Structural Similarities among the Protective Antigens of Sporozoites from Different Species of Malaria Parasites*

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Circumsporozoite (CS) proteins are the protective antigens of several species of malaria parasites. Monoclonal antibodies to CS proteins immunoprecipitate two additional intracellular polypeptides (IS1 and IS2) from extracts of parasites metabolically labeled with \[^{35}S\]methionine. We compared CS and IS proteins from *Plasmodium berghei*, *Plasmodium knowlesi*, *Plasmodium cynomolgi*, and *Plasmodium falciparum* by twodimensional electrophoretic analysis (O'Farrell technique) and tryptic peptide analysis carried out by reverse-phase high performance liquid chromatography. Under reducing and nonreducing conditions, CS, IS1, and IS2 from these species of parasites have apparent M. between 42,000 and 67,000. Their pI values range from 4.7 to 6. The isolated CS and IS proteins from each species react with the respective monoclonal antibodies and yield very similar peptide maps. These results support previous evidence indicating that IS proteins are intracellular precursors of CS proteins. Moreover, they suggest that the processing of IS proteins involves the cleavage of peptides relatively rich in basic residues, since the pI values of the CS proteins are consistently lower than those of the corresponding IS proteins.

Several \[^{35}S\]methionine-labeled peptides with identical retention times were found in chromatograms of digests of CS or IS proteins from different species of parasites. Taken together with previously observed antigenic cross-reactivities between these membrane proteins, these findings suggest that CS and IS polypeptides have structural homologies and probably belong to a family of proteins.

Protective immunity against malaria infection has been obtained following inoculation of animals with radiation-attenuated sporozoites (1). Studies using monoclonal antibodies against rodent (2-4), simian (5), and human (6) malaria sporozoites, as well as epidemiological surveys (7), suggest that the protective antigens are circumsporozoite proteins covering the entire surface membrane of the parasite. Therefore, CS proteins could perhaps be used as malaria vaccines. However, these proteins can be obtained only from sporozoites purified from the salivary glands of infected mosquitoes. There are two possible approaches to obtain large amounts of CS proteins, that is, by genetic engineering or by synthesis of the portions of the molecules which contain the relevant antigenic determinants. As an initial step in this direction, in this investigation we undertook a comparison of the structural features of the CS proteins from various malaria parasites.

Monoclonal antibodies to CS proteins also react with two intrasporozoite polypeptides (5, 8) named IS1 and IS2, in order of increasing molecular weight upon electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. We used two-dimensional electrophoretic analysis and tryptic peptide analysis to compare CS and IS proteins of four species of mammalian malaria, including the human malaria parasite *Plasmodium falciparum*. We show suggestive evidence that these polypeptides are structurally related and belong to a family of homologous proteins.

**MATERIALS AND METHODS**

**Source of Sporozoites**—Sporozoites of human (*P. falciparum*, Thai strain), simian (*Plasmodium knowlesi*, H strain), and rodent (*Plasmodium berghei*, strain NK65) malaria were obtained from mosquito salivary glands 18-20 days after an infective blood meal (9). Purification of the parasites was performed as previously described (2).

**Monoclonal Antibodies**—The production and characterization of monoclonal antibodies against CS proteins of *P. falciparum* (3DE6), *P. knowlesi* (2G3), and *P. berghei* (3D11) are described elsewhere (3, 5, 6).

**\[^{35}S\]Methionine Labeling of Sporozoites**—Viable sporozoites were incubated for 2 h in methionine-free RPMI 1640 medium (Microbial Associates, Walkersville, MD) to which were added 200 μCi of \[^{35}S\]methionine, specific activity >800 Ci/mmol (New England Nuclear), using previously described methodology (8). After addition of protease inhibitors, the sporozoites were solubilized with 1% Nonidet P-40 (Particle Data Laboratories). Insoluble material was removed by centrifugation.

**Immunoprecipitation and Electrophoretic Analysis**—CS and IS proteins were immunoprecipitated from sporozoite extracts with the corresponding monoclonal antibody as described by Kessler (10) using a 10% suspension of formaldehyde-treated *Staphylococcus aureus*, Cowan 1 (Calbiochem-Behring). Monoclonal 2G3 was also used to immunoprecipitate CS and IS proteins from *P. cynomolgi*. Precipitates were washed twice in a buffer containing 15 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Nonidet P-40, pH 8.6, and twice in the same buffer, but with 0.05% sodium deoxycholate instead of Nonidet P-40.

For SDS-PAGE (11), the immunoprecipitates were eluted in a buffer containing 2% SDS, 10% glycerol, 10% β-mercaptoethanol and 6 M urea. The elution buffer for two-dimensional electrophoresis consisted of 1% Ampholines, pH 3.5-10 and 5-7 (LKB Instruments, Inc.), 5% β-mercaptoethanol, 2.5% Nonidet P-40, and 9 M urea. Two-dimensional electrophoresis was carried out by a modification (12) of the O'Farrell technique (13). Labeled samples were loaded at the

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*This work was supported by A.I.D. Grant DPE-0453-C-00-3002-00, UNDP/World Bank/WHO Special Program for Research and Training in Tropical Medicine, and The Rockefeller Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.*
overnight at 37 °C to extract the labeled protein, and pelleted for 15 min.

Samples were coarsely minced and homogenized in a tissue homogenizer in a 2-ml final volume of buffer with 0.1 ml of β-mercaptoethanol per band. Gel suspensions were then boiled for 5 min, shaken, and centrifuged (48,000 g, 15 min at 4 °C) to extract the labeled protein, and pelleted for 15 min at 27,000 × g. To each supernatant, 80 μg of bovine γ-globulin were added and the proteins were precipitated overnight at 0 °C with trichloroacetic acid (final concentration of 20%). Precipitated proteins, recovered by centrifugation for 15 min at 48,000 × g, were washed twice with cold acetone and dried.

Samples were carboxymethylated prior to trypsin digestion as previously described (14). First, the dried pellet was incubated at 50 °C for 30 min with 0.1 ml of 0.5 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride. Then, 50 μl of 0.1 M dithiothreitol in 0.5 M Tris-HCl were added and incubation continued for 2 h at 50 °C. Finally, 55 μl of 0.2 M iodoacetamide in the same buffer were added, and the tube was left in the dark for 30 min at room temperature. The proteins were reprecipitated overnight in 20% trichloroacetic acid, washed twice with cold acetone, dried, and dissolved in 0.15 ml of 0.05 M NH4HCO3 buffer, pH 7.9.

Digestion with 1-[(5-aminooctyl)-2-phenyl]ethylchloromethyl-ketone-treated trypsin (Worthington) was done at room temperature for 20 h with additions of 30 and 20 μl of a trypsin solution (1 mg/ml in 0.05 M ammonium bicarbonate buffer, pH 7.9) at 0 and 16 h incubation time, respectively. The digest was then diluted in 1.5 ml of distilled water, lyophilized, redissolved in 2 ml of water, and relyophilized. Dried digested samples were finally dissolved in 0.2-0.3 ml of distilled water and chromatographed.

HPLC Peptide Mapping—Reverse-phase HPLC was carried out on a Bondapak C18 columns using a Waters Associates system composed of two M-6000 A solvent delivery units, a sample processor (WISP), an M450 variable wavelength detector, an M720 system controller, and an M730 data module.

Solvents were filtered using a 0.45-μm cellulose acetate filter (micro filtration system) for aqueous solutions and a 0.45-μm regenerated cellulose filter for acetonitrile. Solvent A (95% glass-distilled water, 5% acetonitrile) was degassed with stirring under vacuum for at least 20 min, while solvent B (45% distilled water, 55% acetonitrile, with 1 ml/liter of 85% H3PO4 added) was degassed for 1 min prior to use.

Chromatography was carried out at room temperature and effluents were monitored at 240 nm. Injection volume was 0.1 ml and the elution system (flow rate 1 ml/min) was isocratic for 5 min using 100% of solvent A, and this was followed by a 25-min linear gradient until 100% of solvent B was reached.

Eluates were collected in 60 fractions of 0.5 ml/tube (2 fractions/min) and counted with Aquasol (New England Nuclear) in a Beckman LS 7500 scintillation counter. Radioactivity of each fraction was expressed as a percentage of total recovered counts.

Table I

| Species        | CS protein | IS proteins* | M, g | pH (range) |
|----------------|------------|--------------|------|------------|
| P. berghei     | Pb44       | Pb54, Pb52   | 44,000 | 4.7 |
| P. knowlesi    | Pk42       | Pk54, Pk52   | 42,000 | 4.9 |
| P. cynomolgi   | Pc48       | Pc58         | 48,000 | 4.8-5.0 |
| P. falciparum  | Pf58       | Pf56, Pf57, Pf58 | 48,000 | 5.3 |

*In the text the higher and lower M, IS proteins are designated IS2 and IS1, respectively. There is no direct evidence yet that in these parasites the lower molecular weight polypeptides are on the surface membrane of the sporozoites and the higher molecular weight polypeptides are located intracellularly.

Fig. 1. Two-dimensional electrophoretic analysis of CS and IS proteins. Sporozoites of P. cynomolgi (Pc), P. falciparum (Pf), P. knowlesi (Pk), and P. berghei (Pb) were metabolically labeled with [35S]methionine, solubilized with Nonidet P-40, and immunoprecipitated with monoclonal antibodies. The solubilized precipitates were subjected to isoelectric focusing followed by SDS-PAGE. Anodes are to the left. The two-dimensional gel from P. berghei is reproduced from Ref. 8.

Fig. 2. HPLC of trypsin peptides of the [35S]methionine-labeled IS (Pc58) and CS (Pc48) proteins of P. cynomolgi. Experimental procedures are as described under "Materials and Methods." The percentage of the total number of counts recovered per fraction is represented on the ordinate. Peaks were numbered according to retention times. The diagonal broken line represents the increase in the percentage of solvent B. Top, shows the similar chromatograms of Pc48 (broken line) and Pc58 (solid line) peptides injected separately. Bottom, a mixture containing an equal number of counts of both digests was injected. This chromatogram is almost indistinguishable from those shown (top).
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RESULTS

Properties of CS, IS1, and IS2 Polypeptides—We used monoclonal antibodies to precipitate antigens from metabolically labeled extracts of sporozoites of *P. berghei*, *P. knowlesi*, *P. cynomolgi*, and *P. falciparum*. Each monoclonal antibody reacted with three polypeptides (CS, IS1, and IS2). Under reducing or nonreducing conditions, CS, IS1, and IS2 have apparent *M*, between 42,000 and 67,000 as determined by SDS-PAGE (Fig. 1 and Table I). Differences in *M* between IS1 and IS2 proteins of each species were small, only about 2,000, and sometimes these proteins could not be resolved in radioautography of acrylamide gels. The relative intensity of these two bands varied in different preparations, but in most instances the IS2 band was much stronger than the IS1 band.

Following IEF in acrylamide gels, the IS2 protein (and sometimes IS1) appeared as a series of bands with PI values ranging from 5 to 6. The PI values of CS proteins were always lower than those of the corresponding IS proteins.

To determine whether the immunoprecipitated proteins of each *Plasmodium* species share similar or identical epitopes, CS and IS2 + IS1 polypeptides from *P. berghei*, *P. knowlesi*, and *P. cynomolgi* were purified by SDS-PAGE, eluted from the gels, and reacted separately with the respective monoclonal antibodies. In every case we specifically reprecipitated 85% or more of the original counts.

Labeled peaks were numbered according to retention time, the higher numbers representing the longer retention times.
Peptide Maps of CS and IS Proteins—Due to the severe limitations in the number of sporozoites available for metabolic labeling, we compared only peptide maps of \(^{33}\)S-methionine-labeled CS and IS proteins. This amino acid was chosen because it is available with high specific activity, and we had previously shown that it is well incorporated into IS and CS proteins (5, 8). To minimize possible errors associated with the preparation of the tryptic peptides, all CS and IS proteins were isolated, carboxymethylated, and digested simultaneously. Moreover, to control reproducibility of the procedure, chromatographic separations were performed sequentially on the same day, and after each comparison a mixture was analyzed containing an equal number of counts from each preparation. An additional control was to compare the optical density patterns of the chromatograms of the digested protein carrier (bovine \(\gamma\)-globulin) included in each sample. These were almost identical in all runs and the occasional variation in the retention times of some peptides was less than 0.2 min.

Two distinct sets of labeled peptides from CS and IS proteins were detected in the chromatograms; one set, relatively hydrophilic, was retained to a small extent by the C18 column, and the other set was more hydrophobic. In preliminary experiments we used various types of elution gradients in an effort to resolve both sets of peaks simultaneously, but the major species was presumably IS2 since IS1 was barely visible in the radioautographs.

Figs. 2, 3, and 4 are chromatograms of CS and IS proteins derived from \(P.\) cynomolgi, \(P.\) knowlesi, and \(P.\) berghei. It is clear that the peptide maps of the CS and IS proteins of each parasite are almost identical.

Next, we compared the CS or IS proteins of different species of parasites. The results, illustrated in Figs. 5, 6, and 7 and summarized in Table II, show peptides with similar, and others with different retention times. Only peak \(i\) is common to all species of IS and CS proteins, but it represents the flow through of the column and may contain more than one pepi-

![Fig. 6. Interspecies structural relationships detected by HPLC of \(^{33}\)S-methionine-labeled \(P.\) cynomolgi (Pc58) and \(P.\) falciparum (Pf67) IS tryptic digests. Experimental conditions are as described in the legend to Fig. 2 and under "Materials and Methods." When the digests were injected individually (top), similarities (peaks 1, 2, and 7) and differences (peaks 4, 5, 6, 8, and 9) were detected. When the peptides were injected together (bottom), all peaks were present. As expected, in this case the heights of peaks 4, 5, 6, 8, and 9 are about half of those obtained with separate injections.](http://www.jbc.org/)

![Fig. 7. Interspecies structural relationships detected by HPLC of \(^{33}\)S-methionine-labeled \(P.\) cynomolgi (Pc58) and \(P.\) falciparum (Pf67) IS tryptic digests. Experimental conditions are as described in the legend to Fig. 2 and under "Materials and Methods." The top contains the chromatograms of peptides of \(P.\) cynomolgi and \(P.\) falciparum when each digest was injected separately. Peaks 1 and 5 have identical retention times in both chromatograms, while peaks 2, 3, and 7 differ in their retention times. Simultaneous injections of a mixture containing the same number of counts of each peptide preparation (bottom) provided the same peaks and, as expected, peaks 2, 3, and 7 have about half the heights of the corresponding peaks shown (top).](http://www.jbc.org/)

**Table II**

| Species          | Peak No. | min of retention |
|------------------|----------|-----------------|
|                  | 1 (4)    | 2 (5)           |
| \(P.\) berghei   | ++       | +               |
| \(P.\) knowlesi  | +        | +               |
| \(P.\) cynomolgi | +        | +               |
| \(P.\) falciparum| +        | +               |

* +, present; --, absent.*
tide. Peaks 2, 7, and 8 were present in the proteins of *P. berghei*, *P. knowlesi*, and *P. cynomolgi*, while peak 5 was common to *P. cynomolgi*, *P. falciparum*, and *P. berghei*. On the other hand, peak 3 was found only in *P. falciparum* and peak 4 only in *P. knowlesi*. This analysis was repeated several times with samples from different digests. Although small variations were observed in the various runs, the overall patterns were very similar to those shown in the figures.

**DISCUSSION**

The major conclusion of this paper is that the protective antigens of sporozoites (CS and IS proteins) of several different species of *Plasmodium* (*P. berghei, P. knowlesi, P. cynomolgi,* and *P. falciparum*) are structurally related and probably belong to a family of homologous proteins. This conclusion is based on the present findings and on previously observed similarities between their biosynthetic, immunological, and physicochemical properties, and characteristic distribution in malaria parasites.

CS proteins are stage-specific. They are found mainly in mature salivary gland sporozoites and are uniformly distributed on the surface membrane (4, 8). They are not found in the blood stage (8). CS and IS proteins constitute some of the major biosynthetic products of salivary gland sporozoites. Between 5–20% of the [35S]methionine incorporated into proteins by mature sporozoites of *P. berghei* (8), *P. knowlesi* (5), and *P. falciparum* (6) can be specifically precipitated by monoclonal antibodies to the homologous CS proteins.

Pulse-chase experiments performed to examine the relationship between IS1 and IS2 and CS proteins of *P. berghei* and *P. knowlesi* suggested that IS are intracellular precursors of CS (5, 8). This idea is supported by the present results (Figs. 2, 3, and 4) which show that the peptide maps of IS and CS proteins of each species are very similar. However, more than one peptide may be present in the breakthrough peak 1, and insoluble peptides are not represented in the chromatograms. Moreover, structural differences residing in non-methionine-containing peptides would not be revealed in the chromatograms.

Comparison of tryptic digests of CS and IS proteins of different malaria species shows that several [35S]methionine-labeled peptides have identical RT (Table II). This is significant since analysis was performed by reverse-phase HPLC, a method which enhances differences at the sequence level. For example, a single substitution in the sequence of a peptide may change the RT (15, 16), and it has been estimated that proteins with less than 80% homology at the primary level will show completely different chromatograms (17). The discriminatory power of this method is greatly increased when the comparison involves peptides containing methionine, a relatively infrequent amino acid in proteins. Therefore the finding that IS and CS [35S]methionine-labeled peptides from various parasites have identical retention times strongly suggests that these proteins have structural homologies and belong to a single family.

This idea is also supported by the overall similarities between the two-dimensional gels of IS and CS proteins of several malaria species, and by previously observed antigenic cross-reactivities. For example, monoclonal antibodies against CS proteins of *P. knowlesi* bind CS protein of *P. cynomolgi* and *P. falciparum* (5, 6). Of even greater biological significance is the finding that a monoclonal antibody against sporozoites of *Plasmodium yoelii nigeriensis* cross-reacts with sporozoites of *P. berghei* and completely neutralizes their infectivity for mice.2

The results of the two-dimensional gels, summarized in Table I, are also of relevance to the understanding of the processing of IS polypeptides. They indicate that the CS proteins have a lower pi than the respective IS proteins, and that differences in *M* between IS1 and IS2 and between IS1 and CS proteins are constant; that is, about 2000 and 8000, respectively. It seems reasonable to speculate, therefore, that CS protein may be generated from IS2 protein by two sequential cleavages, and that the larger *M* = 8000 moiety removed from IS1 is relatively rich in basic residues.

Finally, the observations that mature salivary gland sporozoites from various species of malaria synthesize relatively large amounts of structurally related proteins which are membrane-associated, and that monoclonal antibodies recognizing these antigens abolish parasite infectivity, suggest that these proteins may have a common function essential for the development of the parasite in the mammalian host. Indeed, recent results indicate that CS polypeptides are involved in the adhesion of parasites to target cells (18). The understanding of the structural basis of this interaction may be of considerable biological and practical interest.

**Acknowledgments**—We wish to thank Rita Altszuler, Marilyn Marcic, Louis Koontz, Douglas Seeley, and Joseph Edelin for their excellent technical assistance, and Joanne Joseph for manuscript preparation. We also thank Drs. Harinasuta, Taphaichari, and Thara, vanj from the University of Mahidol, Thailand, for providing the Thai strain of *P. falciparum*.

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*N. Yoshida, R. S. Nussenzweig, and V. Nussenzweig, manuscript in preparation.*
Structural similarities among the protective antigens of sporozoites from different species of malaria parasites.
F Santoro, A H Cochrane, V Nussenzweig, E H Nardin, R S Nussenzweig, R W Gwadz and A Ferreira

J. Biol. Chem. 1983, 258:3341-3345.

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