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STUDIES ON THE IMMUNOLOGY OF HUMAN MALARIA

I. PRELIMINARY CHARACTERIZATION OF ANTIGENS IN *PLASMODIUM FALCIPARUM* INFECTIONS

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SUMMARY

Extracts of erythrocytes infected with *Plasmodium falciparum* have been prepared from densely infected human placental tissue. Gel-filtration of such extracts on Sephadex G-200 readily separated two types of antigen, designated α and β, respectively. In addition, soluble antigens were detected in the sera of several children and parturient mothers immediately following attacks of malaria. These soluble antigens were found to be immunochemically similar to the α group of placental antigens. Combined histochemical and immunochemical tests indicated that the α antigens were probably proteins.

INTRODUCTION

Despite the fact that malaria is still the most important epidemic disease in the world, knowledge of the immunity acquired towards it remains rudimentary. Indeed, for the immunologist, immunity towards parasitic diseases represents one of the major challenges of the present day. Recently, broad aspects of parasitic immunity have been considered in a special World Health Organization report (1965) while the present state of knowledge relating to malarial immunity has been reviewed at two international workshops held at the Walter Reed Army Institute (1964, 1966) and by a World Health Organization Scientific Group (1968).

Much interest has centred on simian and rodent malarias since these are easily managed within the laboratory. In contrast there has been little work on naturally acquired human malaria and practically none on the antigenic structure of the human parasites. The recently reported application of the gel diffusion test to human malaria (McGregor *et al.*, 1966) has provided a simple tool for the study of certain antigen–antibody systems and this communication describes the application of this test to the study of antigens obtained from human donors infected with *Plasmodium falciparum* in Gambia, West Africa.

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Antigen extracts were obtained from densely infected placental tissue and subjected to gel-filtration on Sephadex G-200. Two groups of antigens differing in elution position were readily identified. In addition, soluble antigens were detected in the sera of several children and parturient mothers immediately following attacks of malaria. Some characteristics of these antigens have been investigated and are discussed in this report.

MATERIALS AND METHODS

Malarial antigens

Except where otherwise stated the malarial antigens investigated were obtained by a method we have already described from placental blood heavily infected with *Plasmodium falciparum* (McGregor *et al.*, 1966). Parasitized erythrocytes together with non-parasitized red cells and leucocytes were disintegrated in an X-Press, or more usually, a Hughes Press, and the clear supernatant obtained after centrifuging for 20 min at 28,000 *g* was taken as antigen. Placental blood was selected because of the high density *P. falciparum* may attain in it and because it constitutes virtually the only accessible source of mature asexual forms of this parasite.

Blood from three different infected placentae was studied. Parasites recovered from the first placenta (M1) were the most mature being predominantly well developed schizonts. Those from the second placenta (M2) comprised schizonts and late stage trophozoites in approximately equal numbers. From the third (M3) the parasites were almost exclusively large uninucleate asexual forms (late trophozoites) in which pigment had coalesced to form a single plaque. In all three instances the parasite content of blood immediately prior to disintegration was high. With respect to material from donors M1 and M2 about 50% of erythrocytes were parasitized. In the case of M3 about 40% of erythrocytes were infected.

Gel-filtration

Gel-filtration was performed on a column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), particle size 40–120 μ, dimensions $2{\cdot}4 \times 77{\cdot}5$ cm. A buffer of 0·2 M-Tris–HCl + 0·2 M-NaCl + 0·1% sodium azide (pH 8·0) and a flow rate of 20 ml/hr were used except for the fractionation of placental extracts from Patients M2 and M3 in which the flow rates were 12 and 10 ml/hr, respectively. The ambient temperature was 25–27°C.

Three-millilitre volumes of sample were fractionated by descending chromatography. Sucrose (3%) was added to facilitate layering and serum samples were previously centrifuged at 14,000 rev/min for 20 min to remove lipid.

The column effluent was continuously monitored at 254 nm (mμ) using a Uvicord I recording spectrophotometer (LKB Instruments, Stockholm) before collection with a time operated fraction collector. Fractions were pooled as indicated in Figs. 2 and 3—otherwise in pairs—and concentrated by ultrafiltration through 8/32 in. Visking tubing (Union Carbide Co., Chicago, Illinois, U.S.A.) prior to further analysis.

Immunoeletrophoresis

Immunoelectrophoresis according to Grabar & Williams (1953) was performed using LKB 'Immunophor' equipment and 1·5% Special Difco Noble Agar in barbitone buffer ($I = 0{\cdot}05$, pH 8·6).

Agar gel electrophoresis

Agar gel electrophoresis was performed on microscope slides using LKB 'Immunophor' equipment and 1·5% Special Difco Noble Agar in barbitone buffer (I = 0·05, pH 8·6), the sample being applied to a slot instead of a well.

Gel diffusion analysis

Double diffusion analyses were made according to Ouchterlony (1958) using 1·5% Special Difco Noble Agar in 0·3 M-phosphate buffer (pH 8·0). After drying and staining with Amido black the gels were photographed.

Antisera

All the antisera used (except anti-β_{1C}-globulin) were raised in rabbits following courses of immunization consisting of at least three injections of emulsified antigen in complete Freund's adjuvant. Usually antigen at a concentration of 3 mg/ml was used.

Anti-IgA (*α-chain specific*)

A serum A-myeloma protein isolated after block electrophoresis and DEAE-cellulose chromatography as described by Fahey & McLaughlin (1963) was used as antigen. The antiserum was shown to be specific by immunoelectrophoresis and Ouchterlony analysis after absorption with a Cohn Fraction II 1:2 preparation of IgG at 1·5 mg/ml.

Anti-IgM (*μ-chain specific*)

A pathological macroglobulin was isolated following block electrophoresis and gel filtration on Sephadex G-200 as described by Fahey & McLaughlin (1963). The antiserum produced to this antigen was absorbed with a Cohn Fraction II 1:2 preparation of IgG at 2 mg/ml and was shown to be specific by both immunoelectrophoresis and Ouchterlony analysis.

Anti-IgG (*γ-chain specific*)

A γ-chain preparation obtained from Cohn Fraction II 1:2 IgG by the sulphonation technique of Franěk & Zikán (1964) was used as antigen. The antiserum was shown to be specific for heavy chains and Fc fragment by immunoelectrophoresis and by Ouchterlony analysis after absorption with a preparation of Fab fragments at 0·2 mg/ml.

Anti-β_{1C}-globulin

This antiserum (kindly provided by Dr R. Thompson, Department of Experimental Pathology, University of Birmingham) was raised in rabbits following a course of four intravenous injections, given at 3-day intervals, of washed alexinated proteus–anti-proteus complex. The animals were bled 5 days after the last injection and the antiserum obtained was shown to be specific for β_{1C}-globulin by immunoelectrophoretic analysis.

Polyvalent antiserum

A polyvalent antiserum against human serum proteins was supplied by Burroughs Wellcome & Co., London.

Acrylamide gel electrophoresis

Disc electrophoresis (according to the method of Ornstein and Davis) was performed using the Canalco Model 6 system (Canalco Industrial Corporation, Bethesda) in 5-mm diameter cylinders of 7% acrylamide gel at pH 8·9 in Tris–chloride buffer as described by Davis (1964). Columns (4·5-cm) of 7% gel were surmounted by 1·5-cm columns of 3·5% 'stacking' gel in Tris–HCl buffer, pH 6·7, containing 20% sucrose. The 7% separating gels were polymerized with *N,N,N′,N′*-tetramethylethylenediamine using a catalyst of 0·14% (w/v) ammonium persulphate. For the stacking gels the proportion of *N,N,N′,N′*-tetramethylethylenediamine was doubled and polymerization effected by the addition of riboflavin and 30 min exposure to a fluorescent light. The sample gels consisted of 5μl of sample solution gelled in an aliquot of the stacking gel (150–200 μl).

Six tubes were electrophoresed simultaneously between two electrode tanks containing Tris–glycine buffer, pH 8·3. One millilitre of tracking dye (0·005% bromophenol blue) was added to 250 ml of buffer in the upper electrode tank. A current of 4 mA/tube was used throughout. Electrophoresis was continued for about 45 min after the samples had entered the separating gel. In this time the bromophenol blue marker had migrated about 3·0–3·5 cm from the inter-gel boundary. Gels were then stained for protein (nigrosin), carbohydrate (periodic acid–Schiff), nucleic acid (Feulgen) and lipid (lipid crimson) using standard histochemical techniques (see below).

Histochemical tests

Differential staining of acrylamide gels was carried out in large test tubes using a minimum of 20 ml of reagent or stain per gel.

Nigrosin (protein). Gels were stained with a solution of 0·05% nigrosin in 2·5% acetic acid and allowed to stand overnight. Background stain was removed using 2·5% acetic acid.

Periodic acid–Schiff (carbohydrate). The gel was first fixed in 7·5% acetic acid for 1 hr and then placed in freshly prepared 0·2% periodic acid in water (4°C for 1 hr). Periodic acid was removed electrophoretically by destaining in 7·5% acetic acid for 1 hr and the gel was then placed in Schiff's reagent at 4°C for 2–3 hr. Non-specific background reaction was removed by repeatedly washing the gel in sulphite rinses (equal parts 10% potassium metabisulphite and N-HCl plus ten parts of water). The gel was stored in the rinse solution.

Feulgen (DNA). The freshly electrophoresed acrylamide gel was placed in ice cold N-HCl for 30 min, hydrolysed in N-HCl at 57°C for 12 min and then washed in cold N-HCl. The gel was then transferred to Schiff's reagent at room temperature and left until positive (approximately 2 hr). The gel was then washed repeatedly with a sulphite rinse (as for periodic acid–Schiff) and stored in the same solution.

Lipid Crimson (lipid). The gel was fixed for 1 hr in 3% acetic acid and then stained overnight in a saturated solution of lipid crimson in methyl alcohol (diluted to 60% with water before use). An acid–Teepol wash was used to remove non-specific background stain.

Agar gel slides were treated as described below.

Feulgen stain (DNA). The wet gel was placed in N-HCl for 30 min, hydrolysed at 57°C in N-HCl for 12 min and then washed in cold N-HCl. The gel was then transferred to Schiff's reagent at +4°C for 2 hr, rinsed in six changes of sulphite water over several hours and finally dried under sulphite saturated filter paper.

Pyronin (DNA and RNA). The technique of Uriel & Avrameas (1961) was followed. The agar slide was fixed in 50% ethanol containing 2% acetic acid and then dried. After staining with 2% pyronin solution (pH 4·7) for 30–60 min, the slide was rinsed in acetate buffer (pH 4·7) until the background cleared. Finally the slide was dried at 80°C for a few minutes.

Acridine Orange (DNA and RNA). The wet gel was placed in phosphate–citrate buffer, pH 6·4, for 10 min and then transferred to the acridine orange working solution for 4 min. After two 5-min rinses the gel was examined for fluorescence under ultraviolet light.

Nigrosin (protein). After fixing and drying the slide was immersed in a solution of 0·5% nigrosin in 2·5% acetic acid and allowed to stand overnight. Background stain was then removed by prolonged washing in 2·5% acetic acid.

RESULTS

The interaction of malarial antigen and antibodies in agar gels is readily demonstrated, and a number of distinct antigen–antibody systems have been detected. The number and appearance of precipitin lines is, however, somewhat variable. This study of human malarial antigens utilized a serum (K.S.) from an adult female Gambian believed to be immune to malaria. This serum reacted well with two antigenic constituents in the first placental

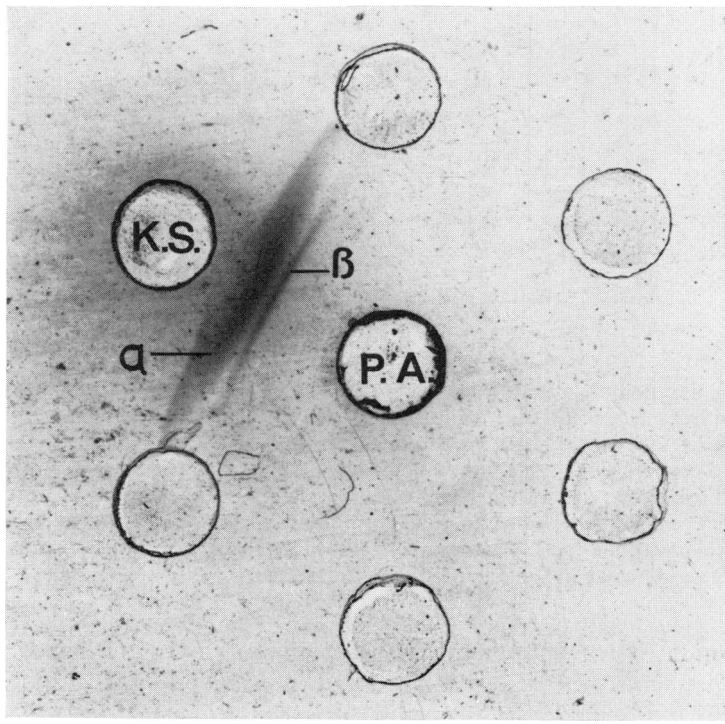

FIG. 1. Gel-diffusion analysis of unfractionated antigens from placenta (P.A.) of mother M1 infected with *P. falciparum*. Immune serum from an adult female Gambian (K.S.) was used as antiserum.

extract studied (obtained using an X-Press). The donor of this placenta was referred to as patient M1. These antigens were arbitrarily designated α and β as shown in Fig. 1. The immune serum K.S. was subsequently used to titrate antigen activity in all the experiments described herein.

An attempt to fractionate antigens according to their size was made using the technique of gel filtration. Fig. 2 shows the result of gel filtration on Sephadex G-200 of a placental extract from mother M1. Three peaks of ultraviolet absorbing material were resolved. The second and largest peak was associated with a reddish brown coloration indicating the elution position of haemoglobin. The fractions obtained in this separation were pooled as indicated in Fig. 2 and concentrated by ultrafiltration through 8/32 in. Visking membrane. The distribution of antigen activity in Pools 1–6 was then determined by gel diffusion using

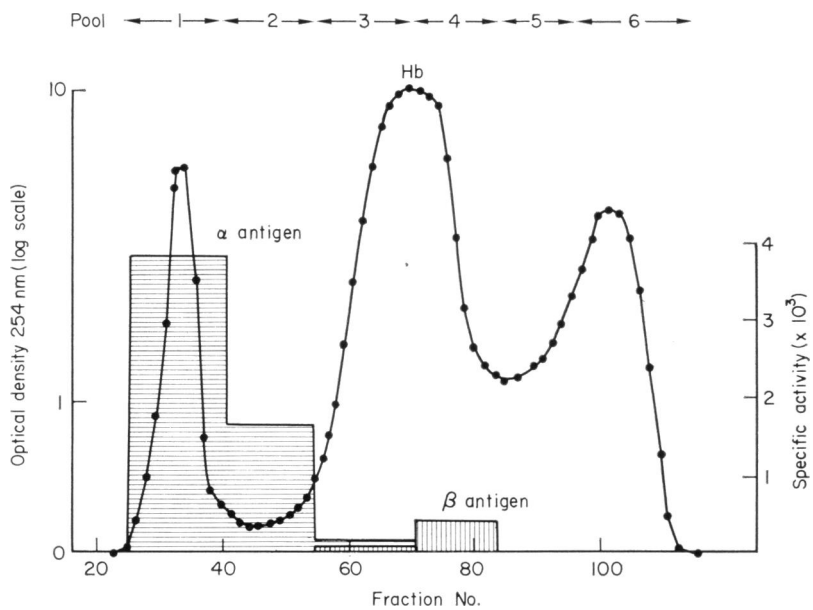

FIG. 2. Gel filtration of antigens extracted (by X-Press) from placenta of mother M1 heavily infected with *P. falciparum*. Three millilitres of sample were applied in 3% sucrose to a column of Sephadex G-200 (2·4 × 77 cm). Elution was performed with a buffer of 0·2 M-Tris–HCl + 0·2 M-NaCl containing 0·1% NaN_3 at a flow rate of 20 ml/hr and at a temperature of 27°C. Fractions were collected at 10-min intervals. Fractions were pooled and concentrated as indicated and antigen activity in each pool is expressed as specific activity (titre multiplied by concentration factor of pool). Titres were established by gel-diffusion using a doubling dilutions system and human immune serum K.S. as antiserum.

a doubling dilutions system and serum K.S. as immune serum. In Fig. 2 antigenic activity is expressed as specific activity (titre multiplied by concentration factor of pool). As can be seen in this figure the α antigen (subsequently termed α1) was associated mainly with Pools 1 and 2 whereas the β antigen (subsequently β1) was found mainly in Pool 4. Some overlapping of these activities occurred in Pool 3.

Following the extraction of antigen from placenta M1 a residue of undisintegrated material

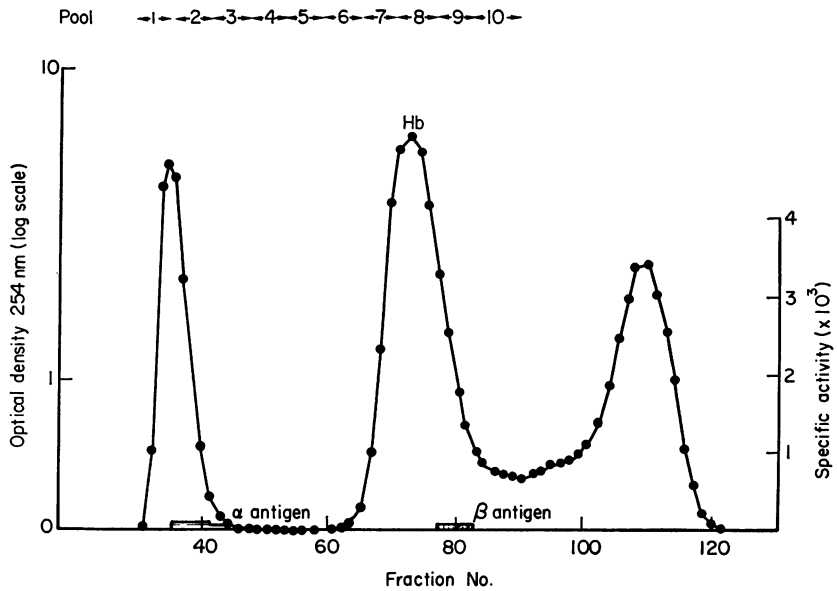

FIG. 3. Gel-filtration of antigens obtained following a second extraction (by Hughes Press) of residue remaining after the initial X-Press extraction (cf. Fig. 2). Three millilitres of sample were fractionated as in Fig. 2.

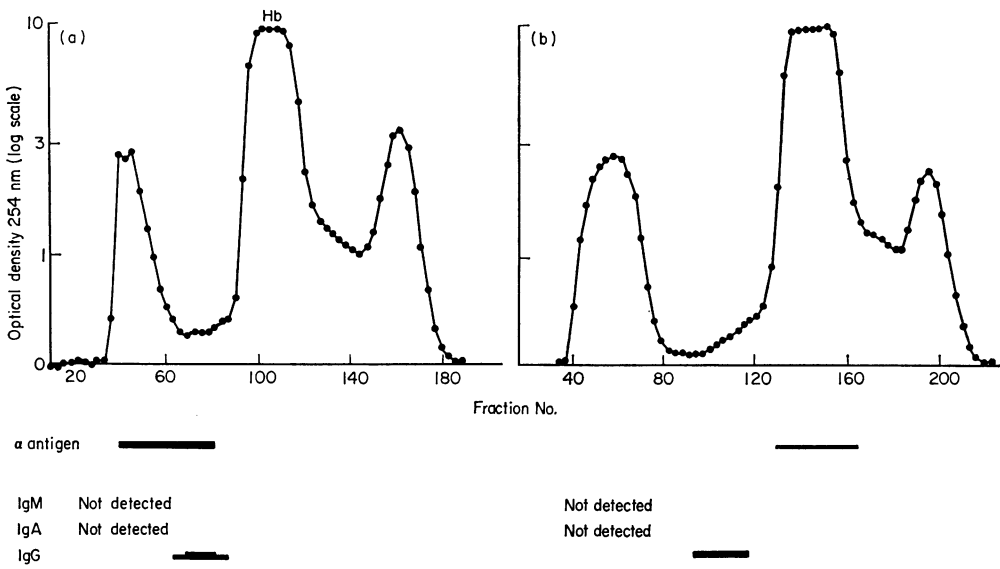

FIG. 4. (a) Gel filtration of antigen extract from placenta of patient M2. Three millilitres of sample were fractionated as in Fig. 2. The distribution of α antigen was determined by gel diffusion using immune serum K.S. as antiserum. Fractions were also examined by gel diffusion for immunoglobulins IgM, IgA and IgG using antisera specific for these proteins. (b) Gel filtration of antigen extract from placenta of patient M3. Three millilitres of sample were fractionated as in (a). The distribution of β antigen was determined by gel diffusion using human immune serum K.S. as antiserum.

remained in the X-Press. This was resuspended and re-extracted in a Hughes Press giving a second antigen supernatant. Fig. 3 shows a Sephadex G-200 fractionation of this extract. Three ultraviolet absorbing peaks were again resolved and the $\alpha 1$ and $\beta 1$ antigens were again detected in the first and second peaks, respectively. In this case, however, the recovery of both antigens was poor. Compared with the initial extraction only 3% of the α antigen activity and 15% of the β antigen activity were recovered.

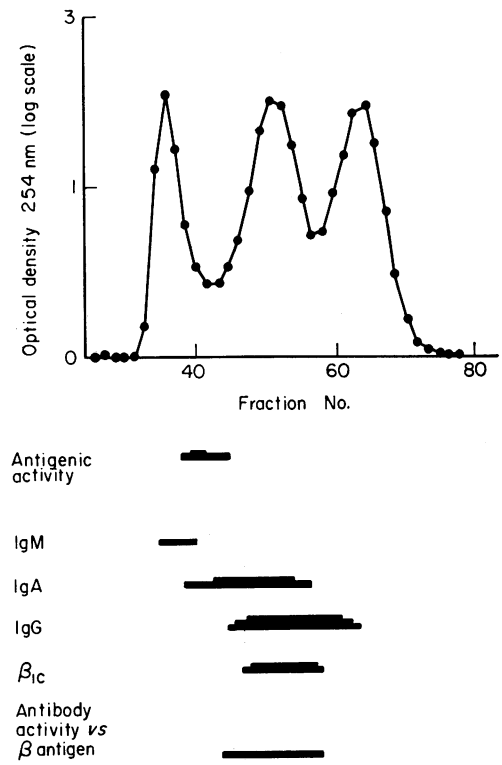

FIG. 5. Gel filtration of serum from patient M1 (donor of placental extracts in Figs. 1 and 2). Three millilitres of serum were fractionated as in Fig. 2. Fractions were pooled in pairs and concentrated for antigenic analysis. Antigenic activity was determined in gel diffusion tests using human immune serum K.S. Plasma protein distributions were established using antisera specific for those proteins.

Two more placental extracts (obtained using the Hughes Press) were examined and found to react with the K.S. immune serum. Although both extracts produced multiple precipitin lines, Fig. 4(a) shows that following a Sephadex G-200 fractionation of placental extract from mother M2 only an α antigen ($\alpha 2$) was detectable and Fig. 4(b) shows that following Sephadex G-200 fractionation of placental extract from mother M3 only a β antigen ($\beta 2$) was detectable. IgG was detected in the eluates from both the M2 and the M3 supernatants. This was presumed to be maternal protein which had not been removed during the preliminary wash stages. There was no evidence of IgA or IgM in the extracts of M2 and M3 although IgA was detected, together with IgG, in Pool 2 from the fractionated M1 supernatant (Fig. 2).

During the investigation of the antigens in the placental extracts it was shown that the serum of the mother M1 contained, at the time of parturition, a component which reacted in agar gel with the immune serum K.S. When serum M1 was subjected to gel-filtration on Sephadex G-200 the component was readily identified in the trailing edge of the first peak (Fig. 5). Thus the elution position was similar to that of the α antigens identified in the placental extracts. No component having an elution position similar to the β antigen was observed. This serum was, in fact, shown to contain antibodies to the β antigen.

The discovery of an apparently free circulating malarial antigen in the serum following an attack of malaria prompted a search for yet other examples of this phenomenon. Another

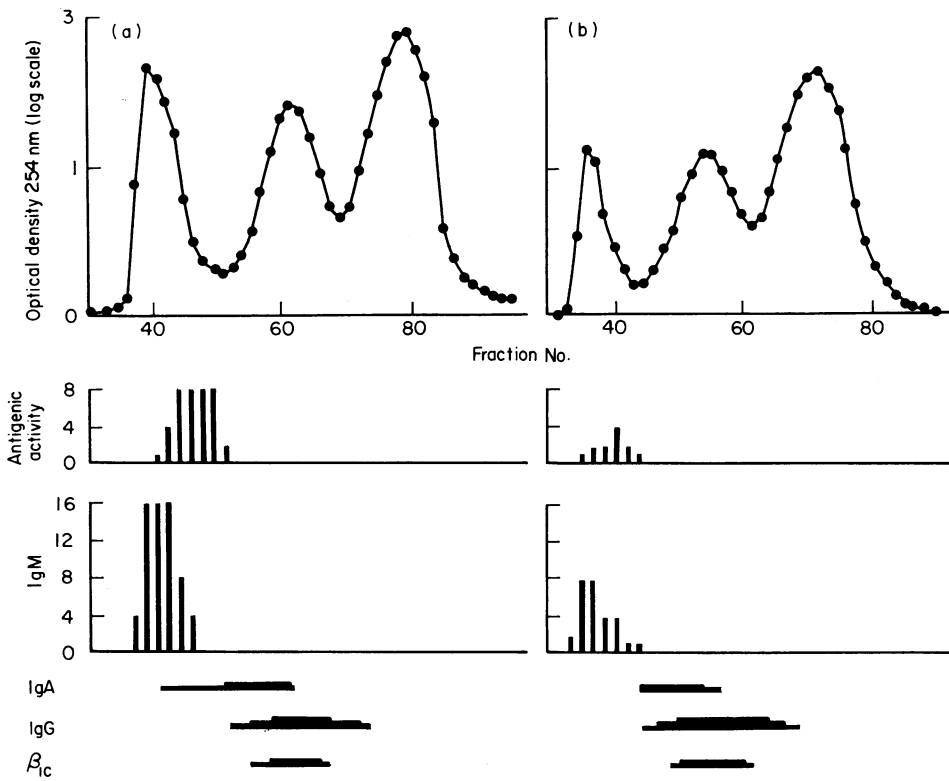

FIG. 6. Gel filtration of sera from patients M.N. (a) and M.J. (b)—both children suffering from severe *P. falciparum* malaria. Three millilitres of serum were fractionated in each case. Fractions were pooled in pairs and concentrated for antigenic analysis. Antigenic activity was determined in gel diffusion tests using a doubling dilution system and human immune serum K.S. as antiserum. Plasma protein distributions were established using antisera specific for those proteins.

parturient woman (M2) was found to have a similar component and also several children who had been admitted to the wards at the M.R.C. Laboratories with very severe malaria (e.g. 40% of red cells parasitized).

Fig. 6 shows Sephadex G-200 fractionations of sera from two children, M.N. and M.J. The distribution of immunoglobulins and β_{1C}-globulin is shown beneath the elution profiles together with the distribution of antigen activity (titrated in a doubling dilutions

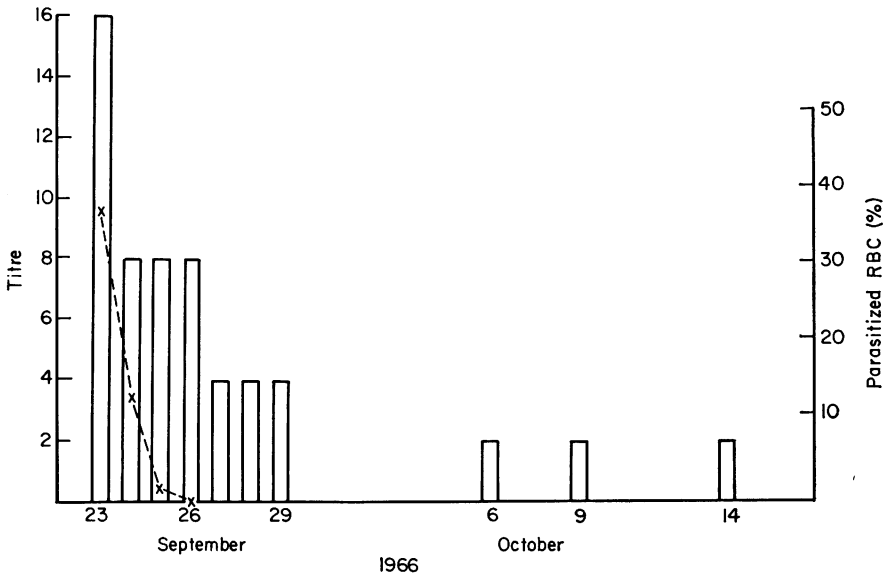

FIG. 7. Showing the persistence of serum antigen (vertical bars) in patient M.J. following heavy *P. falciparum* parasitaemia. Titrations of antigen were performed by gel diffusion tests using a doubling dilutions system and human immune serum K.S. as antiserum. The percentage of parasitized erythrocytes is shown by the broken line.

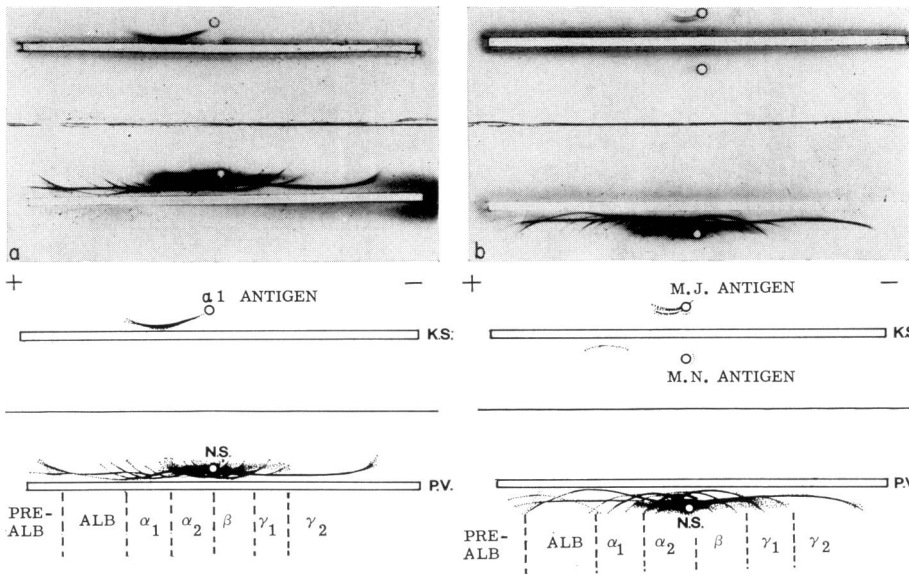

FIG. 8.(a) Immunoelectrophoretic analysis of α1 (placental) antigen in barbitone buffer, pH 8·6. The precipitin arc was developed with human immune serum (K.S.). For comparison a normal serum (N.S.) was electrophoresed and developed with a polyvalent antiserum (P.V.). (b) Immunoelectrophoretic analysis of sera from patients M.J. and M.N. (containing soluble antigen). The precipitin arcs were developed with human immune serum (K.S.).

system). As with patient M1 the antigen activity was detected in the trailing edge of the first peak slightly behind the IgM peak. Usually the antigen disappeared swiftly after effective chemotherapy, but in the case of patient M.J. it continued to circulate in the serum for 3 weeks after eradication of parasitaemia. Fig. 7 shows the persistence of the antigen over this period. The child did not apparently go on to produce any antibodies to this component and

TABLE 1

Antigen		Electrophoretic mobility*
'High molecular weight' antigens	α1 antigen	α_1–α_2
	α1 antigen (second extraction)	α_1–α_2
	M2 antigen	α_2
	α2 antigen	γ_2
	M.J. antigen	α_2
	M.N. antigen	α_1
'Low molecular weight' antigens	β1 antigen	Pre-albumin
	β1 antigen (second extraction)	Not detected

* Mobilities are expressed in terms of the plasma protein classes, i.e. pre-albumin, albumin, α_1, α_2, β, γ_1 and γ_2.

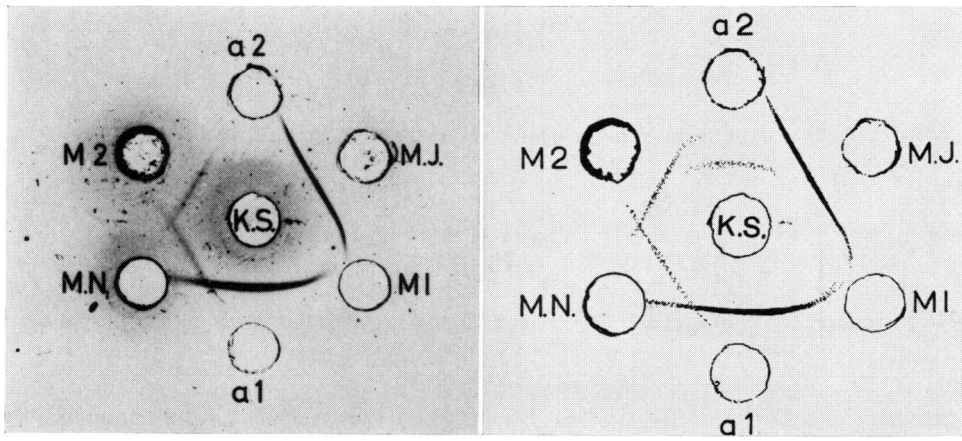

FIG. 9. Gel diffusion analysis in 0·3 M-phosphate buffer pH 8·0, of various malarial antigens. Except for the antiserum K.S. all wells contained Sephadex G-200 fractions enriched with antigen. Samples M.J. and M.N. were from children. Samples M1 and M2 were from mothers whose placental antigens α1 and α2 were extracted and studied.

was subsequently re-admitted with another heavy infection during the course of which a similar antigenic component reappeared.

The free circulating antigens found in the sera of parturient women and several children following severe attacks of malaria had similar elution positions on Sephadex G-200. This

suggests (but does not necessarily prove) that all were of a similar molecular size. It was therefore of interest to investigate other physico-chemical and immunochemical parameters of these antigens. Fig. 8 shows immunoelectrophoretic analyses of the placental α1 antigen (Fig. 8a) and the M.J. and M.N. antigens (Fig. 8b). It can be seen that mobility differences existed between these three antigens and indeed among all the antigens under study. Table 1 shows the electrophoretic mobility (in terms of plasma protein classes) of all the high and low molecular weight antigens studied.

Immunochemical comparison of concentrated Sephadex G-200 fractions containing the high molecular weight antigens also revealed a degree of heterogeneity. Thus some antigens such as those from the serum of mother M1 and her placental extract α1 were completely

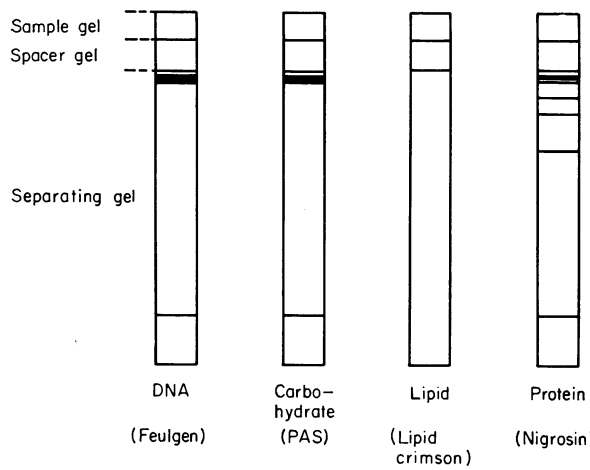

FIG. 10. Disc electrophoresis in acrylamide gel of the α1 antigen. Gels were subsequently stained as indicated for DNA, carbohydrate, lipid and protein.

identical with each other and also with the M.J. antigen. In contrast antigen from M.N. showed non-identity with this group and also with antigen from mother M2. Finally, although difficult to interpret, it was noted that the placental extract α2 antigen was apparently not related to the maternal M2 antigen. Some structural difference was also indicated by the electrophoretic mobilities of the M2 and the α2 antigens (α_2 and γ_2, respectively—see Table 1). This disparity is discussed more fully later.

Definitive chemical identification of the antigens described in this communication was made difficult by the crude nature of the starting material and indeed was not attempted in the case of the β antigens which were always associated with large amounts of haemoglobin. Some preliminary studies have been made on the α1 antigen since this was available in sufficient quantity. A spectrophotometric analysis of Pool 1 from Fig. 2 (rich in α1 antigen) showed a prominent peak of absorbence at 260 nm indicating a high content of nucleic acid. There was no evidence of a secondary peak at 280 nm. The presence of DNA in Pool 1 was confirmed by specific Feulgen staining following acrylamide gel electrophoresis of the α1 antigen (Fig. 10). A heavily stained band was observed in the separating gel close to the interface with the spacer gel (DNA and nucleoprotein are macromolecules and would not be expected to penetrate the gel readily). A periodic acid–Schiff stain for carbo-

hydrate was positive in the same region—presumably reflecting the pentose sugar content of nucleoprotein. Nigrosin was positive for protein in this region and also stained three faster components whereas a lipid crimson stain failed to detect any lipid in the gel.

Further histochemical studies were made following agar gel electrophoresis on microscope slides. Aliquots of the α1 antigen were electrophoresed and one sample was developed as an immuno arc by conventional techniques. The remaining slides were then examined for DNA (Feulgen), DNA and RNA (Pyronin and acridine orange) and protein (Nigrosin). The nucleic acid migrated far forward in the albumin region whereas the protein bands coincided in mobility with the immuno-precipitin arc. This indicated that the α1 antigen was probably a protein although the possibility of its being a glycoprotein could not be excluded.

DISCUSSION

It is clear that a wide spectrum of antigenic substances must be released during the course of a malarial infection and that many of these are capable of promoting an immune response. In recent years Cohen & McGregor (1963) have established that humoral mechanisms make an important contribution to man's acquired immunity to malaria and several immunodiagnostic tests have been developed for the detection of antibodies to malaria. In contrast, practically nothing is known about the relative importance of cellular immunity in malaria and, finally, there is a large gap in our knowledge about the nature of malarial antigens.

We describe in this report an attempt to fractionate and study some of the antigens of *Plasmodium falciparum* malaria. The empirical choice of gel-filtration for fractionation was based on the postulate that all antigens were unlikely to have the same elution characteristics. Furthermore, the method is technically simple and one of the least likely to bring about conformational changes in molecular structure. For the detection of antigen we were able to make use of the gel diffusion test which we had previously developed (McGregor *et al.*, 1966).

We were fortunate to have available an homologous human immune serum which made the interpretation of gel diffusion data relatively simple. This serum reacted with two groups of antigenic components called α and β which were subsequently separated by gel filtration. There was considerable evidence to support the view that apart from size these two antigens were fundamentally different. The extraction and subsequent re-extraction of the antigen from the placenta of mother M1 indicated that the α antigen was readily extracted and little (3%) was recovered in the re-extract. In contrast, the β antigen was less easily extracted and in the re-extract 15% of the original yield was obtained. These observations might indicate a subcellular plasmodial location for the β antigen and a more superficial membranous location for the α antigen (such as a host–cell complex). Alternatively the α antigen may represent a metabolite of the parasite—an exo-antigen—and this view would certainly help to explain its similarities to the free circulating soluble antigens also studied in this investigation. Equally it may represent exposed hidden antigenic determinants of the erythrocytes themselves.

It is of interest that as early as 1939 Eaton reported the existence of soluble complement-fixing antigens in the sera of monkeys with acute infections of *Plasmodium knowlesi.* Cox (1966) has also demonstrated an antigen in the serum of monkeys acutely infected with *P. knowlesi* and showed by gel precipitin tests that it reacted with sera from animals con-

valescent from malaria infection. To the best of our knowledge the observations of soluble antigen in the serum of the children M.J. and M.N. and the mother M1 were the first examples of this phenomenon in human malaria. In a separate investigation (McGregor *et al.*, 1968) we have studied the frequency of such soluble antigens in young malarious children and of antibodies reacting with these in the population of the Gambian village of Keneba. In brief, our findings indicated: (a) that soluble antigens were detectable in the serum of approximately 25% of all children presenting with acute malaria, (b) that antibodies to the antigens were rarely seen in children under 6 years old but were mainly confined to adults (although never more than 15% of the adult population were positive for any one antigen), and (c) the persistence of circulating antigen was of variable duration. The observation that the patient M.J. still had demonstrable soluble antigen several weeks after the eradication of parasitaemia and did not subsequently manufacture antibodies to that antigen is clearly intriguing and has been noted in our preliminary presentation of this data (McGregor, 1967; Turner, 1967). We have speculated that the failure to detect antibodies to circulating soluble malarial antigens, after their elimination, may indicate that they are poorly immunogenic—perhaps because they derive from the host itself. An alternative theory put forward by Weir (1967) in a more general context suggests that soluble antigens characteristically induce immune tolerance.

The failure to detect β antigens in the separation of M2 extract (Fig. 4a) and α antigen in the separation of M3 extract (Fig. 4b) could be interpreted as indicating that considerable heterogeneity in antigen classes is to be anticipated when extracts from different infected placentae are compared. However, we believe that this failure could equally be explained on purely technical grounds. Investigations of the type described here are severely limited by the potency and specificity of the reagent—in this case the antiserum K.S. The precipitin reactions with M2 and M3 extracts were much weaker than those given by the M1 extract and the failure to detect both α and β antigens after gel filtration of each placental extract may have been due to the inability of the antiserum to detect the lower concentration of antigens.

We were able to investigate in more detail the possibility of antigenic variation within the α group. Firstly, there was undoubtedly electrophoretic heterogeneity. Thus if one accepts the view, as seems likely, that these are protein antigens then there must be marked differences in the amino acid composition of members within the group. Secondly there was immunological heterogeneity as revealed by gel diffusion tests. It is, in this context, interesting to note that the $\alpha 1$ placental antigen and the soluble antigen in the serum of mother M1 were antigenically identical whereas the $\alpha 2$ placental antigen appeared to be distinct from the soluble antigen in the serum of mother M2. From a consideration of the results shown in Fig. 9 it seems probable that two distinct antigens were present in both the serum and the placental extract but the concentration of the serum antigen was low in the placental extract and the concentration of the placental antigen was low in the serum. This conclusion is supported by the observation that the precipitin line between antiserum K.S. and the $\alpha 2$ placental antigen is deviated by a similar component in serum M2. Similarly, the precipitin line between K.S. and serum M2 is apparently deviated by a related component in the $\alpha 2$ placental antigen. A possible indication of molecular lability was obtained when the placental $\alpha 1$ antigen was subjected to analytical ultracentrifugation after storage at $-70°C$ for approximately 1 year. A single component was resolved and shown to have a sedimentation coefficient (S_{20w}) of 6·3S. There is no direct evidence that this component was the

α-antigen but if so it is difficult to reconcile the ultracentrifugal findings with the elution position of the antigen on Sephadex G-200 which suggested a molecular weight in the range 300,000–900,000. However, it is unwise to attempt an evaluation of exact molecular weight on the basis of a gel filtration elution position. The factors governing elution position are molecular size and shape rather than molecular weight and the presence of carbohydrate side-chains in a molecule may also influence elution position. Since nothing is known about the molecular dimensions nor whether carbohydrate is present in either of the antigens studied we can only regard the estimations of molecular weight as approximate at this stage. Thus the terms 'high molecular weight' antigen and 'low molecular weight' antigen are used cautiously in both this paper and the next (Turner & McGregor, 1969). Obviously further investigations on the molecular size of these antigens is urgently needed.

At this point it is pertinent to compare our findings with the recent investigations of Diggs (1966) and Chavin (1966). These authors have independently studied the antigenic constituents of *Plasmodium berghei* (a rodent parasite). Both authors found that the majority of plasmodial proteins migrated in the β to albumin range and there were no cathodically migrating proteins. In contrast, Zuckerman (1964), in a study of *P. berghei* extracts, reported proteins of cathodal as well as anodal migration. In our present studies antigens were mostly of α_1 or α_2 mobility (Table 1). However, one (the α2 antigen) migrated cathodically in the γ_2 region. It should be noted that this antigen is the one which appeared on gel diffusion analysis (Fig. 9) to be unrelated to the maternal soluble antigen from patient M2. The latter migrated in the α_2 position thus providing confirmation for the view that the two antigens differed structuraliy.

Chavin (1966) also investigated gel filtration as a possible method of fractionating the antigens of *P. berghei*. He found that on Sephadex G-200 all the antigens were eluted as a single protein peak immediately beyond the void volume. This is comparable with the elution position of the α group of antigens which we have studied. It is interesting that Chavin (1966) also noted a disparity between his gel filtration data and the results of analytical ultracentrifugation. He obtained a sedimentation constant of 3·3S (confirmed by sucrose density centrifugation) which agreed well with a value of 3·4S obtained by Sherman & Hull (1960) for the proteins of *Plasmodium lophurae*. Chavin was unable to find a satisfactory explanation for the discrepancy between the analytical ultracentrifugation and gel filtration data but suggested that some type of protein–protein interaction might have occurred in gel filtration.

Finally, our data should be compared with that of Williamson (1967). Using thin layer gel filtration to study antigens extracted from the Simian parasite *P. knowlesi*, this investigator found that the bulk of such antigens migrated in the same region as haemoglobin. Thus in this case the antigens examined seemed to resemble most closely the group which we have called the β antigens.

There can be no doubt that many of the unanswered questions raised here will be subjected to experimentation in the near future. It is particularly desirable that much more highly purified antigen should be made available for meaningful immunochemical investigation. Our own work was only possible because of the chance availability of a good human antiserum which enabled us to detect antigen unambiguously even when grossly contaminated. A start has been made in fractionating the antigens of various malarial parasites and some of the results are difficult to understand at this stage. Only further investigations using more precisely defined material will help to resolve these difficulties.

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