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Assessment of CD8, CD4/CD8 Ratio, Serum Perforin and Granzyme-B Levels in Chronic HIV Infection

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Abstract

The immune system is a sophisticated network of chemicals and cells that protects the organism's integrity by getting rid of anything that is deemed harmful. The importance of CD8+ T-cells in the suppression of HIV infection has long been recognized. The research was a descriptive study to determine the mean values of CD4, CD8, CD4/CD8 ratio, perforin and granzyme B among HIV patients

on HAART in part of Southern region of Nigeria, using HIV seropositive patients visiting clinic at Irrua Specialist Teaching Hospital and Central Hospital, Uromi Edo State. Information on the HAART status and clinical state of the study participants were obtained from their medical records. A total of 88 participants comprising of 58 HIV sero-positive patients on HAART (test) and 30 apparently healthy participants (control) were recruited for this study. The seropositive patients were further classified into stage I (30) and stage II (28). The HIV positive participants were patients with chronic HIV infection who have been on HAART for a period ranging from 1 year to over 10 years. All participants have had one or two set of ART drug combination; Zidovudine + Lamivudine + Nevirapine and/or Lamivudine + Efavirenz + Tenofovir. The CDC staging method with CD4 count was used to classify the study participants (test group) into stages I and II; stage I (CD4 count ≥ 500 cells/µl), stage II (CD4 count 200 to 500 cells/µl). Stage I participants had significantly ($p = 0.000$) higher mean CD4 values (880.36 ± 334.60 cells/µl) than stage II subjects (400.09 ± 68.03 cells/µl), similarly the mean CD4 values for stage II was significantly ($p = 0.000$) lower than the control group (965.10 ± 129.41 cells/µl). However, there was no significant difference ($p = 1.000$) between mean CD4 values for stage I and control group. The results from the mean CD4/CD8 ratios revealed that stage I had a significant ($p=0.02$) higher ratio than stage II. Also, stage II patients showed significantly ($p<0.05$) lower ratio than the control group. However, there was non-significant difference between stage I group and the control group. There was no significant difference ($p = 1.000$) in serum PEF values between stages I (163.75 ± 23.93 pg/ml) and II (167.21 ± 18.12 pg/ml). However, the sero-positive groups showed significantly ($p < 0.05$) higher PEF values than the control group (137.01 ± 36.71 pg/ml). Granzyme B followed the same pattern with perforin. In conclusion, the result of the mean CD4/CD8 ratio in stage II subjects may reveal possible significant immunological failure and a higher risk of non-AIDS related complications for that group of patients.

Keywords: CD8; CD4; Perforin; Granzyme-B; HIV

Introduction

The immune system is a sophisticated network of chemicals and cells that protects the organism's integrity by getting rid of anything that is deemed harmful. Cellular immunity and particularly cytotoxic T lymphocytes (CTLs) are the main actors in immune response to HIV infection. The importance of CD8+ T-cells in the suppression of HIV infection has long been recognized [1-4]. Because circulating CD8+ T-cells have a high capacity for cytotoxicity, they depend on the expression of CD107a, a common marker of degranulation ability, and the lytic granule contents, specifically granzymes and perforin [5]. Cytotoxic T lymphocytes eliminate virally infected target cells mostly by the exocytosis of cytotoxic granules containing granzymes and perforin. Studies have indicated that genetic mutations or deletions in perforin result in reduced cellular cytotoxicity and severe immunodeficiency [6]. Highly active antiretroviral therapy (HAART) has been demonstrated to be effective in preventing AIDS-related death in people living with HIV since its introduction [7]. However, Kroeze et al. [8] reported that despite HAART-induced viral suppression, only partial immune reconstitution has been seen. Whereas antiretroviral medication comes with side effect and the predominant adverse effect include; prolonged immunological activation and inflammation due to co-infections or residual virus replication [9,10]. HIV-positive individuals have higher levels of immune activation and inflammation, which has an impact on CD8+ T-cells. These changes include: (i) consistently high absolute counts [11]; (ii) an increase in the total memory cell pool with a skewed differentiation [12]; (iii) high expression of the exhaustion marker Programmed Death (PD)-1 and the activation markers HLA-DR and CD38 [13]; and (iv) low cytotoxic ability [3,14]. According to Perdomo-Celis et al. [15]; Tanko et al. [16], HAART can partially restore some of these defects, like the persistent increase in their absolute counts, activation state, and maturation status. During chronic HIV infection, HIV-specific CD8+ T-cell pool exhibit reduced differentiation, decreased functionality, enhanced exhaustion, and little-to-no expression of perforin [4,17,18]. According to some research, one of the factors leading to the progression of HIV infection is the decrease of HIV-specific CD8+ T cell cytolytic capability during chronic infection [17]. Moreover, persistent CD8+ T cell activation in the lack of potent antiviral action may exacerbate the illness and ultimately boost viral replication [19]. Many patients experience expansion of CD8+ T-cell compartment during ART due to bystander activation and continuous residual viral replication, which prevents the CD4/CD8 ratio from normalizing [20,21]. A low CD4/CD8 ratio is indicative of immunological failure, and patients with this ratio also have a greater risk of non-AIDS morbidity and death [22]. Monitoring the CD4, CD8, CD4/CD8 ratio, perforin, and granzyme-B may potentially assist in preventing unfavorable disease outcomes.

Materials and Methods

Research Design and Study Area

The research was a descriptive study to determine the mean values of CD4, CD8, CD4/CD8 ratio, perforin and granzyme B among HIV patients on HAART in part of Southern region of Nigeria, using HIV seropositive patients visiting clinic at Irrua

Specialist Teaching Hospital and Central Hospital, Uromi Edo State. Information on the HAART status and clinical state of the study participants were obtained from their medical records. This study was carried out in Esan Central and Esan North-East LGA in Edo State, Nigeria. This area is located between latitude 6º 10' and 6º 45' north of the equator and between longitudes 6º 10' and 6º 30' east of the Greenwich Meridian [23].

Study Population

A total of 88 participants comprising of 58 HIV sero-positive patients on HAART (test) and 30 apparently healthy participants (control) were recruited for this study. The seropositive patients were further classified into stage I (30) and stage II (28). The HIV positive participants were patients with chronic HIV infection who have been on HAART for a period ranging from 1 year to over 10 years. All participants have had one or two set of ART drug combination; Zidovudine + Lamivudine + Nevirapine and/or Lamivudine + Efavirenz + Tenofovir. The CDC staging method with CD4 count was used to classify the study participants (test group) into stages I and II; stage I (CD4 count ≥ 500 cells/µl), stage II (CD4 count 200 to 500 cells/µl) [24,25]. There was limited number of stage III participants (CD4 count ≤ 200 cells/µl), hence were excluded from the study.

Sample Collection

Ten milliliters (10mls) of venous blood were collected from each patient and dispensed into plain container (5ml) and EDTA container (5ml). The samples in plain container were centrifuged and serum obtained for the determination of Perforin and Granzyme B. The EDTA samples were used for the estimation of CD4+ T cells and CD8+ T cells.

Determination of Serum Perforin

Serum human perforin was estimated using human PF; PFP ELISA Kit (Melsin Medical Co., Limited).

Principle: The assay uses purified human perforin (PF; PFP) antibody to coat microtitre plate wells, make solid-phase antibody, then add PF; PFP to the wells. Combined antibody which with HRP labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add TMB substrate solution. TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of PF; PFP in the samples is then determined by comparing the O. D. of the samples to the standard curve.

Determination of Serum Granzyme B

Serum Granzyme B was estimated using human Gzms-B ELISA Kit (Melsin Medical Co., Limited).

Principle: The assay uses purified human Gzms-B antibody to coat microtitre plate wells, make solid-phase antibody, then add Gzms-B to the wells. Combined antibody which with HRP labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add TMB substrate solution. TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of Gzms-B in the samples is then determined by comparing the O. D. of the samples to the standard curve.

Determination of CD4 and CD8 level

The CD4/CD8 count was determined using the BD FACSCount™ System [26].

Principle: The BD FACSCount™ System work based on the principle of a direct two-colour immunofluorescence method. When whole blood is added to the tubes of a sample reagent pair, the fluorochrome-labelled antibodies bind specifically to antigens on the surface of lymphocytes (CD4 or CD8). The FACSCount instrument detects two colours and measures relative cell size. The CD3 cells will fluoresce red and the CD4 and CD8 cells will fluoresce yellow when analysed on the FACSCount instrument. A known number of reference beads is contained in each reagent tube and functions as fluorescence and quantitation standard for calculating the absolute counts for the CD4+, CD8+, and CD3+ T lymphocytes. Fixative solution is added to the stained samples prior to analysis to preserve the integrity of the antibody binding. No lysing is necessary

Statistical analysis

The results were presented as mean ± standard deviation. The differences in mean values across the groups were analyzed using one-way ANOVA. SPSS statistical package version 22 was used in data analysis. Significant levels were considered at $p < 0.05$.

Results

Table 1 shows the socio-demographic characteristics of the study population. The participants comprised of 58 HIV sero-positive and 30 HIV sero-negative (control) participants. The HIV participants were classified according to the Centre for Disease Control C4 staging pattern, into stage I and II with 30 and 28 study participants respectively. The age range of the participants was 20 years and above, with majority between 41 to 60 years for the sero-positive groups and 20-40 years for the control group. Stage I had 7 (23.3%) male and 23 (76.7%) female, stage II had 6 (21.4%) male and 22 (78.6%) female, while control group had 10

(33.3%) male and 20 (66.7%) female. The participants were mostly those with chronic HIV infection who have been on ART for a period ranging from 1 year to over 10 years. All participants have had one or two sets of ART drug combination; Zidovudine + Lamivudine + Nevirapine and/or Lamivudine + Efavirenz + Tenofovir. Table 2 shows the level of CD4, CD8, CD4/CD8, PEF and GRZM in the HIV positive and control groups (mean ± SD). Stage I participants had significantly ($p = 0.000$) higher mean CD4 values (880.36 ± 334.60 cells/µl) than stage II subjects (400.09 ± 68.03 cells/µl), similarly the mean CD4 values for stage II was significantly ($p = 0.000$) lower than the control group (965.10 ± 129.41 cells/µl). However, there was no significant difference ($p = 1.000$) between mean CD4 values for stage I and control group. The table shows that there was no overall significant difference ($p = 0.530$) in the mean values of CD8 cells count across the groups, even though stage II participants recorded the least values (685.09 ± 366.39 cells/µl) while stage I participants had the highest values (817.77 ± 349.90 cells/µl). Stage I participants showed significantly ($p = 0.020$) higher CD4/CD8 ratio (1.26 ± 0.63) than the stage II (0.73 ± 0.36) patients whereas when compared to the control (1.26 ± 0.27) group, participants in both HIV stages showed no significant difference ($p > 0.05$). There was no significant difference ($p = 1.000$) in serum PEF values between stages I (163.75 ± 23.93 pg/ml) and II (167.21 ± 18.12 pg/ml). However, the sero-positive groups showed significantly ($p < 0.05$) higher PEF values than the control group (137.01 ± 36.71 pg/ml). Granzyme B followed the same pattern with perforin.

Discussion

The present work was designed to determine the mean values of CD4, CD8, CD4/CD8 ratio, perforin and granzyme B among HIV patients on HAART in parts of Edo state. The mean CD4 levels across the three groups showed that stage I had a considerably ($p=0.000$) higher count than stage II. Comparing stage II to the control group, there was a substantial ($p=0.000$) decrease in count. This finding could be the consequence of an increased viral load and T-helper cell depletion that occurs when HIV illness progresses (from Stage I to II) [27,28]. The decrease in the quantity of CD4+ T cells appears to be caused by the effects of prolonged immunological activation as well as the immune system's progressive inability to produce new T cells in chronic infection [29]. There was however non-significant ($p>0.05$) difference between mean CD4 of stage I and control group. This outcome aligns with the findings of Gray et al. [30], which suggests that antiretroviral therapy can decelerate the progression of the illness and perhaps result in a life expectancy approaching normal [9].

***Table 1**: Socio-Demographic Characteristics of the Study Population.*

Variables		**CDC Stage I n(%)**	**CDC Stage II n(%)**	**Control n(%)**
Age (years)	20-40	13(43.3)	7(25.0)	24(80)
	41-60	16(53.3)	17(60.7)	6(20)
	≥61	1(3.3)	4(14.3)	0(0)
	Total	**30(100)**	**28(100)**	**30(100)**
Gender	Male	7(23.3)	6(21.4)	10(33.3)
	Female	23(76.7)	22(78.6)	20(66.7)
	Total	**30(100)**	**28(100)**	**30(100)**
ART combination	(Zidovudine Lamivudine Nevirapine)	Yes	Yes	No
	(Lamivudine Efavirenz Tenofovir)	Yes	Yes	No
Keys: CDC = Centre for Disease Control; ART = Antiretroviral Therapy; M = Mean; SD = Standard Deviation; Yrs = Years, Yes = Subjects have had the ART combination; No = Subjects have not had the ART combination				

***Table 2**: CD4, CD8, CD4/CD8, PEF and GRZM values of the study groups (mean ± SD).*

Group(n)	**CD4 (cells/µl)**	**CD8 (cells/µl)**	**CD4/CD8**	**PEF(pg/ml)**	**GRZM(pg/ml)**
Stage I(30) (A)	880.36±334.60	817.77±349.90	1.26±0.63	163.75±23.93	1.92±0.38

Stage II(28) (B)	400.09±68.03	685.09±366.39	0.73±0.36	167.21±18.12	1.90±0.20
Control(30) (C)	965.10±129.41	782.10±109.56	1.26±0.27	137.01±36.71	1.29±0.19
f- value	16.888	0.645	4.553	4.415	16.174
p-value	<0.0001*	0.530	0.017*	0.019*	<0.0001*
A vs B	<0.0001*	0.792	0.020*	1.000	1.000
A vs C	1.000	1.000	1.000	0.032*	<0.0001*
B vs C	<0.0001*	1.000	0.048*	0.035*	<0.0001*

The clinical management of HIV infection has been led during the past thirty years by CD4 count monitoring. These cell counts have been utilized in clinical settings to guide diagnostic investigations, identify whether to start antiretroviral medication, and determine whether to treat opportunistic infections prophylactically. The mean CD8 values for stage I patients was not significantly ($p>0.05$) different from stage II patients. Similarly, the mean CD8 for HIV sero-positive groups showed non-significant difference with the control group. However, it is expected that stage II participants should have a higher CD8 count than stage I and control subjects [31] due to their supposed enhanced immunological activation [32]. The result from this study, however, might be connected to possible immune exhaustion coming from protracted immunological activation [33]. According to Younas et al. [35], HIV-positive patients in a chronic state gradually lose immune function, including CD8+ T-cell exhaustion and immune function loss in lymph nodes and mucosal tissues. These immune system dysfunctions increase the patient's vulnerability to opportunistic infections and cancer. The results from the mean CD4/CD8 ratios revealed that stage I had a significant ($p=0.02$) higher ratio than stage II. Similarly, stage II patients showed significantly ($p<0.05$) lower ratio than the control group. However, there was non-significant difference between stage I group and the control group. According to Parekh et al. [29], stage II HIV infection is linked to a more severe form of the disease than stage I, and the low ratio in stage II may be explained by persistent immunological activation and a gradual decrease in CD4+ T cell counts. The CD4/CD8 ratio measurement is crucial in identifying a subset of patients with significant immunological failure and a higher risk of non-AIDS related pathology. These individuals may benefit from more active care of risk factors for age-related disorders and more thorough screening for conditions unrelated to AIDS [15,36]. Statistical data from this study showed that the mean serum perforin and granzyme-B for stage I patients did not differ substantially ($p>0.05$) from stage II group. By comparison, the sero-positive groups had significantly ($p<0.05$) higher mean values of serum perforin and granzyme-B than the control group. The substantial increase in these cytolytic molecules for the sero-positive cohort could be linked to the extensive immunological expansion and activation due to HIV infection. According to Baral et al. [31], the CD8+ T-cell pool in HIV infection is highly activated and primed for significant cytotoxic effector activity, but this capacity decreases in the chronic phase of infection. These granule-bound cytolytic chemicals (perforin and granzyme) are secreted by CD8+ T lymphocytes, which subsequently destroy target cells [37]. Perforin creates holes in the cell membrane and facilitating the release of granzyme [6]. Granzyme activates caspase cascade, which eventually induce apoptosis [38].

Conclusion

In conclusion, the result of the mean CD4/CD8 ratio in stage II subjects may reveal possible significant immunological failure and a higher risk of non-AIDS related complications for that group of patients. These individuals may benefit from more active care of risk factors for HIV complication and more thorough screening for non-AIDS pathology and possible secondary infection. The higher mean values of perforin and granzyme in the sero-positive groups than the control group may reflect the vital role of CD8 T-cells' cytolytic function in eliminating the viral pathogen during HIV infection.

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Disclosure of Conflict of Interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Statement of ethical approval

Ethical approval was obtained from the ethics and research committee of Ambrose Alli University, Ekpoma (NHREC/12/06/2013), and informed consent of the patients was obtained before sample collection.

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Authors' Contribution

The entire study procedure was conducted with the involvement of all writers.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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