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MALARIAL ANTIGEN FROM HUMAN BRAIN

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SUMMARY

A method is described for purifying malarial antigen from human brain. The antigen appears to be highly specific for malarial antibodies. On double diffusion in agar gel a precipitin reaction was produced only with sera from persons with malarial parasites in their peripheral blood and those with high malarial fluorescent antibody titre. No precipitin lines were formed with sera from persons who were known to be free from malarial infection.

INTRODUCTION

Since Coggeshall & Kumn (1937) first showed that circulating antibodies occur after infection with malarial parasites, several investigators (Holmes, Stanier & Thompson, 1955; McGregor *et al.*, 1956, 1966; Edozien, Gilles & Udeozo, 1962; Bruce-Chwatt, 1963; Abele *et al.*, 1965) have confirmed this observation.

Various immunological methods have been used to detect and assay circulating antibodies to malaria. Taliafero, Taliafero & Fisher (1927) and Row (1931) used a precipitin ring method, whereas Coggeshall & Eaton (1938), Eaton & Coggeshall (1939) and Mayer & Heidelberger (1946) used the complement-fixation technique; Stein & Desowitze (1964) obtained some satisfactory results with the passive haemagglutination method, and the fluorescent antibody technique was used by Tobie & Coatney (1961), Kuvin *et al.* (1962) and Voller (1962). Recently, McGregor *et al.* (1966), using malarial antigen obtained from heavily infected human placenta, were able to demonstrate circulating malarial antibody by the agar gel double diffusion technique. The present report is concerned with the study of malarial antigen from human brain and its use in the detection of circulating antibodies to *Plasmodium falciparum* by the agar gel double diffusion method.

MATERIALS AND METHODS

Brain tissue from a 4-year-old Nigerian boy was obtained at autopsy, 24 hr after death. The child had been admitted to the University College Hospital, Ibadan with a history of convulsion. He died the day after admission from shock following convulsion and coma. The brain weighed 1300 g. It was oedematous and the cortex had a slaty grey colour characteristic of malaria infection. A brain smear stained with Leishman stain showed

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numerous malarial parasites and characteristic malarial pigment in the cerebral capillaries (Fig. 1).

Extraction of malarial antigen from human brain

Two hundred grams of the brain cortex was obtained. A 20-g portion was used at a time for antigen preparation and the remaining portions were stored at −70°C. The brain was rinsed thoroughly in phosphate buffered saline, pH 7·4 (PBS), to remove all traces of blood and then minced in a Braun's Domestic Homogenizer and suspended in 50 ml cold PBS. The homogenate was filtered through two layers of gauze and then centrifuged at 4500 rev/min for 15 min at 4°C. The reddish tinged supernatant was discarded. The deposit was suspended in 2 volumes of cold PBS in a plastic tube immersed in an ice bath and subjected to sonic disintegration by ten 60-sec bursts from the probe of an ultrasonic power unit

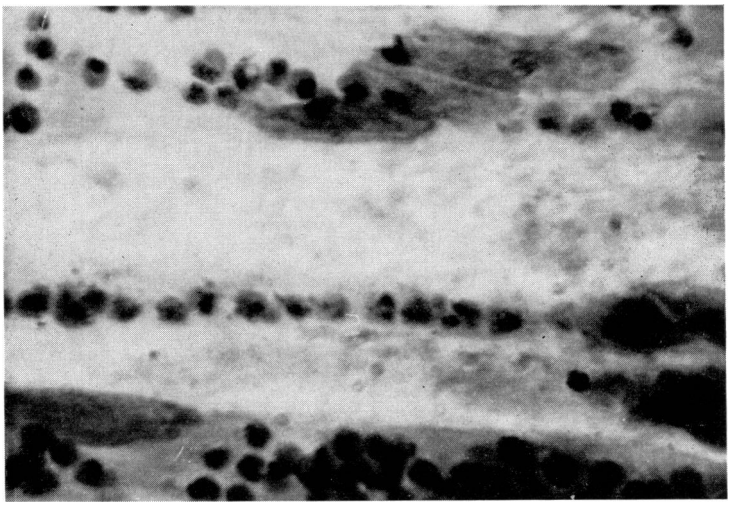

FIG. 1. Smear of brain containing malarial parasites stained with Leishman's stain. The numerous malarial parasites with characteristic malarial pigments in the cerebral capillaries can be readily seen. × 1000.

(M.S.E.) with the power scale setting at 1·5. The sonicated homogenate was centrifuged at 4°C at 10,000 rev/min for 30 min and the light grey supernatant collected and used as the antigen, hereafter referred to as the malarial antigen. Fig. 2 summarizes the steps involved. A control extract from an autopsy brain known to be free from malarial parasite was similarly prepared.

The total protein of the malarial antigen at this stage ranged from 15 to 20 mg/ml as determined by the Biuret method of Gornall, Bardawill & David (1949). This antigen when stored at 4°C retained its antigenic activity for about 2 weeks.

Detection and titration of malarial antibody by the double immunodiffusion in agar

The partially purified malarial antigen was used in estimating malarial antibody in sera (including one cord serum) from Nigerians. One group of twenty-four sera was obtained

during the rainy season from Nigerians who were not taking anti-malarial drugs (McFarlane & Voller, 1966). Eighteen sera served as controls. Twelve of these were obtained from blood donors in Jamaica, known to be free from malaria. The other six control sera were from persons living in Nigeria who were receiving anti-malarial drugs. Two of these were Europeans.

The double diffusion in agar gel was carried out as described by Ouchterlony (1953) using a solution of 1·0% purified agar (Difco Laboratories, Detroit, Michigan) with 0·1% sodium azide, in 0·05 M-barbitone buffer, pH 8·6. Patterns were cut in the agar with a template similar to that described by Gell (1957) and Soothill (1962). About 0·5 ml of antigen was

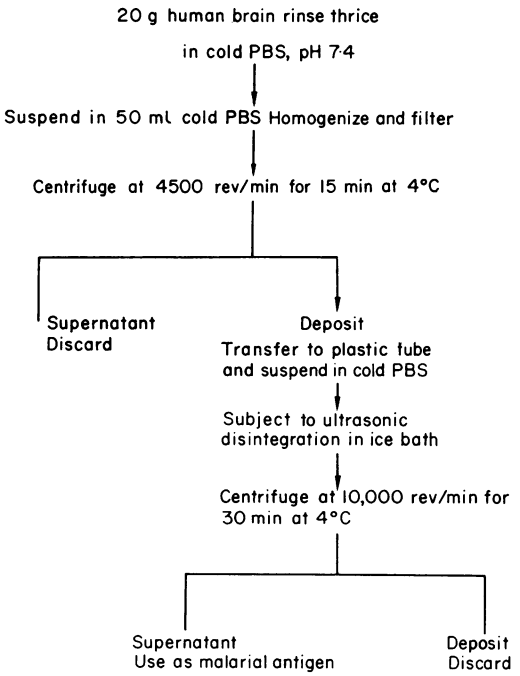

FIG. 2. Steps of malarial antigen preparation.

placed in the large central well and 0·25 ml test serum, in various dilutions, in the peripheral wells. The precipitin lines were allowed to develop in a moist chamber at 18°C, then read on a viewing box at 24, 48 and up to 72 hr.

Fluorescent antibody titres of the human sera were determined by the indirect fluorescent antibody technique of Kuvin *et al.* (1962) and Voller (1962).

Immunoglobulin quantitation was carried out by double diffusion technique of Gell (1957) and Soothill (1962) and by the Hyland Immuno-Plate method.

Fractionation with Sephadex G-200

Three millilitres of the partially purified malarial antigen containing a total protein concentration of 20 mg/ml was passed through a column (2·7 × 100 cm) of Sephadex G-200 (Pharmacia). The column was equilibrated and eluted with 0·15 M-NaCl, pH 7·4, containing

0·1% sodium azide. The flow rate was adjusted to 12–15 ml/hr and 5-ml fractions were collected by means of an LKB fraction collector equipped with a Uvicord assembly, set at 259 mμ and a recorder (L.K.B. Produkter AB, Stockholm, Sweden). Ultraviolet absorption of the effluent fractions was also determined using a Unicam SP 500, at wavelengths throughout the ultraviolet region. Three millilitres of normal human serum was similarly treated.

Analytical ultracentrifugation

This was determined on the Beckman Analytical Ultracentrifuge Model E at 59,000 rev/min at 20°C in PBS, pH 7·4. The first peak which emerged from the Sephadex G-200 column contained the antigen activity, was dialysed against PBS, pH 7·4, for 24 hr and ultracentrifuged. Photographs were taken at 0-min after attaining maximum speed and at 4-min intervals up to 44 min. A second run of the same sample was performed taking photographs at 2-min intervals after attaining maximum speed.

Amino acid analysis

This was carried out on a Beckman/Spinco Model 120B Amino Acid Analyser. One millilitre of malarial antigen containing 4 mg protein was hydrolysed in 1 ml, of 6 N-HCl for 22 hr at 110°C in vacuum sealed tubes (Spackman, Stein & Moore, 1958). The hydrochloric acid was removed under vacuum and the samples taken up in 3 ml of citrate diluent buffer, pH 2·2, before being placed on the ion exchange column.

Peptic digestion

This was performed according to the method of Peperman & Pappenheimer (1941). A few citric acid crystals were added to 2 ml of the malarial antigen until pH became 4·2. Crystalline pepsin (1 mg) was added, the solution left at room temperature for 30 min and then heated at 58°C for 45 min. The mixture was centrifuged at 2000 rev/min to remove any coagulated protein and the supernatant dialysed overnight in the cold against phosphate buffered saline, pH 7·4. The dialysed antigen was used for further studies.

Reduction with 2-mercaptoethanol

This was done according to the method of Hannestad & Mellbye (1967) which is a modification of Deutsch & Morton's (1962) original method. A volume of 3·4 ml of the malarial antigen with a total protein of 20 mg was treated with 0·6 ml of a 1 M-2-mercaptoethanol. The solution was allowed to stand for 6 hr at room temperature, after which it was dialysed against 0·02 M-iodoacetamide (Fluka Ag. Buchs, Switzerland) in PBS, pH 7·4, in the cold for 8 hr, centrifuged and the supernatant tested for antigenic activity.

RESULTS

Table 1 shows the results obtained for the titration of malarial antibody by the double gel diffusion method using malarial antigen prepared from human brain. The fluorescent malarial antibody titres and the immunoglobulin concentration are also shown. Sixteen of the twenty-four sera tested gave a precipitin reaction with the malarial antigen. Twelve formed a precipitin line in agar gel between 12 and 24 hr. Four sera (Nos. 6, 17, 18 and 19, Table 1) produced a precipitin line after diffusion for 48 hr. The other sera gave no precipitin line even after 72 hr. As shown in Table 1, a precipitin line was given by the sera of four of

TABLE 1. Malarial antibody titres and immunoglobulin concentration in some Nigerians (modified from McFarlane & Voller, 1966)

Study No.	Agar gel diffusion test	Immunoglobulins (mg/ml)			Malarial fluorescent antibody titre	Parasitaemia
		IgG	IgA	IgM		
1	+	25·60	2·50	1·12	640	—
2	+	20·00	1·70	0·84	1280	—
3	−	40·00	2·80	2·50	1280	—
6	+	42·00	3·30	4·50	1280	—
7	++	40·00	1·70	1·80	2560	—
8	−	25·00	3·90	2·80	5120	*P. falciparum* 200 mm^3
9	+	49·00	2·85	0·80	5120	—
10	+	36·00	3·90	2·24	640	—
11	+	30·00	2·30	1·12	1250	—
12	++	24·00	4·01	2·80	5120	*P. falciparum* 980 mm^3
13	−	30·00	2·30	1·60	2560	—
14	−	72·00	3·50	3·36	5120	—
15	−	22·00	2·80	0·80	160	—
16	−	27·00	3·90	4·50	5120	—
17	+	40·00	2·30	3·00	2560	—
18	+	68·00	4·40	0·80	640	—
19	+	22·00	1·00	11·05	5120	*P. falciparum* 20 mm^3
20	−	29·00	1·80	1·70	2560	—
21	+	48·00	5·10	3·60	1280	*P. falciparum* 1420 mm^3
22	+	35·50	3·00	7·70	1280	*P. falciparum* 60 mm^3
23	−	20·00	4·00	4·60	5120	*P. malariae* 540 mm^3
24	+	28·00	1·25	1·85	2560	Not determined
25	++	31·00	2·75	2·00	5120	Not determined
26 (cord serum)	++	25·00	Nil	0·17	2560	Not determined
Mean values		34·54 ±2·80	2·806 ±0·55	2·802 ±0·55	2752 ±380	

TABLE 2. Analysis of immunoglobulins and malarial antibody in terms of malarial infection (modified from McFarlane & Voller, 1966)

Blood film	No. of patients with a positive precipitin line	Malarial antibody titre	IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)
Malaria, +	5/6*	3,840±812	29·1±4·5	5·4±1·3	3·50±0·56
Malaria, −	10/17	2,150±387	39·2±3·7	2·4±0·47	2·77±0·063
Difference between groups		$P = 0·05$	$P = 0·1$	$P = 0·1$	$P = 0·1$

* Five out of six patients gave a positive precipitin test.

the five subjects who had *P. falciparum* in their peripheral blood. A precipitin line was given by the sera of nine out of fifteen (60%) of subjects who had no detectable parasites in their peripheral blood. Table 2 shows that five out of six (80%) of subjects with the highest titres of malarial fluorescent antibody together with a positive blood smear gave a precipitin line while ten out of seventeen (60%) of subjects with a negative malarial blood film who had received no anti-malarial drugs also gave a precipitin line.

In contrast, no precipitin line was obtained when control brain extract was tested against twenty-four sera of subjects who had not been taking anti-malarial drugs. Control sera from eighteen subjects who were on regular anti-malarial therapy or living in a community free from malarial parasites produced no precipitin lines with either the malarial antigen or control brain extract.

Fig. 3 shows the results obtained in attempts at titrating the malarial antibody by the double diffusion technique. The serum (No. 12 in Table 1) which showed a marked pre-

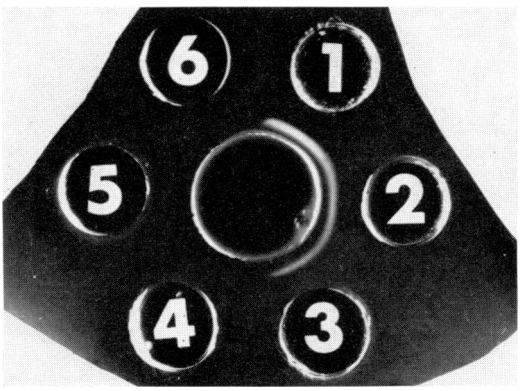

FIG. 3. Titration of malarial antibody precipitin lines produced in agar gel. Centre well contains malarial antigen. Wells 1, 2, 3, 4, 5 and 6 contain 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 dilution, respectively, of serum No. 12 (Table 1). The strong precipitin lines with the 1:4, 1:8 and 1:16 dilutions are clearly visible at 24 hr when this picture was taken. However, on standing for 72 hr fainter precipitin lines appeared at 1:32 and at 1:64. By this time, however, the earlier precipitin lines at 1:4, 1:8 and 1:16 have become very broad and diffuse and difficult to read.

cipitin reaction used in this experiment was obtained from an individual who had 200/mm^3 of *P. falciparum* with markedly elevated malarial fluorescent antibody titre and elevated immunoglobulin levels. This serum, after doubling dilution from 1:4 to 1:128, gave a precipitin line at the 1:64 dilution with the malarial antigen. The last two dilutions, 1:32 and 1:64 produced the precipitin line between 48 and 72 hr, although the stronger dilutions produced a precipitin reaction in less than 24 hr.

When the malarial antigen was heated at 56°C only a weak precipitin line in agar gel was formed with the immune human serum, and after heating at 100°C no precipitin line was formed.

Digestion with pepsin and treatment with 2-mercaptoethanol completely destroyed the antigenic activity of the cerebral malarial antigen.

Fig. 4 shows the elution pattern obtained after the malarial antigen was passed through a column of Sephadex G-200. Two peaks emerged from the column. The first peak which

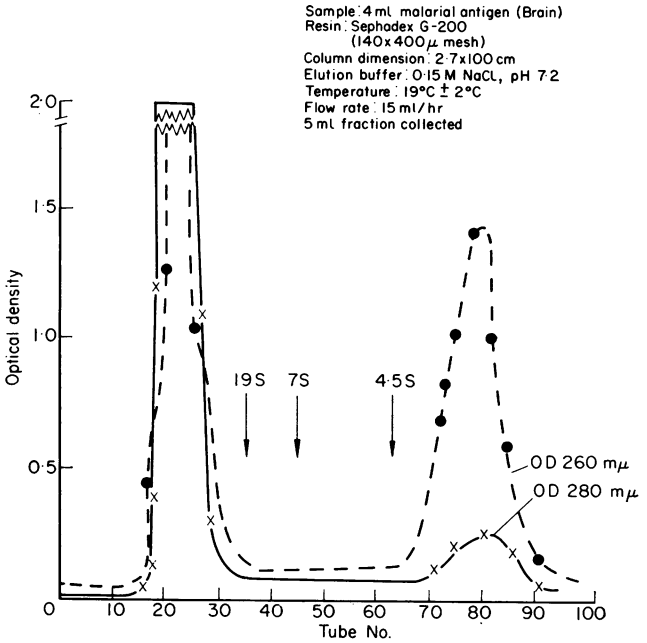

FIG. 4. Sephadex G-200 elution pattern of malarial brain antigen. The positions of the three arrows on the elution pattern mark the relative positions where the first, second and third peaks corresponding to 19S, 7S and 4·5S fractions of whole human serum, eluted in control experiments under identical conditions as described in the text.

TABLE 3. Amino acid analysis of crude malarial antigen extracted from human brain

Amino acid	Total protein (μM/g)
Aspartic acid	154·50
Threonine	86·95
Serine	116·90
Proline	53·10
Glutamic acid	169·15
Glycine	109·40
Alanine	129·90
Leucine	62·15
Glucosamine	113·55
Tyrosine + phenylalanine	20·55
Ethanolamine	10·40
Histidine	30·95

No correction was made for destruction during hydrolysis.

emerged ahead of the 19S position of human serum had maximum absorption at 280 mμ and was antigenic. On ultracentrifugation, this peak revealed typical characteristics of a heavy molecular weight protein, suggesting a sedimentation coefficient between 21S and 30S. The second peak had no antigenic activity and had an absorption maximum at 260 mμ.

Analysis of the crude malarial antigen revealed thirteen amino acids. Table 3 shows their relative concentration.

DISCUSSION

The specificity and ease with which the agar gel double diffusion test can be carried out will make it a very useful tool in the estimation of malarial antibodies in human serum. It can be readily used when a large number of specimens are to be tested simultaneously and can be accurately titrated.

The malarial antigen, purified from human brain, produced a precipitin line with sixteen out of twenty-four sera obtained in the peak of the malarial season in Nigeria, from individuals who received no anti-malarial therapy. This antigen produced no precipitin lines, either with sera from persons who received regular anti-malarial medication, or with sera from persons known to be living in a community free from malarial parasites. The control extract from normal human brain produced no precipitin line with any of the sera. These results indicate that the antigen–antibody reaction which produced the positive precipitin lines in agar gel appears to be due to malarial antigen reacting specifically with circulating malarial antibody.

Although the series is small it should be noted that 80% of the sera from persons with malarial parasites in their peripheral blood gave a positive precipitin reaction with the malarial antigen which seems to be further evidence that the precipitin line is due to the presence of circulating malarial antibodies.

The detection of malarial antibodies in 60% of the group who had no detectable malarial parasites in their blood and who also received no anti-malarial drug provide some indication of the degree of sensitivity and specificity of the precipitin reaction in the present study. The cord serum which contained detectable IgM but no IgA and 25 mg/ml of IgG had a fluorescent antibody titre of 2560 and produced a precipitin reaction with the malarial antigen, suggests that the malarial antibody in the foetus is mainly of the IgG type and confirms previous observations that malarial antibody crosses the human placenta (Edozien *et al.*, 1962; Cohen & McGregor, 1963).

The malarial antigen emerged from a Sephadex G-200 column ahead of the 19S peak of human serum indicating that it is a high molecular weight protein. This conclusion was supported both by the very slow rate of diffusion of the malarial antigen in agar gel and by the rapid rate at which the purified antigen sedimented on ultracentrifugation. This seems to suggest that the sedimentation constant of the malarial antigen is greater than 19S, probably between 21S and 30S having a molecular weight above 1,000,000.

The malarial antigen retained its antigenic activity after gel filtration but this antigenic activity was completely lost following peptic digestion, treatment with mercaptoethanol and heating at 100°C. Some activity was lost after heating at 56°C. This behaviour is characteristic of proteins. Hydrolysis of the malarial antigen followed by chromatography, revealed thirteen amino acids, but since no correction factor was applied, it is possible that one or two amino acids not accounted for may have been lost during hydrolysis.

Accurate titration of specific malarial antibodies as described in this study might provide a useful index of prognosis in the treatment of malarial infection, particularly in resistant strains. The simplicity and ease with which this method can be carried out in the estimation of circulating malarial antibodies compares favourably with the malarial fluorescent antibody test.

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