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Soluble Serum Antigens of *P. falciparum* in Nigerians

II. Immunochemical Studies

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Summary. Malarial soluble serum antigens (MSSA) were studied in five sera obtained from young Nigerian children during acute *P. falciparum* infection and in one serum from an *Aotus* monkey artificially infected with *P. falciparum*, using physicochemical and immunochemical techniques.

MSSA were detected by precipitation with adult human sera in gel diffusion tests. Fractionation of sera by Sephadex G-200 chromatography, gel diffusion tests in 1·5 and 7% agar, absorption tests with antisera to human immunoglobulins, and treatment of sera with mercaptoethanol and citric acid demonstrated the presence of MSSA in two forms: MSSA free and MSSA bound in a complex with immunoglobulin M.

Résumé. Le sérum soluble d'antigenes paludéens (MSSA) a été étudié dans cinq séra obtenus des jeunes enfants Nigerians pendant l'infection grave de *P. falciparum* et aussi dans un sérum d'un singe du groupe *Aotus* artificiellement infecté de *P. falciparum* en utilisant les techniques physicochimique et immunochimique.

MSSA a été détecté en precipitant les séra de l'adulte d'homme dans la reaction de diffusion du gel. Les séra fractionnés par Sephadex G-200 chromatographie, la réaction de diffusion du gel dans 1·5% et 7% d'agar-agar, les reactions d'absorption avec antisera a l'immunoglobulines humaines, le traitement de séra avec mercaptoethanole et l'acide citrique ont démontré la présence de MSSA en deux formes: MSSA libre et MSSA attaché en complexité avec immunoglobuline M.

INTRODUCTION

The malarial soluble serum antigens (MSSA) released into the circulation during the course of acute *P. falciparum* infection in children were originally described by McGregor and his collaborators (McGregor *et al.*, 1968). They later demonstrated that these serum antigens are thermostable and are similar to the 'S' class of malarial antigens extracted from the

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human placenta (Wilson *et al.*, 1969). Their findings were confirmed by the demonstration of MSSA in Nigerian children acutely infected with *P. falciparum* in our previous study, where we also investigated the specificity of *P. falciparum* antigens in the MSSA (Williams & Houba, 1972).

The fact that the sera of these children contained both malarial soluble antigen as well as malarial antibodies strongly suggests the possibility of circulating soluble immune complexes in excess of antigen. Although Wilson *et al.* (1969) found no evidence of immune complexes in the sera of Gambian children, we decided to investigate their possible presence in the sera of some Nigerian children with acute *P. falciparum* infection.

MATERIALS AND METHODS

Source of serum antigens

Five of the children's sera known to contain MSSA, as found in our previous study (Williams & Houba, 1972), were selected for detailed immunochemical investigation. All sera were obtained from young Nigerian children with acute *P. falciparum* infection. The details of these sera (number, age, parasite counts and antibody titres to *P. falciparum* antigens using conjugates specific to human IgG and IgM) are given in Table 1.

TABLE 1. Details of age, parasite count and fluorescent antibody titres of the five MSSA analysed

Number	Age	*P. falciparum* parasite count/mm³	Fluorescent antibody test against *P. falciparum** IgG	IgM
3	18 months	250,000	80†	80
4	16 months	200,000	1280	320
18	3 years	850,000	1280	80
30	15 months	100,000	160	80
55	15 motnhs	280,000	1280	1280

* *P. falciparum* slide antigens from artificially infected *Aotus* monkeys were prepared and kindly supplied by Dr A. Voller, Nuffield Institute of Comparative Medicine, London.

† Reciprocals of serum dilution.

One serum from an *Aotus* monkey artificially infected with a Nigerian strain of *P. falciparum*, collected at the peak of infection, was kindly provided by Dr A. Voller.

Immune sera from adults

Four sera from adult Nigerians known to be immune to malaria were chosen from a panel of sera known to have positive precipitating activity with MSSA, Nos. 7, 14, 83 and 384 (Williams & Houba, 1972).

Fractionation of MSSA by Sephadex G-200 chromatography

Four millilitres of each child's serum (1·5 ml. of *Aotus* monkey serum) with 500 mg

sucrose added, were fractionated separately by passage through a column (2·7 × 100 cm) of Sephadex G-200 (Pharmacia, Sweden). The column was equilibrated and eluted with 0·1 ml phosphate-buffered saline, pH 7·4 (PBS), containing 0·1% of sodium azide. The flow rate was adjusted to 15–20 ml/hr, and 5 ml fractions were collected by means of LKB fraction collector equipped with a Uvicord assembly set and recorder (LKB Producter, Stockholm, Sweden). The protein content was determined by optical density at 280 nm. The effluents were pooled, labelled alphabetically and concentrated × 20 by negative pressure dialysis to about 1 ml. The concentrates were tested for MSSA with relevant immune adult sera, and for IgG, IgA, IgM and the C_3 component of complement (beta-1-C/beta-1-A) with specific antisera, in Ouchterlony plates.

Precipitation of MSSA with ammonium sulphate

One volume of saturated ammonium sulphate was added drop by drop under stirring to one volume of serum at 4°C; the mixture was left to precipitate for 1 hr with occasional shaking and centrifuged in refrigerated centrifuge (4°C) for 1 hr. The precipitate was washed with saturated ammonium sulphate, dissolved in a minimal amount of PBS and dialysed against PBS (visking tubing 8/32″) at 4°C for 2 days (PBS changed twice a day). The supernatants were also dialysed under the same conditions and concentrated by negative pressure dialysis. Both the precipitate and supernatant were tested for MSSA activity by gel diffusion.

Gel diffusion tests

The Ouchterlony technique (1953) was applied, using Noble-agar (Difco Laboratories, U.S.A.). Two different concentrations of agar (1·5 and 7%) were used. The agar plates were incubated in humid chambers at room temperature in an air-conditioned room (22°C ± 2°C) and the results read twice a day up to 4 days.

Absorption of MSSA with antisera to human immunoglobulins

For absorption experiments the specific antisera to human immunoglobulins IgG and IgM were loaded first into the wells and after their diffusion into agar the wells were filled with MSSA sera. Different dilutions of antisera to human immunoglobulins were applied in order to achieve the optimal proportions for absorption and to avoid the excess of antiserum.

Treatment of MSSA with mercaptoethanol and citric acid

To nine volumes of MSSA was added one volume of 1 M mercaptoethanol so that the final concentration of mercaptoethanol was 0·1 molar. The mixture was left at room temperature for 18 hr and tested in gel diffusion test. One volume of 2 M citric acid to nine volumes of MSSA sera brought their pH to 2·5–3·0. The mixture was left at room temperature for 18 hr, neutralized to pH 7·0–7·5 by adding solid TRIS (trishydroxymethylaminomethane) and tested in gel diffusion tests.

Antisera to human immunoglobulins and C_3 component of complement

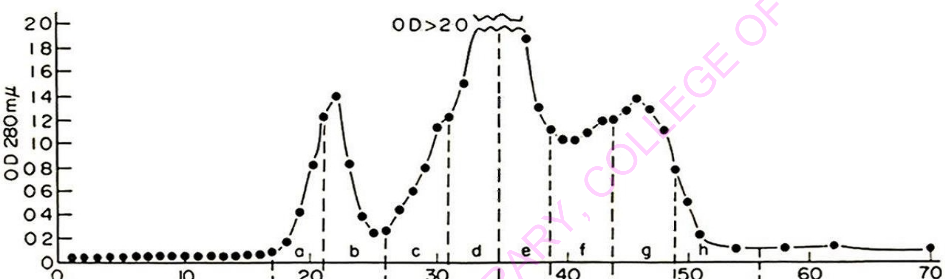
Sheep and/or goat antisera to human immunoglobulins IgG, IgA and IgM were kindly provided by Dr D. S. Rowe, WHO Research Centre for Immunoglobulins, Lausanne, Switzerland. The antisera showed single precipitin lines for IgG, IgA or IgM with whole

human serum and/or monkey serum after immunoelectrophoresis. Goat antiserum to human beta-1-C/beta-1-A (Hyland Laboratories, U.S.A.) was used.

RESULTS

The MSSA Nos. 3, 4, 18, 30 and 55 varied in their *ability to precipitate* with immune sera from adults in the Ouchterlony test using (1·5% agar): MSSA No. 18 gave precipitin line with adult immune serum 83 only, but not with the other adult immune sera Nos. 7, 14, and 384. Similar property was found in MSSA No. 55 which precipitated with adult immune serum 384 only. In contrast, MSSA No. 3 precipitated with three immune sera from adults, i.e. Nos. 14, 83, 384 but not with No. 7. The *Aotus* monkey serum precipitated with adult immune sera Nos. 7 and 83.

The results of fractionation of the MSSA sera on Sephadex G-200 showed two different patterns of MSSA distribution: The first is represented by MSSA No. 18 illustrated in Fig. 1.



	a	b	c	d	e	f	g	h
MSSA activity	±	+	+	−	−	−	−	−
IgM activity	+	+	+	−	−	−	−	−
IgA activity	−	±	+	+	+	±	−	−
IgG activity	−	±	+	+	+	+	−	−
βIC/βIA activity	−	−	±	+	+	−	−	−

FIG. 1. Sephadex G-200 elution pattern of malarial soluble serum antigen (MSSA) No. 18. Sample: 4 ml MSSA (No. 18) + 500 mg sucrose; resin: Sephadex G–200 (140 × 400 μm mesh); column dimension: 2·7 × 120 cm; buffer: 0·1 M *tris*-HCl in 0·5 M NaCl pH 8·0; temperature: 19°C ± 2°C; flow rate: 15 ml/hr; volume: 5 ml fractions collected per tube.

Note the distribution of MSSA activity in the first peak fractions.

Three peaks of proteins were recorded by measuring the optical density at 280 mμm. The activity of MSSA demonstrated by diffusing the concentrated fractions in 1·5% agar gel with the corresponding adult immune serum was found in the first peak and in the ascending portion of the second protein peak. The distribution of immunoglobulins IgG IgA and IgM as well as C_3 component of complement corresponded to the distribution found in normal human sera. Similar distribution of MSSA activity, immunoglobulins and C_3 component of complement were found in all human MSSA. Figure 2 represents the second type of distribution: *Aotus* monkey MSSA (only 1·5 ml. used for fractionation and, therefore, low peaks of proteins) in which MSSA precipitin activity was found only in the second protein peak.

All the MSSA sera were precipitated with *saturated ammonium sulphate*. The MSSA precipitin activity demonstrated by Ouchterlony test with appropriate immune sera was found only in the precipitates; there was no MSSA activity in any of the supernatants.

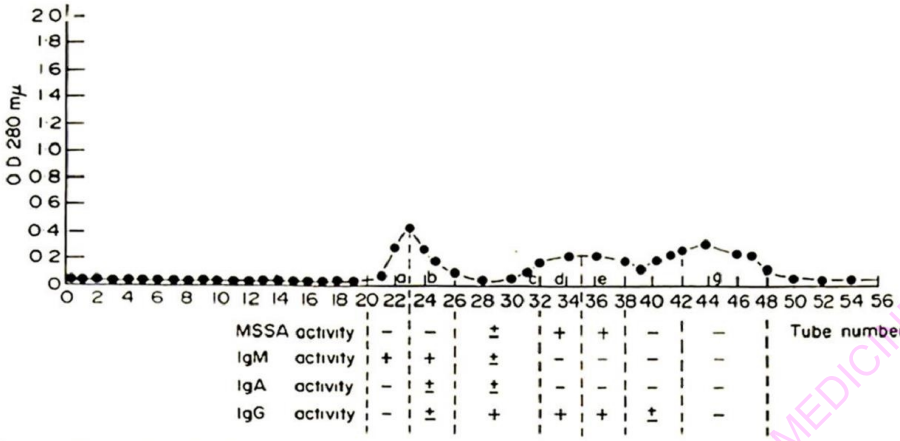


FIG. 2. Sephadex G-200 elution pattern of *Aotus* monkey MSSA. Sample: 1·5 ml monkey MSSA + 200 mg sucrose; resin: Sephadex G-200 (140 × 400 μm mesh); column dimensions: 2·7 × 100 cm; buffer: 0·1 M *tris*-HCl in 0·5 M NaCl pH 8·0; temperature: 19°C ± 2°C; flow rate: 15 ml/hr; volume: 5 ml fractions collected per tube.

Note the distribution of MSSA activity in the second peak fractions.

The *absorption experiments* showed distinct differences among the MSSA sera with regard to their ability to precipitate with the corresponding immune sera in 1·5% agar diffusion tests. The precipitin lines of MSSA sera Nos. 4, 18 and *Aotus* monkey did not disappear after absorption of sera with antisera to IgG and IgM— and the precipitin lines which developed after absorption gave lines of identity when compared to the lines developed by non-absorbed MSSA. The MSSA activity of serum No. 55 was also not absorbed by antiserum to IgG and was only partially absorbed by antiserum to IgM (faint precipitin line appeared after 70 hr of incubation). The precipitin activity of sera MSSA No. 3 and 30 was completely abolished after absorption with antiserum to IgM but remained after absorption with antiserum to IgG.

The absorption experiments suggested that MSSA Nos. 4, 18 and *Aotus* monkey serum contained MSSA not bound to immunoglobulins but MSSA sera Nos. 3 and 30 (and perhaps serum 55) contained MSSA bound to IgM. In order to confirm this difference, 7% agar was used for diffusion tests (low molecular substances not more than 7S will diffuse in 7% agar, but higher molecular particles such as 19S are not able to diffuse—Houba, unpublished observation). The MSSA were also cleaved by treatment with mercaptoethanol and citric acid; the products of the cleavage were also diffused in 7% agar.

MSSA sera Nos. 4 and 18 gave faint precipitin lines in 7% agar after 70 hr incubation when tested with corresponding adult immune sera in all instances, that is MSSA before treatment as well as after treatment with mercaptoethanol and/or citiric acid. The capacity of these MSSA (treated or untreated) to form precipitin lines was not influenced by absorption with antisera to IgG and/or IgM. *Aotus* monkey serum showed similar properties but the precipitin lines were much stronger.

In contrast MSSA sera Nos. 3 and 30 showed no precipitin lines when tested against corresponding adult immune sera in 7% agar even after 90 hr of incubation. However, after treatment with mercaptoethanol and/or citric acid these MSSA sera produced precipitin

TABLE 2. Immunochemical properties of the various MSSA studied

Products used for testing and their treatment		Precipitin reaction of MSSA sera					
		18	4	3	30	55	*Aotus* monkey
Gel diffusion test in 1·5% agar	Sera precipitated with ammonium sulphate:						
	Precipitate	+	+	+	+	+	+
	Supernatant	−	−	−	−	−	−
	Unabsorbed original sera	+	+	+	+	+	+
	Sera absorbed with:						
	Anti-IgG	+	+	+	+	+	+
	Anti-IgM	+	+	−	−	±	+
Gel diffusion test in 7% agar	Sera non-treated unabsorbed	±	±	−	−	−	+
	Sera treated with:						
	(a) Mercaptoethanol						
	Unabsorbed	±	±	±	±	±	+
	Absorbed with:						
	anti-IgG	±	±	±	±	±	+
	anti-IgM	±	±	±	±	nd*	+
	(b) Citric acid						
	Unabsorbed	±	±	±	±	±	+
	Absorbed with:						
	Anti-IgG	+	+	+	+	nd	nd
	Anti-IgM	±	±	±	±	nd	nd

* nd, Not done.

FIG. 3. Agar double diffusion test of MSSA No. 30 and corresponding adult immune serum No. 7. Central well, adult immune serum No. 7; 1, MSSA No. 30 absorbed with anti-IgG diluted 1 : 3; 3, MSSA No. 30 absorbed with anti-IgM diluted 1 : 3; 5, MSSA No. 30 absorbed with anti-IgM diluted 1 : 5; 2, 4 and 6; MSSA No. 30 unabsorbed.

Note that the anti-IgM completely absorbed the MSSA activity whereas anti-IgG has no such effect.

lines with corresponding adult immune sera in 7% agar after 48–70 hr. The precipitin activity of both MSSA Nos. 3 and 30 treated with mercaptoethanol and/or citric acid was not influenced by absorption with either antiserum to IgM.

MSSA serum No. 55 presented findings partially different from the previous groups: this serum gave no precipitin line with corresponding adult serum in 7% agar and after the treatment with mercaptoethanol or citric acid a very faint line appeared after 90 hr incubation. The absorption experiments with antisera to immunoglobulins were not done due to shortage of the MSSA No. 55.

The properties of MSSA sera described above are summarized in Table 2, and the typical results of absorption experiment is given in Fig. 3.

DISCUSSION

Our observations have shown some of the different properties of MSSA released into the circulation during acute attack of *P. falciparum* infection in young Nigerian children and their heterogeneity with regard to precipitin formation when diffused against immune sera from immune adults.

Some of the physicochemical properties were common to all MSSA including the *Aotus* monkey MSSA: MSSA activity in all of these sera was thermostable at 100°C for 5 min, stable at low pH (citric acid pH 2·5 for 18 hr at room temperature) and precipitable with half-saturated ammonium sulphate. These properties are consistent with physicochemical properties of *P. falciparum* 'S' antigens described by Wilson *et al.* (1969).

A distinct difference among MSSA sera was demonstrated by absorption experiments with antisera to human immunoglobulins. The ability of MSSA Nos. 4 and 18 as well as *Aotus* monkey MSSA to precipitate with corresponding adult immune sera was not influenced by previous absorption with the antisera to human IgG or IgM whereas the activity of MSSA Nos. 3, 30 and partially 55 was abolished by absorption with antiserum to IgM. The absorption effect might be explained by cross-reactivity, e.g. that MSSA in the latter sera have had some common or similar antigenic determinants with heavy chains of IgM. However, the experiments with 7% agar after treatment of the sera with mercaptoethanol and citric acid did not support this possibility.

The increased concentration of agar in gel diffusion test reduces diffusion of high molecular weight substances to such an extent that 19S molecules such as IgM cannot diffuse through 7% agar under the conditions described but the low molecular substances of 7S or lower (sub-units of IgM) do diffuse—(Houba, unpublished data). This experiment in combination with the reductive cleavage technique by mercaptoethanol and the dissociation of immune complexes at acid pH by citric acid has been applied in our studies in order to confirm the differences found in absorption tests. MSSA Nos. 4, 18 and *Aotus* monkey MSSA diffused through 7% agar regardless of their treatment with mercaptoethanol and/or citric acid but MSSA Nos. 3, 30 and 55 did not diffuse through 7% agar unless treated.

Reductive cleavage by mercaptoethanol split the molecules of immunoglobulins, e.g. IgM in our case, into small units and probably released the MSSA which diffused through 7% agar. This observation is consistent with the loss of serological activity of IgM after treatment with mercaptoethanol (Houba & Allison, 1966). The direct effect of mercaptoethanol on MSSA seems unlikely.

The treatment of MSSA Nos. 3 and 30 with citric acid at low pH split the antigen–

antibody complex, the antigen diffused through 7% agar and their precipitating ability with immune sera was no longer influenced by absorption with antisera to IgM. We have not experienced any recombination effect during neutralization of acid-treated MSSA sera and, therefore, the special techniques for separation of antigen or antibody were not used. The findings described have shown that MSSA may exist in free form (Sera Nos. 4, 18 and *Aotus* monkey) or bound with IgM in the form of soluble immune complex (sera Nos. 3, 30, partially 55). This would suggest that the free form of MSSA should have smaller molecular size than the bound MSSA, but as shown by fractionation of sera by Sephadex G-200 chromatography, only *Aotus* monkey MSSA would fit into this hyphothesis. This monkey serum antigen differed from the MSSA found in the children in one aspect: non-immune *Aotus* monkey was artificially infected with *P. falciparum* and the serum was collected in relatively short period before production of antibodies. On the other hand, all the children sera examined were from natural infections and had shown a certain level of humoral immunity (antibody titres to *P. falciparum*).

MSSA from all the children sera were eluted in earlier fractions of Sephadex G-200 but only some of them demonstrated MSSA bound in form of immune complex. This discrepancy is difficult to explain as the exact nature and structure of MSSA is not known (glycoproteins) and similar discrepancy of MSSA molecular size estimated by two different techniques has been reported (Wilson *et al.*, 1969). The possibility exists that MSSA from the children sera are bound in immune complexes with different degree of dissociation which may depend on avidity of antibody or on multivalency of antigens. Complexes with very low avid antibodies may dissociate in gel diffusion test at pH 8·6 whereas complexes with higher avidity of antibodies may be stable. The importance of differences between low and high avidity malarial antibodies in formation of immune complexes and their biological significance has been recently discussed (WHO, 1971). Unfortunately, the limited quantities of sera available from very young children as well as some technical difficulties did not allow the further study of these possibilities.

The activity of C_3 component of complement in MSSA was found in the second protein peak in Sephadex G-200 chromatography which corresponds to the position found in normal human sera. This is evidence that complement has not been bound in the immune complex because if it were, its activity would be located in the first peak together with antigen (Soothill & Hendrickse, 1967).

In conclusion, our observations have shown that MSSA may be detected in sera of young Nigerian children during acute *P. falciparum* infection in two forms: free and bound in complex with IgM.

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