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Immunoglobulin Characteristics of Antibodies to Malarial S-antigens in Man

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Summary. The electrophoretic mobility of antibodies to malarial S-antigens was usually γ_1. The same antigen-stimulated antibodies with different mobilities in different individuals. IgG antibodies and others probably belonging to the IgM class were detected. IgG dissociated from immune complexes contained light chains of λ type only in two cases and predominantly κ type in a third. This antibody cross-reacted with an antiserum to IgG2. Antibodies to other malarial antigens had restricted mobilities in the γ_2 region.

INTRODUCTION

Different kinds of malarial antigens have been described in extracts of human blood infected with *Plasmodium falciparum* (Wilson, McGregor, Hall, Williams and Bartholomew, 1969). Those called S-antigens show considerable serological diversity and are found in the circulation during acute infections. Precipitating antibodies to these antigens are rarely detected in children examined in cross-sectional studies of village communities but their prevalence increases with age (McGregor, Turner, Williams and Hall, 1968). The presence of antibodies to S-antigens is associated with increased serum concentrations of IgM (McGregor and Wilson, 1971), and we have suggested that the relatively brief persistence of some of these antibodies may be because they belong to this class of immunoglobulin. Fractionation of sera from immune adults showed, however, that IgM and IgG-like antibodies to S-antigens occur with equal frequency and we report here further studies on IgG antibodies to S-antigens with special reference to their electrophoretic mobility. This was found to be restricted and different from that of some other malarial antibodies.

MATERIALS AND METHODS

S-antigens

Africans with heavy *Plasmodium falciparum* infections (usually $\geqslant$ 100,000 parasites/cc blood) or in whose blood a combination of anaemia, pigmented leucocytes and malarial parasites indicated a recent infection, were bled by venepuncture prior to treatment. The blood was centrifuged and the serum stored in 0·5-ml aliquots at $-70°$ with a small quantity of sodium azide added as a preservative.

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Malarial extracts

Extracts of placental blood (P26 and P66) heavily infected with *P. falciparum* were made according to McGregor, Hall, Williams, Hardy and Turner (1966). The final cell suspension of P26 contained 69 per cent parasitized cells which were mostly late stage trophozoites. Blood from P66 contained 46 per cent parasitized cells of which 53 per cent were trophozoites and 47 per cent schizonts. Saline extracts were made and stored at $-70°$.

Malarial antisera

Sera were obtained from immune African adults from the villages of Keneba (K) and Manduar (M) in the West Kiang District of the Gambia. Sera from a group of adults, K601, M224, M262, M358 and M547, were used in the present studies as these were known from previous tests with numerous S-antigens to be amongst the most reactive in the population studied (McGregor and Wilson, 1971).

Gel diffusion

Double diffusion by the method of Ouchterlony (1958) was carried out in 1·5 per cent Noble agar buffered in 0·05 μ veronal, pH 8·6. Antigen- and antibody-containing sera were single or double loaded into wells and diffusion permitted for 48 hours at 5°. Subsequently, gels were washed, dried and stained with Amido Black using standard procedures.

Gel filtration

Gel filtration was done in columns (2·4 × 77·5 cm) with Sephadex G-200 (particle size 40–120 μm). The buffer used was 0·2 M Tris-HCl saline, pH 8·0, to which traces of sodium azide were added as a preservative. Serum fractionation and concentration of eluates were carried out essentially as described by Turner and McGregor (1969).

Immunoelectrophoresis

Immunoelectrophoresis in 1·5 per cent Noble agar in veronal buffer (0·05 μm), pH 8·6, was carried out according to Grabar and Williams (1953).

Crossed electrophoresis

Electrophoretic separation of S-antigens was carried out in a gel consisting of 5 per cent acrylamide and 0·8 per cent agarose in Tris glycine buffer, pH 8·7, as described by Uriel (1966). After the initial electrophoresis a second well was punched in one end of the gel and loaded with antigen-containing serum labelled with Bromophenol Blue as a marker. A second eletrophoresis was then performed at right angles to the first so that the components in the acrylamide/agarose gel migrated into a second gel of 1 per cent agarose containing antiserum at a dilution of 1/100. The gels were washed in M/15 phosphate buffer, pH 8·0 and stained with Amido Black in the wet state prior to photography.

Formation and dissolution of immune complexes

Sera containing S-antigen or antibody were clarified by centrifugation at 40,000 rev/min for 20 minutes. Subsequently, they were held at 5° for 3–7 days but cryoprecipitates were not observed with the sera used below. Samples of 1 or 0·5 ml of serum containing S-antigen were mixed and incubated for 2–4 days at 5° with sufficient of the appropriate

antiserum (usually an equal volume) to give slight antibody excess. The precipitates were separated by centrifugation and washed two or three times in M/15 phosphate buffer, pH 8·0. Attempts to dissociate two of the antigen–antibody complexes were made by resuspending the washed pellet in a small volume of saline and adding traces of HCl (N/20, N/10 or N). This resulted only in slight clearing. In the third system we examined, the immune precipitate dissolved immediately on adding one drop of N/20 HCl.

None of the immune precipitates dissolved when they were suspended in 5 or 10 M urea but they did so in acidified urea. The addition of an equal volume of 28 per cent Na_2SO_4 to precipitates dissolved in this way gave a further precipitate which was separated by centrifugation and overlayed twice with water to reduce the concentration of salt and urea. This precipitate was partly dissolved in a small volume of M/15 phosphate buffer and the resulting solution was tested by gel diffusion for the presence of immunoglobulin, antibody activity and S-antigen determinants.

Antisera to immunoglobulins

Rabbit antisera specific for γ chain and μ chain of human Ig were those prepared and described by Rowe, McGregor, Smith, Hall and Williams (1968). Another rabbit antiserum to the Fab piece of human IgG, was kindly provided by Dr D. Catty of the Department of Experimental Pathology, University of Birmingham, England. The rabbit was immunized with Fab which had been prepared from pooled normal IgG and homogenized in Freund's complete adjuvant. Lyophilized rabbit antisera to Bence-Jones proteins of κ and λ type were obtained from the Institute for Sera and Vaccines, Praha, Czechoslovakia. Antisera to human IgG heavy chain subclasses were prepared by Dr G. Virella, as described by Virella and Parkhouse (1970). A horse antiserum to whole human serum proteins was obtained from Wellcome Laboratories, Beckenham, England.

RESULTS

ELECTROPHORETIC MOBILITY OF ANTIBODIES

Immunoelectrophoresis showed that antibodies to S-antigens usually have restricted electrophoretic mobilities in the γ_1-region. A spectrum of antibodies of different mobilities was found when sera from immune adults were tested against a variety of S-antigens (Fig. 1). These antibodies tended to be fast migrating but occasionally antibodies with slower electrophoretic mobilities were found. Antibodies from children also had electrophoretic mobilities that were restricted compared to total IgG or IgM. In one experiment there was an inverse relationship between the electrophoretic mobilities of antibodies to a fast migrating (pre-albumin) and a slow migrating (γ_2) S-antigen. This was not apparent with various S-antigens of intermediate mobility and their antibodies.

Antibodies to other malarial antigens were also found to be restricted but in the γ_2-region (Fig. 2). Detailed studies on these antibodies remain to be done so they will not be discussed further at the present time.

SERUM FRACTIONATION ON SEPHADEX G-200

Two months after treatment for malaria an 18-month-old girl (case No. 784) produced antibody to an S-antigen which had been circulating in her blood. The serum, which contained much lipid, was stored at 4° until tested. It was centrifuged at 40,000 rev/min

for 20 minutes prior to fractionation on Sephadex G-200 and the pooled concentrated fractions were tested against the original antigen-containing serum (Fig. 3). Most of the antibody was in pool 2 and in pools 4 and 5 but the reactions produced by pools 4 and 5 were rapidly lost when the gel was washed at room temperature (25°), either in 1 per cent

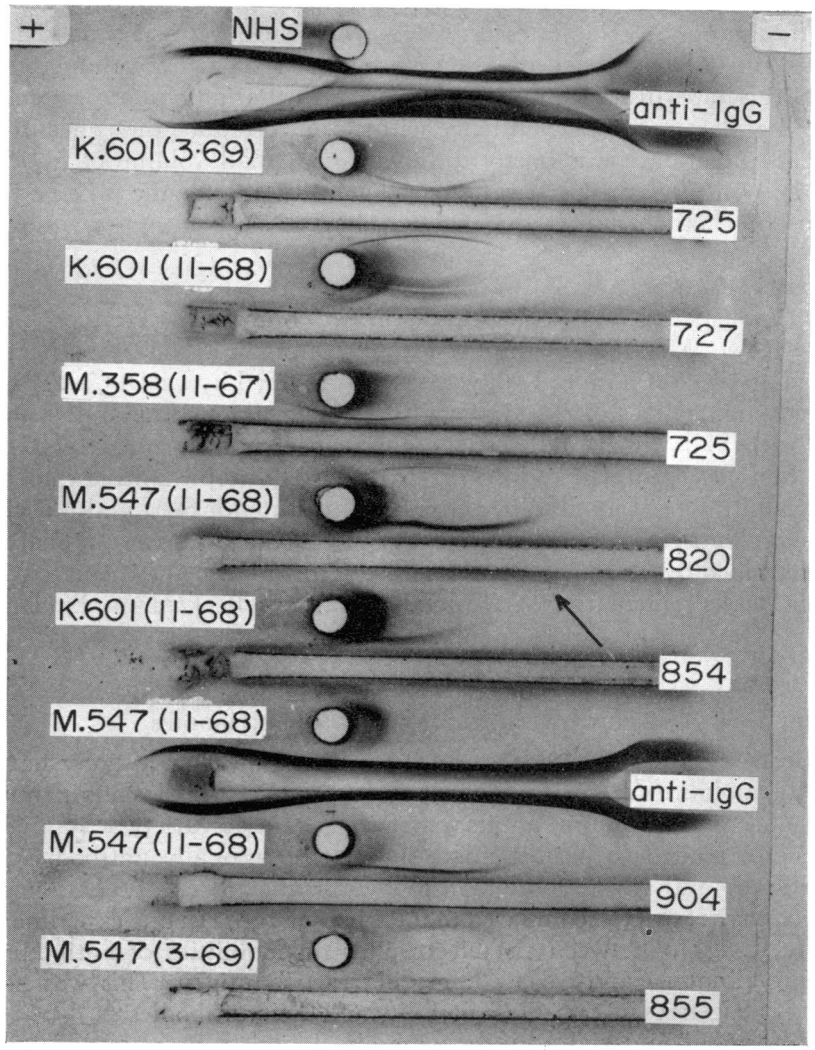

FIG. 1. Immunoelectrophoresis of antibodies in serum samples from three immune adults (K601, M358 and M547) to S-antigens in sera from six infected children. Note the restricted electrophoretic mobility of the different antibodies. Arrow indicates a slower moving antibody.

saline or in M/15 phosphate buffer, pH 8·0. The antibody in pool 2, which was directed against the same antigen, produced a precipitate which did not dissolve on washing, as was also the case with antibody from an immune adult (K601). Further experiments showed that the reactions produced by pools 4 and 5, as well as by pool 2, were relatively

stable when washed and dried in the cold (5°), but the precipitating activity of all the pools was inhibited when the reactants were diffused at 37°.

In contrast to the serum of this child, gel filtration of serum from the immune adult woman, K601, revealed that antibodies to the mixtures of S-antigens which we tested were confined to the second protein peak which was rich in IgG (Fig. 4). The antibodies to S-antigens again had discrete electrophoretic mobilities and produced reactions which were stable when the gels were washed and dried at room temperature prior to staining.

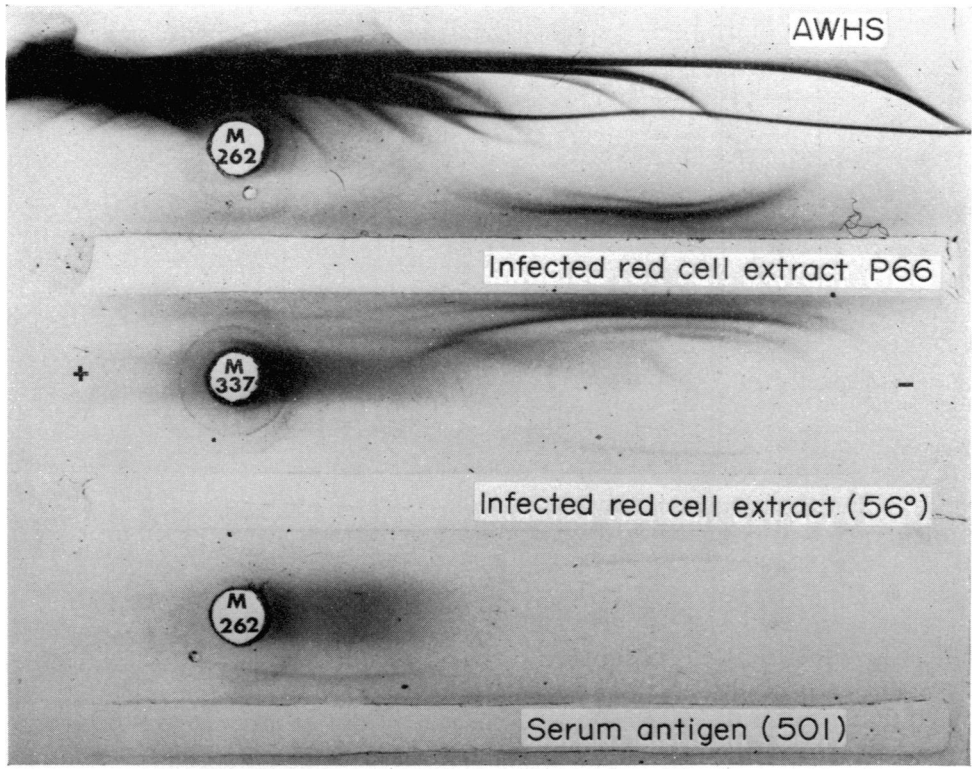

FIG. 2. Antibody to an S-antigen (501) was compared with antibodies to other malarial antigens which were in an extract of infected red cells (P66). Heating the cell extract at 56° inactivated some but not all of the malarial antigens (see Wilson *et al.*, 1969). Two different malarial antisera were used, M262 and M337. IgG and other serum proteins were precipitated with an antiserum to whole human serum (AWHS). The electrophoretic mobility of antibody to the S-antigen was γ_1 but antibody to other malarial antigens was γ_2.

IDENTIFICATION OF THE Ig CLASS

Attempts were made to identify the immunoglobulin (Ig) class of certain anti-S antibodies using sheep and goat antisera specific for IgG, IgM and IgA. These experiments were not successful when the whole immune serum was examined as the malarial precipitin line passed without apparent deviation into the strong reactions produced by the anti-globulin sera. Absorption of Ig from these African sera (which usually have elevated levels of IgG and IgM (Rowe *et al.*, 1968) was always incomplete and without obvious effect on the malarial reactions. In other gel diffusion and absorption experiments, rabbit

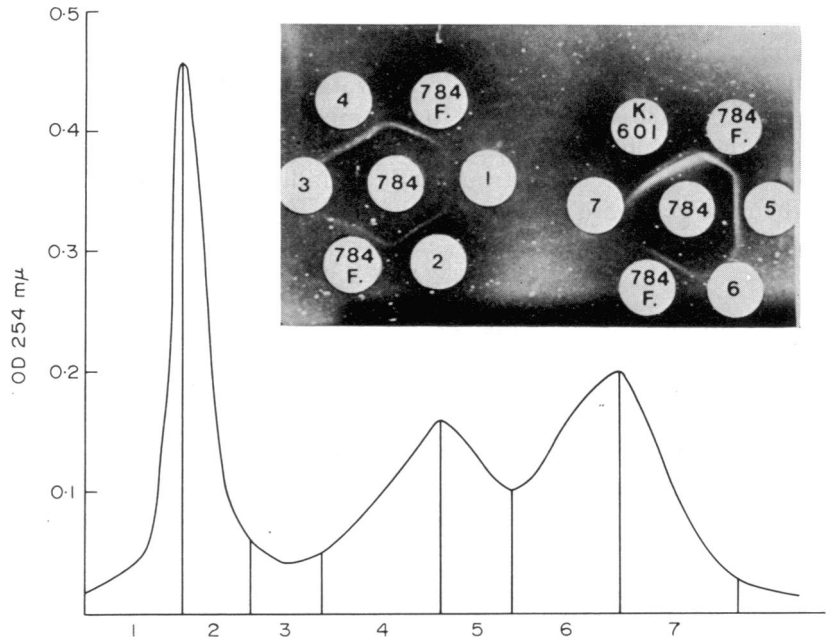

FIG. 3. Gel filtration of a child's serum containing antibody to an S-antigen which had previously been circulating in its blood. Antibodies in the whole serum (784F) and pooled eluates are shown in the inset. The original serum with S-antigens is no. 784. Antibodies were present in the IgM- and IgG- rich fractions.

antisera to either the Fab portion of IgG or to Bence-Jones proteins of κ and λ type, again failed to interact detectably with anti-S components in whole immune serum.

IDENTIFICATION OF IgG SUBCLASSES

We tried to identify the IgG subclass of an antibody (M224) to an S-antigen (1201) using specific antiglobulin sera. The electrophoretic mobility of the malarial antibody resembled that of IgG2 or IgG4 more than that of IgG3, but the arc of each IgG subclass overlapped the position of the malarial reaction to some extent (Fig. 5). The malarial antiserum (M224) contained more IgG4 than two other immune Gambian sera with which it was compared (Table 1), but gel diffusion experiments showed that the reaction between M224 and antigen 1201 cross-reacted with one of two antisera to IgG2 but not with antisera to IgG4 or IgG3 (Fig. 6).

TABLE 1
INVERSE TITRE OF SUBCLASSES OF IgG* IN THREE GAMBIAN SERA

	IgG1	IgG2	IgG3	IgG4
M547	80	10	40	0
K621	160	20	20	0
M224	80	20	20	40

* Titres were determined by the Ouchterlony method using doubling dilutions of the Gambian sera.

IDENTIFICATION OF L CHAIN TYPE

In experiments with three different S-antigen–antibody complexes, the immune precipitates were dissociated in acid-urea (see Materials and Methods) or where possible in acid alone, and the products examined by gel precipitation for Ig and L-chain determinants as well as for antibody and antigen activity. The acid-urea treatment of salt precipitated globulins (14 per cent Na_2SO_4) from whole serum yielded L chain determin-

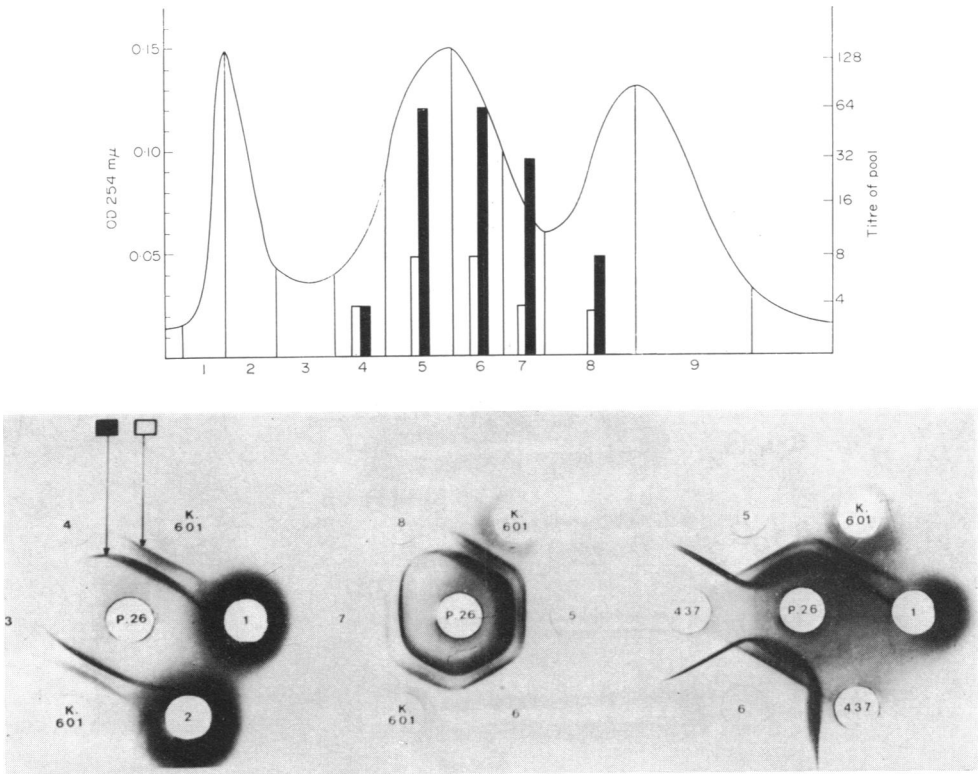

FIG. 4. Gel filtration of an adult's serum (K601) showed that only fractions rich in IgG had antibodies to S-antigens in an extract of infected blood (P26) and to related antigens in an infected child's serum (437). Antigens other than those of the S class were first destroyed by heating P26 at 100° for 5 minutes (Wilson *et al.*, 1969).

ants of both κ and λ types, as well as γ chain determinants (Fig. 7). In contrast, an immune precipitate made with antibody from the serum of K601 and S-antigen serum No. 387, yielded L chain determinants of λ type only (Fig. 8a), as well as traces of γ chain determinants (not shown). The dissolved complex did not deviate the precipitin line formed between anti-Lk and normal human serum. Similarly, an immune precipitate made with antibody from another adult (M214) and another serum which contained S-antigen (M123) was found, within the limits of the test, to contain L chain determinants of λ type only (Fig. 8b), as well as traces of γ chain. Although not shown in the illustrations, traces of 'free' malarial antigen were found in the salt supernatant of these preparations. The third antigen–antibody complex which we examined (M224-versus-1201) was dissolved in acid alone. It yielded L chains which were predominantly of κ type, as well as γ chain

determinants (Fig. 8c). No free antigen was apparent when the precipitate which had been dissolved in acid was tested by gel diffusion at pH 8·6. Probably this was because most of it was recomplexed in the precipitate which rapidly developed in and around the well. There was, however, some free antibody specific for antigen 1201.

When this third antigen–antibody complex was dissociated in acid-urea, no Ig determinants were recovered in the salt precipitate but the supernatant contained L chain determinants of κ type (Fig. 8d). There was also γ chain activity. Malarial antigen was

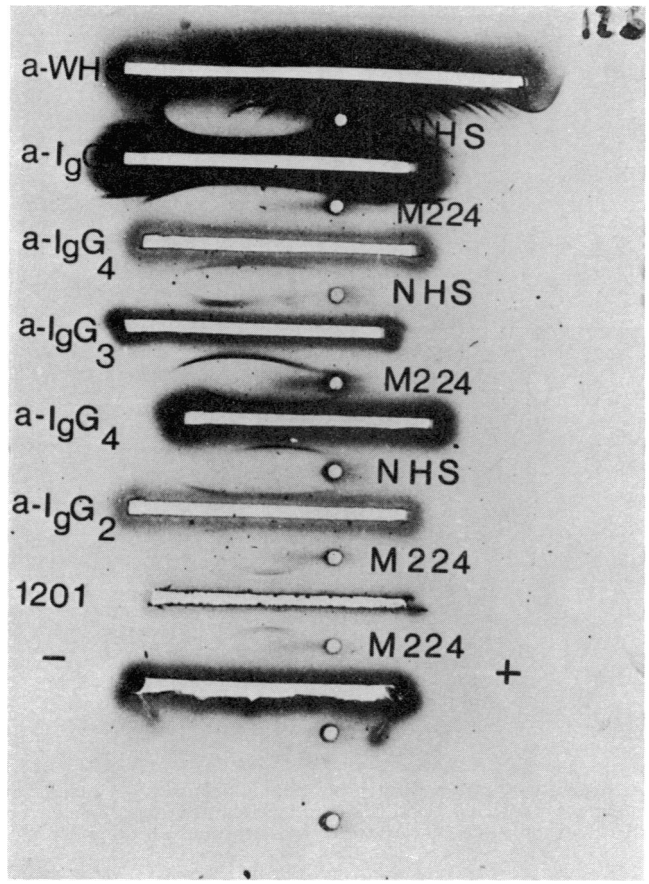

FIG. 5. An electrophoretic comparison of the subclasses of IgG present in the malarial antiserum M224 and the reactions it produced with the malarial S-antigens in serum 1201.

recovered predominantly in the salt supernatant there being only traces in the precipitate. As can be seen in Fig. 8d, the strong reaction produced by this 'free' malarial antigen and the whole serum M224, was not deviated by the diffuse L chain reactions on either side of it. This might have been because the precipitin system for L chains was unbalanced.

Dissolution of the immune complex (M224-versus-1201) by acid, or by the combined treatment of acid-urea and salt, indicated that a mixture of L chains of κ and λ type was present. This could be explained if the antiserum (M224) contained a mixture of antibodies which had combined with a single S-antigen, but it was also possible that 1201 contained

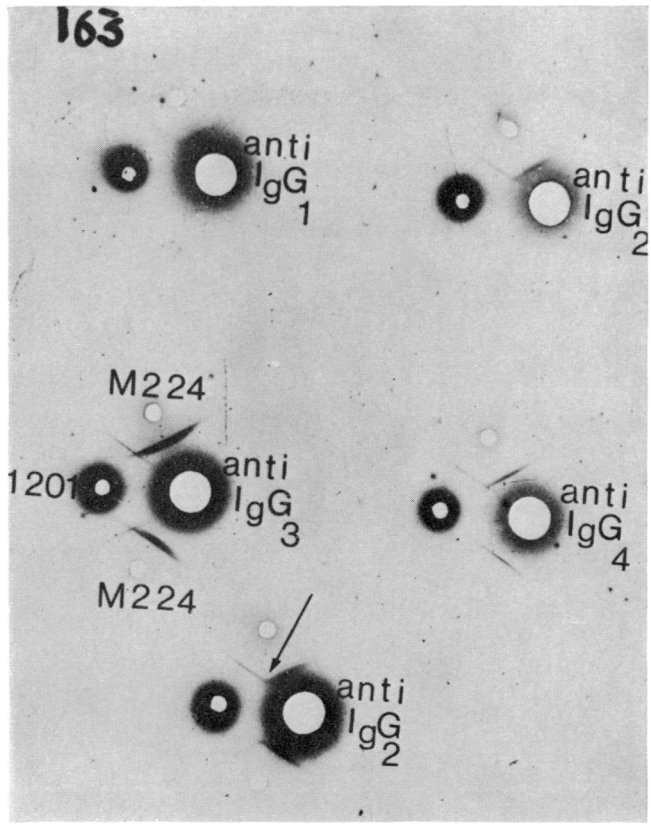

FIG. 6. Antibody precipitated by the malarial S-antigen 1201 cross-reacted with an antiserum to IgG2 (arrow) but not with antisera to IgG3 or IgG4. Two different sheep antisera to IgG2 were tried. Serum 1201 which contained the heat-stable malarial antigens was boiled prior to diffusion to remove unnecessary globulins.

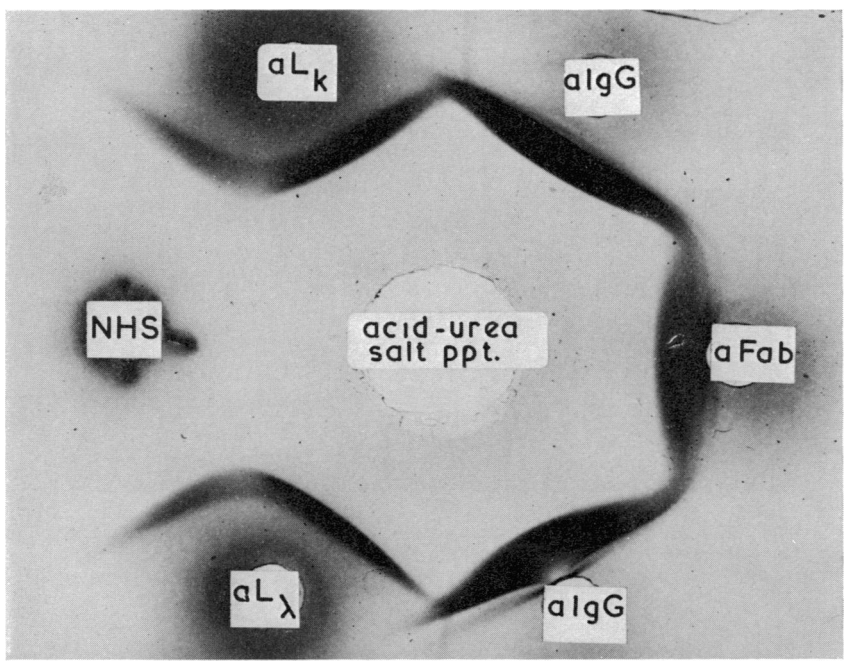

FIG. 7. Globulins precipitated from normal human serum in 14 per cent Na_2SO_4 were dissolved in acidified 10 M urea. A second salt fractionation gave a precipitate which contained IgG and L chain determinants of both λ and κ type.

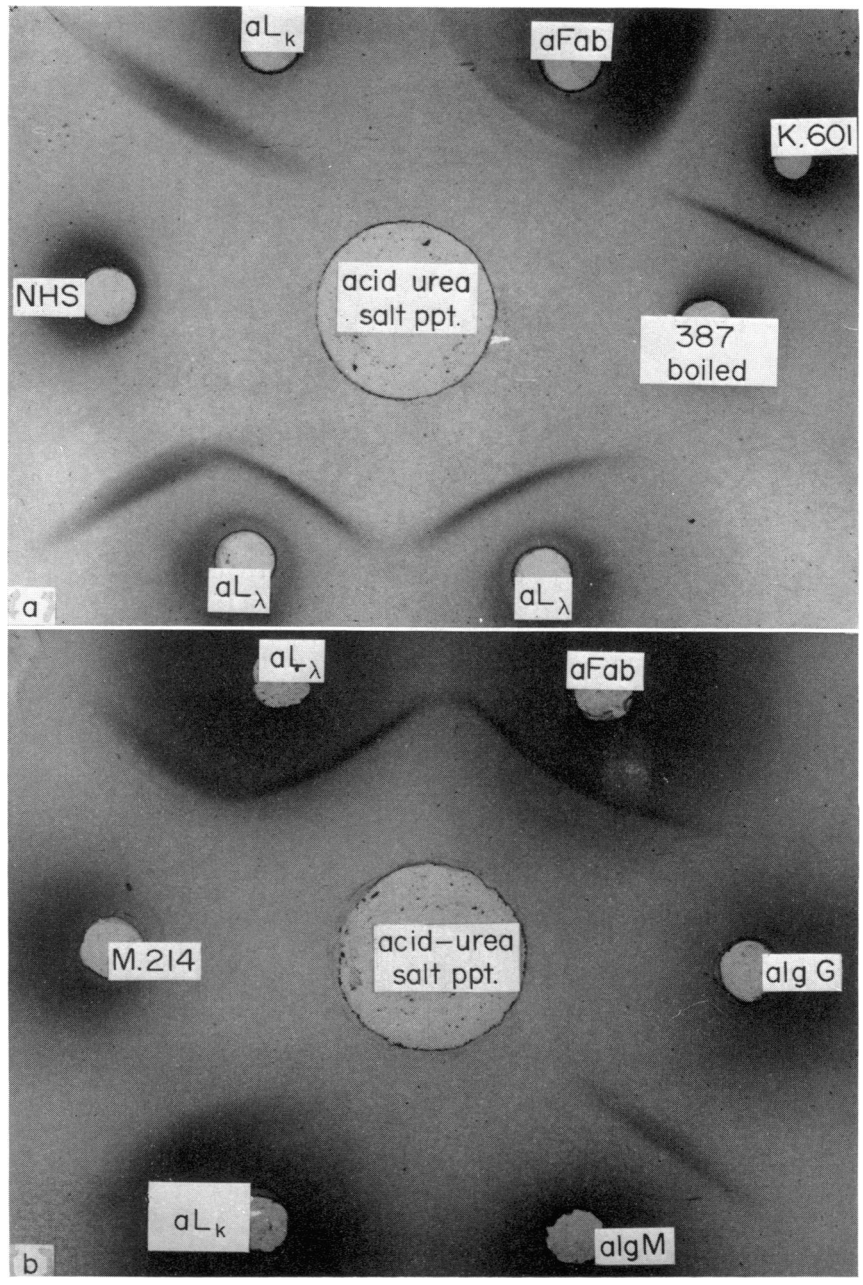

FIG. 8. Recovery of H and L chain determinants from immune complexes of malarial antibody and S-antigen. The central well was filled as follows: (a) K601 plus antigen 387 dissolved in acid urea. (b) M214 plus antigen M123 dissolved in acid urea.

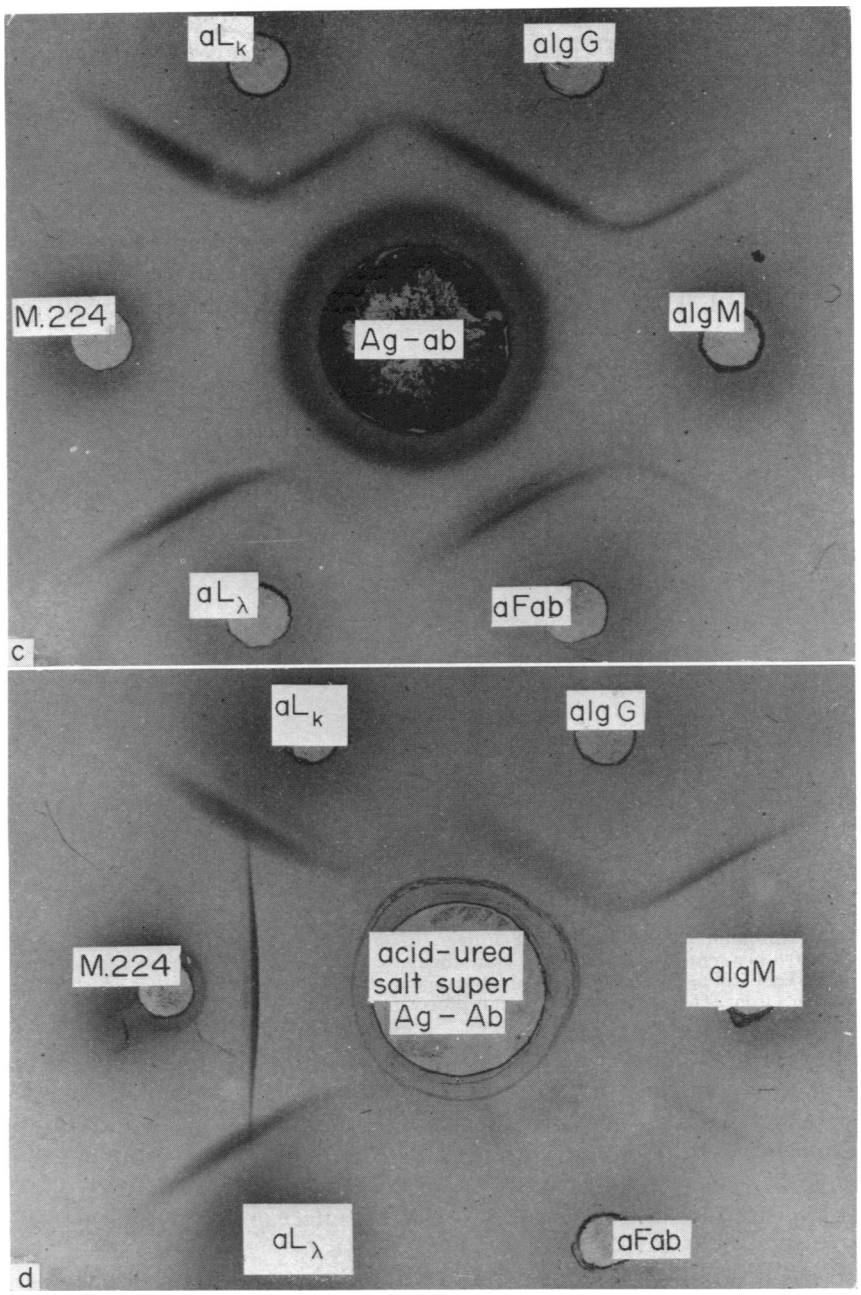

FIG. 8. (c) M224 plus antigen 1201 dissolved in acid. (d) M224 plus antigen 1201 dissolved in acid urea. L chains of λ type were found in (a) and (b) and predominantly of κ type in (c) and (d).

different antigens each of which reacted with antibodies of a single L chain type. Crossed immunoelectrophoresis in acrylamide/agarose gels clearly showed that at least two different antigens were present in 1201 (Fig. 9). One of these (antigen 1) appeared to be heterogeneous in its size and/or its charge.

DISCUSSION

Beside the range of antibody specificities due to the heterogeneity of S-antigens (Wilson *et al.*, 1969) we now have evidence that each specific antibody has other individual characteristics. Immunoelectrophoresis of sera from immune individuals confirmed the single observation of Turner and McGregor (1969) that antibodies to one of their

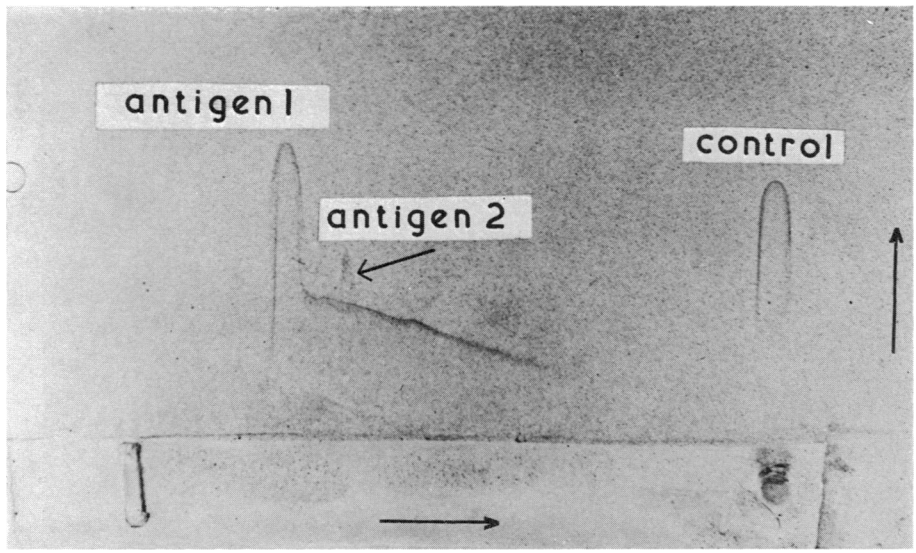

FIG. 9. Crossed immunoelectrophoresis of S-antigens in serum 1201. Initial separation was in 5 per cent acrylamide/0·8 per cent agarose. Antigens were then electrophoresed in 1 per cent agarose containing antiserum M224 diluted 1/100. Two distinct antigens were demonstrated one of which was heterogeneous with a front of fast-moving components preceding the main peak.

α-antigens appeared to have restricted electrophoretic mobility. In keeping with their finding we have shown that the majority of antibodies to S-antigens migrate in the γ_1 region. Different antibodies, however, do not have the same mobility and differences were seen in a comparison of the mobilities of antibodies from different individuals against the same antigens. The electrophoretic mobility of S-antigens, which can vary widely (Wilson *et al.*, 1969) appeared to have only a slight selective effect on the mobility of their specific antibodies.

We tested the possibility that antibodies to S-antigens may be produced preferentially in certain subclasses of IgG. Electrophoretic comparison suggested that the malarial antibodies behaved like IgG2 or possibly IgG4 and by means of the Ouchterlony technique, it was found that antibodies to one S-antigen gave a reaction of partial identity with IgG2.

Tests for InV activity might be useful in the study of antibodies to S-antigens as this

determinant in detectable in κ type L chains of γ_1 and γ_3 subclasses of IgG (Terry, Fahey and Steinberg, 1965). Curtain and Baumgarten (1965) employing an indirect fluorescent antibody test, found no InV (A^+) activity in the malarial antibody of eighteen of thirty-four Melanesian donors whose serum was InV (a^+). This result and a similar restriction which they reported for Gm (a^+) and Gm(b^+) specificities supports our present evidence for selective production of antibodies to S-antigens. Attention should be drawn, however, to the fact that the mixtures of antimalarial antibodies which were measured by Curtain and Baumgarten in their tests, were probably limited from the outset by the selection of antigens present in the malarial blood smears which they used.

The immune complexes which we have examined involved S-antigens from three different children's sera and antibodies from three immune adult sera. Two children's sera, M123 and 387, were subsequently found to contain antigens of identical specificity. It is of interest that L chains of λ type only were found in the antibodies to the S-antigens which were of the same specificity but derived from different sources. Further comparisons of this kind might show whether a particular S-antigen always induces the same restricted humoral response. The L chains of the antibodies which were precipitated in the third, unrelated antigen–antibody system were predominantly of κ type, although traces of λ type were also present. In this instance, it was shown that at least two distinct antigens were precipitated but it remains to be demonstrated whether each antigen reacted with antibodies of one L chain type or with a mixture of antibodies of both L chain types.

The electrophoretic mobility of the antibodies known from the above studies to have L chains of κ or λ type were similar, so that selection of L chains by these criteria alone does not explain the small electrophoretic differences seen amongst antibodies to S-antigens. The antibodies selected for the studies of immune precipitates were chosen because of their strong gel precipitation reactions and all were found subsequently to be of the IgG class. Similar studies with IgM antibodies, which tend to give weaker reactions with S-antigens, have yet to be done.

A parallel may be drawn between the response to malarial S-antigens and other antibody responses in man which involve restriction of antibody molecules. Thus cold agglutinins to red cell antigens of I-specificity regularly were found to be IgM molecules with L chains of type κ (Harboe, van Furth, Schubothe, Lind and Evans, 1965). These L chains showed restricted banding in urea-starch gels (Cooper, 1968). This and other chemical differences were taken to indicate their monoclonal origin (Cohen and Cooper, 1968). In a similar way, antibodies to Dextran, levan or techoic acid may not only belong to different classes of immunoglobulin but the L chain bands seen in urea-acrylamide gels are restricted in a manner resembling that seen in myeloma globulins (Dorner, Yount and Kabat, 1969). Selection also affected the IgG subclass of some of these antibodies as well as genetic markers such as the Gm allotypes (Yount, Dorner, Kunkel and Kabat, 1968). The benign increase of clones producing electrophoretically restricted antibodies to bacterial carbohydrate antigens has also been shown in immunized rabbits (Krause, 1970).

It is possible that the antibody response to S-antigens might be of a similar nature and if these antibodies could be purified sufficiently they could be examined for H and L chain restrictions. If these were found it could be argued that the increased prevalence of anti-S antibodies in adults compared to children is due to the increase in size of individual clones of cells during the years of repeated malarial stimulation which the indigenous villagers whom we have studied are subjected to. As natural stimulation is by the intra-

vascular route this may be a significant factor in the production of restricted malarial antibodies in man.

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