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Assessment of Leucocytes and CD4+ T-cells in HIV Seronegative Tuberculosis Patients

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Abstract

Background: Tuberculosis is a major global health problem associated with large mortality. The burden of tuberculosis is particularly high for the African region, mainly due to the high prevalence of Human Immunodeficiency Virus infection. While several studies have focused on the immunological responses of human host to HIV seropositive tuberculosis infection, this study aims to determine the immunological responses (CD4+ and leucocyte cell counts) of human host to HIV seronegative tuberculosis infection. Methods: The study used a cross-sectional study design for population that consisted of 100 study subjects who presented with HIV seronegative tuberculosis infection at diagnosis as well as 40 apparently healthy volunteers who were HIV and tuberculosis negative as control in Central Hospital, Benin City. Results: The result indicated a statistically significant CD4+ lymphocytopaenia, leucocytosis, neutrophilia and monocytosis. Lymphocyte count was not statistically significant despite lymphocytopaenia observed in 28% of the study subjects. It was observed that 25 (96.2%) of leucocytosis, 19 (100%) of neutrophilia, 26 (92.9%) of lymphocytopaenia and 26 (92.9%) of monocytosis were patients having CD4+ lymphocytopaenia. The feminine gender had the highest prevalence rate of CD4+ lymphocytopaenia, leucocytosis, neutrophilia, lymphocytopaenia and monocytosis. Furthermore, disease severity, age and gender seemed to play important role in determining the cellular immunity of tuberculosis patients. Conclusion: CD4+ lymphocytopaenia, leucocytosis, neutrophilia and monocytosis were statistically significant in the study. Interestingly, females appear to be more prone to having CD4+ lymphocytopaenia, leucocytosis, neutrophilia, lymphocytopaenia and monocytosis while these conditions could be occurring in ascending order of age groups.

Keywords: Tuberculosis, HIV Seronegative, Leucocytes, CD4+ T-Cells

1. Introduction

1.1 Background

It is a well-known fact that tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), has become a serious global health menace which is associated with large number of deaths, usually more than that from any other single infectious disease and has remained one of the top ten causes of death globally [1-2]. Mtb is an obligate aerobic, intracellular pathogen, which has a penchant for the lung tissue rich in oxygen supply. The tubercle bacilli gain access into the body via the respiratory route and spread from the site of initial infection in the lungs through the lymphatics or blood to other parts of the body, the apex of the lungs and the regional lymph nodes being preferred sites [3].

An estimated 10.4 million people fell ill with TB in 2016 with about 1.3 million and 374,000 TB deaths occurring among HIV negative and positive people respectively. Africa and South-East Asia accounted for 82% of the deaths among HIV negative people and 85% in both HIV negative and positive people [2]. WHO report classified Nigeria as one of the 22 high TB-burden countries (HBCs) and the 22 HBCs accounted for over 80% of the world's TB cases. According to the report, Nigeria has a mortality rate of 94 among HIV negative and 49 among HIV positive cases, prevalence rate of 326 and incidence rate of 338 cases per 100,000 population in 2013 [4]. While in 2017, WHO had classified Nigeria as one of the 14 high burden countries for combined TB, TB/HIV and MDRTB [2].

It has been reported that most humans who get infected with Mtb do not exhibit progression to the disease and that about one-third of exposed HIV negative individuals become infected, and of this number 3 to 5% develop TB in the first year, while an additional 3 to 5% of those infected develop TB later in their lives, it is thought that most adult TB in non-HIV infected patients is caused by reactivation of pre-existing infection [5-6]. It is documented that HIV positive persons infected with Mtb have a 50% chance of developing reactivation (post-primary) TB at some point in their lives, these individuals and others who are immune-suppressed can also be newly infected with Mtb and in many cases show rapid progression to active disease [5-6]. Adult TB, whether resulting from activation or new infection in HIV infected patients, is almost always pulmonary and is associated with differing degrees of lung involvement and damage [1].

On the immunological responses to Mtb invasion, cell-mediated immunity have been implicated to play active role in the body's defense mechanism against Mtb. The cells involved are lymphocytes (CD4+, CD8+ and γ δ T lymphocytes), neutrophils, monocytes/macrophages, natural killer (NK) cells, dendritic cells and B cells [7-8]. Infection with Mtb is thought to induce distinct antigen-specific CD4+ T-cells endowed with effector functions [9], while monocytes and lymphocytes have been implicated in the induction of immune responses and their levels in peripheral blood is thought to reflect the state of an individual's immunity to infection. Monocytes are an essential component of the innate immune response that acts as a link to the adaptive immune system through antigen presentation to lymphocytes [10]. Studies have demonstrated the functions of neutrophils to include contribution to the generation of effector T-cells, participation in the formation of granuloma, and also play active roles in tissue necrosis, destruction, and infection dissemination [11]. Various haematological disorders have been reported in TB to include anaemia, lymphocytopaenia, neutrophilia, monocytosis, monocytopaenia, thrombocytopenia and thrombocytosis [12].

Several studies have focused on the immunological responses of human host to HIV seropositive tuberculosis infection, this present study aims to determine the immunological response of human host to HIV seronegative tuberculosis infection. Specifically, the study was designed to determine the CD4+ counts, total leucocyte counts (TLC), neutrophil counts (NC), lymphocyte counts (LC) and monocyte counts (MC) in HIV seronegative TB patients attending Central Hospital, Benin City.

1.2 Rationale for Study

TB infections have remained a major public health challenge all over the world because of a large potential reservoir of infection, lack of an effective vaccine to prevent the infection or disease and a long chemotherapeutic regime with multiple chemotherapeutic agents that are difficult to maintain, with significant morbidity and mortality especially with HIV co-infection, multidrug resistant and extensive drug resistant TB infections. There seems to be dearth of data on HIV seronegative tuberculosis infection among victims in Benin City as focus is on HIV co-infected tuberculosis disease. Thus, it is imperative that research focuses on understanding the immune response to this infection. Hence, we were interested in finding out the host immune response to tuberculosis infection in HIV seronegative patients, especially the responses of CD4+ and leucocytes (total and differential) cell counts.

1.3 Scope of Study

This research was restricted to HIV seronegative TB positive (Microscopy and/or GeneXpert) patients at Central Hospital TB Laboratory in Benin City (samples were collected from these patients at the point of diagnosis before the commencement of anti-tubercular therapy-ATT). This was due to time, resources and manpower available for the study. However, the demographic restriction of Central Hospital TB laboratory within Benin City may generate data which may be a true reflection of disease profile in Benin City as well as Edo State and beyond.

2. Materials and Methods

2.1 Ethical considerations

The Ethics Review Committee of Central Hospital, Benin City reviewed and approved the study protocol (HA.577/Vol.11/76). In addition, information sheet about the study was given or explained to each study participant to have a full understanding of the research as well as what is expected of them. In the scenario where a potential study participant was not literate, the information sheet was explained in simple English, Pidgin English or local dialect (Bini). Thereafter, consent (written or verbal) was sought from study participants.

2.2 Study location and Population

The research study was conducted among patients attending Central Hospital, Benin City, Edo State, Nigeria. The study population consisted of one hundred and forty subjects (140 participants).

2.3 Sample collection and processing

Six milliliter (6ml) of blood sample was aseptically drawn from each of the participants by veni-puncture; 4ml of the blood sample was dispensed into Ethylene diamine tetra-acetic acid (EDTA) anticoagulated container and mixed properly to avoid clotting and the remaining 2ml was dispensed into plain container and allowed to clot. Serum was separated from the clotted blood sample in the plain container and stored at 4^{o}C which was then used for HIV screening. The stored serum sample prior to use was allowed to attain room temperature.

CD4+ count: CD4+ count was determined by the Partec flow-cytometric method using an EDTA whole blood sample. The instrument uses fluorescent-labelled anti-CD4 monoclonal antibodies to capture CD4+ T-cells from whole blood and allows automated CD4+ counting.

Twenty Microliter (20µl) of CD4+ monoclonal antibody was introduced into Rohren test tubes and 20µl of well mixed whole blood of study subjects was added, this was mixed and incubated in the dark for 15 minutes at room temperature. Thereafter, 800µl of CD4+ buffer was added, mixed and read on the Cyflow. Partec™ Flow Cytometers (CyFlow® and PAS®) is a compact flow cytometer for diverse flow cytometry analysis whereby cells in suspension is differentiated and counted according to the cell size and internal structure.

Total/differential leucocyte count: Full blood count (complete blood count) was determined in EDTA blood containers using Sysmex kx-21N haematology auto-analyzer instrument. The equipment was switched on and set to the whole blood mode, sample information was appropriately imputed into the machine while the blood sample was mixed sufficiently in the EDTA container. The tube was then set to the sample probe and in that condition the start switch was pressed and the

system executes automatic analysis and displays the result on the LCD screen. A printed copy of the result was obtained at the end of the analysis from which total and differential leucocyte cell counts were extracted from the parameters.

The instrument analyzes whole blood samples by flow cytometry using an argon laser as the light source, whole blood samples are stained with a fluorescent dye and passed through laser beam in the sheathed flow cell. The fluorescently labeled cells were irradiated using an argon laser beam, the forward scatter and the side fluorescence emitted from each cell in the sheath flow were detected while the former (forward scatter) was used as an indicator of relative cell size, the later (side fluorescence) was used as an indicator of RNA content. All red blood cells in the sample were haemolyzed while the white blood cells were denucleated and their nuclei shrunk for differential enumeration.

2.4 HIV Screening

The 140 subjects were screened for HIV using Alere Determine ™ HIV-1/2 to detect HIV seronegative cases. The reagents and samples were removed from the refrigerator and allowed to stand for about 25minutes in order to attain room temperature. Test device numbering 140 were labelled appropriately, a precision pipette was used to apply 50µl of the serum to the sample pad and allowed to migrate by capillary action through the conjugate pad and then through the nitrocellulose membrane. A timer was set and the results were read in 25minutes to determine HIV positive and negative cases. HIV negative cases were included in the study while the positive cases were excluded.

2.5 Selection criteria

Subjects used in this study included TB positive (microscopy and/or GeneXpert) patients without HIV infection. For the purpose of this study, the following groups of people were excluded:

1. HIV seropositive TB patients.
2. Children who are below the ages of 15 years of age due to their inability to pass sputum for TB test.
3. Pregnant female patients to avoid interference of pregnancy with leucocyte count.

2.6 Statistical analysis

The data generated from the laboratory analysis of blood samples collected from the study and control subjects were grouped according to the value presentation, that is, normal values and low/high values. In order to test the hypotheses stated for the study, the student's unpaired t-test (Microsoft Excel Package, 2013 version) was used for analyses. The P-value was taken as 0.05, 95% confidence interval, such that $P < 0.05$ indicated a significance.

3. Results

As shown in Table 1, 43% of the study subjects presented with CD4+ lymphocytopaenia, while 26%, 19%, 28% and another 28% presented with leucocytosis, neutrophilia, lymphocytopaenia and monocytosis respectively.

Table 1: Result/Statistical Analysis of CD_4^+ and Leucocyte counts of study subjects

Parameter	NV (%)	Ab (%)	P-value
CD_4^+	57	43 (CD4+ lymphocytopaenia)	0.000
TLC	74	26 (Leucocytosis)	0.002
NC	81	19 (Neutrophilia)	0.049
LC	72	28 (Lymphocytopaenia)	0.658
MC	72	28 (Monocytosis)	0.003

KEY: TLC- Total leucocyte count**; NC-** Neutrophil count**; LC-** Lymphocyte count; **MC-** Monocyte count; **NV-** Normal values; **Ab-** Abnormal values.

Table 2 showed that 43% of the study subjects had CD4+ counts < 500cells/µl (min-max: 378-496 cells/µl), 51% had between 500cells/µl and 999cells/µl while 6% had greater than 1000cells/µl (min-max: 1076-1301 cells/µl). It is also seen that 25(96.2%) of the abnormal values of TLC, 19(100%) of the abnormal values of NC, 26(92.9%) of the abnormal values of LC and 26(92.9%) of the abnormal values of MC occurred within the abnormal values of CD4+ count of the study subjects while only 1(3.8%) of the abnormal TLC value, 2(7.1%) of the abnormal LC value and 2(7.1%) of the abnormal MC value occurred within the normal values of CD4+ count.

Table 2: Summary of CD_4^+ counts and distribution of abnormal leucocyte counts of study subjects

CD_4^+ count	Subjects (n, %)	Ab. TLC (n, %)	Ab. NC (n, %)	Ab. LC (n, %)	Ab. MC (n, %)
<500	43(43)	25(96.2)	19(100)	26(92.9)	26 (92.9)
500-999	51(51)	1(3.8)	-	2(7.1)	2 (7.1)
>1000	6(6)	-	-	-	-
TOTAL	**100(100)**	**26(100)**	**19(100)**	**28(100)**	**28 (100)**

KEY: TLC- Total leucocyte count**; NC-** Neutrophil count**; LC-** Lymphocyte count; **MC-** Monocyte count**; Ab-** Abnormal values; n- Number

As shown in Table 3, CD4+ lymphocytopaenia was observed in 32(52.5%) females and 15(38.5%) males while 29(47.5%) females and 24(61.5%) males had normal CD4+ count.

Table 3: Gender distribution of CD_4^+ counts of study subjects

CD_4^+ Count	F (n, %)	M (n, %)	TOTAL
<500	32 (52.5)	15 (38.5)	43
500-999	26 (42.6)	21 (53.8)	51
>1000	03 (4.9)	03 (7.7)	06
TOTAL (%)	**61 (100)**	**39 (100)**	**100**

KEY: F- Female; **M-** Male, **n-** Number

Table 4 showed that 29.5% of females and 20.5% of males had leucocytosis, 21.3% of females and 15.4% of males had neutrophilia, 29.5% of females and 25.6% of males had lymphocytopaenia, and 32.8% of females and 20.5% of males had monocytosis.

Table 4: Gender distribution of leucocyte counts of study subjects

Parameter	Abnormal values			Normal values		
	No	F (n, %)	M (n, %)	No	F (n, %)	M (n, %)
TLC	26	18(29.5)	8(20.5)	74	43(70.5)	31(79.5)
NC	19	13(21.3)	6(15.4)	81	48(78.7)	33(84.6)
LC	28	18(29.5)	10(25.6)	72	43(70.5)	29(74.4)
MC	28	20(32.8)	8(20.5)	72	41(67.2)	31(79.5)

In relation to age, it was observed from table 5 that age group 51-60 years had highest CD4+ lymphocytopaenia (54.5%), followed by 41-50years (48.3%), 31-40years (46.2%), 21-30years (25%) and age group <20years (20%). For TLC distribution in relation to age group, it was observed that 51-60years had the highest leucocytosis (36.4%), followed by 31-40years (28.2%), 41-50years (27.6%) and 21-30years (18.8%). Age group 31-40years had the highest record of neutrophilia (25.6%), followed by 51-60years (18.2%), 41-50years (17.2%) and 21-30years (12.5%). Age group 51-60years had the highest record of lymphocytopaenia (36.4%), followed by 31-40years (33.3%), 41-50 years (27.6%) and 21-30years (18.8%). Age group 51-60years had the highest record of monocytosis (36.4%), followed by 31-40years (33.3%), 41-50years (27.6%) and 21-30years (18.8%). Age group less than 20years had no record of abnormal leucocyte values.

Table 5: Normal and abnormal values among age groups of study subjects

Age	<20 (n, %)	21-30 (n, %)	31-40 (n, %)	41-50 (n, %)	51-60 (n, %)
Abnormal values					
CD4+	1(20)	4(25)	18(46.2)	14(48.3)	6(54.5)
TLC	0	3(18.8)	11(28.2)	8(27.6)	4(36.4)
NC	0	2(12.5)	10(25.6)	5(17.2)	2(18.2)
LC	0	3(18.8)	13(33.3)	8(27.6)	4(36.4)
MC	0	3(18.8)	13(33.3)	8(27.6)	4(36.4)
Normal values					
CD4+	4(80)	12(75)	21(53.8)	15(51.7)	5(45.5)
TLC	5(100)	13(81.2)	28(71.8)	21(72.4)	7(63.6)
NC	5(100)	14(87.5)	29(74.4)	24(82.8)	9(81.8)
LC	5(100)	13(81.2)	26(66.7)	21(72.4)	7(63.6)
MC	5(100)	13(81.2)	26(66.7)	21(72.4)	7(63.6)
TOTAL	**5**	**16**	**39**	**29**	**11**

KEY: TLC- Total leucocyte count**; NC-** Neutrophil count**; LC-** Lymphocyte count;
MC- Monocyte count**; n-** Number

4. Discussion

TB is a major health problem throughout the world and remains the single largest infectious disease causing high mortality in humans [1-2]. The immune response to this infection relies on cell-mediated immunity which is evident in CD4+ and CD8+ T-lymphocyte responses to Mtb antigens [7-8].

This study was to enumerate CD4+ lymphocytes and leucocytes (total and differential) in HIV seronegative TB positive (Microscopy and/or GeneXpert) patients before the commencement of anti-tubercular therapy (ATT). CD4+ count was found to be significantly reduced (CD4+ lymphocytopaenia) below the 500cells/µL threshold mark in 43% of the study subjects while 57% had CD4+ counts above 500cells/µL. The statistical analysis of the CD4+ counts against control subjects showed a statistically significant ($P<0.05$) CD4+ lymphocytopaenia.

The findings from this study are in agreement with the findings of other researchers [13-18]. A similar study conducted in Ethiopia [16] reported a substantial prevalence of low CD4+ cell counts

before the initiation of ATT with 25% having CD4+ cell counts below 500cells/µL and 10% had CD4+ cell counts lower than 350cells/µL. The present study did not have any record of CD4+ cells below 350cells/µL, this may probably be due to the severity of disease between the two studies as the present study focused on patients with less severe tuberculosis infection as none of the patients were on clinical admission and not on ATT at the time of the study so as to prevent the interaction of drugs (ATT) with the outcome of the study. The researchers correlated CD4+ cell strata with clinical variables and concluded that low CD4+ cell counts are associated with TB disease severity, a situation that cannot be determined in this present study due to the fact that all the study subjects were those coming for TB diagnostic test for the first time and severity of disease was not the focus of the study.

In another study in Iran [14] and Saudi Arabia [15], the researchers reported significantly ($P<0.05$) lower CD4+ counts among TB infected HIV negative patients as compared with control. Another study carried out in Ethiopia [17] and similar studies conducted in Nigeria [18-19], supported the CD4+ lymphocytopaenia findings from this present study.

Sixty one (61) females and 39 males were used for the study, 32 (52.5%) females and 15(38.5%) males had CD4+ lymphocytopaenia (table 3). It can be observed from the findings of this study with regards to gender that more females were prone to CD4+ lymphocytopaenia compared to males. A similar study [18] reported a significant higher mean of CD4+ cells in female TB patients on DOTS than their male counterparts but reported no significant difference in gender of TB patients not on ATT. The CD4+ lymphocytopaenia observed in this study from patients suffering from tuberculosis can be a sign of CD4+ T-cell suppression.

The haematological profile of HIV seronegative tuberculosis patients carried out in this study before the commencement of ATT showed a statistically significant ($P<0.05$) leucocytosis when compared with those of control subjects. The results showed 26% leucocytosis among study subjects, there was however no record of leucopaenia in this study. The findings of this study support the findings of other researchers who also reported significant leucocytosis ($P<0.05$) in their study [18, 20-21]. However, the statistically significant leucocytosis reported in this study is at variance with the findings of other researchers who found normal leucocyte values in their study [22-23], the disparity in the findings could be due to normalization of leucocytes as a result of therapy as the researchers study subjects included those already on ATT, while other researchers like Shafee and colleagues [24] reported 14% leucopaenia in their study. The leucocytosis observed in our study is probably due to the increased response of leucocytes as a result of its phagocytic activity to Mtb infection.

The neutrophils counts of HIV seronegative tuberculosis patients observed in this study showed a statistically significant ($P<0.05$) neutrophilia when compared with those of control subjects. The results showed that 19% of the study subjects had neutrophilia, there was no neutropaenia recorded in this study. The findings of this study supports the findings of other researchers [18, 20-21] and contrary to the normal values reported in another study [23].

The lymphocyte counts of HIV seronegative tuberculosis patients carried out in this study before the commencement of ATT was not statistically significant ($P>0.05$) despite the lymphocytopaenia recorded in 28% of the study subjects. This is in support of the findings of other researchers [18, 20-21, 23]. However, there was no record of lymphocytosis in this study contrary to the 6% lymphocytosis reported in one study [25].

The monocyte counts of HIV seronegative tuberculosis patients carried out in this study before the commencement of ATT showed a statistically significant ($P<0.05$) monocytosis, the results showed that 28% of the study subjects had monocytosis, there was however no monocytopaenia recorded in this study. The findings of this study agrees with previous studies [20-21], but contrary to the no significant difference between study and control groups reported in another study [23].

It can be seen that the leucocytosis observed in this study is due to neutrophilia and monocytosis, the roles of neutrophils and monocytes in tuberculosis infection cannot be undermined as they are soon mobilized to the site of infection to provide cellular immune cover for the host. Another observation from our study (table 2) is that CD4+ lymphocytopaenia in HIV seronegative tuberculosis

subjects affects the total and differential leucocyte counts of sufferers as study subjects with normal CD4+ cells had relatively stable total and differential leucocyte counts compared to study subjects with CD4+ lymphocytopaenia.

CD4+ lymphocytopaenia was found to be variedly distributed among different age. While, age group 51-60years had the highest prevalence rate (54.5%) age group <20years had the lowest prevalence rate (20%). An increasing prevalence rate of CD4+ lymphocytopaenia was observed in ascending order of age groups, this is suggestive of diminishing cellular immunity with increase in age from age group <20years to 51-60years. This findings corroborates the findings of other researchers [26-27]. Age group 51-60years was found to have the highest prevalence rate for leucocytosis, lymphocytopaenia and monocytosis while age group 31-40years had the highest prevalence rate for neutrophilia. This observation is suggestive of reduced cellular immunity with regards to increase in age.

5. Conclusion

This study highlights the importance of cellular immunity conducted by CD4+ T-cells and leucocytes in the outcome of tuberculosis. The findings from this study indicated a statistically significant CD4+ lymphocytopaenia, leucocytosis, neutrophilia, monocytosis and a non-statistically significant lymphocytopaenia despite 28% lymphocytopaenia recorded in the study. The feminine gender had the highest prevalence rate of CD4+ lymphocytopaenia, leucocytosis, neutrophilia, lymphocytopaenia and monocytosis. An increasing prevalence rate of CD4+ lymphocytopaenia was observed in ascending order of age groups.

6. Recommendation

The response of cellular immunity should always be considered in approach to patients with tuberculosis and the need for modalities such as effective vaccination and immunotherapy should be implemented while CD4+ and leucocyte values should always be monitored during the course of ATT.

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