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Original article

Serum Levels of anti-BCG, Albumin and Packed Cell Volume and White Blood Cell Count in Subjects with HIV and Malaria Co-morbidity

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Abstracts: The present study was designed to determine anti-BCG titres, serum albumin, packed cell volume, white blood cell count and malaria parasite density in HIV infected subjects in a malaria endemic area with stable transmission. For this study 75 participants aged between 17 and 70 years (females=45; males=30) were enlisted and grouped into: (i) HIV/AIDS subjects (n=21) on anti-retroviral therapy (ART), 12 of these subjects had malaria co-infection; (ii) HIV seropositive subjects (n=29) not on ART, 13 of whom had malaria co-infection, and (iii) HIV seronegative control subjects (n=25), 15 of whom had malaria parasiteamia. Serum albumin, anti-BCG and PCV, WBC and malaria parasite density were determined in all participants. The results showed that all participants had detectable anti-BCG, but the titre was lowest in HIV/AIDS on ART followed by HIV seropositives and control subjects ($f=16.878$; $p<0.001$). The results also showed that serum albumin (g/l) was significantly different among the HIV seropositives, HIV/AIDS and control subjects ($f=8.043$; $p<0.001$). This pattern was also true for the PCV ($f=17.505$; $p<0.001$). When the above parameters were considered for subjects with malaria co-infection, a similar pattern of results was observed. There was no within-group difference in those with or without malaria for the respective groups except for WBC count, which was significantly reduced in HIV/AIDS subjects with malaria, compared with those without malaria. The positive association between WBC count and malaria parasite density was consistent in all groups. The present study thus indicates that, although there is evidence of prior exposure to bacterial infection, serum anti-BCG, serum albumin and PCV are mostly impaired by HIV infection even in cases of malaria co-morbidity in endemic areas.

Key words: Malaria, HIV/AIDS, BCG, PCV, Albumin

INTRODUCTION

One of the most important problems facing people living with HIV is the clinical resurgence of *Mycobacterium tuberculosis*, a chronic bacterial infection. The resurgence of the clinical disease in most HIV infected subjects' remains a difficult problem to solve [1, 2]. Similarly, HIV and malaria co-infection has been reported in both stable and unstable malaria endemic areas of sub-Saharan Africa [3, 4, 5, 6]. Therefore the possibility of all three infections occurring in HIV infected subjects is not low. Although bacillus calmette guerin (BCG) vaccine for protection against tuberculosis has long been available [2], this is not the case for malaria. Although BCG produces some antibody-mediated responses against tuberculosis in the recipient, the focus has always been on the cell-mediated immunity, which provides protection against the disease [7, 8, 9]. The present study was designed to determine anti-BCG titres in HIV infected subjects as a sign of exposure either through vaccination or exposure to a wide variety of strains. Since the study was carried out in a malaria endemic area with stable transmission, the possible impact of malaria infection on the anti-BCG titre was assessed in addition to defining the biochemical and/or nutritional stability using serum albumin levels and packed cell volume.

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MATERIALS AND METHODS

Subjects

A total of 75 participants (male=30 female=45) aged between 17 and 70 years were involved in the present study. They were recruited at the Voluntary and Counselling Unit (VCT) of Nnamdi Azikiwe University Teaching Hospital, Nnewi Nigeria between May and August 2007, and all underwent HIV and *Plasmodium falciparum* screening. Based on the results, the participants were grouped as follows according to the World Health Organization staging for HIV.

(i) HIV/AIDS subjects (n=21) on anti-retroviral therapy (ART). Twelve of these subjects had malaria co-infection. The anti-retroviral therapy (ART) included Stocrin (efavirenz), Lamivudine and Viread (tenofovir), all in company recommended doses. The participants, who were HIV stage two category with no evidence of tuberculosis, had been on the ART for periods between 3 and 6 months.

(ii) HIV seropositive subjects (n=29). They were not on ART. Thirteen of the subjects had malaria co-infection. The participants were HIV stage one category with no evidence of tuberculosis.

(iii) HIV seronegative subjects (n=25). Fifteen of these subjects had malaria parasiteamia.

Blood samples were collected from all the participants, and the serum component was extracted and stored at −20℃ until analysis. The serum samples were used for the analyses of albumin and determination of anti-BCG titre, while the whole blood was used for the analysis of packed cell volume, WBC count and parasite density. The participants provided informed consent, and the Board of the Ethics Committee approved the study design.

Methods

Detection of antibodies to HIV-1 and HIV-2 in human plasma

Two different methods were used, namely, Abbott determine ™ HIV 1 & 2 kit, which is an *in vitro* visually read immunoassay (Abbott Japan Co ltd. Tokyo, Japan), and HIV 1 & 2 STAT-PAK Assay kit, which is an immunochromatographic test for the qualitative detection of antibodies to HIV-1 and HIV-2 in human plasma (CHEMBIO Diagnostic system, INC, New York, USA). For the Abbott determine ™ HIV-1 & 2, the procedure described by the manufacturer was used for the analysis. Briefly, 50 µl of participants' plasma samples separated from corresponding whole blood samples in EDTA were applied to the appropriately labeled sample pad. After 15 minutes but not more than 60 minutes of sample application, the result was read. This method has inherent quality control that validates the results.

For the immunochromatographic method for HIV 1 & 2, the procedure described by the manufacturer was used for the analysis. In brief, 5 µl of participants' plasma was dispensed into the sample well in the appropriately labeled sample pad. Three drops of the buffer supplied by the manufacturer was added into the appropriately labelled sample wells. The results of the tests were read at 10 minutes after the addition of the running buffer. This method has inherent quality control that validates the results. The HIV seropositive results obtained using these two methods were used to classify participants presenting with HIV infection.

Diagnosis of *P. falciparium* malaria

Whole blood was used for the diagnosis of *P. falciparium* malaria using the Malaria Plasmodium Falciparium Rapid Test Device (Para check, Orchid Biomedical Systems, Vena Goa, India) and Giemsa stained thick blood smears for microscopic detection of P. falciparum parasites. The parasite density was determined as previously reported [10] and the density was expressed per litre of blood. However, the principle of the *P. falciparum* antigen detection is based on a rapid chromatographic immunoassay for the qualitative detection of circulating *P. falciparium* antigen in the whole blood. This method utilizes gold conjugate to selectively detect *Plasmodium* antigen. The procedure described by the manufacturer was used. Briefly, a 10 µl whole blood specimen was transferred into appropriately labelled specimen cassettes containing a sample well. Subsequently, 3 drops of buffer supplied by the manufacturer or approximately 120 µl was added to the sample wells. After 15 min the results were read. The test device has inherent quality control that validates the result. The presence of two pink lines at the region of the control and test sample signifies presence of *P. falciparum* malaria infection, while the presence of only 1 pink line in the control region signifies absence of *P. falciparum* malaria.

Determination of serum anti-BCG levels

Serum levels of anti-BCG were determined by the haemagglutination technique. In brief, pooled O (Rhesus (D) positive) blood samples collected from blood donors were used for the 10% red cell suspension in normal saline after several washings in normal saline. BCG for vaccination was used to coat or sensitize the washed red cells by incubating the BCG-red cell mixture at 37℃ for 90 minutes. This allows for adsorption of the BCG antigens on the red cell surface. After the incubation period, the BCG sensitized red cells were washed to remove any unbound BCG antigen. Subsequently 10% suspension of the BCG-sensitized red cells was prepared for the purposes of the

haemagglutination test. For the haemagglutination test, the serum samples were diluted accordingly and equal volumes of the BCG-sensitized red cells were added to the various dilutions of the samples in order to determine the anti-BCG titer for each serum. The serum-BCG-sensitized red cells were incubated at 37℃ for 120 minutes. The maximum serum dilution providing a positive haemagglutination result on the basis of the presence of macroscopic and/or microscopic agglutination was recorded.

Serum albumin concentration was determined using bromocresol green (BCG) kit (Randox Laboratories Limited UK) and the method described by Doumas et al [11]. Meanwhile, PCV was determined by the micro-haematocrit method described by Dacie and Lewis [12]. Similarly the white blood cell count was determined using Teurk's solution as described by Dacie and Lewis [12].

Statistical analysis

The variables were expressed as mean (±sd). Both student t-test and analysis of variance (ANOVA) were used to assess significant mean differences. The spearman's correlation coefficient was used to assess the level of association between two variables. A difference of <0.05, <0.01 and <0.001, respectively, was considered significant.

RESULTS

The mean values of serum anti-BCG, PCV and serum albumin were significantly different among the HIV/AIDS, HIV seroposiitve and HIV seronegative control subjects (f= 16.878, P<0.001; f=8.043, P<0.001; and f=17.505, P< 0.001). However, no significant mean difference was observed for WBC count, malaria parasite density or age of subjects among these groups. Similarly, no significant difference was observed in CD4 count between the HIV/AIDS and HIV seropositive subjects. See Table 1.

Similarly, when the subjects with malaria parasites were compared separately for the above parameters among the HIV/AIDS, HIV seroposiitve and HIV seronegative control subjects, the mean values of serum anti-BCG, PCV and serum albumin were significantly different (f=7.035, P< 0.01; f=6.538, P<0.01; and f=6.094, P<0.01). However, no significant mean difference was observed for WBC count or malaria parasite density, although the age of the subjects in these groups was significantly different. Similarly, no sig-

Table 1: (mean±sd) Serum anti-BCG (titre), PCV (%), serum albumin (g/l), WBC count ($x10^6$), malaria parasite density (10^5/l) and age (yrs) of HIV/AIDS subjects, HIV seropositive subjects and control subjects.

Parameters	HIV/AIDS	HIV seropositive	Control	f-value	p-value
BCG-titre	5.0±6.5 (n=21)	7.8±12.5 (n=29)	286±339 (n=25)	16.878	<0.001
PCV (%)	35.9±4.8 (n=21)	31.4±4.9 (n=29)	35.6±3.6 (n=25)	8.043	<0.001
Albumin (g/l)	46.6±9.1 (n=21)	37.4±8.4 (n=29)	53.0±10.4 (n=25)	17.505	<0.001
WBC ($x10^6$/l)	4.47±1.44 (n=21)	4.46±1.63 (n=29)	4.66±1.69 (n=25)	0.122	>0.1
Parasite density ($x10^5$/l)	6.0±3.6 (n=12)	6.6±4.6 (n=13)	8.9±3.8 (n=15)	1.995	>0.1
Age (yrs)	38±9 (n=21)	36±10 (n=29)	32±9 (n=25)	2.746	>0.05
CD4 (/MM^3)	360±210	276±159	-	-	>0.1

Table 2: (mean±sd) Serum anti-BCG (titre), PCV (%), serum albumin (g/l), WBC count ($x10^6$), malaria parasite density (10^5/l) and age (yrs) of HIV/AIDS subjects, HIV seropositive subjects and control subjects with malaria parasiteamia.

Parameter	HIV/AIDS (n=12)	HIV seroposiitve (n=13)	Control (n=15)	f-value	P-value
BCG-titre	5.1±8.5	8.9±17.0	278±359	7.035	<0.01
PCV (%)	35.2±4.1	30.5±5.8	36.5±3.7	6.538	<0.01
Albumin (g/l)	44.9±7.4	37.3±9.3	50.3±11.1	6.094	<0.01
WBC ($x10^6$/l)	3.87±1.17	4.48±1.54	4.69±1.61	1.097	>0.1
Parasite density ($x10^5$/l)	6.0±3.7	6.6±4.6	8.9±3.8	1.995	>0.1
Age (yrs)	39±9	38±11	28±9	5.280	<0.01
CD4 (/MM^3)	345±231	328±205	-	-	>0.1

nificant difference was observed in CD4 count between the HIV/AIDS and HIV seropositive subjects. See Table 2.

When a within-group comparison was made for HIV/AIDS subjects with or without malaria, no significant difference was observed for any parameter except WBC count, which was significantly reduced in those with malaria compared to those without malaria ($P<0.05$). See Table 3.

Similarly, when a within-group comparison was made for HIV seropositive subjects with or without malaria parasitaemia no significant difference was observed for any of the parameters. See Table 4. This was also the pattern for the control subjects with or without malaria parasitaemia, except that the age of those without malaria parasitaemia was higher than those with malaria parasitaemia. See Table 5.

The packed cell volume was negatively associated with age ($r = -0.437$; $P<0.05$) while the WBC was positively associated with malaria parasite density ($r = 0.798$; $P<0.01$) in the HIV/AIDS subjects on ART. Similarly, the anti-BCG titre was negatively associated with age ($r = -0.392$; $P<0.05$) while WBC count was positively associated with malaria parasite density ($r = 0.758$; $P<0.01$) in HIV seropositive subjects. Finally, the WBC count was positively associated with malaria parasite density ($r = 0.525$; $P<0.05$) in the control subjects.

DISCUSSION

The present study examined the presence of anti-BCG in the sera of 75 participants and revealed widespread exposure either due to BCG vaccination or exposure to a variety of strains. This indicates that the resurgence of tuberculosis

Table 3: Mean (±sd) anti-BCG titer, PCV, serum albumin, WBC count, parasite density and age of HIV/AIDS participants with or without malaria.

Parameter	Malaria positive (n=12)	Malaria negative (n=9)	p-value
Anti-BCG titer	5.1±8.5	4.8±2.4	>0.1
PCV (%)	35.1±4.0	36.8±5.7	>0.1
Albumin (g/l)	44.9±7.4	49.0±11.2	>0.1
WBC ($X10^6$/l)	3.86±1.17	5.27±1.41	<0.05
Parasite density ($x10^5$/l)	6.0±3.6	-	
Age (yrs)	39.0±9.2	37.0±8.3	>0.1
CD4 (/MM^3)	345±231	379±189	>0.1

Table 4: Mean (±sd) anti-BCG titer, PCV, serum albumin, WBC count, parasite density and age of HIV seropositive participants with or without malaria.

Parameter	Malaria positive (n=13)	Malaria negative (n=16)	p-value
Anti-BCG titer	8.9±17.0	6.9±7.6	>0.1
PCV (%)	30.4±5.7	32.3±4.1	>0.1
Albumin (g/l)	37.2±9.3	37.5±7.9	>0.1
WBC ($X10^6$/l)	4.48±1.54	4.43±1.75	>0.1
Parasite density ($x10^5$/l)	6.6±4.6	-	
Age (yrs)	37.8±11.1	34.7±10.0	>0.1
CD4 (/MM^3)	328±205	234±98	>0.1

Table 5: Mean (±sd) anti-BCG titer, PCV, serum albumin, WBC count, parasite density and age of control participants with or without malaria.

Parameter	Malaria positive (n=15)	Malaria negative (n=10)	p-value
Anti-BCG titer	278.4±359.5	298.6±324.7	>0.1
PCV (%)	36.5±3.6	34.4±3.4	>0.1
Albumin (g/l)	50.3±11.1	57.0±8.1	>0.1
WBC ($X10^6$/l)	4.69±1.61	4.60±1.89	>0.1
Parasite density ($x10^5$/l)	8.93±3.84	-	
Age (yrs)	28.13±8.7	37.2±7.1	<0.05

in some HIV-infected subjects in this part of Nigeria may not be due to a lack of previous exposure, since there has been an almost 100% BCG vaccination coverage at childhood for more than 5 decades. However, none of the participants showed clinical signs of tuberculosis. A significant difference in serum anti-BCG titres was observed among the HIV/AIDS subjects on ART, HIV seropositive subjects and control subjects. The lowest titre was observed in the HIV/AIDS subjects while the highest titre was observed in the control subjects. The serum anti-BCG titre followed a similar pattern as explained above even among the subjects with malaria parasiteamia in the respective groups. The dramatic drop in anti-BCG titre in both HIV/AIDS subjects on ART and HIV seropositive subjects compared with the titre in the control subjects suggests the impact of viral infection on BCG antibody response. No improvement was observed with the use of ART. When the within-group BCG titre was considered for those participants with or without malaria, no significant difference was observed among HIV/AIDS, HIV seropositive and control subjects. This clearly shows that malaria parasitaemia in endemic areas does not play any role in the drop in anti-BCG response in HIV-infected subjects. That is, the titres were similar between those with malaria parasiteamia and those without malaria parasiteamia within each group.

In most developing countries, HIV and tuberculosis have been observed as a co-infection [13, 14, 15]. It is believed that the cellular immune response is more important in protection against tuberculosis [16]. Thus the reduced anti-BCG titre in HIV-infected subjects observed in the present study probably reflects the impact of viral infection on the immune system of the infected host and also previous exposure. The impact in HIV/AIDS or HIV seropositive cases is severe because the titre is elevated in the control participants. This might be an indication of progressive impairment of specific immune responses.

Previous studies on BCG level have shown different titre ranges in children and adults with detectable anti-BCG titre. The titre range in the present study was 1:2 to 1:32 in HIV/AIDS subjects; 1:1 to 1:64 in HIV seropositive subjects and 1:16 to 1:1024 in the control subjects.

The serum anti-BCG titre was negatively associated with age in HIV seropositive subjects, indicating that the ability to produce anti-BCG response declines with age in HIV-infected subjects. Hence, if the anti-BCG titre is used as an index for specific immune response status, older citizens might be highly prone to tuberculosis in this area.

The significant differences in mean serum albumin concentration, which was lowest in the HIV seropositive subjects and highest in the control subjects, suggest that commencement of ART may help to revert synthetic capacity to produce albumin in the infected host. This evidence of reduced albumin in HIV seropositive subjects is an indication of the impact of viral infection on the nutritional stability of the host, irrespective of malaria parasiteamia. Previous studies have shown that albumin is a good index to monitor recovery and predict prognosis in HIV infected subjects [17, 18]. The PCV was lowest in the HIV seropositive subjects suggesting that aneamia is one of the consequences of this distorted nutritional status, irrespective of malaria parasiteamia. However, no significant difference was observed within group for these parameters between subjects with and without malaria parasitaemia.

The WBC count was significantly reduced in HIV/AIDS subjects with malaria parasitaemia compared with those without malaria parasitaemia, but this was not the case for the HIV seropositive or control subjects. Nevertheless, WBC count was positively associated with malaria parasite density in all the groups suggesting that the WBC count is dependent on the malaria parasiteamia and perhaps explaining the lack of significance observed in mean WBC count between the groups. However, the negative association observed between age and packed cell volume in HIV/AIDS subjects on ART suggests that susceptibility to anaemia increases with age due to the impact of the ART.

In conclusion, the present study indicates that, although there is evidence of prior exposure to bacterial infection, serum anti-BCG, serum albumin and PCV are mostly impaired by HIV infection even in cases of malaria co-morbidity in endemic areas.

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