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STUDIES ON THE IMMUNOLOGY OF HUMAN MALARIA

II. CHARACTERIZATION OF ANTIBODIES TO *PLASMODIUM FALCIPARUM* INFECTIONS

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SUMMARY

Gel precipitin and fluorescent antibodies to human *Plasmodium falciparum* malaria have been investigated using techniques of comparative immunoelectrophoresis, gel-filtration and DEAE-cellulose chromatography. Antibody activity was generally associated with the IgG class but some gel precipitin antibodies of IgM type were identified. Findings in regard to antibody activity in the IgA class were inconclusive.

INTRODUCTION

There has existed for many years a demand for a suitable simple serological test to aid in the diagnosis and treatment of malaria. In the past complement fixation (e.g. Kingsbury, 1927; Eaton & Coggeshall, 1939), agglutination (e.g. Eaton, 1938; Brown & Brown, 1965), fluorescence (e.g. Tobie & Coatney, 1961; Voller, 1964) and haemagglutination (e.g. Stein & Desowitz, 1964; Mahoney, Redington & Schoenbechler, 1966) techniques have each been evaluated in their turn with varying degrees of success. In the field of human malaria the fluorescence test has been particularly valuable in epidemiological surveys (McGregor *et al.*, 1965). Recently a further technique, that of gel diffusion, has been used to demonstrate antigen–antibody interactions in human malaria (McGregor *et al.*, 1966).

This communication describes an investigation of fluorescent and gel precipitin antibodies to *Plasmodium falciparum* malaria. In particular, an attempt has been made using techniques of comparative immunoelectrophoresis, Sephadex gel filtration and DEAE-cellulose chromatography to define which of the major immunoglobulin classes (IgG, IgA or IgM—see World Health Organization, 1964) contains such antibodies.

MATERIALS AND METHODS

Malarial antigens

With the exception of the fluorescence experiments the antigens studied were derived as

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described in Paper I (Turner & McGregor, 1969). Those which are specifically referred to in this paper are:

(1) *α-Antigen.* A concentrate of 'high molecular weight' antigen obtained following Sephadex G-200 gel-filtration of a disintegrated *Plasmodium falciparum* infected placenta (α1 antigen in Paper I).

(2) *β-Antigen.* A concentrate of 'low molecular weight' antigen obtained following Sephadex G-200 gel-filtration of a disintegrated *P. falciparum* infected placenta (β1 antigen in Paper I).

(3) *M.J. antigen.* A concentrate of 'high molecular weight' antigen obtained following Sephadex G-200 fractionation of serum taken from patient M.J. following a severe attack of *P. falciparum* malaria.

(4) *P5 antigen.* Whole unfractionated placental antigen fully described in our earlier publication (McGregor *et al.*, 1966).

Immunoelectrophoresis

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

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.

Gel filtration

Gel filtration was performed on columns of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) particle size 40–120 μ. The dimensions of the column used for the fluorescence experiments was 2·4 × 56·5 cm; elsewhere a column measuring 2·4 × 77·5 cm was used. A buffer of 0·2 M-Tris–HCl + 0·2 M-HCl + 0·1% sodium azide (pH 8·0) and a flow rate of 20 ml/hr was used throughout. The ambient temperature was 25–27°C.

Fractionations of 3-ml volumes of sample were performed 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.

In the studies on fluorescent antibodies the effluent was collected in a volumetric fraction collector and absorbency at 280 nm (mμ) determined by spectrophotometric measurements. In all other experiments effluent was continuously monitored at 254 nm with a Uvicord spectrophotometer (L.K.B. Instruments, Stockholm, Sweden) before collection with a time operated fraction collector. Fractions were then pooled in pairs and concentrated by ultrafiltration through 8/32 in. Visking tubing (Union Carbide Co., Chicago, Illinois, U.S.A.) prior to further analysis.

DEAE-cellulose chromatography

For chromatography on diethylaminoethyl(DEAE)-cellulose 5 ml of serum were dialysed against 0·015 M, pH 8·0, potassium phosphate buffer and applied to a 2 × 22 cm column containing Whatman DE-52 cellulose equilibrated with the same buffer. Elution was performed with 500 ml of buffer in a gradient from 0·015 M to 0·3 M at pH 8·0 (Fahey &

McLaughlin, 1963). The effluent was continuously monitored at 254 nm with a Uvicord spectrophotometer before collection with a time operated fraction collector. Fractions were then pooled in pairs and concentrated by ultrafiltration with 8/32 in. Visking tubing.

Quantitative determinations of immunoglobulins

The antibody–agar technique of Fahey & McKelvey (1965) was used for the quantitative measurement of individual serum immunoglobulins. Results were expressed as a percentage of a standard Caucasian serum.

Antisera

The antisera used in this investigation are fully described in the accompanying paper (Turner & McGregor, 1969).

Malarial fluorescent antibody titrations

The titration of fluorescent antibodies in the fractions obtained following Sephadex gel-filtration was performed essentially as described by Voller (1964) using a range of doubling dilutions for each fraction.

Mercaptoethanol sensitivity of fluorescent antibodies

An attempt to assess the mercaptoethanol sensitivity of the fluorescent antibodies in the Sephadex fractions was made using the technique of Rockey & Kunkel (1962).

Ten microlitres of 2-mercaptoethanol were added to 1-ml volumes of fractions to give a final concentration of 0·1 M. After mixing, the samples were incubated at room temperature for 4 hr. The mixtures were then dialysed (using 8/32 in. Visking tubing) against three changes of 0·02 M-iodoacetamide in 24 hr. Control samples were treated exactly as above except for the substitution of 10 μl saline for 2-mercaptoethanol. Finally, all samples were re-titrated for fluorescent antibody activity.

RESULTS

Gel-precipitin antibodies

Comparative immunoelectrophoresis was used to show that the gel precipitin lines previously observed (McGregor *et al.*, 1966) were indeed due to γ-globulins in the immune serum. Fig. 1 shows an electrophoresis of a serum from an adult female Gambian, believed to be immune to malaria, developed against a specific anti-IgG serum in the lower trough. The same serum produced two precipitin lines of similar mobility to the IgG line when diffused against a malarial antigen extract in the upper trough. This strongly suggests that one or other of the immunoglobulin classes is involved in this reaction.

Fig. 2 shows another comparative immunoelectrophoresis experiment in which serum (again an adult female Gambian, K.S., believed to be immune to malaria) was electrophoresed and developed against a polyvalent anti-human serum (lower trough). The labelling of the IgM and IgA arcs is based on observations of several such analyses of hyperimmune African sera where the IgM component is often grossly elevated. The upper trough contained the α1 antigen (see Paper I) and produced a precipitin band of γ_1 mobility, the shape of which did not appear to be deviated by either the IgA or IgM precipitin arcs. It was concluded, therefore, that the precipitin line was produced by IgG antibodies of restricted

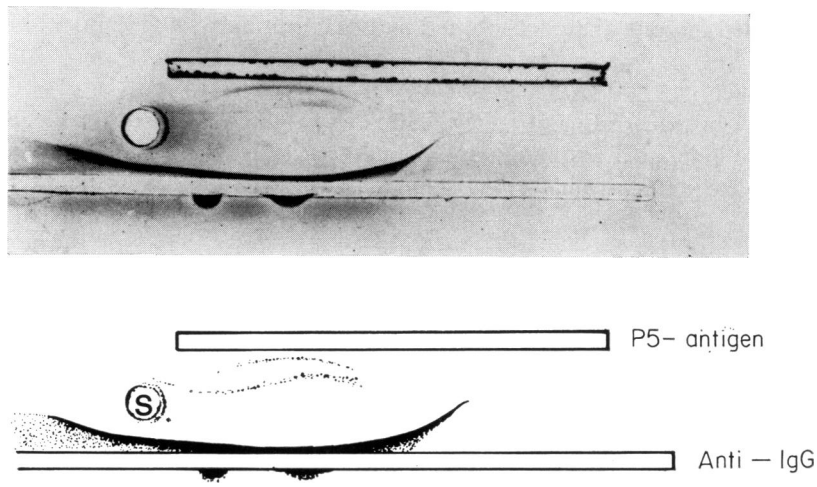

FIG. 1. Immunoelectrophoresis of an immune serum (S) from an adult Gambian. The lower trough contained a specific anti-IgG serum and the upper trough contained placental antigen P5.

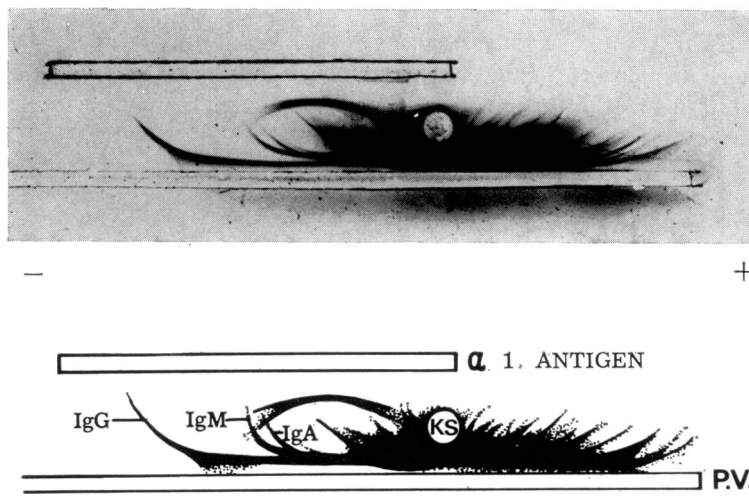

FIG. 2. Immunoelectrophoresis of immune serum from subject K.S. (an adult female Gambian). The lower trough contained a polyvalent antiserum (PV) and the upper trough contained the α1 placental antigen.

electrophoretic mobility. Gel filtration experiments (see below) confirmed the IgG nature of these antibodies in K.S. serum.

A Sephadex G-200 fractionation of serum from an adult male Gambian (Y.S.) is shown in Fig. 3(a). As with all human sera, three main peaks were resolved. The profile is typical of an immune adult Gambian in that the second peak is quantitatively the largest because of the content of IgG.

The distribution of the major immunoglobulins and β_{1C}-globulin are also shown in Fig. 3(a) beneath the elution diagram. These distributions do not differ significantly from

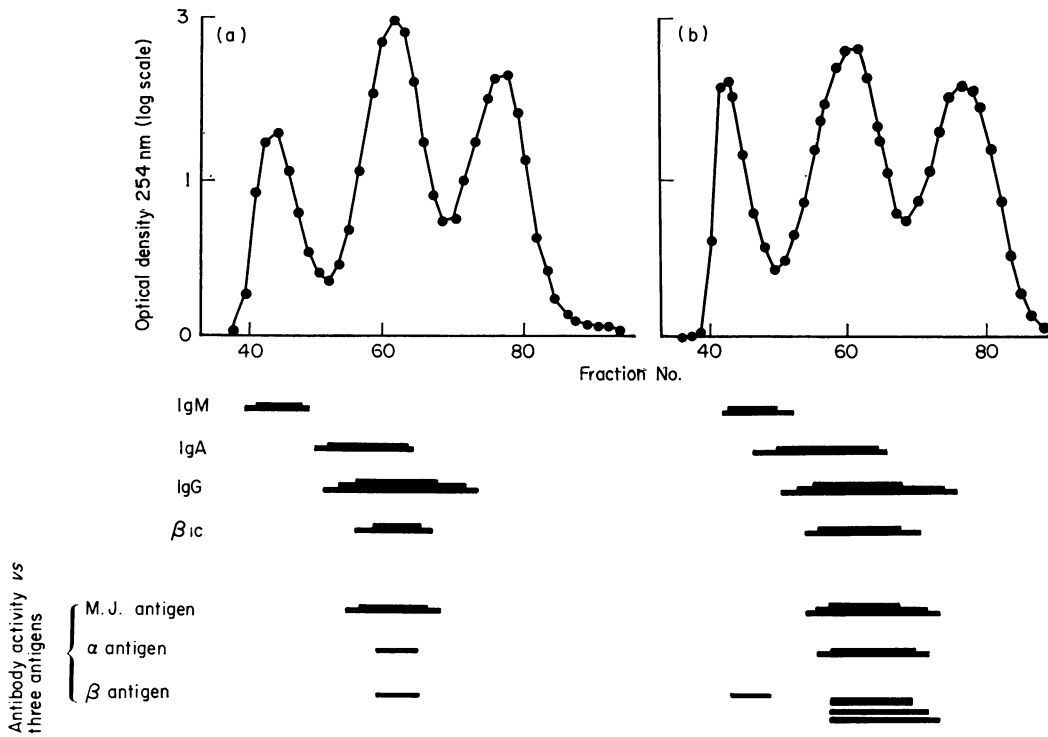

FIG. 3. (a) Gel filtration on Sephadex G-200 of serum from subject Y.S. (an adult male Gambian believed to be immune to malaria). Three millilitres of serum were fractionated as described in the text. Fractions were pooled in pairs and concentrated for antigenic analysis. Plasma protein distributions were established using antisera specific for those proteins. The antibody activities indicated were determined using concentrated antigen from patient M.J. and concentrates of the α and β placental antigens. (b) Gel filtration on Sephadex G-200 of serum subject K.S. (an adult female Gambian believed to be immune to malaria). Three millilitres of serum were fractionated as described in the text. Fractions were subsequently treated as in (a).

those of a normal Caucasian subject. The IgM and IgG proteins were confined to the first and second peaks, respectively, whereas IgA was associated with the leading edge of the second peak. β_{1C}-Globulin was detected in the second peak. Accurate evaluations of the levels of these proteins was not attempted but a semi-quantitative indication based on the relative intensities of the precipitin lines is given by the thickness of the horizontal bars in

Fig. 3(a). Thus, although IgA was detectable in the trailing edge of the first peak it was present only in low concentrations and most of the protein was associated with the leading edge of the second peak.

Fig. 3(a) also shows the reactivity of the various fractions in gel precipitin tests with antigen preparations. These are also plotted semi-quantitatively and it can be seen that the mid-point of each distribution bar is coincident with the mid-point of the IgG distribution bar indicating that the antibodies to all three antigens are of IgG class.

Fig. 3(b) shows a Sephadex G-200 fractionation of serum form subject K.S. (an adult female Gambian). As with subject Y.S. the protein profile is typical of an adult Gambian.

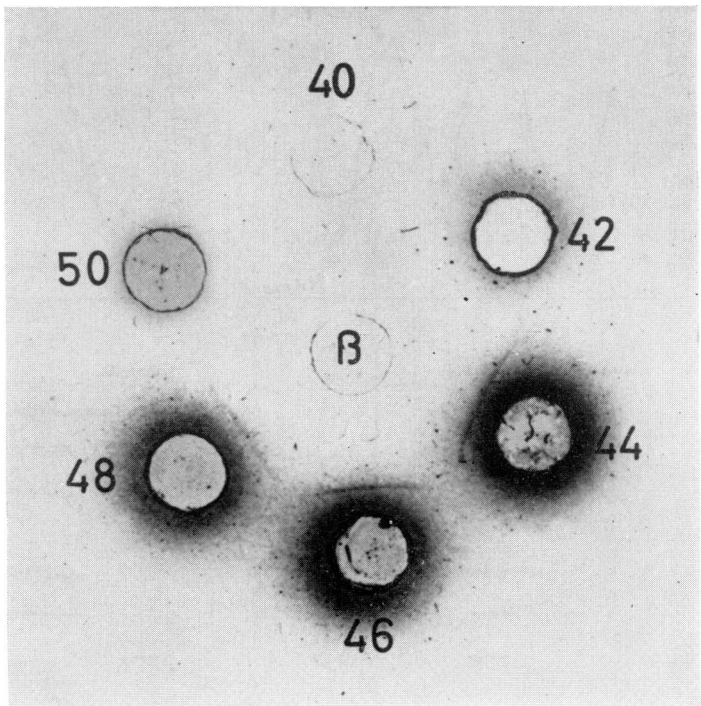

FIG. 4. Ouchterlony analysis of serial concentrated fractions from the macroglobulin region of a Sephadex G-200 fractionation of serum from subject K.S. (see Fig. 3b). Centre well contained the β placental antigen.

Protein distributions and antibody activity against the same three antigens are again shown below. Antibodies to the M.J. antigen and against the α antigen were symmetrical with the distribution of IgG. In contrast the antibody activity against the β antigen was of a more complex pattern and indicated a considerable degree of both antigen and antibody heterogeneity. Four distinct precipitating systems were resolved—three in the IgG region and one in the IgM region. In addition to their Sephadex elution position the macroglobulin nature of the antibodies in the IgM region was further indicated by the position and shape of the precipitin lines (Fig. 4). Thus they were close to the antibody well and convex to the antigen well.

Further information on the nature of the gel-precipitin antibodies was obtained following chromatography of another sample of adult Gambian serum on DEAE-cellulose. Fig. 5 shows the result of this experiment. The protein profile is similar to that shown by any normal serum with a characteristic first peak of IgG, a second peak corresponding to

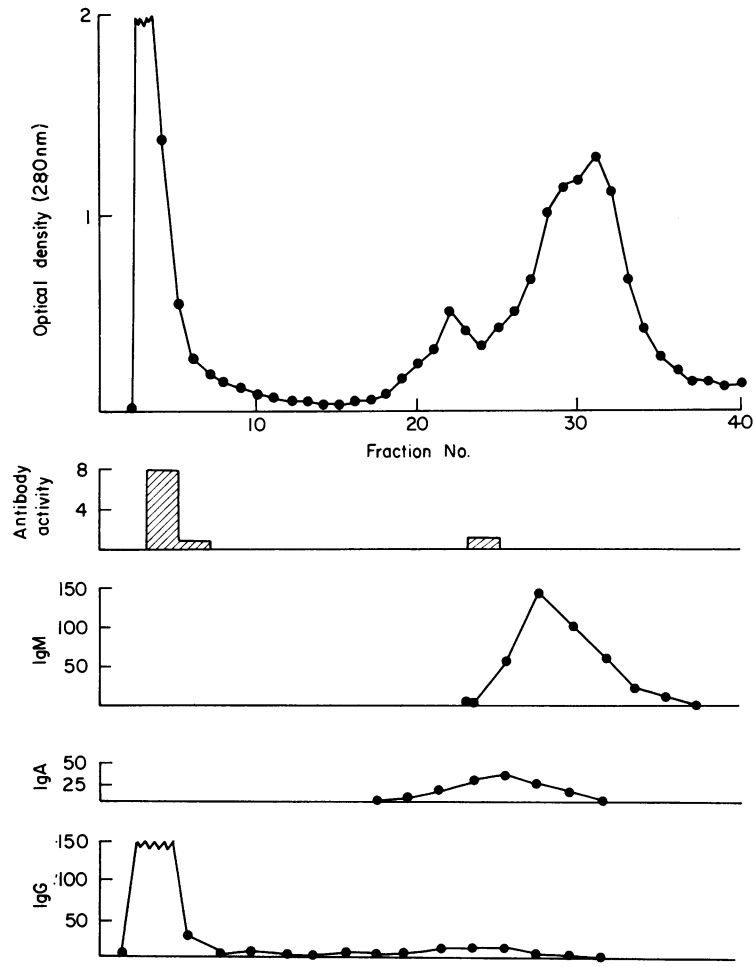

FIG. 5. Distribution of gel-precipitating antibody activity and immunoglobulins of an immune adult Gambian serum following chromatography on DEAE-cellulose. Elution was performed with pH 8·0 potassium phosphate buffer in a gradient from 0·015 M to 0·3 M. After determination of optical density, fractions were pooled in pairs and concentrated ten-fold by ultrafiltration, and their concentration of immunoglobulins then determined. Antibody titrations were performed after further ultrafiltration (final concentration sixty-fold).

transferrin and a third peak containing the bulk of the serum albumin. The distribution of the three major immunoglobulins IgM, IgA and IgG was determined by the technique of Fahey & McKelvey (1965). IgG was associated with the first protein peak but a second subsidiary peak was also eluted in fractions 20–30. IgA and IgM were eluted as single peaks

with maxima in fractions 25 and 28, respectively. The fractions were concentrated sixty-fold and antibody activity of the β antigen was assayed. Fractions associated with the IgG peak were the most active but activity was also detected in fractions 23–25. This latter activity was clearly too early to be associated with the IgM peak. Although the possibility that this activity was associated with IgA could not be excluded it seemed most probable that it was correlated with the second IgG peak. Thus, on the basis of this single separation ion-exchange chromatography appears to confirm the results obtained with gel-filtration; namely, that the major part of the human gel precipitating antibodies to *Plasmodium falciparum* malaria are of IgG class.

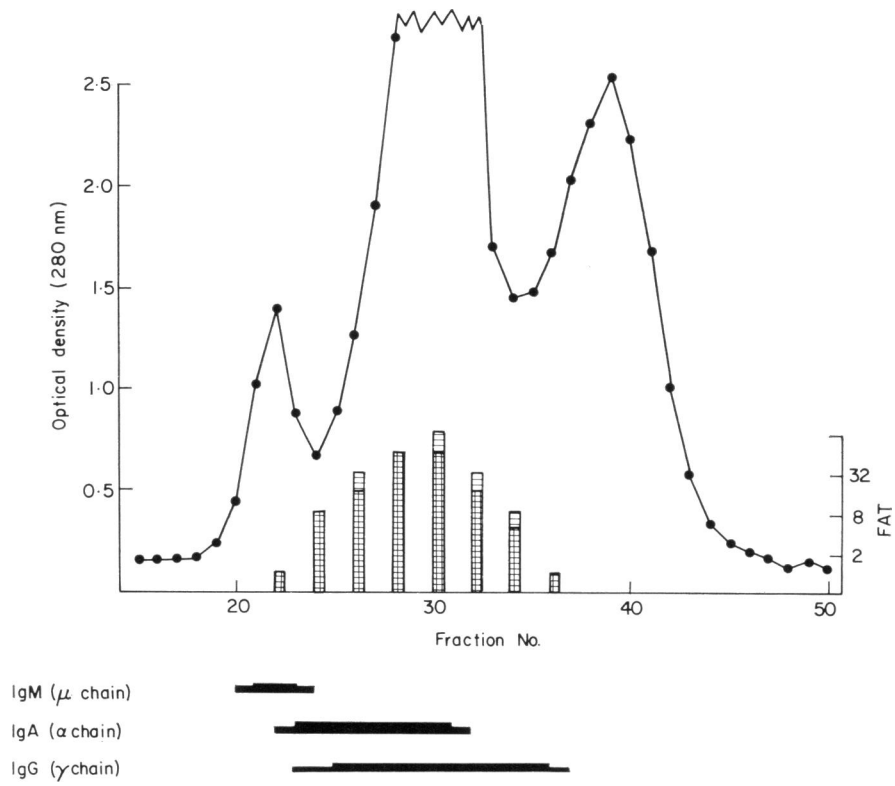

FIG. 6. Gel filtration on Sephadex G-200 of serum from an adult Gambian having a high titre of fluorescent antibodies to malaria. Three millilitres of serum were fractionated as described in the text. Serum protein distributions in the eluates were established using antisera specific for those proteins. Alternate fractions were evaluated for fluorescent antibody titres (vertical bars with horizontal hatching). Vertical hatching indicates the fluorescent antibody titre following treatment with 0·1 M-2-mercaptoethanol.

Fluorescent antibodies

Fig. 6 shows a Sephadex G-200 fractionation of a serum having a high (1:3200) titre of malarial fluorescent antibodies. The antigenic distribution of IgM, IgA and IgG is again shown by the horizontal bars beneath the elution profile. The distribution of fluorescent

antibodies is superimposed on the elution profile (indicated by the horizontally hatched columns). A symmetry with the IgG distribution is again apparent but some activity was present in early fractions containing IgM.

The experiments with 2-mercaptoethanol (which inactivates IgM antibodies but not IgG) were initiated in an attempt to characterize the antibody activity in the earlier fractions. As can be seen in Fig. 6 the difference in fluorescent antibody titre (FAT) between fractions treated with 2-mercaptoethanol and untreated control fractions is not significant. Thus, in the case of this particular sample of serum the greater part of the fluorescent antibody activity was apparently confined to the IgG class.

DISCUSSION

The immune response of the human organism to the complicated dynamic infection of malaria is not well understood. Classically immune processes are a combination of cellular and humoral components and those of malaria are no exception. The total humoral response to the relatively large protozoan organism undergoing a succession of developmental phases must be compounded of numerous antibodies having different specificities. Some of these antibodies may act against some phases of the parasite but not against others, while some may be directed against products of parasitic metabolism rather than against the parasite itself.

A precise clarification of malarial antibodies according to function is clearly not feasible at the present time and until our knowledge is more complete the use of broad terms such as 'protective' and 'non-protective' seems indicated. The former should perhaps not be restricted to antibodies which are effectively antiparasitic but should include those which, by acting against substances formed in the course of parasitaemia, may diminish the clinical illness associated with parasitism. As no laboratory method currently exists whereby such antibodies may be differentiated from others, comprehensive evaluation of the data obtained with any antibody detection system is not possible without additional *in vivo* experimentation.

This communication is concerned only with one aspect of the humoral response. Firstly it was important to demonstrate that the gel precipitin reaction between malarial antigen extracts and immune sera was indeed a reaction involving the immunoglobulins of those sera. The technique of immunoelectrophoresis made this possible by electrophoresing the serum and then developing precipitin arcs with: (a) antisera to human serum proteins, and (b) the antigen extract.

Two samples of serum examined in this way reacted with malarial antigen in the γ-globulin region. The serum K.S. was of further interest because the antigen precipitin arcs did not appear to be deviated by adjacent IgM and IgA lines although having identical mobilities. There was other evidence (Sephadex gel-filtration experiments) to suggest that the gel precipitin antibodies to α antigen in K.S. serum were of IgG class and it is, therefore, of interest to speculate on the significance of their restricted electrophoretic mobility. One possible explanation (which we were not able to verify experimentally) is that all the antibodies of this particular specificity belong to one of the subclasses of IgG. Examples of this have already been reported. Thus Kabat (1967) showed that the anti-levan antibodies from one individual belonged exclusively to the subclass IgG2 (or γG2—see World Health Organization 1966). It is noteworthy that the subclasses of IgG have distinctive electro-

phoretic mobilities (Terry, 1964) and IgG2 has a γ_1 mobility similar to that shown by the antibodies in K.S. serum reacting with the α antigen.

Sephadex gel filtration and DEAE-cellulose chromatography both indicated that the greater part of the gel precipitin antibodies to malaria are of IgG class but there was also some evidence of activity in the IgM class. These findings are similar to those of Abele and co-workers (1965) who used the fluorescent antibody test to study human antibodies in primary malarial infection. IgM antibodies appeared first and increased swiftly following the onset of parasitaemia. Later the IgM titre fell as IgG antibodies appeared and rose in titre.

The immunoglobulin levels of African populations exposed to frequent malarial infections have been studied by Turner & Voller (1966) and Rowe *et al.* (1968). Both groups found that the mean values for IgG and IgM were significantly higher than those of Caucasian subjects of similar age. Rowe and co-workers also showed that levels of IgM tended to rise at an earlier age than other immunoglobulins and IgG levels were of adult proportions by the age of 5 years. These findings may be consistent with Abele's observations that malarial antibodies are initially IgM but later IgG.

The IgG class of Gambian immunoglobulin has been shown to contain antibodies that effectively reduce *Plasmodium falciparum* parasitaemia (Cohen, McGregor & Carrington, 1961) but it has not been fully established whether IgM has a similar effect. Clearly it will be important to establish whether gel precipitin antibodies are protective or non-protective. At this stage it seems that they might be both. There is some evidence that the α complex of antigens studied in this work may represent either a parasite metabolite or a host–cell complex especially as it appears to be very readily released in the extraction procedure adopted and relatively few individuals produce antibodies against it. Conversely the β complex of antigens is somewhat more difficult to extract suggesting that it might be truly plasmodial and is the antigen group against which most adult individuals seem to possess antibodies.

ADDENDUM

Since the preparation of this manuscript Collins & Contacos (personal communication 1968) have produced evidence that human fluorescent antibodies to *Plasmodium falciparum* and *Plasmodium vivax* antigens occur in all the major immunoglobulin classes, i.e. IgG, IgM and IgA.

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