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Association of CD4 cell count and antiretroviral therapy antibody production in Human Immunodeficiency virus seropositive subjects on antiretroviral drugs in Benin City, Nigeria.

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Abstract
Antiretroviral therapy (ART) is widely used and subjects on ART were reported to develop antibodies against these drugs when used as monotherapy. The aim of this study was to determine the proportion of HIV-sero positive subjects on antiretroviral therapy who developed antibodies to the drugs used as combination therapy and to associate the presence of antibodies to CD4 cell count which is a marker of disease progression. A total of 300 subjects were randomly selected for the study, consisting of 100 HIV-sero positive subjects already on ART, 100 newly diagnosed HIV-sero positive ART-naive subjects and 100 HIV-sero negative individuals who were monitored as controls. CD4 cell count and complete blood count were assayed using BD FASCOUNT CD3/CD4 and Sysmex KX-21N autoanalyzer respectively. Antigen-Antibody agglutination technique was used to detect antibodies produced against antiretroviral drugs in HIV -sero positive subjects on ART. The results showed that antibodies to antiretroviral drugs (tenofovir disoproxil fumarate/lamivudine Efavirenz + lamivudine/ nevirapine/zidovudine combination) were detected in 33(33%) out of the 100 HIV-sero positive subjects on ART studied. Significantly higher CD4 cell count ($p<0.001$), PCV ($p<0.001$) and white blood cell count ($p<0.001$) were recorded among control group than HIV positive subjects whether they were on ART or not. However, a significantly lower level of platelet was observed in HIV-sero positive subjects on ART who developed antibodies. The findings from this study showed that antibody to antiretroviral drugs was detected in 33(33%) out of the 100 HIV- sero positive subjects on ART combination studied. The use of combination therapy has led to a smaller number of subjects on ART who developed antibody as against a higher percentage on monotherapy that was previously reported. Significantly lower level of platelet was observed among HIV-sero positive subjects who developed ART antibodies.

Keywords: ART antibodies, HIV, CD4 cell count, Haematological parameters.

Introduction

Antiretroviral therapy (ART) involving the use of a combination of antiretroviral drugs made up of two nucleoside reverse transcriptase inhibitors (NRTI) and an important non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitors (PI) has become the gold standard in the management of patients with HIV infection unlike the use of monotherapy which was the practice many years ago (Odunukwe *et al*., 2005). According to Andrade and Cotter (2006), disorders like abnormalities in lipid metabolism lipodystrophy, insulin resistance have been associated with protease inhibitors. This has led to a decline in the use of protease inhibitors as antiretroviral therapy. Antiretroviral therapy has been reported to improve packed cell volume of patients infected with HIV, though there are evidences that these drugs have cytopenic effect when used as monotherapy (Odunukwe *et al*., 2005). The use of ART has led to decreased mortality of infected subjects since it was introduced in 1996 (Gea-banacloche *et al*., 1999).

It was reported that numerous individuals living with HIV that were on ART treatment still developed anaemia (Mildvan, 2003). Antiretroviral therapy treatment was reported as one of the causes of anaemia in HIV infected subjects (Moyle, 2004). The antiretroviral drugs cause anaemia by inhibition of haemoglobin synthesis (Wood *et al*., 2002) and by production of antibodies against the drugs or their metabolites (Omoregie *et al.,* 2008). It was previously reported that 81% HIV-sero positive subjects on ART had antibodies to one or more of the ART drugs when used as monotherapy (Omoregie *et al.,* 2008).

The use of multiple drug therapy has been shown to be more effective in the management of patients with HIV infection (Barlett *et al*., 2006; Thompson, 2010). The proportion of individuals who developed antibodies to ART monotherapy was reported in Benin City, Nigeria (Omoregie *et al.,* 2008). But no study to the best of our knowledge has been conducted to evaluate antibody development against ART-combination drug therapy. The objective of this study therefore was to evaluate the proportion of the HIV-sero positive subjects on ART-combination therapy who developed antibodies to the drugs and also to associate the presence of these antibodies with the measured immunological and haematological parameters.

Materials and Methods

Study parameters:

A total of three hundred (300) individuals were enrolled, consisting of 100 HIV-sero positive subjects already on ART for 3 -6 months (24 males and 76 females), 100 newly diagnosed HIV-sero positive naive subjects (27 males and 73 females) 100 HIV-sero negative individuals (volunteer donors consisting of 42 males and 58 females) who served as controls, were recruited for this study. The HIV positive individuals were attending HIV clinic at Central Hospital, Benin City. Subjects who had physical manifestation of full-blown AIDS were excluded from this study.

Ethical consideration: The protocol for the study was reviewed and approved by the ethical committee of Edo State Ministry of Health with reference number: HA.577/VOL.2/153.

Sample collection and analysis: Ten millimeters of blood was obtained for analysis from each subject. Five (5) milliliters of blood were dispensed into Ethylene Diamine Tetra acetic acid (EDTA) (0.1ml of EDTA solution to 5mls of blood sample) specimen container, mixed and analyzed immediately while the remaining blood sample was dispensed into plain specimen container. The sample in the plain container was allowed to clot, and the serum separated into a pre-labeled plain container, the serum was stored frozen at -20^{o}C and was only thawed when needed for analysis. The measured haematological parameters included: packed cell volume (PCV), haemoglobin concentration, lymphocyte count, platelet count and white blood cell count (WBC). Analysis for full blood count was carried out using the Sysmex KX-21N auto analyzer (Sysmex Corporation Kobe, Japan). CD4 cell count was estimated using the BD FASCOUNT CD3/CD4 auto analyzer (BD Biosciences, California, USA).

Detection of Antibodies to ART Drugs

Antigen-antibody agglutination method demonstrated by Petz was used (Petz, 1986). A tablet of Tenofovir Disoproxil Fumarate/ lamivudine /Efavirenz and lamivudine/ nevirapine/zidovudine were dissolved in 10 milliliters of normal saline in a test tube, the drug suspension was centrifuged. Equal volumes of group O washed cells (3%) and solution of Tenofovir Disoproxil Fumarate/lamivudine /Efavirenz and lamivudine/nevirapine/ zidovudine were placed in a test tube and then incubated at 37^{O}C for 30 minutes. The drug-red blood cell mixture was washed four times to remove excess drug. Equal volume of patient's serum and drug-red blood mixture was placed in a clean test tube and incubated at 37^{O}C for 1 hour. The mixture was washed three times, Antihuman globulin (AHG) was added and centrifuged and haemolysis or agglutination was observed for. The controls were similarly treated, ie

I. Drug red blood cell complex without patient's serum.
II. Patient's serum and group O red blood cells without drug.
III. Both results were negative.

Statistical Analysis
Results were expressed as mean±SEM and were analyzed by Student's t-test. P-values of < 0.05 were considered statistically significant and Pearson correlation coefficient was calculated to determine the association between antibodies produced and $CD4^+$ cell count.

Results
The results obtained in this study are shown in Tables 1-4. A total of 33(33%) out of the 100 HIV-sero positive subjects on ART-combination therapy had antibodies to the drugs used. The mean levels of CD4 cell count, platelets and white blood cell were significantly higher ($p<0.001$) in HIV-sero positive subjects who developed antibodies against HAART than those who did not develop antibodies. Conversely the mean level of PCV was significantly lower ($p<0.001$) in subjects who developed HAART antibodies than their counterparts who did not develop antibodies. The differences in the mean lymphocyte count and Hb values between the two groups were not statistically significant (table 1).

The comparison of the values of measured variables between HIV positive naïve, on ART and healthy HIV—negative controls are shown in table 2. The correlation of CD4 cell count with measured parameters among HIV subjects on ART and controls showed that CD4 cell count significantly correlated with lymphocyte count ($R=0.526$, $p<0.001$), PCV ($R=0.279$, $p<0.01$) and haemoglobin ($R=0.309$; $p<0.05$) in HIV-sero positive subjects on ART. Among the controls, CD4 count correlated positively with lymphocyte ($R=0.527$, $p<0.001$), PCV ($R=0.221$, $p<0.05$) and hemoglobin ($R=0.254$, $p<0.05$).

Table 1: Comparison of CD4 Cell Count and some Haematological parameters among HIV-Seropositive subjects in the absence or presence of ART antibodies

Measured variables	Mean±SEM		P-value
	HIV-sero positive subjects on ART who developed antibodies	HIV-sero positive subjects on ART who did not develop antibodies	
Number of subjects	33	67	
CD4 (cell/µl)	561.33 ± 68.77	485.96 ± 36.36	<0.001
Platelets ($x10^3$/µl)	231.04 ± 14.07	213.26 ± 9.40	<0.001
WBC ($x10^3$/µl)	5.32 ± 0.22	5.21 ± 0.28	<0.001
Lymphocyte ($x10^3$/µl)	2.26 ± 0.21	2.02 ± 0.10	>0.05
PCV (%)	29.63 ± 0.10	30.59 ± 0.53	<0.001
HB (g/L)	10.15 ± 0.37	10.56 ± 0.22	>0.05

Key: $p \leq 0.05$- Significant; $p \geq 0.05$- Not significant
WBC: White Blood Cells
PCV: Packed Cell Volume
HB: Haemoglobin

Table 2: Comparison of CD4 Cell Count and some haematological parameters of the studied population

Measured variables	Mean ± SEM			P-value
	ART naïve HIV Sero-positive	HIV Sero-positive on ART	HIV Sero-negative (controls)	
Number of subjects	100	100	100	
CD4 (cell/µl)	$355.63^{b} \pm 28.14$	$503.52^{a} \pm 32.16$	$914.23^{c} \pm 25.41$	<0.001
Platelets ($x10^3$/µl)	$257.76^{b} \pm 11.52$	$217^{a}.52 \pm 7.90$	$206.10^{a} \pm 6.79$	<0.001
WBC ($x10^3$/µl)	$5.39^{a} \pm 0.23$	$5.25^{a} \pm 0.22$	$7.43^{b} \pm 0.18$	<0.001
Lymphocyte ($x10^3$/µl)	$2.19^{a} \pm 0.10$	$2.09^{a} \pm 0.09$	$1.96^{a} \pm 0.05$	>0.05
PCV (%)	$32.21^{b} \pm 0.67$	$30.59^{a} \pm 0.49$	$33.73^{b} \pm 0.34$	<0.001
HB (g/dL)	$11.04^{a} \pm 0.23$	$10.51^{a} \pm 0.19$	$10.96^{a} \pm 0.37$	>0.05

Key: p≤0.05- Significant; p≥0.05- Not significant
WBC: White Blood Cells; PCV: Packed Cell Volume; HB: Haemoglobin
Values with different superscripts indicate significant different (ANOVA).

Table 3: Correlation of CD4 cell count with studied parameters among HIV- sero positive subjects on ART

Parameters	CD4(Cell/µl)	P-value
Platelet ($\times10^3$/µl)	-0.073	>0.05
WBC ($\times10^3$/µl)	0.108	>0.05
Lymphocytes ($\times10^3$/µl)	0.526	<0.001
PCV (%)	0.279	<0.01
HB(g/L)	0.309	<0.05

Key: p≤0.05- Significant; p≥0.05- Not significant
WBC: White Blood Cells
PCV: Packed Cell Volume
HB: Haemoglobin

Table 4: Correlation of CD4 count with studied parameters among HIV-sero negative subjects

Parameters	CD4(Cell/µl)	P-value
Platelet ($\times10^3$/µl)	0.044	>0.05
WBC ($\times10^3$/µl)	0.147	>0.05
Lymphocytes ($\times10^3$/µl)	0.527	< 0.001
PCV (%)	0.221	<0.05
HB(g/L)	0.254	<0.05

Key: p≤0.05- Significant; p≥0.05- Not significant
WBC: White Blood Cells
PCV: Packed Cell Volume
HB: Haemoglobin.

Discussion

The presence of antibodies to tenofovir disoproxil fumarate/lamivudine efavirenz and lamivudine/ nevirapine/zidovudine were detected in 33(33%) out of the 100 HIV-sero positive subjects on ART combination therapy. The implication of this finding is that treatment of HIV- sero positive subjects with tenofovir disoproxil fumarate/lamivudine Efavirenz and lamivudine/nevirapine/zidovudine combinations has brought about a decrease in the occurrence of ART- antibody development among subjects when compared with previous finding (Omoregie *et al*., 2008) which reported that a total of 81(81%) out of the 100 HIV- sero positive subjects on ART developed antibodies to ART monotherapy. Studies have shown that the use of antiretroviral drugs may cause anaemia by producing antibodies against the drugs or their metabolites. The mechanism could be the attachment of the drugs or their metabolites to red blood cells surface which then results in antibody production against the antiretroviral drugs. The antibody will in turn attach to the red cell membrane. These sensitized red cells either activates complement and are lysed or are damaged by the reticulo-endothelial system (Petz, 1986). This may had resulted in the significantly lower level of PCV between HIV-sero positive subjects on ART who developed antibodies compared to their counterparts who did not develop antibodies.

In this study, we observed a significantly lower level of PCV in subjects on HAART than HAART naïve and control. This observation is at variance with previous study (Odunukwe *et al*., 2005) that reported that HAART improved haematocrit values. Our finding is however in agreement with a previous report which reported a significantly higher PCV in the control group compared to subjects on HAART and HAART (Omoregie *et al*., 2008). The causes of anaemia infection are many and include bone marrow suppression by HIV and HAART as haemoglobin synthesis was reported to be inhibited by Zidovudine especially in Africans. Inadequate nutrition could be a major factor in HIV infection but was not considered in this study.

The reasons for significantly higher levels of CD4 cell count, platelets and WBC in HIV positive subjects who developed antibodies compared with those who did not develop antibodies is not clear. The severity and progression of HIV infection could also influence anaemia. The CD4 cell count which is a measure of HIV infection progression correlated positively with PCV ($r=0.279$; $p<0.01$) and Hb ($r=0.309$; $p<0.05$).

Thrombocytopenia was reported among HIV subjects. Immune thrombocytopenic purpura has been recognized as a diagnostic criterion of AIDS related complex (Cooper and Merigan, 1996). Studies have shown that megakaryocytes may be target for HIV infection as well as reservoir for the virus in the bone marrow, suggesting a potential mechanism for defective thrombocytopoiesis (Nubila *et al*., 2012). In this

study there was a significant decrease ($p<0.001$) in platelet count among the HIV-sero positive subjects. Furthermore, CD4 cell count significantly correlated with lymphocyte count ($r=0.526$, $p<0.001$) in HIV-sero positive subjects on ART group. This finding also agrees with previous study (Idowu *et al*., 2013).

Conclusion

The findings from this study showed that antibody to antiretroviral drugs (tenofovir disoproxil fumarate/lamivudine Efavirenz + lamivudine/nevirapine/zidovudine combination) was detected in 33(33%) of the 100 HIV-sero positive subjects on ART. The number of subjects who developed antibody to combination ART therapy was significantly lower than previously reported.

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