ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2004) 22: 213-218

Leucocyte Migration and Nitroblue Tetrazolium Assay in Nigerian Children with Bacteremia and Malaria Parasitemia

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SUMMARY The prevalence of malaria parasitemia, bacteremia, certain hematological parameters, leucocyte migration index and nitroblue tetrazolium dye reduction were determined in 147 Nigerian children (4.24 ± 2.88 years of age). Sixty (40.8%), 28(19.1%) and 26(17.7%) had malaria parasitemia only, bacteremia only and both malaria parasitemia and bacteremia, respectively. Four genera of bacteria, i.e *E. coli, Proteus, Staphylococcus and Salmonella*, were detected in subjects with both malaria parasitemia and bacteremia. The 4 bacterial genera and *Klebsiella* were detected in subjects with bacterial infection only. *P. falciparum* (68%), *P. malariae* (25%) and *P. ovale* (7%) were the species of malaria parasites identified in our subjects. Bacteremia was most prevalent in subjects with hemoglobin AA (HbAA) (60.7%) followed by HbAC (21.45%). Packed cell volume (PCV) and Hb concentration were similar in all groups but mean counts of red blood cells (RBC) and white blood cells (WBC) were statistically significantly lower in subjects with malaria parasites only compared to the controls. Leucocyte migration was significantly reduced in children with bacteremia only or both malaria parasitemia and bacteremia compared to controls, while the nitroblue tetrazolium assay was significantly reduced in children with bacteremia only. It may be concluded that malaria parasitemia significantly affects both leucocyte migration and nitroblue tetrazolium assay.

Immunity to malaria is complex and undoubtedly results in low-level parasitemia.[1] Also, immunity to bacterial infections is mediated by both cellular and humoral mechanisms.[2] However, complete knowledge of the immune status of individuals hosting both bacteria and malaria parasites is lacking. Such knowledge is necessary for a rational design of treatment programs and vaccination protocols.

Several specific disturbances in the immune system in association with *P. falciparum* malaria infections have been described. *In vivo*, antibody responses to specific antigens including tetanus toxoid and the "O" antigen of *Salmonella typhi* were reduced during acute malaria episodes.[3] *In vitro*, monocyte derived macrophage functions were severely impaired by the ingestion of parasite-derived hemozoin.[4] Bacteria express many different surface antigens and secrete a variety of virulence factors that may trigger or inhibit immune responses.[5]

Published epidemiological data on the co-existence of both malaria parasites and bacteria are few. Prada *et al.*[6] showed that 8 out of 50 Nigerian children with cerebral malaria had bacteremia while O`Dempsey *et al.*[7] suggested that non-typhoid *Salmonella* species were particularly associated with *P. falciparum* infection. The association of bacteria species with malaria infection may pose a challenge in the management of pyrexia and treatment

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of malaria in children of malaria endemic zones since the two pathogens of different phylogeny may stimulate or synergistic different courses of immune responses.

The specific objective of this study was to reveal the co-existence of bacteria with malaria infections in Nigerian children, to establish their immune status (leucocyte migration and bactericidal activity) and their hematological parameters.

MATERIALS AND METHODS

Subjects

Informed consent was obtained from the parents of the 147 children (4.24 ± 2.88 years of age) recruited for the study and from the authorities of the hospitals (Local Government Clinics in Inalende and Idi-Ogungun areas of Ibadan, Nigeria and the Adeoyo State Hospital, Ibadan, Nigeria) where the subjects were recruited. Venous blood samples were collected by venipuncture and aliquoted into sterile bottles containing nutrient broths (for microbiological analysis), EDTA bottles (for hematological analysis), heparinized bottles (for immunological analysis) and on microscope slides as thin film (for parasitological analysis). Based on laboratory investigations, the subjects were grouped into controls (healthy children without malaria parasites or bacteria), subjects with malaria parasites only, subjects with bacteria only and subjects with both bacteria and malaria parasites.

Parasitological investigation

A thick blood film was prepared on a glass slide to investigate the status of *Plasmodium* infection of all subjects while a thin blood film was prepared for identifying the species of *Plasmodium*. The latter was stained with a 4% Giemsa stain before viewing with a compound microscope under an oil immersion lens. All species of malaria parasites seen were counted and the densities recorded as number of parasites per 200 WBCs.[8]

Microbiological investigation

For bacteremia, blood samples collected by venipuncture were put on a blood agar broth in a sterile glass bottle. The content was thoroughly mixed and incubated at 37°C for 7 days. Each day the content was examined for turbidity. A turbid culture was inoculated onto a blood agar plate, chocolate agar and MacConkey plate and incubated at 37°C overnight. The characteristic appearance of the organism grown on each plate was noted, necessary biochemical tests were performed and identification of the colony was carried out as described by Cowan and Steel.[9]

Hematological investigation

Venous blood in EDTA bottles was used for hematological parameters determined by standard operating procedures.[10] Such parameters included hemoglobin (Hb) genotype, packed cell volume (PCV), total WBC count, RBC count and hemoglobin concentration.

Immunological parameters

Nitroblue tetrazolium dye reduction index (% NBT): this was based on the method of Feigin *et al.*[11] For a stimulated NBT procedure, 50 µl of NBT solution, 25 µl heparinized blood and 25 µl of stimulant solution (non-viable bacterial extract) were mixed gently, incubated at 37°C for 10 minutes followed by another 10 minutes at 26°C. A thick smear of the mixture was prepared, air dried for 10 minutes, treated with undiluted Wright stain for 15 seconds and diluted Wright stain (1:1) for 30 seconds before rinsing in water and air-drying. One hundred neutrophils were counted under an oil immersion objective and the neutrophils showing dark formazan deposit were recorded as positive. The percentage bacterially stimulated NBT index was calculated as previously described:[11]

Neutrophils with dark formazan deposit (positive)/Total neutrophil count x 100

Percentage leucocyte migration index: this was described elsewhere.[12] Briefly, leucocytes were separated from whole blood using 3% dextran. The result was washed thrice in Krebs Ringer solution, filled into capillary tubes, anchored into a migratory chamber filled with either Krebs Ringer solution or Krebs Ringer solution and antigen (purified protein derivative of *M. tuberculosis*). This was incubated for 18 hours at 37°C and the area of leucocyte migration in the chamber-containing antigen was compared to the area of migration in a chamber without antigen. The percentage MI due to the antigen was calculated thus:

Area of migration in antigen solution/Area of migration in the medium alone x 100

A migration index value of 80% or less was considered positive.[12]

Statistical methods

The data generated were analyzed for mean, standard deviation, one-way analysis of variance (ANOVA) (F- test) and Student test (t- test). The statistical significance is at 95% confidence interval ($p < 0.05$).

RESULTS

Out of a total of 147 children, 60 (40.8%), 28 (19.1%) and 26 (17.7%) had malaria parasitemia only, bacteremia only and both malaria parasitemia and bacteremia, respectively. Four genera of bacteria were detected in subjects with both malaria parasitemia and bacteremia. These were *Escherichia, Proteus, Staphylococcus* and *Salmonella* while five genera of bacteria were detected in subjects with bacterial infection only. These included the four above and *Klebsiella* (Table 1).

Table 2 shows that *Plasmodium* parasitemia was most predominant in individuals with hemoglobin AA (HbAA) genotype followed by those with HbAS genotype. Bacteremia was most prevalent in subjects with HbAA (60.7%) followed by HbAC (21.45%). Packed cell volume (PCV) and Hb concentration were similar in all groups but mean counts of RBC ($t = 2.13$, $p > 0.05$) and WBC ($t = 2.87$, $p > 0.02$) were statistically significantly lower in subjects with malaria parasites only compared to the controls (Table 3).

Mean % MI was statistically significantly reduced in subjects with both malaria parasitemia and bacteremia compared to the controls ($t = 6.42$, $p < 0.01$) or children

Table 1 Prevalence of bacterial genera in subjects with bacteremia alone and in those with both malaria and bacteremia

Type of bacteria	Malaria and bacteremia N (%)	Bacteremia alone N (%)
E. coli	5 (19.2)	13 (46.4)
Proteus	4 (15.4)	2 (7.1)
Staphylococcus	16 (61.5)	10 (35.7)
Salmonella	1 (3.9)	2 (7.1)
Klebsiella	0 (0)	1 (3.6)

Table 2 The distribution of different Hb genotypes in subjects with malaria and/or bacteremia

Hb genotypes	Malaria alone N (%)	Bacteremia alone N (%)	Malaria + Bacteremia N (%)
AA	41 (68.3)	17 (60.7)	17 (65.4)
AS	11 (18.3)	3 (10.7)	6 (23.1)
AC	8 (13.3)	6 (21.4)	3 (11.5)
SC	0 (0)	0 (0)	0 (0)
SS	0 (0)	2 (7.1)	0 (0)
Total	60	28	26

Table 3 Comparison of hematological parameters in the control and test groups

Groups	N	PCV (%)	Hemoglobin (g/dl)	RBC (x 10^6 cells/μl)	WBC (x 10^3 cells/μl)
Control	93	28.15 ± 5.26	9.65 ± 1.83	3.68 ± 0.75	9.87 ± 5.75
Malaria only	60	26.27 ± 5.44	9.09 ± 1.68	3.44 ± 0.73*	7.51 ± 3.38*
Malaria and bacteremia	26	26.31 ± 5.42	9.1 ± 1.78	3.57 ± 0.76	8.03 ± 2.26
Bacteremia only	28	27.93 ± 5.8	9.51 ± 1.89	3.6 ± 0.08	10.50 ± 6.49
F, *p*-values		1.91, 0.13	1.43, 0.24	2.9, 0.10	2.45, 0.06

The statistical significance is at 95% confidence interval.
*Significant difference between controls and malaria only.

with bacteremia only ($t = 6.33$, $p < 0.01$) or children with malaria parasitemia alone ($t = 9.55$, $p < 0.01$). Children with malaria parasitemia only had statistically significantly lowered % MI compared to the controls ($t = 6.75$, $p < 0.01$). The mean % NBT assay was statistically significantly reduced in subjects with bacteremia only ($t = 11.10$, $p < 0.01$) or subjects with both malaria parasitemia and bacteremia ($t = 9.23$, $p < 0.01$) compared to the controls (Table 4).

Table 4 Comparison of percentage leucocyte migration and NBT assay in control and patient groups

Groups	N	% MI	% NBT
Controls	93	61.01 ± 16.76	75.44 ± 5.62
Malaria only	60	42.94 ± 15.12[1, 2]	76.27 ± 8.10[2]
Malaria and bacteremia	26	38.61 ± 11.06[1,3]	63.31 ±6.78[1]
Bacteremia only	28	60.79 ± 17.44[2,3]	60.90 ± 7.84[1]
F, *p*-values		25.60, 0.00(s)	55.08, 0.00(s)

F- value = One-way analysis of variance (ANOVA). The statistical significance is at 95% confidence interval; % MI = percentage migration index of leucocytes; % NBT = percentage bactericidal index of leucocytes.
[1] % MI or % NBT were significantly reduced compared with the control group;
[2] % MI or % NBT was significantly raised compared with subjects having both malaria parasitemia and bacteremia;
[3] % MI was significantly different from subjects having malaria only

DISCUSSION

Acute malaria is extremely common in developing countries and severe malaria is a major cause of hospitalization in many parts of Sub-Saharan African for children under five years of age.[1,13] Few data on the co-existence of malaria parasitemia and bacteremia exist. Moreover, immunological reasons to explain these occurrences are scanty. Our present data established that *Staphylococcus* was the most prevalent bacterial species in subjects with both malaria parasites and bacteremia, but *E. coli* was the most prevalent in children with bacteremia only. Subjects with HbAA genotype (followed by HbAS and HbAC) had the highest rate of malaria parasitemia and bacteremia, thus confirming the protective role of HbS genotype from malaria.[14] Although the precise mechanism for the protection conferred by HbS remains unclear, two plausible mechanisms are: reduced parasite replication within the erythrocyte and increased clearance of non-parasitized RBC along with parasitized RBC by the reticuloendothelial activity of the spleen.[14] Friedman and Trager[15] suggested that parasites in infected HbS cells develop normally until the cells are sequestered in the tissues where there is low oxygen tension and intracellular pH. These conditions sickle the host cells, and reduce the potassium levels resulting in parasite death. Alternatively, infected cells might sickle while circulating rather than while being sequestered and are eliminated by the filtering action of the spleen or through phagocytosis by the cells of the reticuloendothelial system.

The observed reduction of the total WBC count in children with malaria infection only corroborates earlier studies on malaria infected Nigerians.[16,17] Although the mechanism of this leucopenia in subjects with malaria only is unknown, it could be due to a sequestration of leukocytes in specific areas of some lymphoid organs or due to an uncompensated decrease in certain subtypes of T-cells as earlier detected by Wells *et al.*[18] The finding of lower RBC numbers and consequently lower PCV and Hb concentration in subjects with malaria only could be in support of previous findings: accelerated clearance of circulating infected RBC through phagocytosis, auto-immune destruction of RBC, bone marrow hypoplasia, increased RBC destruction during endo-RBC schizogony and RBC sequestration brought about by cytoadherence and rosetting.[19,20] Sera from subjects with malaria have been shown to enhance phagocytosis of *P. falciparum*-infected RBC by monocytes and polymorphonuclear leucocytes.[21] Thus, it is possible that phagocytosis of circulating infected RBC may contribute to reduced RBC counts.

Richard *et al.*[22] suggested that a major pathway of cell mediated immunity in *Plasmodium* infection involves the release of lymphokines which stimulate mononuclear phagocytes to exert anti-parasite effects. Leucocyte migration inhibitory factor (LMIF) inhibits random migration of leucocytes during inflammation. It also plays a central role in the control of both innate and antigen-specific immunity.[23] In the present study, the reduced % MI in subjects with malaria parasitemia only could be a

result of an increased secretion of LMIF. Similarly the % MI was further reduced in subjects with a co-infection of malaria and bacteria but not in those with bacteremia only. There are several mechanisms employed by bacteria to evade phagocytosis: preventing interaction between phagocytes and C3b on the bacterial surface, binding of *Streptococcus pyogenes* protein M to complement factor H, molecular mimicry and masking with host antigen.[24] It is likely that a reduction of LMIF production is another mechanism used by bacteria to escape host immunity.

Killing of ingested antigen by phagocytes is primarily accomplished by toxic molecules, particularly reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). The present study measured the production of ROIs since reduction of NBT dye depends on the amount of H_2O_2 produced during intracellular killing. Many bacteria and *Plasmodium* are susceptible to reactive oxygen intermediates.[25] Mean % NBT dye reduction in subjects with malaria parasitemia alone is similar to that of the controls. It is possible that adequate amounts of H_2O_2 were generated in subjects with malaria parasitemia. Significantly reduced % NBT in subjects with bacteremia only and in those with both malaria parasitemia and bacteremia indicated depressed production or over usage of H_2O_2. This observation may explain the higher density of malaria parasites in subjects with a co-infection of bacteria and malaria (53,145 ±18,897/μl of blood) than in those with malaria parasitemia only (33,967 ± 27,442/μl of blood). This study shows that leucocyte migration inhibition factor (LMIF) secretion is raised in subjects with malaria parasitemia only while production of H_2O_2 is reduced in subjects with bacteremia only, thus indicating that both malaria parasitemia and bacteremia affect cell-mediated immunity.

ACKNOWLEDGEMENT

This work was sponsored by The University of Ibadan, Nigeria, College Research Grant.

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