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LEUCOCYTE PHAGOCYTOSIS IN CHILDREN WITH URINARY SCHISTOSOMIASIS AND ASYMPTOMATIC MALARIA PARASITEMIA

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In the participants considered for this study, leucocyte migration, neutrophil candidacidal activity and ability to generate reactive oxygen were determined as percentage migration index (%MI), candidacidal phagocytic index (%CI) and bacterial stimulated nitroblue tetrazolium (NBT) dye reduction index (%NBT) respectively. Also, malaria density was counted from thick blood film of glass slide stained with Giemsa stain. The participants were 54 school children having urinary schistosomiasis without malaria parasites (USS-M), 18 children with both urinary schistosomiasis and malaria parasites (USS+M), 46 children with malaria parasites without urinary schistosomiasis (M-USS) and 29 controls. The mean %MI was least while %NBT index was highest in USS+M subjects but M-USS subjects had least %CI. Malaria density was higher in M-USS subjects than USS+M subjects. The results of this study showed that low prevalence and reduced severity of malaria parasites in children with urinary schistosomiasis may be due to adequate production of LMIF and reactive oxygen species.

Keywords: Leucocyte phagocytosis, malaria, schistosomiasis, Nigeria

INTRODUCTION

The epidemiology of malaria coincides geographically with that of schistosomiasis in many region of the world. However, immune correlates of resistance during schistosomiasis or malaria remains unclearly defined (1). Also little is known about the immune responses of individuals with concurrent infections of schistosome or malaria parasites with other parasites/pathogens. Co-infection of malaria parasite with certain pathogens/parasites was found to result in both susceptibility and protection against cerebral malaria.

Protection against cerebral malaria was observed in CBA/J mice inoculated with attenuated third stage larva of *Brugia pahangi* (2) and in C57BL/6 mice infected with both *Plasmodium berghei* and murine leukemia virus (3). In contrast, others (4, 5) reported increased mortality of mice infected with *Leishmania mexicana* and *Plasmodium yoeli* or *Plasmodium inuei* and hepatitis B virus.

Also, the outcomes of co-infection in schistosoma parasite with other pathogens/parasites are diverse. Susceptible AKR mice acquired the capacity to resolve *Trichuris muris* infection when co-infected with *Schistosoma mansoni* (6) whereas increased parasitaemia of *Plasmodium chabauddi* was seen in malaria resistant C57BL/6 mice concurrently infected with both *S. mansoni* and *P. chabauddi* (7) Marshall *et al* (8) concluded that *S. mansoni* infection caused a significantly higher mortality in *Toxoplasma gondii* infected C57BL/6mice.

Before now, co-existence or immunological studies in humans infected with both *Plasmodium spp* and *Schistosoma spp* was not considered. This is important considering increased treatment failure being recently experienced in malaria and schistosomiasis subjects. Therefore, the present study determined leucocyte migration, ability to kill *Candida* and to produce reactive oxygen intermediates in

Nigerian school children having urinary schistosomiasis (USS) with or without malaria parasites. Such knowledge will be beneficial in rational design of treatment programme and optimization of vaccination protocols.

MATERIALS AND METHODS

Subjects

The study was approved by the Parent Teachers Association of St John's Primary School, Mokola, Ibadan, Nigeria where the subjects were recruited. Informed consent was obtained from the pupils, parents/guardians of the pupils, teachers and head-teachers before sample collection. All subjects (n=147, age 6-14 years) gave consents of participation and were grouped as:

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

Diagnosis of USS

USS was diagnosed by identification of terminally spined eggs of *S. haematobium* in urine sediments following centrifugation at 2000 rpm for 5 minutes. The urine sample was obtained from each subject in a clean 50 ml plastic tube with the assistance of the class teachers between 10.00hrs-12.00hrs after brief exercise. The sediment was examined using 40 X objective lens of a binocular microscope (Wild Heerbrugg).

Diagnosis/classification of malaria

The thick blood films on glass slides were stained with 4% Giemsa stain and examined for 100 high-power fields under oil-immersion objective lens. All stages of malaria parasites seen were counted and the densities recorded as number of parasites per 200 white blood cells (WBCs). Children with no malaria 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 genotype as confirmed by the method of Marengo-Rowe (9). Also excluded were those on antimalaria or anti-schistosoma chemotherapy between 1-30 days to the time of sample collection.

% Leucocyte migration index (%MI)

The method described by Hudson and Hay (10) was followed. Five ml of heparinized blood was mixed with equal volume of 3% dextran solution. The capillary tubes filled with packed white blood cells were anchored into migration chamber containing either medium (15% foetal calf serum) or antigen (PPD)-medium solution (1:20 dilution). A drop of streptomycin was added to each well and incubated at 37°C in CO_2 for 18 hours. The percentage MI due to the antigen was calculated thus: % MI=T/C X 100, where C is the area of migration in the medium alone and T is the area of migration in antigen solution. The migration index value of 80% or less was taken as positive (11).

Preparation of neutrophil suspension

Neutrophil suspension was prepared from fresh unclotted whole blood as described by Strober (12). Five ml of blood was mixed with equal volume of 6% dextran in Kreb Ringers solution at 20°C for 45 minutes. The leucocyte-rich plasma was gently layered on 5ml Ficoll-Hypaque

solution and spun at 1400rpm for 40 minutes leaving neutrophil / red blood cells pellet. The RBCs were lysed with 10ml cold 0.2% NaCl for 30 seconds, after which 10ml ice cold 1.6% NaCl was added to restore the isotonicity. The neutrophil number was adjusted to 5 X 10^6 cells / ml.

Percentage Candidacidal index (%CI)

The percentage CI was determined using the ability of neutrophils to kill *Candida albicans* as previously described (14). A saline-wash concentrated suspension of a 24-hr culture of *C. albicans* was made in Krebs Ringer solution. This was adjusted to 5 x 10^6 cells/ml of Krebs Ringer solution and the viability of the cells was confirmed to be 95% by the Trypan blue dye. To a mixture of 0.25 ml of autologous plasma, 0.25ml of 5 x 10^6 neutrophil suspension, and 0.25 ml of Krebs Ringer solutions; Candida (0.25 ml at 5 X 10^6/ml of Krebs Ringer solution) was added.

A similar set up was made for the control tube except that neutrophil suspension was omitted. The tubes containing the mixture were incubated for 1hr with shaking at every 15 minutes. At the end of this period, 0.25ml of 2.5% sodium deoxycholate was added to each mixture to lyse neutrophil but not *Candida*. Four ml of 0.01% of methylene blue was added for 10 minutes to stain dead *Candida*. This was carefully removed leaving about 0.5ml to resuspend the organism. The % of dead *Candida* was determined using haemocytometer.

Nitroblue tetrazolium (%NBT) dye reduction

The method described by Strober (14) was followed. A drop of 10g/ml Phorbol myristate acetate was spread and dried on a glass coverslip. A drop of non-coagulated blood was added, incubated at 37°C for 45 minutes in humidity chamber until clot and 2 drops of NBT were added for 20 minutes. This was fixed with 100% methanol for 1 minute and counterstained with Safranin for 10 minutes. The % NBT positive cells in a total 200 neutrophils were determined using microscope at 40 X objective. Neutrophils that had undergone respiratory burst showed a granular blue black cytoplasmic formazan staining were taken as NBT-positive. NBT-negative neutrophils exhibited no such cytoplasm staining.

Cell viability test

Cell viability was determined by mixing 10µl of cell suspension with 10µl of 0.5% Trypan blue and 180 µL of Krebs Ringer solution. The number of cells excluding the stain was expressed as a percentage of the total number of cells counted. Cell suspension used in this study had viability greater than 90%.

Statistical analysis

Statistical methods employed in the analysis of data generated include mean, standard deviation, Student's t-test and Chi-square (2 X 2 Contingency) test.

RESULTS

The overall prevalence of subjects with co-infection of urinary schistosomiasis and asymptomatic malaria (USS+M) in the study population was 12% while the prevalence of subjects with asymptomatic malaria without urinary schistosomiasis (M-USS) was 31%. Malaria parasite numbers were lower in USS+M subjects (23-2924 malaria parasites numbers per µl blood) compared with M-USS subjects (63-4942 malaria parasites per µl blood). Statistical analysis showed significant difference. The most prevalence specie of *Plasmodium*

among M-USS or USS+M subjects was *P. falciparum* followed by *P. malariae*. Few of the M-USS subjects had *P. ovale* only (4%) or *P. ovale* + *falciparium* (2%). None of the USS+M subjects had *P. ovale* only, *P. falciparum* + *P. ovale* or *P. falciparum* + *P. malariae* (Table 2). Table 3 shows that mean % MI was significantly reduced in M-USS subjects, USS-M and USS+M compared with the controls. % MI was least in USS+M subjects, NBT dye reduction index was similar in M-USS subjects and the controls, but the value in USS+M was the highest (Table 3). % CI was comparable in USS-M, USS+M and the controls but the value was least in M- USS subjects (Table 3).

Table 1: The prevalence of asymptomatic malaria, USS or both among pupils attending St John's Primary School, Ibadan, Nigeria

		USS		
		With	**Without**	**p**
Asymptomatic Malaria	**With**	18(12%) 109 (23-2624)*	46(31%) 319(63-4942)*	<0.001
	Without	54 (37%)	29(20%)	<0.001

* = Mean and (range) of malaria parasite number per µl of blood

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

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

X^2 = 1.82, p >0.10

Table 3: Leucocyte migration indices (%MI), Candidacidal index (%CI) and NBT dye reduction index (%NBT) in M-USS, USS-M, USS+M compared with the controls

Subjects	**n**	**%MI**	**% CI**	**% NBT index**
Controls	29	69+/-28	26:2+/-6.2	86.9+/-10.4
M-USS	46	54+/-20.3*	20+/-36*	80.1+/-18.9
USS-M	54	55± 29.4*	24.8± 5.9^{0}	88.0± 20*
USS+M	18	48+/-15.9$^{*0\#}$	24.3+/4.1^{0}	89+/-17.3^{*0}

* Significantly different from the controls (p<0.005), ^{0}Significantly different from M-USS (p<0.05), $^{\#}$Significantly different from USS-M (p<0.05)

DISCUSSION

The result of the present study indicates that malaria density is less in USS+M subjects (23-2624 malaria parasites/µl blood) compared with M-USS subjects (63-4942 malaria parasites/µl blood). Moreso, co-existence of *Plasmodium spp* and *S. haematobium* in same host alters leucocyte migration, neutrophil candidacidal activity and ability to generate reactive oxygen intermediates. This contradicts the results of Lewinsohn (13) and Lwin *et al* (14) that observed no change in malaria parasitaemia and activities of CD4+ T lymphocytes in mice infected with both *S. mansoni* and *Plasmodium* parasite.

In the present study, leucocyte migration was significantly reduced in all test subjects (particularly USS+M) compared with the controls. This may be a result of increased production of leucocyte migration inhibitory factor (LMIF). LMIF that inhibit random migration of leucocytes is produced by activated lymphocytes. Increased LMIF production retards leucocyte migration with resultant reduced % MI. This is supported by a report that LMIF has the ability to retain leucocytes at the site of infection or inflammation *in vivo* (11). From the present results, it could be proposed that lymphocytes in malaria or / and USS subjects produced excess LMIF and that leucocytes from them respond appropriately.

The percentage CI were similar in USS-M, USS+M and the controls indicating that *S. haematobium* infection do not alter candidacidal activity which is based on production of reactive oxygen species. Nitric oxide production has been proposed as important mechanism of protective immunity to malaria (14, 16) and schistosomiasis (17). In this study, the efficiency of superoxide generation as shown by % NBT index was significantly higher in USS+M compared with either the controls or M-USS subjects. The ability USS+M subjects to generate adequate amount of superoxide might have interfered with the growth of *Plasmodium,* thus low malaria parasite density in USS+M subjects. Adult worms of schistosome attract RBCs to cover their surfaces (18). These RBCs are lysed so as to release intracellular malaria parasites for phagocytosis. This is in support of low malaria parasitaemia in USS+M subjects.

The present study revealed that leucocyte phagocytosis is not adversely affected during co-infection of malaria with schistosomiasis but *Plasmodium* infection alone reduces neutrophil Candidacidal activity by reducing the production of reactive oxygen species.

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