
International Journal of Advanced Multidisciplinary Research (IJAMR)

ISSN: 2393-8870

www.ijarm.com

Research Article

Comparative study of leukopoietin levels in human immunodeficiency virus infection in Umuahia, Nigeria

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Abstract

This study was carried out to determine the comparative study of leukopoietin levels in human immunodeficiency virus infection in Umuahia, Nigeria. A total number of ninety (90) subjects within the age of 18-60 years were used for the study. The subjects were divided into three (3) groups of thirty (30) subjects in each group. Group I comprised of control subjects. Group II were HIV- subjects not on therapy, and group III were AIDS- subjects not on therapy. Blood samples were collected into commercially prepared dipotassium EDTA vacutainer for both test and control subjects after informed consent. These were used in determining their Leukopoietin levels. After the analyses of the blood samples, the following results were observed. The level of leukopoietin of group II (1.9 ± 0.9) was slightly higher than that of group I (1.2 ± 0.9) but not statistically significant at $P < 0.05$. The Leukopoietin of group III (5.4 ± 1.5) was statistically higher when compared with those of groups I (1.2 ± 0.9) and II (1.9 ± 0.9) respectively.

Keywords: Comparative study, Leukopoietin level, Human Immunodeficiency Virus.

Introduction

Human immunodeficiency virus (HIV) is a lentivirus (slowly replicating retrovirus) that causes acquired immunodeficiency syndrome (AIDS) (Weiss, 1993; Douek *et al.*, 2009) in humans, with progressive failure of the immune system allowing life-threatening opportunistic infections and cancers to attack the body. Infection occurs by body fluid contact like blood, semen, vaginal fluid, pre-ejaculate, and breast milk. Within these bodily fluid, HIV is present as both free virus particles and virus within infected immune cells. The four main routes of transmission are unsafe sex, contaminated needles, breast milk, and perinatal transmission. The primary means of HIV sexual transmission is through vaginal or anal

intercourse, but it can also be transmitted through oral sex (Williams and Steven, 2007).

HIV infection is characterised and associated with destruction of a subset of lymphocytes known as CD4 cells that are responsible for maintaining the immunity of the body. This situation results to a fall in the ability of the immune system to prevent diseases leading to life-threatening opportunistic infections through a number of mechanisms including: apoptosis of uninfected bystander cells (Garg *et al.*, 2012), direct viral killing of infected cells, and killing of infected CD4+ T cells by CD8 cytotoxic lymphocytes that recognize infected cells (Kumar, 2012).

This immune system plays a central role not only in health maintenance but also in pathogenesis. It is a system of biological structures and processes within an organism that protects against disease. For it to perform its functions, it must ensure continuous reception and processing of information about the antigenic state of the organism. This perception must allow an evaluation of how serious is any divergence from the norm whose notion varies during the development of the organism and between different organisms (Igor *et al.*, 2010). Surveillance of the human body is accomplished in part by means of patrolling white blood cells or leucocytes (Darius *et al.*, 2003). There are two major arms of the immune system, one which works through antibodies, which are produced by B-cells and plasma cells, and the other that works through direct cellular action and which relies heavily on CD4+ T-cells. The first is called antibody-mediated or humoral immunity, and the second is called cell-mediated immunity. It is the cell-mediated arm of the immune system that is found to be profoundly suppressed in people diagnosed with AIDS. The antibody-mediated arm of the immune system, however, is usually hyper stimulated in the early stages, with "increasing levels of humoral antibodies and plasma cells". The fact that antibody levels are increased is what allows the HIV antibody screening tests to use serum that has been diluted 400 times, unlike other antibody tests that usually use straight, undiluted serum. In these early stages the lymph nodes may grow in size and be chronically enlarged. In late stages, however, both the cell-mediated and antibody-mediated arms of the immune system begin to fail and lymph node atrophy results (Irwin, 2001). The CD4/CD8 ratio is considered a marker of disease progression in HIV and AIDS, and is often found to be inverted meaning that there are less CD4 cells than CD8 cells, resulting in a ratio of less than 1. CD8 cells are often increased, especially in less advanced stages of AIDS, and this combination of lowered CD4 counts and increased CD8 counts are commonly thought to occur only in people diagnosed HIV-positive. Another finding that is common in people diagnosed HIV-positive is reduced lymphocyte activity and function, as measured by their responsiveness to foreign antigens. This can result in a state of "anergy", where people's skin fails to respond when antigens are injected under it (Irwin, 2001).

CD8 T cells are powerful components of the adaptive immune system, yet were not formally recognized

until long after the discovery of antibody. CD8 T cells contribute to the eradication of intracellular infections and to the control of many chronic infections (Masopust *et al.*, 2007). The CD8 (cluster of differentiation 8) which is a transmembrane glycoprotein that serves as co-receptor for the T-cell receptor binds specifically to class I major histocompatibility complex (MHC) molecule. Its responses in HIV infection can be divided into (1) the lytic response (Cytotoxic T Lymphocytes, CTLs) which make use of proteins in their cytoplasm such as perforin and granzymes for cell lysis (direct killing response) and (2) Non-lytic responses (chemokines) which are soluble substances secreted by CD8 cells e.g. cytokines that work by either inhibiting HIV replication or inhibiting viral entry into target cells changing the viral set point. During many infections, all T lymphocytes regardless of specificity may undergo cytokine-driven phenotypic changes –so called bystander activation, but only those T-cells that recognize pathogen-encoded antigen go through multiple rounds of replication to generate enormous numbers of CTL (Cytotoxic T Lymphocytes) effector progeny that are the foot soldiers of the adaptive immune response (Nu and Michael, 2011).

Leukopoietin on the other hand, is a hypothetical substance presumed to be humoral means of regulating leucopoiesis. The discovery of erythropoietin, a humoral substance regulating production of erythrocytes has raised the question whether humoral factors for control of leucocytes exist. Probably, there are many leukopoietic stimulants specific for granulocytes, lymphocytes, monocytes etc, possibly even for neutrophilic, eosinophilic, or basophilic granulocytes. Almost certainly these stimulating substances are interrelated not only among themselves, but also with erythropoietins and thrombopoietins to maintain a well balanced hematopoietic population (Bierman, 2006). According to Kakhelidze and Dolgina, (1972) these endogenous substances capable of inducing leucocytosis or of stimulating leucopoiesis have been given many names depending on the conditions and method used for their detection. Also options differ regarding the site of formation of these substances. These leukopoietins are glycoproteins and of a molecular size that can readily traverse capillary and cell membrane en route to the target site based on the prompt initiation and promotion of leucopoiesis in the marrow by circulating stimulant. Examples include Granulocyte

Colony Stimulating Factor (G-CSF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Colony -Stimulating Factor-1(CSF-1), CFU-preB etc. Although it is plausible that these growth factors may play important roles in activation of granulocytes and non granulocytes as well as supporting final maturation stages at locations of inflammation. There is no evidence at present that production of granulocytes or non- granulocytes is regulated systemically through a mechanism that senses the mature cell numbers in the body and causes elaboration of a factor that works in the marrow.

Aim

To comparatively study leukopoietin levels in HIV /AIDS subjects and apparently healthy subjects.

Materials and methods

Study Area

The study was conducted at the HIV/AIDS clinic of Federal Medical Centre (F.M.C.) Umuahia which is located at the heart of Abia State capital territory. Umuahia covers a land mass of 245km², with Latitude of 5.5267 (decimal degree) North and Longitude 7.48959 (decimal degree) East with a population of 264,662 (Mongobay, 2012).

Advocacy, Pre-Survey Contacts and Ethical Considerations:

With a well detailed research proposal and a letter of Introduction from the department, the head, Health Research and Ethics Committee of the Institution was met. After their meeting and thorough perusal of the protocols of the research, an ethical approval was given for the study.

Study Population and Enrolment:

A total number of Ninety (90) subjects were used which was calculated based on the prevalence rate of HIV infected subjects according to Araoye, (2004). These subjects with the age of 18-60 years were grouped into three (3) of thirty (30) each. Group I was made up of control subjects while groups II and III were made up of HIV subjects and AIDS subjects respectively. The HIV subjects were selected from donors that came to donate blood at the hospital and

those that came for premarital counselling and testing after testing only positive to HIV screening. The AIDS patients were selected from ART clinic at F.M.C. Umuahia. The control subjects were selected from donors who were fit and from voluntary workers at the laboratory department F.M.C Umuahia.

Selection Criteria

Both oral and written consent were obtained from the subject who also accented to the sample collection. The group I (controls) were selected on the basis that they were apparently healthy and showed no signs and symptoms of any viral (hepatitis B and C), systemic or cardiovascular diseases from the pre donating screening done on them such as clerking. HIV test, Hepatitis test, etc.

The group II (HIV-patients) were selected having being confirmed of having HIV infection by the standard technique and not reactive to any other viral infections (hepatitis B and C), no other complications associated with the HIV infection and that are not sicklers or immunocompromised from their CD4 cell count.

Group III (AIDS- Patients) were picked on the basis that they were confirmed of having AIDS and showed all AIDS indicator conditions (but no history of tuberculosis or reactive to any other viral infection.

Exclusion Criteria:

The subjects showing any underlying signs and symptoms of diseases other than HIV and AIDS for the test subjects were excluded from the study. While the control groups reactive to any viral infections including HIV/AIDS or that are immunocompromised were excluded.

Sample Collection

About 6ml of venous blood was aseptically collected from the patients using a standard venipuncture technique. About 3ml was dispensed into a commercially prepared dipotassium EDTA Vacutainer (Beckon, Dickson and Company) while the remaining 3ml was dispensed into a dry plain plastic tube and allowed to clot. The samples were centrifuged at 3000 rpm for 10minutes to separate the plasma and the serum respectively.

The plasma was used for Leucopoietin analysis after the whole blood was used for analysis of CD8 while the serum was used for confirmation of HIV and other viral infections.

Laboratory Reagents and Kits:

All the reagents and kits were bought commercially from a reputable company as the standard operating procedures were strictly adhered to.

Leukopoietin Analysis:

The analysis was done using ELISA method.

Principle:

Analyte (sample) was adsorbed onto a solid phase which is immobilized, a liquid sample (Biotin-conjugate) was added onto the stationary solid phase with special binding properties and was followed by multiple liquid reagents (the substrate and stop solution) that were sequentially added, incubated and washed followed by some optical change in the final liquid in the well from which the quantity of the analyte was measured.

Procedure

About 50ul of sample and 50ul of sample diluents were added to the sample well. 100ul of sample diluent was added to the blank well while 100ul of standard was added to the standard well. 50ul of Biotin-conjugate was added to all the wells covered with an adhesive and incubated at room temperature for 3 hours.

After the incubation the adhesive was removed, micro wells emptied and washed 6 times with the wash buffer. The micro wells were tapped several times on an absorbent paper to remove water. The 100ul of diluted streptavidin-HRP to all the wells was added. It was covered with an adhesive and incubated at room temperature for 20minutes. After which the adhesive was removed and wells washed 6 times. Then 100ul of TMBS substrate solution was added to all the wells and incubated for 10 minutes, after which 100ul of stop solution was added to all the wells and absorbance read at 405nm spectrophotometrically and the results calculated using concentration of standard.

$$\text{Calculation: } \frac{\text{Abs of test} \times \text{conc. of STD}}{\text{Abs of STD}}$$

Where: Abs. = Absorbance
STD = Standard (0.303 iu/ml)
Conc. = Concentration (50 iu/ml).

HIV testing

The HIV status of the patients was done using both screening kits (Stat pak and Determine test kit) and confirmatory kit (Orenic Immunocomb II HIV 1&2 confirm).

HIV screening test

Chembio HIV 1/2 STAT-PAK (USA)

The chembio HIV 1/2 STAT-PAK assay is a single use, immunochromatographic test used to detect antibodies to Human Immunodeficiency Virus Type 1 (HIV-1) and Type 2 (HIV-2) in finger stick, whole blood, venous whole blood, serum and plasma specimens.

Principle of Chembio HIV 1/2 Stat-Pak test kit

This employs a unique combination of a specific antibody binding proteins, which is conjugated to colloidal gold dye particles, and HIV- 1/2 antigens, which are bound to the membrane solid phase. The sample is applied to the sample(s) well followed by the addition of running buffer. This facilitates the lateral flow of the released products and promotes the binding of antibodies to the antigens. If present, the antibodies bind to the gold conjugated antibody binding protein. In a reactive 9sample, the dye conjugated- immune complex migrates on the nitrocellulose membrane and is captured by the antigens immobilized in the test (t) area, producing a pink /purple line while in non-reactive sample, the pink/purple colour is absent but there will always be a pink/purple colour in the control region containing immunoglobulin G antigens indicating a proper performance.

Procedure:

With the sample loop provided, 5ul of the sample was taken and applied on the sample pad of the device.

Then three drops(105ul) of the running buffer were added on the sample well also. The result was then read after 10minutes.

Interpretation of test performance

When the test was completed, a pink/purple line appeared in the control (C) area of the test device on nonreactive as well as reactive samples. This control line served as an internal control and gave confirmation of sample addition and proper test performance. Pink/purple lines in both the TEST (T) and CONTROL (C) areas indicated a reactive sample. Specificity= 100%, and sensitivity= 100%

Alere Determine HIV- 1/2 Kit (JAPAN)

Alere Determine HIV- 1/2 Kit is an in vitro, visually read qualitative immunoassay for the detection of antibodies to HIV- 1 and HIV- 2 in human serum, plasma or whole blood. The test is intended as an aid to detect antibodies to hiv-1/ hiv-2 from infected individuals.

Principle of Determine test kit:

Alere Determine hiv-1/2 is an immunochromatorapic test for the qualitative detection of antibodies to hiv-1/hiv-2. As sample is added to the sample pad and allowed to migrate through the conjugate pad, it reconstitutes and mixes with the selenium- colloid-antigen conjugate. The mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptide at the patient's window site. If antibodies to HIV-1 and/or HIV -2 are present in the sample, they bind to the antigen- selenium colloid and to the antigen at the patient window forming a red line at the patient's window site, but if the antibodies to HIV-1 and HIV -2 are absent, the antigen-selenium colloid flows past the patient window, and no red line is formed at the patient window site.

Procedure:

The desired number of test units from the 10- test card was removed by bending and tearing at the perforation. The protective cover from each test was removed. 50ul of sample (serum) was added to the sample pad and allowed to flow through the solid

phase. The result was read within a minimum of 15 minutes.

Interpretation of Result

Positive result (two bars)

Red bars appeared in both the control window (labelled " control") and the patient window (labelled "patient") of the strip. Visible red colour in the patient window was interpreted as positive.

Negative result (one bar)

One red bar appeared in the control window of the strip (labelled " control") and no red bar appeared in the strip (labelled "patient").

Invalid (No bar or one bar only in patient window)

If there is no red bar in the control window of the strip and even if a red bar appears in the patient window of the strip, the result is invalid and should be repeated. Specificity=99.75% and Sensitivity=100.00%

HIV confirmatory test

The HIV confirmatory testing was done using an indirect solid phase Enzyme Immunoassay (EIA) kit (Organic Immunocomb II HIV 1&2 combfirm kit).

Principle

The ImmunoComb® II HIV 1 & 2 CombFirm test is an indirect solid-phase enzyme immunoassay (EIA). The solid phase is a card with 12 projections ("teeth"). Each card has 6 pairs of teeth, with six antigen spots per pair (3 spots on each tooth). The left tooth of each pair carries an upper spot sensitized with human immunoglobulin (Internal Control), and the two protein markers p24 (*gag*) and p31 (*pol*). The right tooth has three *env*-derived protein spots gp41, gp120 and gp36.

The Developing Plate has 6 rows (A-F), each row containing a reagent solution ready for use at a different step in the assay. The test is performed stepwise, by moving the Card from row to row, with incubation. To start the test, serum or plasma specimens are added to the diluents in the wells of row A of the Developing Plate. The Card is then inserted in

the wells of row A. Anti-HIV antibodies, if present in the specimens, will specifically bind to the HIV antigens on the teeth of the Card. Unbound components are washed away in row B. In row C, the anti-HIV IgG captured on the teeth, and the human immunoglobulin on the upper spots (Internal Control), will react with anti-human IgG antibodies labelled with alkaline phosphatase (AP). In the next two rows, unbound components are removed by washing. In row F, the bound alkaline phosphatase will react with chromogenic components. The results are visible as gray-blue spots on the surface of the teeth of the Card.

Kit Contents

Cards

The kit contains 3 plastic Cards. Each Card has 6 pairs of teeth, one pair for each test (Figure 3.1). Both teeth of a pair carry identical numbers. Each pair of teeth is sensitized with six reactive areas.

Developing Plates

The kit contains 3 Developing Plates, covered by aluminium foil. Each developing Plate contains all reagents needed for the test. The developing Plate consists of 6 rows (A–F). Row A is divided into 6 wells, whereas the other rows are divided into 12 wells each. The contents of each row are as follows:

- Row A - specimen diluent
- Row B - washing solution
- Row C - alkaline phosphatase-labeled goat anti-human IgG
Antibodies
- Row D - washing solution
- Row E - washing solution
- Row F - chromogenic substrate solution containing
5-bromo-4-chloro-3-indolyl phosphate (BCIP) and
nitro blue tetrazolium (NBT).

Statistical analysis:

The results were expressed as mean and standard deviation ($\bar{x} \pm SD$) the analysis was done using student's t-test and Pearson correlation analysis with the statistical package for social science (SPSS) version 13. The level of significance was at $P < 0.05$.

Results

The results of the Ninety subjects (90) used for the study was as tabulated below and the results obtained have been summarized under three (3) tables.

30	apparently healthy subjects	group I
30	HIV- infected adults-	group II
30	AIDS- patients	group III

The table shows that the Leukopoietin of the group II (1.9 ± 0.9) was slightly higher when compared with the control group I (1.2 ± 0.9) but not statistically significant. But the mean value of group III (5.4 ± 1.5) was statistically higher than both the group I and II.

Discussion

The mean value of leukopoietin was significantly higher in group III (AIDS patients) when compared with group I and II. Though there was a slight increase in the mean value of leukopoietin in group II (HIV patients) when compared with group I (control) but it was not statistically significant. The increased mean value of leukopoietin observed in group II could be as a result of continuous production of naïve T cells as a response to the immune cells depletion. The production of these immune cells continues to a point when there is a total breakdown of the body's immune system due to increased viral load with consequent opportunistic infections. At this stage, there is leucopoietic dysfunction possibly as a result of defects in leukopoietin production, and the cells gradually decline numerically and functionally.

This study showed increase in proliferation of T lymphocytes measured by leukopoietin concentration in HIV infection, therefore it would be of immense health benefit to include leukopoietin level determination to serve as a prognostic guide in virologic treatment and management of HIV/AIDS patients.

Conclusion

The leukopoietin levels of HIV positive patients, when compared with that of apparently healthy controls showed no statistical difference but when the leukopoietin levels of AIDS patients were compared with that of HIV positive asymptomatic patients and apparently healthy subjects, there was statistical difference in both.

Table 1 Mean values of Leukopoietin of the studied groups (I, II, and III).

Groups	Leukopoietin (iu/ml)	Sex		Age Distribution (years)		
		Male	Female	(18 -35)	(36 -45)	(46 - 60)
I	1.2 ± 0.9	20	10	20	09	01
II	1.9 ± 0.9 ^{NS}	25	05	15	10	05
III	5.4 ± 1.5**	05	25	05	15	10

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