Sporozoites*. Partially purified *P. berghei* sporozoites from salivary glands were incubated at room temperature in the presence of [35 S]methionine. At various time intervals aliquots were taken, precipitated with TCA, washed as described in Materials and Methods, and counted for radioactivity. The incorporation of [35 S]methionine into protein increased during 90 min of incubation. At this time the incorporation was \sim 10 times greater in the preparations containing sporozoites compared with those obtained from noninfected mosquitos (Fig. 1).

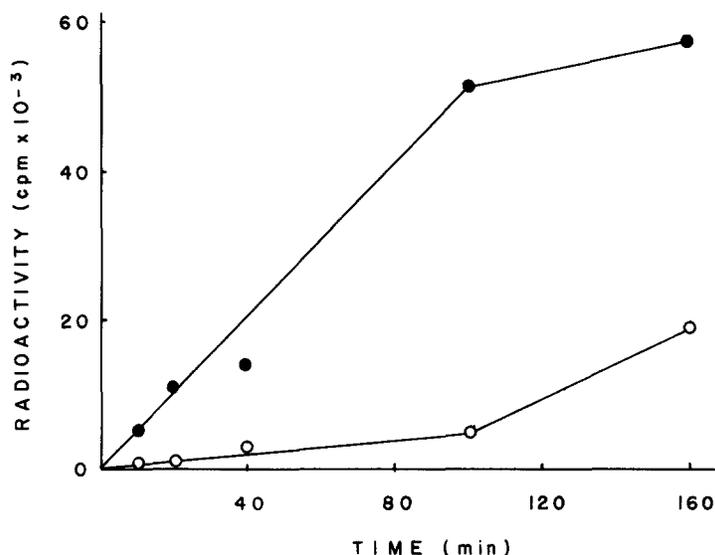


FIG. 1. Incorporation of [³⁵S]methionine by *P. berghei* sporozoites. Parasites were purified from infected mosquito salivary glands by using a lectin column as described elsewhere (2). Salivary glands of noninfected mosquitoes, serving as control, were processed the same way. The purified sporozoites (●) and the noninfected salivary gland preparation (○) were incubated at room temperature in the presence of 200 μ Ci of [³⁵S]methionine. At various time intervals aliquots were taken, precipitated with TCA, washed several times, and the radioactivity counted. Note that the incorporation of [³⁵S]methionine, presumably by contaminating microorganisms present in the salivary gland preparation of normal mosquitoes, was minimal during the first 90 min of incubation.

The incorporation of [³⁵S]methionine was almost completely inhibited by puromycin, at a concentration of 20 μ g/ml. It was not affected by adding immune serum or the monoclonal antibodies 3D11 (final concentration, 1 mg/ml) to the incubation medium.

Fig. 2 shows that the incorporation of [³⁵S]methionine by the sporozoites is temperature dependent and more rapid at 37° than at 25°C. However, in the experiments to be described, the parasites were incubated with the radiolabeled amino acid at 25°C, because at the lower temperature the sporozoites retained more of their infectivity and gave strong circumsporozoite reactions² (15) after 2 h of incubation, whereas at 37°C the parasites lost their viability within 15 min.

Synthesis of Pb44-related Products by Sporozoites. The incorporation of [³⁵S]methionine into products antigenically related to Pb44, the protective antigen of *P. berghei*, was evaluated as follows. Purified parasites incubated in medium containing [³⁵S]methionine for 30 min at 25°C were extracted with NP-40 and incubated with an excess of the monoclonal antibody 3D11 (2). In repeated experiments, 5–15% of the counts were specifically immunoprecipitated. The solubilized immunoprecipitates were subjected to SDS-PAGE followed by radioautography. The results were identical whether the electrophoresis was performed under reducing or nonreducing conditions. When

² When mature salivary gland sporozoites are incubated with antibodies to Pb44, a precipitate which progressively increases in size appears at the posterior end of the parasite. As shown elsewhere (3), this phenomenon, called circumsporozoite reaction, represents the shedding of Pb44 following its cross-linking by antibody. Losses in infectivity of sporozoites are closely associated with diminished circumsporozoite reactions (A. Cochrane and R. S. Nussenzweig, unpublished observations).

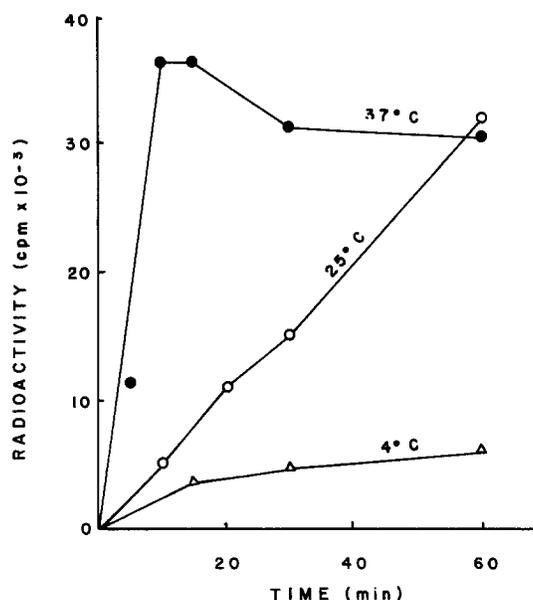


FIG. 2. Effect of temperature on the incorporation of [³⁵S]methionine by *P. berghei* sporozoites. The purified sporozoite preparation was divided in three samples which were incubated at 4°, 25°, and 37°C in the presence of 200 μCi of [³⁵S]methionine. At various time intervals aliquots of the labeled parasite suspension were taken, precipitated with TCA, washed, and the radioactivity of the precipitate counted. The incorporation of [³⁵S]methionine by the parasites is temperature dependent.

the incorporation of [³⁵S]methionine was performed for <30 min, the radioautograph (Fig. 3, track 2) revealed the presence of two strong specific bands with ~52,000 and 54,000 mol wt (*Pb52 and *Pb54) and a weak specific band with a 44,000 mol wt (*Pb44). If the incorporation was prolonged for 120 min, the results were qualitatively identical; that is, the same three polypeptides were specifically immunoprecipitated. However, the relative intensities of the *Pb52 and *Pb44 bands varied from experiment to experiment, and were inversely related. Sometimes *Pb44 was much stronger than *Pb52, as shown, for example, in track 5 of Fig. 3.

When run side by side on a slab gel, the position of *Pb44 coincided with that of ¹²⁵I-labeled Pb44 obtained by immunoprecipitation of extracts from surface iodinated sporozoites (tracks 4 and 5, Fig. 3).

*Evidence That *Pb52 Is a Precursor of *Pb44.* We used the pulse-chase technique to investigate the relationship between the sporozoite proteins recognized by the monoclonal antibody. The parasites were first incubated for 15 min with medium containing [³⁵S]methionine at 25°C. At this point excess cold methionine was added, and the incubation continued for an additional 120 min. Samples were removed during the second incubation, and the respective extracts were immunoprecipitated and subjected to SDS-PAGE followed by radioautography. The radioautographs of the slab gels of two separate experiments are shown in Fig. 4. *Pb52 and *Pb54 appear as strong bands after the pulse labeling for 15 min while *Pb44 appeared as a very weak band (tracks 1 and 6). With increasing time after chase, the intensity of the *Pb44 band increased while the intensity of *Pb52 correspondingly decreased (tracks 2 and 7, 60 min after the chase; tracks 3 and 8, 120 min after the chase). These results suggest a

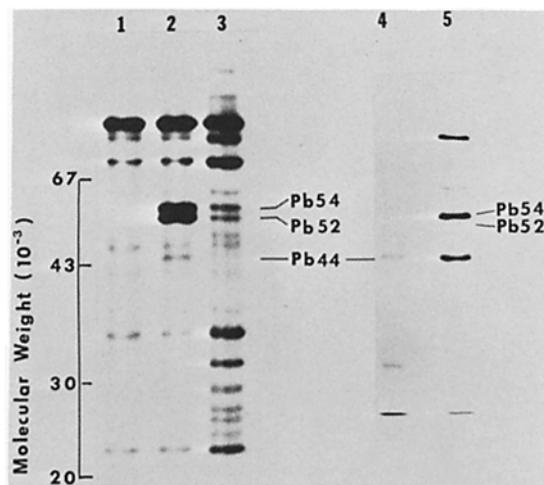


FIG. 3. SDS-PAGE of immune precipitates of [^{35}S]methionine-labeled *P. berghei* sporozoite extracts. Purified sporozoites were incubated at room temperature for 30 min in the presence of 200 μCi of [^{35}S]methionine. The NP-40 extract was immunoprecipitated with supernate from plasmacytoma P3U1 culture (negative control, track 1) and with supernatant cultures of hybridoma 3D11, producing antisporozoite monoclonal antibodies (track 2). The pattern of the total sporozoite extract is shown in track 3. Three bands with apparent molecular weights of 44,000, 52,000, and 54,000 were specifically immunoprecipitated. Note that *Pb52 and *Pb54 are prominent bands of the total extract. The additional tracks show that *Pb44 obtained by metabolic labeling (track 5) and ^{125}I -Pb44 obtained by surface iodination of sporozoites (track 4) have identical molecular weights. In this instance the source of *Pb44 was an extract of parasites which had been incubated for 120 min with [^{35}S]methionine, and the intensity of the *Pb44 band is far greater than that of *Pb52. Surface iodination to obtain ^{125}I -Pb44 was performed as described by Yoshida et al. (2) and the immunoprecipitation carried out with 3D11. The additional bands are contaminants which bind nonspecifically to Staph A.

precursor—product relationship between *Pb52 and *Pb44. In contrast, the intensity of the *Pb54 band only diminished slightly at 120 minutes after the chase.

*Evidence That *Pb54 is Recognized by the Monoclonal Antibody 3D11.* To demonstrate that *Pb54 is recognized by 3D11 and not merely coprecipitated with the other polypeptides, we attempted to isolate it from slab gels. To avoid contamination of *Pb54 with *Pb52, salivary gland sporozoites were labeled with [^{35}S]methionine for 15 min, an excess cold methionine was added, and the incubation was prolonged for 120 min. The NP-40 extracts were immunoprecipitated with 3D11 and subjected to SDS-PAGE.

As expected from the results shown in the previous section, after the pulse-chase the only prominent bands on the slab gel were *Pb54 and *Pb44 (Fig. 5, track 1). The bands were excised from the gel and the proteins eluted as described previously. The eluates were then immunoprecipitated with 3D11 and again subjected to SDS-PAGE and radioautography. We calculated that 50–70% of the total cpm loaded onto the gels were eluted from the two bands and ~50% of each of the eluted proteins were specifically immunoprecipitated by 3D11. Fig. 5, tracks 2 and 3, shows the analysis of the immunoprecipitates by SDS-PAGE. It is clear that both purified polypeptides were recognized by 3D11.

*Further Evidence That Metabolically Labeled *Pb44 and Pb44, the Protective Antigen of P. berghei, are Identical.* In previous studies we characterized the protective antigen of

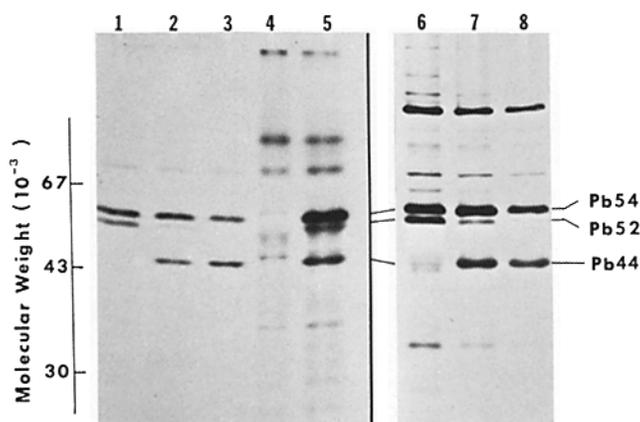


FIG. 4. Pulse-chase experiments to study the relationship among *Pb54, *Pb52, and *Pb44. The salivary gland sporozoites were pulse labeled with 200 μ Ci of [35 S]methionine for 15 min at room temperature and chased with an excess of cold methionine for differing periods of time. The labeled sporozoites were treated with NP-40 and the extracts were incubated with the serum from mice bearing the 3D11 hybridoma-producing monoclonal antibody to Pb44. The immunoprecipitates were subjected to SDS-PAGE and radioautography. Tracks 1-5 and 6-8 show the results of two separate experiments. Tracks 1 and 6 contain immunoprecipitates of extracts obtained from parasites at the time of initiation of the chase (0 min); tracks 2 and 7, immunoprecipitates obtained 60 min after the chase; tracks 3 and 8, immunoprecipitates obtained 120 min after the chase. Note that in both experiments the intensity of the *Pb52 and *Pb44 bands is inversely related, and that 60 min after the chase the intensity of *Pb44 is comparable to that of *Pb52 at time zero. Controls contain extracts of parasites incubated for 120 min with [35 S]methionine and immunoprecipitated with normal mouse serum (track 4) or with the serum of mice bearing the hybridoma 3D11 (track 5).

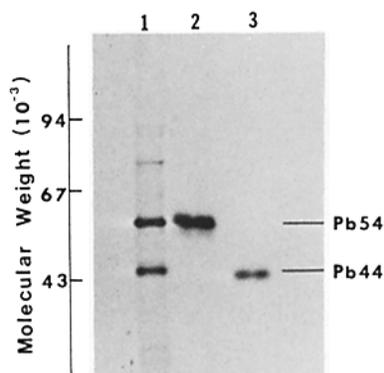


FIG. 5. Specific immunoprecipitation of purified *Pb54 and *Pb44 by the monoclonal antibody 3D11. Sporozoites were incubated with 200 μ Ci of [35 S]methionine for 2 h at room temperature. The NP-40 extracts were immunoprecipitated with 3D11 and subjected to SDS-PAGE and radioautography. The most prominent bands were identified as *Pb54 and *Pb44 (track 1). The corresponding areas of the gels were excised and the proteins eluted as described in Materials and Methods. The purified material was then immunoprecipitated with 3D11 and subjected to a second SDS-PAGE and radioautography. Tracks 2 and 3 show the results obtained with purified *Pb54 and *Pb44, respectively.

sporozoites of *P. berghei* as a membrane protein with 44,000 mol wt, which could be labeled by lactoperoxidase-catalyzed radioiodination of viable parasites (2). The results of the present investigation have demonstrated that the metabolically labeled

product *Pb44 has the same apparent molecular weight as Pb44 and is also recognized by the monoclonal antibody 3D11. An additional argument supporting the idea that *Pb44 and Pb44 are identical proteins is that they have the same pI under denaturing conditions, as shown in Figs. 6 and 7. Indeed, when immunoprecipitated extracts of surface-iodinated (Fig. 6) or metabolically labeled parasites (Fig. 7) were analyzed by the O'Farrell technique, *Pb44 and Pb44 focused at the same pI of 4.7. Results were not changed if the extracts were pretreated with neuraminidase before subjecting them to IEF. Also of interest is the observation that the other two proteins, *Pb52 and *Pb54, could barely be distinguished in the two-dimensional gels of the metabolically labeled material; that is, *Pb52 and *Pb54 have not only similar molecular weights, but also similar pI's, between 5.1 and 5.3 (Fig. 7).

To verify whether *Pb44, similarly to Pb44, is also present on the membrane of sporozoites, the following experiment was performed. Sporozoites were incubated with [³⁵S]methionine for 2 h at room temperature, and then incubated with a solution of trypsin (100 µg/ml) in medium 199 for 10 min at 37°C. The reaction was stopped by adding an equal volume of a solution of soybean trypsin inhibitor (100 µg/ml). As a control, another sample of labeled parasites was incubated with a mixture of enzyme and inhibitor. The sporozoites from both preparations were morphologically unaltered, as determined by phase microscopy. However, trypsin-treated parasites did not give circumsporozoite reactions following incubation with 3D11.

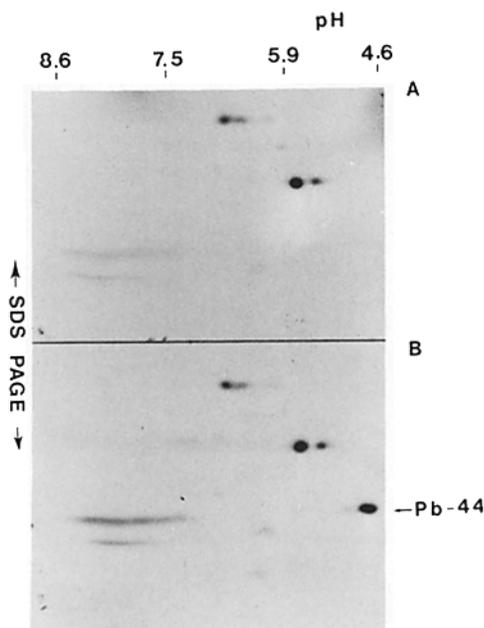


FIG. 6. Two-dimensional analysis of *P. berghei* sporozoites labeled with ¹²⁵I. The membranes of sporozoites were radiolabeled by lactoperoxidase-mediated iodination after which parasites were treated with NP-40. The extracts were immunoprecipitated with serum of mice bearing the 3D11 hybridoma or with normal mouse serum. The immunoprecipitates were subjected to IEF, SDS-PAGE and radioautography as described in Materials and Methods. The figure shows the results of the two-dimensional radioautograph of the immunoprecipitates obtained with normal mouse serum (A) or with monoclonal antibodies 3D11 (B). The only specific spot in B is Pb44, with a pI of 4.7. IEF was performed in the horizontal direction. The pH gradient is shown on top of the figure.

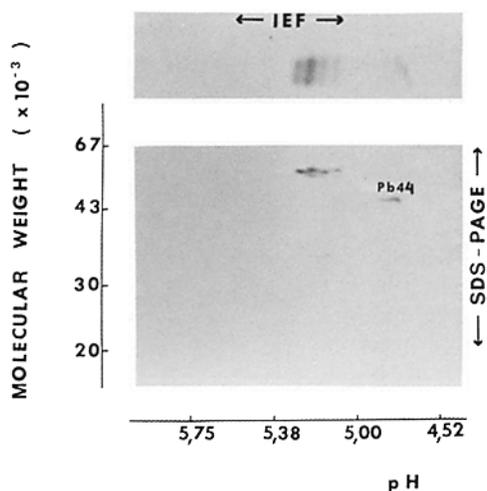


FIG. 7. Two-dimensional analysis of immunoprecipitates of metabolically labeled *P. berghei* sporozoites. Sporozoites were incubated with 200 μ Ci of 35 S-methionine for 2 h at room temperature and then treated with NP-40. The parasite extracts were immunoprecipitated with serum of mice bearing the 3D11 hybridoma or with normal mouse serum. The immunoprecipitates were subjected to IEF and subsequently to a second dimensional separation in SDS-PAGE as described in Materials and Methods. *Pb44 focused at pH 4.7 while components of apparent molecular weight of \sim 53,000 focused between 5.1 and 5.3. The control gel (not shown) had no spots. IEF was performed in the horizontal direction. The pH gradient is represented in the bottom of the figure.

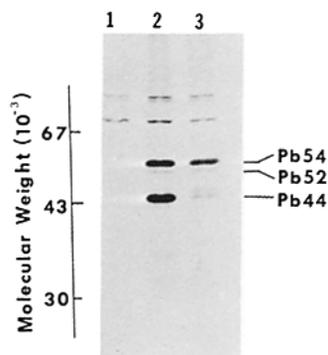


FIG. 8. Effect of trypsin on [35 S]methionine-labeled *P. berghei* sporozoites. Sporozoites were incubated with 200 μ Ci of [35 S]methionine for 2 h at room temperature. Separate samples were treated with 100 μ g/ml of trypsin or with a mixture of 100 μ g/ml of trypsin and soybean trypsin inhibitor for 10 min at 37°C. The parasites were treated with NP-40, and the extracts immunoprecipitated with serum of mice bearing the hybridoma 3D11 or with normal serum. Track 1 is a control which contains the immunoprecipitates of extracts of parasites immunoprecipitated with normal mouse serum. Track 2 consists of parasites treated with trypsin and soybean trypsin inhibitor, and immunoprecipitated with 3D11. Track 3 consists of extracts of parasites treated with trypsin and immunoprecipitated with 3D11. The results show a pronounced decrease in the intensity of the *Pb44 band after trypsinization. In contrast, *Pb52 is unchanged and *Pb54 only slightly affected. An additional weak band in track 3 located between *Pb52 and *Pb44 may represent a fragment of a contaminant or of *Pb54.

Both preparations were then extracted with NP-40 and immunoprecipitated with monoclonal antibody 3D11. The immunoprecipitates were subjected to SDS-PAGE and radioautography. The results are shown in Fig. 8. As compared with the control

(track 2) there was a pronounced decrease in the intensity of *Pb44 after trypsinization (track 3). In contrast, the intensity of *Pb52 was unchanged and *Pb54 was only slightly affected. The most likely explanation for these findings is that *Pb44 is located on the surface membrane of the parasite and is more accessible to the proteolytic activity of trypsin, whereas most or all *Pb52 and *Pb54 are located intracellularly.

Lack of Synthesis of Pb44-related Polypeptides by Midgut Sporozoites and by Blood Forms of P. berghei. Using the same procedures, we assayed the sporozoites from the midgut of mosquitos and the blood forms of *P. berghei* for their ability to synthesize polypeptides recognized by 3D11. In several experiments we found that both forms of the parasites effectively incorporated [³⁵S]methionine into protein. However, in contrast to the results obtained with salivary gland sporozoites, Pb44-related polypeptides were barely detected in extracts of sporozoites derived from midgut oocysts, and not found at all in extracts of the blood forms of the parasite.

Discussion

In this paper we have attempted to study some aspects of the biosynthesis of Pb44. The different developmental stages of *P. berghei* were metabolically labeled with [³⁵S]methionine and the extracts immunoprecipitated with the hybridoma 3D11, which binds specifically to Pb44. The analysis of the immunoprecipitates by SDS-PAGE and radioautography (Fig. 3) showed that mature salivary gland sporozoites contain three proteins recognized by 3D11, with apparent molecular weights of 44,000 (*Pb44), 52,000 (*Pb52), and 54,000 (*Pb54).

One of these proteins, *Pb44, is probably identical to Pb44 present on the cell membrane of surface-iodinated parasites. Both molecules have the same molecular weights and isoelectric points, and are recognized by the monoclonal antibody 3D11. Furthermore, *Pb44 appears also to be located on the cell membrane. Indeed, treatment of metabolically labeled parasites with trypsin selectively affects *Pb44 (Fig. 8), indicating that it is more accessible to the enzyme than the other two polypeptides. The possibility that *Pb52 and *Pb54 are resistant to proteolysis was excluded in experiments in which we treated NP-40 extracts, rather than intact parasites, with trypsin under the same conditions and found that both proteins were readily cleaved (not shown).

It seems that *Pb52 is an intracellular precursor of *Pb44. This was first suggested by the observation that when parasites were incubated with [³⁵S]methionine for different periods of time, the radiolabeled amino acid was incorporated first into the higher molecular weight bands, and only later into Pb44. More important, pulse-chase experiments showed that with increasing time after chase there is an inverse relationship between the intensities of *Pb52 and *Pb44 bands. That is, the *Pb52 band decreases in intensity, while the *Pb44 band correspondingly increases (Fig. 4).

The relationship between *Pb54 and the other two polypeptides is not clear, except that they share an antigenic determinant with *Pb44. This was demonstrated by isolating both proteins from slab gels and showing that they were recognized by the monoclonal antibody 3D11 (Fig. 5). In this way we excluded the possibility that *Pb54 could have been coprecipitated with one of the other polypeptides. It is also clear that *Pb54 and *Pb52 have very similar isoelectric points. In fact, the two proteins can barely be distinguished by the two-dimensional O'Farrell technique (Figs. 6 and 7), further indicating that they may be structurally related. A possible

explanation of these findings is that *Pb54 is a precursor of *Pb52. The results of several pulse-chase experiments failed to substantiate this hypothesis. Although *Pb54 was readily labeled after a few minutes of incubation of the parasites with [³⁵S]-methionine, we found no evidence for its transformation into *Pb52 2 h after the chase. Therefore, if *Pb54 generates *Pb52, the turnover rates at 25°C must be slow. An alternative possibility is that *Pb54, although antigenically related, is functionally independent of the other polypeptides. Additional information clarifying the structural relationship between *Pb54 and the other two proteins may be obtained by peptide mapping. These studies are presently underway.

The present observations demonstrate that the three polypeptides have at least one common epitope, and suggest that the cleavage of *Pb52 generates *Pb44, which is then translocated to the cell membrane. Furthermore, our data show that the synthesis of these proteins is strictly associated with only one developmental stage of the parasite; that is, mature sporozoites. *Pb54, *Pb52, or *Pb44 were not detected in blood forms and were found in only minute amounts in sporozoites from the midgut of mosquitoes.

In contrast, in mature sporozoites they constituted one of the main products of protein synthesis. Indeed, immunoprecipitation with 3D11 brought down between 5 and 15% of the total counts in extracts of metabolically labeled parasites. This finding is supported by the observation that *Pb54 and *Pb52 can always be identified as prominent bands in SDS-PAGE of total extracts of metabolically labeled mature sporozoites (see, for example, Fig. 3, track 3). Therefore, the biosynthesis of proteins in mature sporozoites seems to be in large part committed to the production of a single membrane protein that may, as suggested elsewhere (3), be involved in the penetration of the parasite into the target cell.

From a practical point of view, these observations indicate that mRNA for Pb44 and related proteins is present in relatively large amounts of mature sporozoites. This may render easier the task of cloning the Pb44 gene of *P. berghei*, or of identifying homologous genes in other species of *Plasmodium*, aiming at the production of the protective antigens by the recombinant DNA technology.

Summary

In a previous paper (2) we identified a protective antigen (Pb44) of the surface membrane of sporozoites of *Plasmodium berghei* by means of a monoclonal antibody. Immunoprecipitation of extracts of mature salivary gland sporozoites, metabolically labeled with L-[³⁵S]methionine using the same monoclonal antibody, revealed three specific polypeptides: *Pb44, *Pb52, and *Pb54.

Metabolically labeled *Pb44 is probably identical to the protective antigen previously identified by surface labeling. Both proteins have the same molecular weights and isoelectric points under denaturing conditions, and they share an epitope. Moreover, *Pb44 also seems to be located on the cell membrane.

The results of pulse-chase experiments strongly suggest that *Pb52 is the precursor of *Pb44. The relationship between *Pb54 and the protective antigen is unknown. The three polypeptides seem to be strictly associated with only one of the developmental stage of the parasite. They were not detected in blood forms and were found in minute amounts in sporozoites from the midgut of mosquitoes. In contrast, in mature salivary gland sporozoites they constitute main products of protein synthesis.

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References

1. Nussenzweig, R. S. 1980. Use of radiation-attenuated sporozoites in the immunoprophylaxis of malaria. *Int. J. Nucl. Med. Biol.* **7**:89.
2. Yoshida, N., R. S. Nussenzweig, P. Potocnjak, V. Nussenzweig, and M. Aikawa. 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science (Wash. D. C.)*. **207**:71.
3. Potocnjak, P., N. Yoshida, R. S. Nussenzweig, and V. Nussenzweig. 1980. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J. Exp. Med.* **151**:1504.
4. Cochrane, A. H., R. Gwadz, V. Nussenzweig, and R. S. Nussenzweig. 1981. Monoclonal antibodies against a surface antigen of sporozoites of simian malaria abolish parasite infectivity. *Fed. Proc.* **40**:1011 (Abstr.).
5. Aikawa, M., N. Yoshida, R. S. Nussenzweig, and V. Nussenzweig. 1981. The protective antigen of rodent malarial sporozoites (*Plasmodium berghei*) is a differentiation antigen. *J. Immunol.* **126**:2494.
6. Vandenberg, J. P., R. S. Nussenzweig, and H. Most. 1968. Further studies on the *Plasmodium berghei-Anopheles stephensi* rodent system of mammalian malaria. *J. Parasitol.* **54**:1009.
7. Hubbard, A. L., and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. *J. Cell Biol.* **55**:390.
8. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. D. C.)*. **193**:673.
9. Trager, W., S. G. Langreth, and E. G. Platzer. 1972. Viability and fine structure of extracellular *Plasmodium lophurae* prepared by different methods. *Proc. Helminthol. Soc. Wash.* **39**:220.
10. Kessler, S. W. 1975. Rapid isolation of antigens from cells with staphylococcal protein A antibody absorbent: parameters of the interaction of antibody antigen complexes with protein A. *J. Immunol.* **115**:1617.
11. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680.
12. Ferreira, A. and D. Eichinger. 1981. A modified two-dimensional electrophoretic technique. *J. Immunol. Methods.* **43**:291.
13. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007.
14. Beemon, K., and T. Hunter. 1978. Characterization of Rous sarcoma virus src gene products synthesized in vitro. *J. Virol.* **28**:551.
15. Vandenberg, J., R. S. Nussenzweig, and H. Most. 1969. Protective immunity produced by injections of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. *Mil. Med.* **134** (Suppl.):1183.