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Fractionation and characterization of *Plasmodium falciparum* antigens*

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In human malarial infections, the immunological importance of modified host cell components and of parasite antigens that may be released on spontaneous rupture of infected erythrocytes in vivo *is not known. It is difficult, however, to analyse antigens extracted from entire parasitized red cells because of contamination with normal red cell constituents, notably haemoglobin. To overcome this problem, a simple chromatographic procedure has been developed that concentrates antigens and radiolabelled parasite products in a separate fraction from haemoglobin. Details are also given of investigations of two different kinds of antigen associated with* Plasmodium falciparum. *One antigen known to be immunogenic in man is shown to be associated with membranes. The other antigen is poorly immunogenic in man and other animals and is shown to be a soluble, rather stable, acidic protein.*

In recent years, research on malarial antigens has tended to concentrate on the merozoite stage of the parasite. However, the reproducible preparation in bulk of *Plasmodium falciparum* merozoites entirely freed from red cell contamination has yet to be fully realized and information about possible red cell contaminants would be useful. Furthermore, there is some evidence that modified host cell components, or parasite antigens that are readily lost on the rupture of infected erythrocytes, might be of immunological importance. Also, there are good reasons for investigating and enriching antigen and enzyme systems from forms of the parasite, such as the trophozoite, that are not easily harvested in relatively uncontaminated form like the merozoite. The technique described below for preparing *P. falciparum* antigens in relatively haemoglobin-free extracts of entire infected erythrocytes is based on the method of Bottini & Huehns (*1*) for the preparation of non-haemoglobin constituents of normal red cells. We have found only one other report in the malaria literature of the application of a similar technique; this was used by Sherman et al. (*2*) to study amino acid incorporation by *P. lophurae*.

Particular attention is paid to the characterization of two *P. falciparum* antigens, La_1-antigens and S-antigens. To justify their detailed study it is necessary to point out briefly the contrasting biological and epidemiological properties previously established (*3*) which make these antigens of interest: La_1-antigens are chemically labile, S-antigens are stable: La_1-antigens have a very limited range of specificity, S-antigens are serologically diverse: La_1-antigens are highly immunogenic, S-antigens poorly so. To understand these differences we need to know more about the nature and origin of the antigens.

MATERIALS AND METHODS

Sources of antigen

Infected erythrocytes. Concentrated preparations of schizonts were obtained in the Gambia from human placental blood heavily infected with *P. falciparum*, as described by McGregor et al. (*4*). Mature parasites were collected in a similar way from peripheral blood of *Aotus* monkeys infected with the Palo Alto strain of *P. falciparum*, as well as from rhesus monkeys infected with *P. knowlesi*.

Peripheral blood infected with ring forms of *P. falciparum* was collected from Gambian children voluntarily attending an outpatients clinic in the Gambia. This blood with 10–20% infected erythrocytes was maintained in supplemented Medium 199 in 2-litre flasks for 24–48 h until the parasites matured, as described by Phillips et al. (*5*). Amino acids labelled with ^3H or ^{14}C were added to some cultures (*6*).

Antigens were also obtained from long-term cultures of *P. falciparum* by a modification of the method of Trager & Jensen (*7*). Small-scale Petri dish cultures were transferred to flat-bottomed (75-cm²)

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tissue culture flasks (Falcon) which contained 10 ml of medium (renewed twice daily) and were maintained under an atmosphere comprising 5% O_2, 7% CO_2, and 88% N_2. The medium was RPMI 1640 supplemented with 5% each of normal human serum and heat-inactivated horse serum; this medium supported parasite growth for several weeks. Mature parasites were harvested by the Plasmagel[a] method of Pasvol et al. (*8*).

Infected plasma. Plasma was obtained from Gambian children with acute *P. falciparum* infections or from infected *Aotus* monkeys with heavy parasitaemias.

Extraction of antigens

Large volumes of frozen schizonts were disrupted by means of a Hughes press and then centrifuged at 17×10^3 *g* for 30 min. The supernatant was used in the studies reported here. Small volumes of schizonts were disrupted by freezing (–20 °C) and thawing prior to centrifugation. S-antigens were prepared by heating infected plasma at 100 °C for 5 min and centrifuging at 30×10^3 *g* for 30 min to pellet the insoluble material.

Chromatography

CM cellulose (Whatman) was equilibrated in 0.01 mol/litre phosphate buffer, pH 6.5. Extracts of infected or uninfected erythrocytes were centrifuged at 17×10^3 *g* for 30 min or 100×10^3 *g* for 60 min and the supernatant dialysed overnight against the equilibrium buffer at 4 °C. The length of column required for chromatography varied with the volume of lysate added—up to 3 ml was accommodated on a column 1.5 x 50 cm, up to 1 ml on a column 1.5 x 18 cm, and very small volumes extracted from preparations of cultured schizonts were separated on a column in a 1-ml syringe. After elution of peak I at 4 °C, the bulk of the remaining protein was eluted with 0.05 mol/litre phosphate buffer, pH 8.0 (peak II). Pooled eluates were concentrated by negative-pressure dialysis against 0.06 mol/litre barbital buffer, pH 8.6.

Gel diffusion

Precipitin reactions and autoradiographs of agar gels were carried out as described by Wilson (*6*).

Radiolabelling

Boiled plasma from infected children or *Aotus* monkeys was dialysed against 0.2 mol/litre borate buffer (pH 9.0) and 0.1–0.5 ml mixed with 10–25 µl (^{14}C)-formaldehyde (specific activity: 51.8×10^7 Bq (14 mCi) per mmol) according to the method of Rice & Means (*9*). After the addition of excess borohydride, labelled samples were passed through a G-25 column and concentrated.

Polyacrylamide gel electrophoresis (PAGE)

Slab gels (7.5% acrylamide) were run in Tris/glycine buffer, pH 8.3, or in buffer containing sodium dodecyl sulfate (SDS) as described by Laemmli (*10*). Samples run in SDS gels were first precipitated with acetone and dissolved in Laemmli sample buffer. Portions of gels were stained with 0.15 g/litre (0.015%) Coomassie blue or by the periodic acid Schiff's method (PAS). Other portions of gel were sliced with an automatic slicer and the 1- or 2-mm segments eluted with 0.1 ml of buffer at 4 °C overnight. Slices of gel containing radiolabelled materials were placed in vials with NCS[b] tissue solubilizer (Amersham/Searle) for scintillation counting. Fluorographs of gels containing radiolabelled antigen were prepared as described by Laskey & Mills (*11*).

Isoelectric focusing (IEF)

Preparative flat-bed isoelectric focusing in a granulated gel (Ultrodex) was carried out with the LKB 2117 Multiphor apparatus. Separated proteins were located by staining a filter paper print and relevant areas of gel were removed for elution and pH measurements.

Amino acid analysis

IEF eluates were passed through a G-25 column and freeze-dried. Samples were hydrolysed in 10 mol/litre HCl at 100 °C for 18 h prior to analysis on a Beckman amino acid analyser.

RESULTS

Chromatography of red cell extracts

Extracts of infected red cells were chromatographed on CM-cellulose in 0.01 mol/litre phosphate buffer, pH 6.5. A slightly coloured fraction (peak I) was well separated from the bulk of the haemoglobin (Fig. 1). A similar fraction was obtained from extracts of infected *Aotus* (*P. falciparum*) or rhesus monkey (*P. knowlesi*) red cells, as well as from uninfected human red cells. Peak I material from uninfected human blood maintained in cultures containing radiolabelled amino acids was devoid of activity. On the other hand, there was a significant enrichment (2.5- to 5-fold) of radiolabelled non-dialysable components in peak I material from cultured blood infected with

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P. falciparum (Fig. 1, Table 1). As discussed previously (*6*) much of this label can be presumed to represent radiolabelled parasite products.

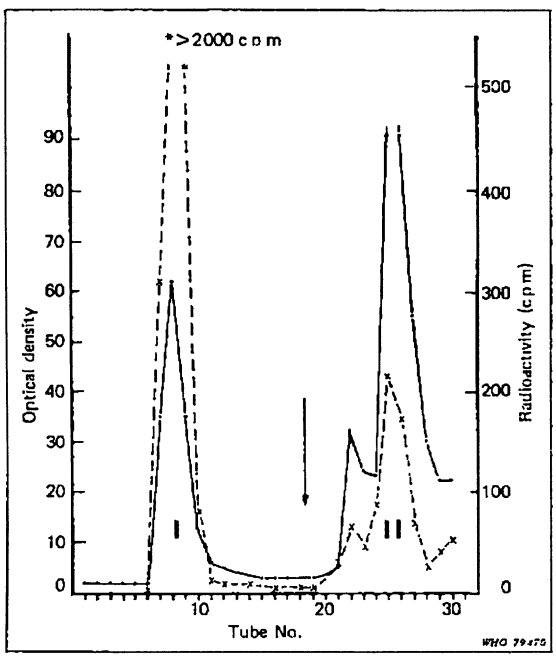

Fig. 1. Fractionation of an extract of infected erythrocytes by CM-cellulose chromatography. Peak I was eluted in 0.01 mol/litre phosphate buffer, pH 6.5, peak II in 0.05 mol/litre phosphate buffer, pH 8.0. Profiles of optical density (solid line) and radioactivity (broken line — cpm of 20 µl-samples per tube) are shown.

Table 1. Enrichment of radiolabelled parasite products by CM-cellulose chromatography[a]

Fraction	Relative activity (cpm/optical density)	Cpm (%)
Total	0 59 (0.45 – 0 95)	100
Peak I	2.09 (1 43 – 2 63)	73 (63 – 94)
Peak II	0 21 (0.04 – 0 35)	27 (6 – 37)

[a] Mean and range of 6 experiments

Peak I was subfractionated by decreasing the pH of the elution buffer. At about pH 5.8 a minor peak was separated, but comparison of the optical density and radioactivity associated with this peak showed that no enrichment occurred and the refinement was considered of little value.

Distribution of parasite antigens

The antigenic activity of material from peaks I and II, extracted from erythrocytes infected with *P. falciparum*, was analysed by gel diffusion with sera from immune adult Gambians. All of the L, R, and S classes of antigens previously identified by this method (*3*) were present in peak I (Fig. 2). Only minor amounts of antigen were detected occasionally in peak II.

Fractionation of peak I by polyacrylamide gel electrophoresis (PAGE) under both non-dissociating and dissociating (SDS) conditions revealed many protein bands but little obvious correlation between these and radioactivity profiles obtained by scintillation counting of sliced gels (Fig. 3). Clearly, non-haemoglobin red cell constituents (as well as serum contaminants) still constituted the bulk of the protein present in peak I in the preparations of infected erythrocytes used.

Specific antigens in fractionated extracts

Two of the known malarial antigens in peak I have been located with certainty following PAGE in non-dissociating gels.

La_1-antigen. This antigen did not penetrate 7.5% acrylamide gels but was recovered exclusively in a slice from the top of the gel containing the origin. Material trapped at the origin was scraped from gels and pelleted by centrifugation at 100×10^3 *g* for 30 min. Examination of the fixed pellet by electron microscopy showed that it was composed almost entirely of smooth membranes (Fig. 4). These formed vesicles of widely different sizes and multilammelar forms were seen. The La_1-antigen in the supernatants of fresh extracts centrifuged at 17×10^3 *g* was not enriched by further centrifugation at 100×10^3 *g* but a tendency for the antigen to form sedimentable aggregates under other conditions has been noted previously (*12*). La_1-antigen has been identified among several other antigens extracted from schizonts harvested from long-term *in vitro* cultures by concentration with Plasmagel.

S-antigens. These antigens were also located after PAGE of peak I material. Frequently S-antigen activity was spread over several gel slices indicating heterogeneity in size or charge. Recovery of S-antigen activity following SDS-PAGE was a significant finding and more detailed studies of S-antigens are given below.

Immunochemistry of S-antigens from plasma

Relative molecular mass. Two S-antigens of different specificity and from different sources (one from infected *Aotus* monkeys and the other from an

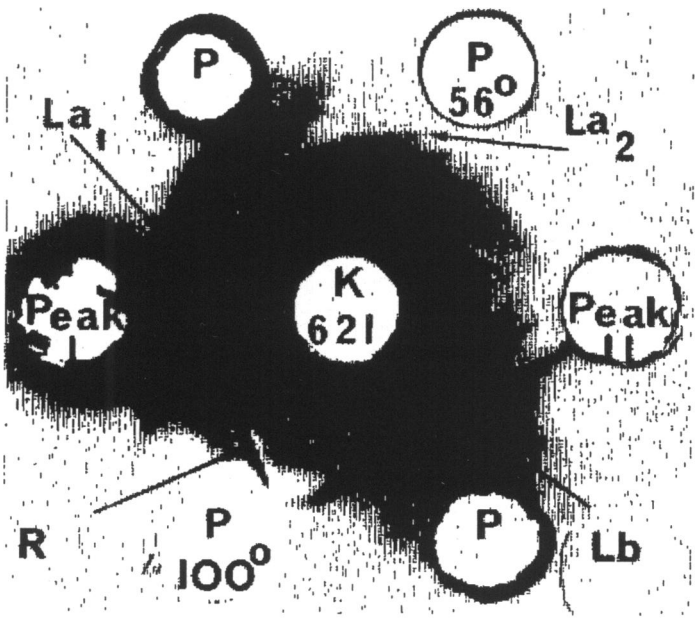

Fig. 2. Comparison of antigens in an extract of infected placental blood (P) and those in peaks I and II. As well as the heat-sensitive antigens (La_1, La_2, Lb) detected by antiserum K621, other antisera detected S-antigen in peak I.

infected child) have been examined in more detail by PAGE.

An S-antigen from infected *Aotus* monkey plasma was labelled with (^{14}C)-formaldehyde by reductive alkylation (Fig. 5). Aliquots of labelled material were absorbed with an equal volume (40 μl) of normal human serum (NHS), horse anti-whole human serum (AWHS), or serum with specific antibody from an immune Gambian (M213). After incubation and centrifugation the supernatants were compared by PAGE. Fluorographs located an antigen-specific band removed by M213, and comparison with other proteins indicated a relative molecular mass of about 130 000 (Fig. 6A). Antigen was eluted from the same region in slices from a gel run in parallel but the spread was greater (110 000–145 000, see Table 2). Coomassie blue but not PAS stained a faint band in the region corresponding to the main antigen band.

Table 2. Relative molecular masses (in thousands) of S-antigens[a]

Antigen source		Ordinary gel				SDS gel	
		Untreated antigen		Boiled antigen			
		Range	Mean	Range	Mean	Range	Mean
Aotus	W17	110 – 158 (2)	134	110 – 145 (2)	128	141 – 155 (3)	148
	W3	128 – 155 (1)	142	136 – 170 (1)	153	121 – 164 (5)	148
	A42	117 – 240 (10)	178	121 – 186 (6)	154	133 – 182 (3)	147
Human	1318A	146 – 220 (3)	183	–	–	154 – 212 (2)	183
	7727	–	–	–	–	310 – 470 (2)	425
	7774	–	–	–	–	370 – 430 (1)	400

[a] Figures in parentheses = number of experiments

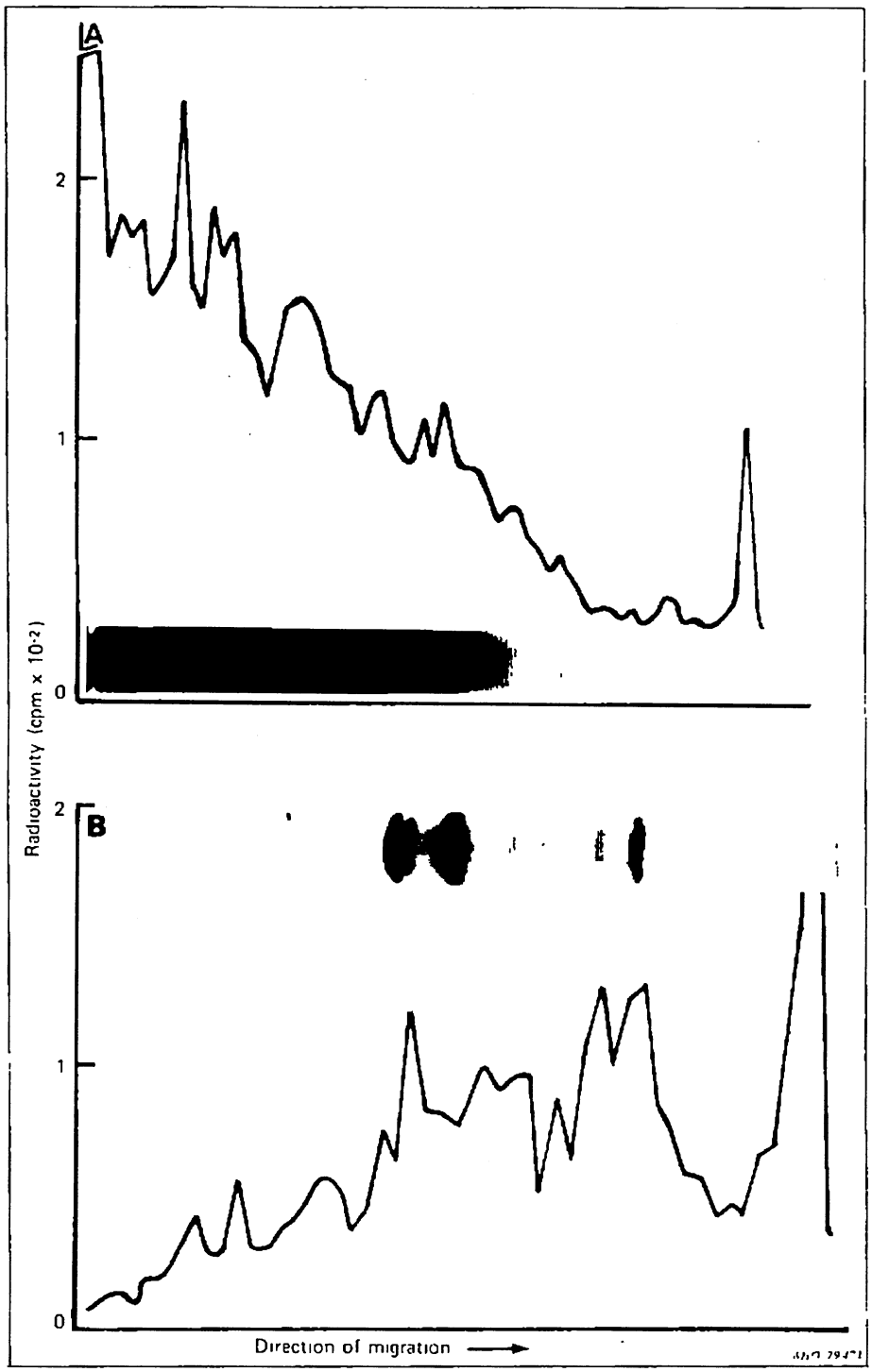

Fig. 3. Polyacrylamide gel electrophoresis of peak I material. A. Non-dissociating gel. B. SDS gel. Radioactivity profiles are compared with protein bands stained with Coomassie blue.

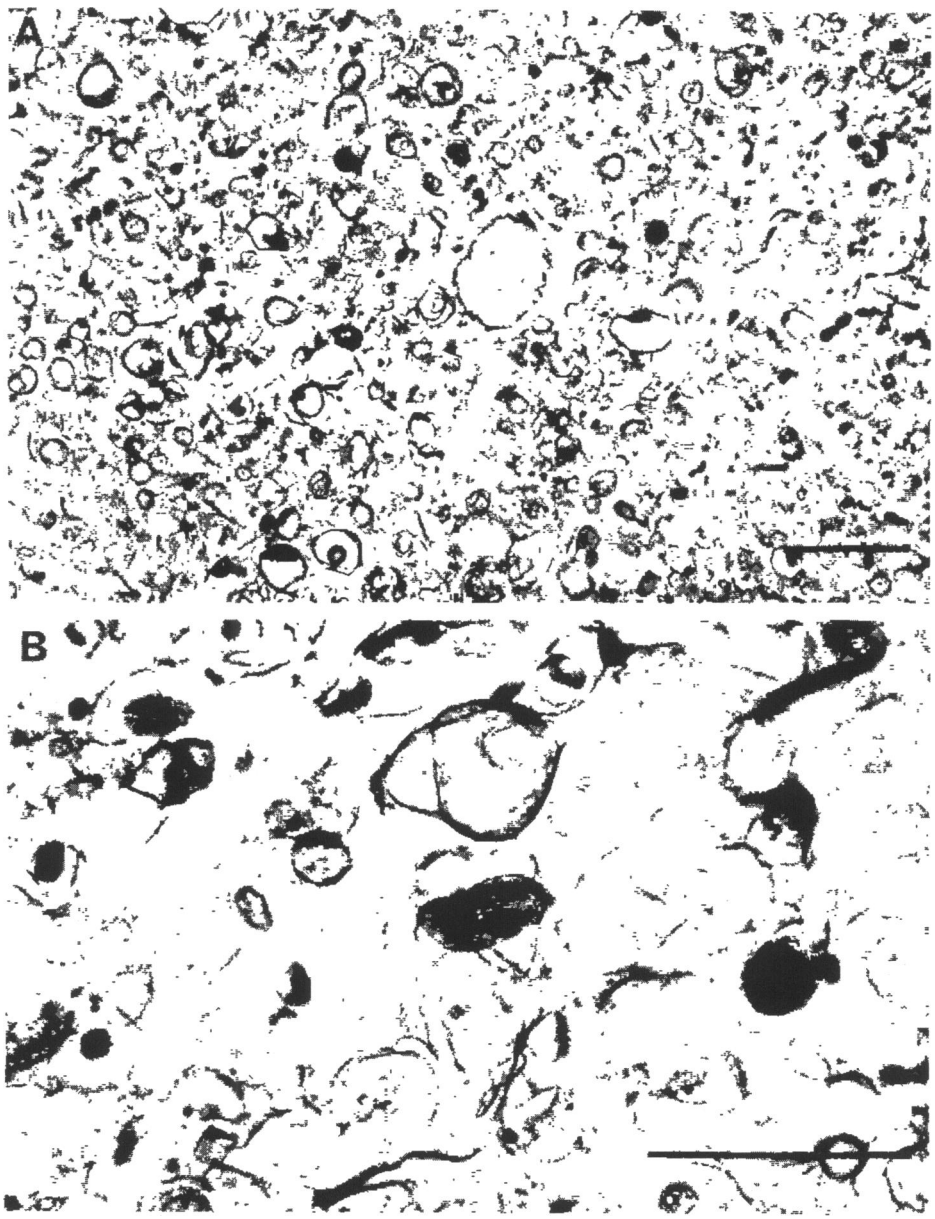

Fig. 4. Electron micrographs of peak I material trapped at the origin on polyacrylamide gel electrophoresis. Scale bars represent 1 μm (A), 0.5 μm (B).

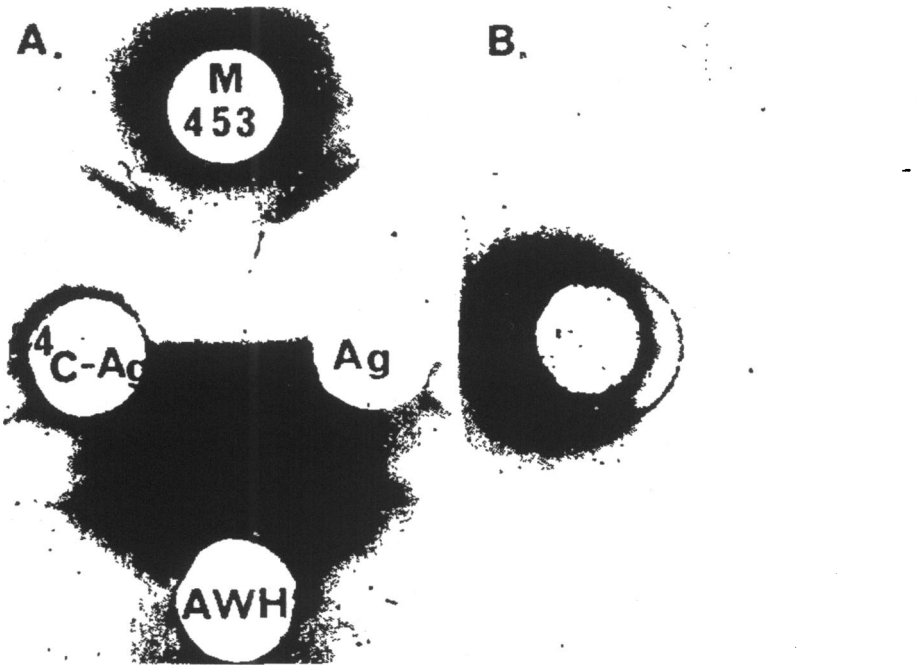

Fig. 5. A. Antiserum M 453 did not detect any change in the serological specificity of a partially purified plasma S-antigen before (Ag) and after radiolabelling (^{14}C-Ag). Neither preparation reacted with anti-whole human serum (AWHS).
B. Autoradiograph of (A)

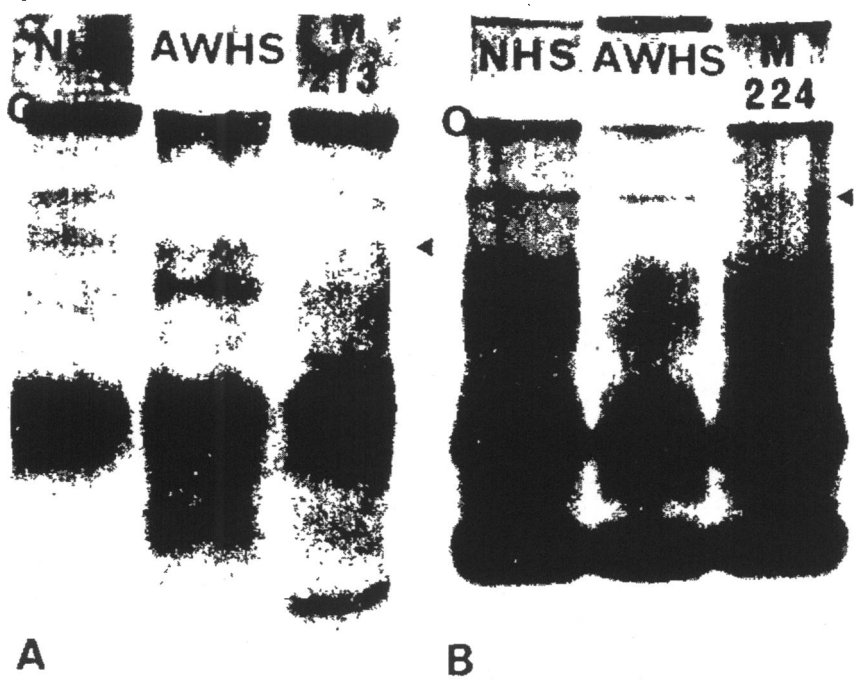

Fig. 6. Identification of radiolabelled S-antigens by antibody absorption and fluorography.
A S-antigen from *Aotus*, absorbed with antiserum M213 (arrow).
B. S-antigen from a child, absorbed with antiserum M224 (arrow).

Several preparations of an antigen were separated by SDS-PAGE and the antigen detected either positively in slices of gel or negatively by fluorography or scintillation counting of preparations absorbed with antibody (Fig. 7). Good agreement was found for a main band having a relative molecular mass of 148 000, with a minor spread downwards from about 180 000 (Table 2).

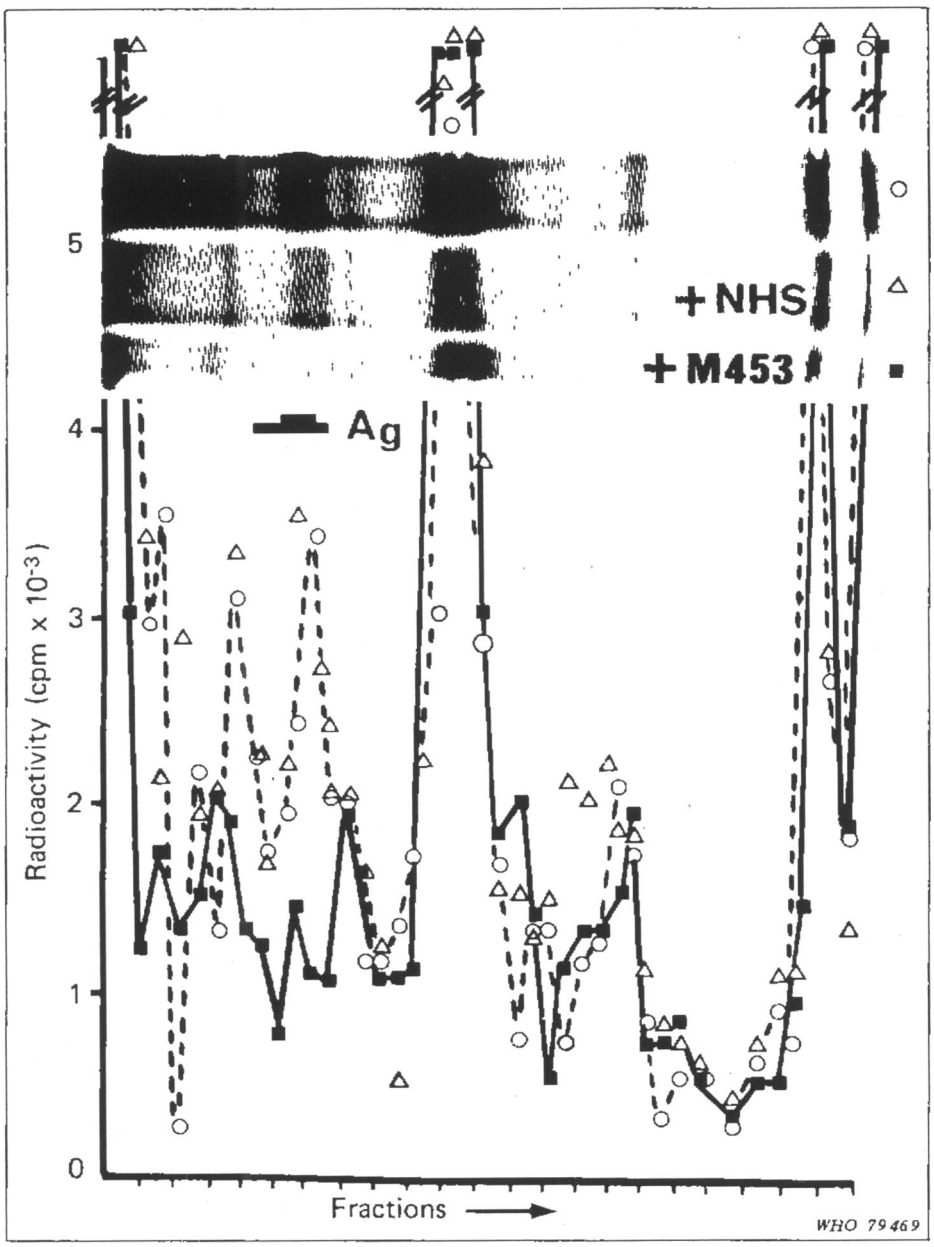

Fig. 7. Identification of (^{14}C)-labelled S-antigen after SDS-PAGE. The labelled antigen preparation (circles) was absorbed with normal serum (triangles), or immune Gambian serum (black squares), and examined after electrophoresis, either by fluorography (inset) or by scintillation counts of sliced gels. Bar shows position of eluted antigen

Similar methods have been used to examine S-antigens from infected children. In the first of these cases summarized in Table 2, fluorography showed that the main antigen-specific band had a relative molecular mass of about 180 000 (Fig. 6B). This was confirmed by antigen elution experiments and by SDS-PAGE but again heterogeneity was observed with a spread from about 150 000 to 210 000. Much higher values (~400 000) were determined for two other human S-antigens obtained by SDS-PAGE (Table 2).

Isoelectric point. Heterogeneity has also been noted in the isoelectric point (pI) of S-antigens in the boiled plasma of infected *Aotus* monkeys. Preparative isoelectric focusing in ampholines in the pH ranges of 3–10 and 2.5–8 gave a mean pH value of 4.5 for the main bulk of antigen, with a variable minor spread from 4.4 to 4.7. The pattern of stained protein bands in such gels was consistent; the antigen lying in a narrow, poorly stained area between a major protein contaminant (pI 4.75) and a minor band (pI 4.4) (Fig. 8).

Amino acid composition. After preparative isoelectric focusing, antigen (possibly contaminated with the minor band) was eluted and hydrolysed for analysis of its amino acid composition (Table 3). Apart from the absence of proline, cysteine, methionine, tyrosine, and possibly phenylalanine, the usual amino acids were present in different proportions. The eluate was richest in glycine and glutamic acid, with leucine, aspartic acid, alanine and lysine being the next most common.

Table 3. Provisional amino acid composition of an S-antigen[a]

Amino acid	Per 100 residues		
	(a)	(b)	(c)
Lys	8.0	9 5	7 1
His	1.0	1 8	2 0
Arg	1 5	3 8	4 1
Asp	12 6	9 5	12 4
Thr	8 0	8 6	6 4
Ser	6.5	4 8	5.5
Glu	12 6	12 4	17 9
Pro	1 0	–	3 0
Gly	6 5	14.3	6 2
Ala	8 0	9 5	8 7
Cys/2	1.5	–	1 0
Val	7.0	5 7	7.3
Isoleu	5 5	3 8	2.8
Leu	14 1	11 4	10 5

[a] The S-antigen fraction (*b*) is compared with two possible serum contaminants of pI 4 4 (*a*) and 4 75 (*c*).

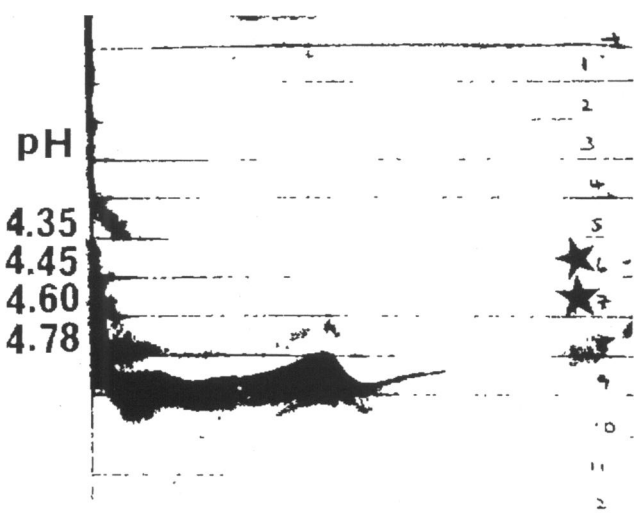

Fig. 8. Stained paper print of proteins separated by isoelectric focusing of partially purified S-antigen. The stars denote fractions with antigenic activity.

DISCUSSION

We have shown that CM-cellulose chromatography of extracts of red cells infected with *P. falciparum* is a good way to separate haemoglobin from a major antigenic fraction (peak I). Radiolabelled parasite products are also concentrated in this fraction. The activity of parasite and red cell acid-proteases is minimal at the elution pH judged to be optimal, namely pH 6.5–6.8 (*12*, and Hempelmann, unpublished results, 1978).

The supernatant (17×10^3 *g*) of the extracts used as the starting point for most of our studies contained both membranous and soluble components with antigenic activity. On the other hand, it had the disadvantage of consisting to a large extent of normal red cell constituents. Preliminary lysis of infected erythrocytes with hypotonic solutions or antiserum would improve this aspect of the fractionation procedure, but the possible loss of antigenic components from the red cell surface or interior following such manipulations has not been assessed. Another improvement would be to minimize contamination with uninfected cells. In the case of parasites harvested from cultures, we have recently tried to synchronize parasite development prior to antigen collection. To do this, schizonts from the penultimate cell division were concentrated by the Plasmagel method and seeded into fresh cultures, in which they gave rise to a synchronous brood of parasites; these, in turn, were harvested when mature.

The association we have shown between La_1-antigens and membranes agrees well with earlier work on their size and sedimentation properties, as well as their tendency to aggregate in media of low ionic strength or after standing for some days at 4 °C (*13*). The immunogenicity of this antigen has been demonstrated by the rapid increase in specific antibody that occurs after antimalarial drug therapy of either infected African children (*14*) or a European with a primary infection (*15*). Whether this is due to the physical association of the La_1-antigen with membranes is a matter for speculation, but the soluble S-antigens seem in contrast to be poorly immunogenic, and we have not been able to produce antibody in rabbits immunized with partially purified S-antigen in adjuvant (unpublished results, 1978). Consequently, serological detection of these antigens still relies on antisera produced in a small proportion of infected children and adults (*16*) or *Aotus* monkeys (*17*).

Unlike the other malarial antigens that have been described, S-antigens can be obtained in reasonable quantities from the plasma of heavily infected subjects. Despite our uncertainty as to the origin of these antigens, their serological diversity is of considerable interest. So also is the consistent association of particular S-antigens with individual isolates of parasites. For example, the S-antigen associated with the Palo Alto strain of *P. falciparum* examined in the present study was found to be unchanged in specificity following cultivation of parasites *in vitro* for one month and then passage through either a *Saimiri* monkey or sequential passage through two semi-immune *Aotus* monkeys (unpublished results, 1978). Such antigens can serve as markers for different isolates of parasites.

Although only provisional, the amino acid composition of the S-antigen-rich fraction prepared by IEF fits well with most of the previous observations made on the immunochemistry of these antigens (*18*). The low cysteine content agrees with the absence of crucial quaternary structure implied by the antigen's stability in various denaturing reagents and its recovery from SDS gels. The negligible absorption at 280 nm agrees with the apparent absence of tyrosine and phenylalanine. The presence of lysine and arginine would be expected from the antigen's known susceptibility to trypsin, and the apparent absence of aromatic amino acids might help to account for the rather unexpected resistance to pepsin. As indicated by the isoelectric point, the content of acidic amino acids was high. The previous failure to label S-antigens in cultures of infected blood containing radiolabelled isoleucine and methionine (*6*) might be explained by the low content of these amino acids and this aspect is now open to further experimentation with the amino acids found in higher concentration. Further experiments are in progress to confirm the amino acid composition and to analyse an additional large peak of unidentified material, which might consist of hexosamines. Comparison of S-antigens of different specificities and from different sources is also planned.

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RÉSUMÉ

FRACTIONNEMENT ET CARACTÉRISATION DES ANTIGÈNES DE *PLASMODIUM FALCIPARUM*

Des extraits d'érythrocytes entiers infectés par *Plasmodium falciparum* ont été analysés par chromatographie en CM-cellulose pour faire apparaître en deux fractions distinctes l'hémoglobine et les produits antigéniques et parasitaires. La fraction antigénique (I) contenant les diverses classes d'antigènes parasitaires déjà identifiées par la technique des anticorps précipitants, ainsi que des constituants marqués non dialysables 2,5 à 5 fois plus concentrés que dans un extrait d'érythrocytes humains non infectés, tout ce matériel étant relativement exempt d'hémoglobine. On a constaté la présence d'antigène La_1 associé à des débris de membranes récupérés à l'origine après électrophorèse en gel de polyacrylamide (PAGE). Cet antigène a aussi été décelé parmi plusieurs autres qui ont été extraits de schizontes récoltés en culture de longue durée. En ce qui concerne l'antigène S, on a procédé à l'évaluation de son poids moléculaire par élution après électrophorèse en gel de dodécylsulfate de sodium-polyacrylamide (SDS-PAGE) ainsi que par absorption de l'antigène radiomarqué avec les anticorps spécifiques et examen fluorographique ou scintigraphique. Dans le matériel provenant de singes *Aotus* infectés, l'antigène S formait une bande majeure de masse moléculaire relative de 148 000; diverses analyses du même ordre ont montré que l'étalement peut varier de 110 000 à 180 000; avec des antigènes S provenant d'enfants ou d'adultes infectés, on a observé des valeurs pouvant être beaucoup plus élevées et une hétérogénéité analogue—également constatée dans les points iso-électriques. Une analyse provisoire des acides aminés a été faite sur un antigène S provenant de singes *Aotus* et fractionné après électrofocalisation préparatoire sur pI 4,5.

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