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Complement factors and circulating immune complexes in children with urinary schistosomiasis and asymptomatic malaria

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Summary
Serum concentrations of circulating immune complexes (CIC), complement factors (Factor B, C4, C8) and complement activities (CH50 and AH50) were determined in Nigerian school children having urinary schistosomiasis with or without symptomatic malaria by polyethylene glycol precipitation method, single radial immunodiffusion and total haemolytic activities respectively. One hundred and forty-seven children were recruited from St. John's Primary School, Mokola, Ibadan, Nigeria.*P. ovale* only, mixed infection of *P. ovale* with *P. falciparum* or mixed infection of *P. malariae* with *P. falciparum* were found in subjects with asymptomatic malaria without urinary schistosomiasis (M-USS) but *P. malariae* or *P. falciparum* was found in subjects with co-infection of urinary schistosomiasis and asymptomatic malaria (M + USS). Mean value of C4 concentration was significantly reduced in M - USS subjects or subjects having both USS and asymptomatic malaria (M + USS) compared with non-infected controls. Serum concentration of Factor B(FB) was significantly reduced while AH50 was significantly increased in urinary schisosomiasis subjects without malaria (USS-M) compared with M-USS subjects or the controls. These observations implied that complement system in USS-M subjects is activated predominantly via alternative pathway (APW) while complement system is activated via classical pathway (CPW) in M-USS or M+USS subjects. The switch of complement activation pathway from alternative type in USS-M subjects to classical type in M-USS subjects may explain the lower malaria parasite densities often found in children harbouring *Schistosoma haematobium* parasites.

Keywords: *Malaria, schistosomiasis, complements, immune complexes.*

Résumé
Les concentrations du sérum de l' immune complexe circulant (ICC), facteurs du complément (Facteur B, C4, C8) et les activités du complément (CH50 et AH50) ont été déterminé dans les écoliers Nigérians ayant la schistosomiase urinaire avec/ sans un symtome du paludisme par une méthode de la précipitation du glycol du polyéthylène, l'immunodiffusion radiaux et les activités de l'haemolytique totales respectivement. Cent et quarante-sept enfants ont été admis de l'ecole 'St. John's primary school, Mokola, Ibadan, Nigeria.'Seul le P. Ovale, l' infection mélangée de *P.* ovale avec le *P.* falciparum ou l' infection mélangée de P. malariae avec le P. falciparum ont été trouvées dans les sujets avec paludisme manifestant la schistosomiase urinaire (M-USS) mais le P. malariae ou le P. falciparum etait trouvé dans les sujets avec le co-infection de schistosomiase urinaire et le paludisme asymptomatique (M + USS). La valeur moyenne de concentration C4 a été réduite dans le sujet de M- USS ou le USS et paludisme asymptomatique (M + USS) comparé avec les contrôles non-infectés. Le concentration du sérum de Facteur B(FB) a été réduit considérablement pendant qu'AH50 a été augmentés dans les sujets du schisosomiase urinaires sans le paludisme (USS-M) comparé avec les sujets du M-USS ou les contrôles. Ces observations impliquent que le système du complément dans les sujets de USS-M est plus actives d'une manière prédominante par le passage alternatif (APW) pendant que le système du complément est activé par le chemin classique (CPW) dans les sujets de M-USS ou les sujets M+USS. Le changement du passage d'activation complémentaire de type alternative dans les sujets de USS-M à type classique dans les sujets M-USS peut souvent expliquer les densités du parasite paludisme inférieure trouvée dans harbouring des enfants ayant des parasites Schistosoma haematobium.

Introduction

Schistosomiasis is found in geographical regions where malaria is endemic [1]. A previous study [2] attributed the co-existence of both malaria and schistosomiasis to socio-economic factors. However, adequate information of immune status of individuals hosting both schistosome and malaria parasites are lacking. Such information is necessary for rational design of treatment programme and vaccination protocols.

Complement system is an innate immune defence mechanism, which provides effector system for specific immunity. Complement system also play important roles in opsonisation, chemotaxis and in the clearance of immune complexes [3]. During malaria or schistosomiasis, reports on some components of complement activation pathway are discrepant. In malaria, depressed C3 [4], increased Factor B [5], reduced or unchanged C4 and C1q [4] have nity and humoral immunity act in concert or sequentially to control and clear a blood-stage malaria infection [4]. The endo-erythrocytic stage of malaria parasite induces a lot of immunological responses. This commences with proliferation of phagocytes of the reticuloendothelial system particularly in the spleen, liver and bone marrow. These cells phagocytose

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parasitised and unparasitised red blood cells, free malaria parasites and malaria pigments. The action is controlled by thymus-derived lymphocytes [5]. During the early phase of malaria infection, both reactive oxygen and reactive nitrogen metabolites produced by non-specific immune cells participate in controlling the primary parasitemia [4,5].

In schistosomiasis subjects, reduced C3 [8], raised C3d [9], elevated alternative pathway (APW) [9], combination of both APW and CPW [10] were demonstrated. Also, host immunity in schistosomiasis subjects does not eliminate adult worm of schistosome but immune attack of the host is directed to the eggs [8]. Antibody dependent eosinophil cytotoxicity is seen against schistosome ova to trap of the eggs in host tissues but not outright death of the eggs [21]. Granuloma formation around schistosome eggs is mediated by Th-2 cellular activity under the modulation of antibody, antigen-antibody complexes and by antigen specific suppressor cells [22].

Studies that determined levels of complement factors and activities in an individual harbouring both malaria and Schistosoma parasites are rare. This study determined the serum levels of Factor B (a component of APW), C4 (a component of CPW), C8 (a member of macromolecular membrane attach complex, MAC), CIC (Index of complement mediated clearance), AH50 (alternative pathway of complement haemolytic activity) and CH50 (classical pathway of complement haemolytic activity) in children having USS with or without malaria. The aim is to determine the influence of co-existence of both malaria parasite and *S. haematobium* on host humoral immune responses.

Materials and methods

Subjects

The subjects were pupils of St. John's Primary School, Mokola, Ibadan, Nigeria, and their participation was approved by Parent Teachers Association. All subjects (n = 147, age = 6 –14 years) recruited gave consents for participation. The school was selected for the study based on the fact that most pupils in the school make regular contact with "Dandaru River" which contains Bulinus snails (*interdediate host of S. haematobium* [24].

1. Children with both urinary schistosomiasis (USS) and asymptomatic malaria (M + USS)(n = 18).
2. Children without USS but with asymptomatic malaria (M-USS) (n= 46).
3. Children with on USS (USS – M) (n = 54).
4. Non-infected and apparently healthy controls © (n = 29).

Diagnosis of USS

USS was diagnosed by identification of terminally spined egges of *S. haematobium* in urine sediments following centrifugation at 1500 X G for 5 minutes, using a clinical centrifuge (02194, Needham Hts, Mass, USA) [2]. The urine sample was obtained from each subject in a clean 50ml plastic tube with the assistance of the class teachers between 10 hours and 12.00 hours after brief exercise. The urine samples were protected from sunlight by wrapping the sampling bottle in a carbon paper to prevent hatching of *S. haematobium* eggs to miracidium larvae. The urine samples were spun within 2 hours of collection. The sediment was examined using 40 X objectives lens of a binocular microscope (Wild Heerbrugg).

Diagnosis and classification of malaria

The thick and thin blood films on glass slides were stained with 4% Giemsa stain and examined for 100 high-power fields under oil-immersion objective lens of a binocular microscope (Wild Heerbrugg). All species of malaria parasites seen were identified, counted and the densities recorded as number of parasites per 200 white blood cells [11]. Children with no malaria parasite detected in their blood samples and without the eggs of *S. haematobium* in their urine samples were considered as controls.

Exclusion criteria

Children with HbSS or HbAS blood haemoglobin genotypes as confirmed by the method of Marengo-Rowe [12] were exculded from the study. Also excluded were those on antimalarial or antischistosomal chemotherapy between 1 day – 30 days to the time of blood collection.

Determination of complement levels and activities:

Venous blood (10ml) was collected by venipuncture in a clean glass bottle without anticoagulant. This was allowed to retract and the serum obtained was used to quantitate the levels of complement factors (FB, C4, C8) by single radial immunodiffusion method [13] using anti-sera (FB, C4, and C8) and their standards. C4 and C8 and their standards were purchased from Behringerwerke, AG, Marburg, Federal Republic of Germany. Antisera to FB and its standard were purchased from Pel-Freez Biologicals, USA. A volume of an optimally diluted monospecific anti-serum was mixed with noble agar and poured on glass plate. Wells of equal diameter were cut in the antibody agar mixture. The wells were filled with test and standard sera. The plates for FB, C4, and C8 were incubated at room temperature for 18 hours. After incubation, the diameters of the precipitin rings were measured with micrometer eyepiece.

Equal volume of standardised sheep and red blood cells (1 x 10^9 sheep RBCs/ml) and 2 minimum haemolytic dose of haemolysin was mixed and incubated at 37°C for 15 minutes with shaking. Diluted (1:50) test serum was added accordingly into each of the tubes at 5.0ml, 2.5ml, 1.5ml, 1.0ml so that each tube contained final volume of 7.5ml. Among the set up were serum blank (5.0ml complement diluent + 2.5ml (1:50) test serum), cell blank (1.0ml sensitized SRBC + 6.5ml diluent) and 10% lysis (1.0ml sensitized SRBC + 6.5ml (1:50 test serum). All tubes were incubated for 1 hour at 37°C in shaking water bath, thereaf-

ter spun at 1,500xG for 5 minutes to separate the supernatant whose optical density (OD) was measured at 541nm wavelength. AH50 or CH50 was extrapolated from standard graph plotted as % lysis against complement dilution.

Determination of circulating immune complexes

The concentrations of CICs in the sera of all the subjects were measured by the polyethylene glycol (PEG) 6000 precipitation method as described by Haskova et al (14). PEG 6000 solution was added to serum in borate buffer to give a final concentration of 3.7% PEG and 1 in 3 (1:3) dilution of serum. After incubation at room temperature, the optical density was measured at 450nm wavelength using spectrophotometer (Milton Roy 1001) to determine immune complex concentrations.

Statistical analysis

Chi-square method was employed to analyse the data on the prevalence of subjects with either or both malaria parasites and USS (Table 1) or prevalence of different species of *Plasmodium* in the subjects (Table 2). Student t-test was used for the analysis of the levels of Complement factors and Complement activities (Table 3 and 4).

Results

The overall prevalence of M+USS subjects in the study population was 12% while the prevalence M-USS subjects was 31%. Parasite numbers were lower in M+USS subjects (23-2924 malaria parasites per µl blood) compared with M-USS subjects (63-4942 malaria parasite per µl blood). Statistical analyses showed significant differences ($p<0.001$) (Table 1). Of all the species of *Plasmodium* identified, *P. falciparum* was most prevalent in M+USS (77%) or M-USS (72%) subjects followed by *P. malariae* (23% for M+USS and 11% for M-USS). In M+USS subjects, *P. ovale* or mixed infection of *Plasmodium* species was not found. However, mixed infection of *P. falciparum* and *ovale* (2%) or *P.falciparum* and *malariae* (11%) was present in M-USS subjects (Table 2).

Table 1: The prevalence of asymptomatic malaria, USS or both among the school children considered for this study

		USS		
		With	Without	p
Asymptomatic malaria	With	18 (12%)	46 (31%)	
		*109 (23-2924)	319 (63-4942)	<0.001
	Without	54 (37%)	29 (20%)	<0.001

**Mean (Range) of malaria parasite number per ul blood.*
USS = Urinary schistosomiasis.

Table 2: The prevalence of different species of *Plasmodium* in M-USS and M+USS subjects

Subjects	*P. falciparum*	*P. malariae*	*P. ovale*	*P. falciparum +P. ovale*	*P. falciparum +P.malariae*
M-USS	33 (72%)	5 (11%)	2 (4%)	1 (2%)	5 (11%)
M+USS	14 (77%)	4 (23%)	0 (0%)	0 (0%)	0 (0%)

$X^2 = 1.82$, $p>0.1$

M-USS = Children without urinary schistosomiasis but with asymptomatic malaria.

M+USS = Children with both urinary schistosomiasis and asymptomatic malaria.

Table 3: Serum levels (mean ± 1S.D) of C4, C8 and FB in children with asymptomatic malaria without urinary schistomiasis (M-USS), urinary schistosomiasis without malaria (USS-M), or asymptomatic malaria with urinary schistoso miasis (M+USS) compared with the controls.

	Complement	Factors	(mg%)	
Subjects:	n	C4	C8	FB
Controls	29	22±76	5.1±2.0	15.1±3.9
M-USS	46	10.4±5.0*•←	4.0±3.3	14.6±7.7
USS	54	29.3±6.3*•	3.1±0.4*•	8.5±4.3*•
M+USS	18	16.4±9.2*	3.9±1.6*•	21.1±10.3*•

n = Number
** = Significantly different from the controls ($p<0.05$)*
• = Significantly different from M-USS ($p<0.05$)
← = Significantly different from M+ USS ($p<0.05$)

Table 3 shows that mean value of C4 was significantly reduced in both M+USS and M-USS subjects compared with the controls ($P < 0.01$ in each case). Also, Table 3 shows that FB values were significantly reduced in USS-M subjects compared with the controls or M+USS subjects. The mean level of C8 was similar in USS-M or M-USS subjects ($P < 0.1$), though lower than in the controls.

Table 4: Serum levels (mean ± 1S.D) of AH50, CH50 and CIC in study groups and the controls.

Subjects:	n	CH50(U/ml)	AH50(U/ml)	CIC(mg%)
Controls	29	27.8±8	31.1±12	13.9±9
M-USS	46	36.4±20*	33±19←	20±9*
USS	54	41±12*•←	34±17←	24±10*•
M+USS	18	34±10*	52±23*	23±18*

N = Number
** = Significantly different from the controls ($p<0.05$)*
• = Significantly different from M-USS ($p<0.05$)
← = Significantly different from M+ USS ($p<0.05$)

The values of CIC and CH50 were similar in M+USS and M-USS ($P>0.2$) but were significantly raised when compared with the controls ($P < 0.01$ in each case). Moreover,

the mean value of AH50 was significantly raised in USS-M subjects compared with other groups of the subjects.

Discussion

Concurrent infection of malaria and USS (12%) was not as high as either malaria alone (31%) or USS alone (37%) among the school children considered for this study. More so, mean malaria parasite density was lower in school children with both malaria and USS (109 malaria parasites per µl blood) compared with those with malaria alone (319 malaria parasites per µl blood)

This study demonstrated that malaria is associated with C4 hypocomplementaemia suggesting that complement activation occurs predominantly via CPW. Immune complex formation between antigens from merozoite stage of *Plasmodium* and complement fixing antibody may play a major role in the consumption of C4 as a result of activation of CPW. Moreso, Topley *et al* (15) showed the presence of complement factors on RBC during malaria, it is possible that this involves the binding of C4.

FB is important in the production of C3 convertase, C5 convertase and functional haemolytic activity [16] The finding of reduced FB in USS-M subjects suggest FB consumption during complement activation via APW.

The terminal sequence of complement activation involves C5b, C6, C7, C8 and C9 that interact sequentially to form a macromolecular membrane complex that disrupts the membrane enabling ions and small molecules to diffuse through freely. Binding of C8 to trimolecular complex C5b67 begins the membrane damage aggravated by C9 [4], thus elucidating the importance of C8 in the lysis of complement. However, if the reaction occurs on an immune complex, C5b67 complex is released and bind to nearby cells causing "innocent-bystander" lysis, which is concomitant to auto-immunity [4]. Autoimmune tissue damage involving innocent-bystander lysis has been implicated in a number of diseases with high serum level of CIC [17] as the present study subjects. In clinical malaria and schistosomiasis, antibodies against immunoglobulin, complement and various tissue components have been described [28, 29, 20].

The reduction in the C8 levels in all test subjects may be in support of rapid consumption of terminal factors in complement activation pathways as a result of stimulation by antigens from both *Plasmodium* and *Schistosoma*. The pathogenesis of clinical features of malaria or schistosomiasis is not completely established, although damage to small blood vessels, activation of kinin system, disseminated intravascular coagulation and inflammatory cytokine have been reported [21]. Complement activation have been directly linked with kinin activation, intravascular coagulation and tumour necrotic factor production [22], thus increased complement activities caused *Plasmodium* or *Schistosoma* may add to the basis of their pathogenesis.

Activation of complement system via classical pathway may be an advantage in M+USS subjects since mean malaria parasite count was lower in them. Antigens from surface coat of adult Schistosoma activates complement pathways efficiently [9,10], such activation may remove certain stages of *Plasmodium* parasites which circulate in the blood stream before escaping into the cells. *Plasmodium* parasitised RBCs may also be lysed by complement activation to expose endo-erythrocytic stages of *Plasmodium* to host immune destruction.

The efficiency of immune-complex formation depends upon the amount of C3b and C4b bound to pre-existing immune complex. The binding of C4b and C3b fragments to immune complexes during CPW aggravates the formation of soluble immune complexes [23]. This is in line with the present study where reduced C4 and elevated CIC were found in the test subjects. In addition, complement mediates the removal of immune complexes through CRI on human RBC. This is transported to the liver and spleen before elimination [23]. Raised levels of CIC in the test subjects when compared with controls, indicates that CIC was formed at a faster rate than being cleared in the test subjects.

It may be concluded that activation of complement system via either CPW or APW may be one of the mechanisms responsible for reduced densities of malaria parasites in Nigerian children with USS.

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