

**ASSESSMENT OF THE IMMUNE STATUS OF HIV
POSITIVE AND HIV NEGATIVE INDIVIDUALS
WITH AND WITHOUT INTESTINAL PARASITIC
INFECTIONS.**

**A THESIS SUBMITTED TO THE
School of Graduate Studies
Addis Ababa University**

In partial fulfilment of
the requirements for the degree
of Master of Science in Biology

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June, 1998.

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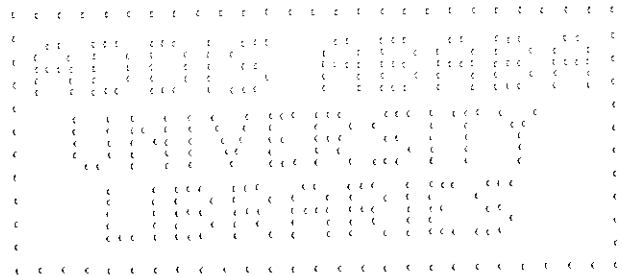
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Acknowledgement

I am most grateful to my advisors, Dr. Beyene Petros, Dr. Tobias Rinke de Wit and Dr. Arnaud Fontanet for their consistent guidance and encouragement and above all for introducing me to the field of Immunology. I would like to extend my deepest gratitude to Mrs. Tsehaynesh Mesele for her invaluable assistance and encouragement during my practical work and for critically reading and commenting on this thesis.

I am grateful to the Ethiopian-Netherlands AIDS Research Project (ENARP) for funding my project and providing me with all laboratory facilities. My heart-felt thanks also go to all members of the staff of ENARP for their endless cooperation. My sincere thanks go to my instructors in the department of Biology and all members of the staff.



ABSTRACT

Several features of the Acquired Immunodeficiency Syndrome (AIDS) epidemic in developing countries make it different from the epidemic in the industrialised world. Faster rate of progression to AIDS and death, and a higher risk of infection after exposure to HIV are reported from developing regions of the world. Differences in natural history of HIV infection in developing versus industrialised countries might be related to differences in the immune status and responses of the patients. This might be due to exposure to multiple pathogens in the former countries, among which intestinal parasitic infections are the most common. The objective of this cross-sectional study is to assess the immune status of HIV infected individuals with and without intestinal parasites and to determine the effect of treating parasitic infections on some immunological markers of the patients. Various immune cell subsets were examined by means of flow cytometry, using an extensive panel of monoclonal antibody combinations on HIV seropositive subjects and healthy controls with and without parasitic infections (n=80). Pre and post parasite treatment analysis was carried out in HIV seropositive (n=5) and seronegative (n=6) individuals. Stool specimens of all study subjects were examined for intestinal parasites using both direct and concentration methods. Decreased proportions of CD4⁺ cells and increase of CD8⁺ T cells were observed in the HIV seropositive subjects. As would be expected CD4/CD8 ratio was highly reduced in HIV seropositives when compared with the HIV seronegative individuals (mean, 0.34 vs 1.23). Major changes in the proportions of T cell subsets were seen in the CD8 cells. This was characterized by a progressive decrease of effector cells and an increase in memory cells. Intestinal parasitic infections had no statistically significant effect on the CD4 and CD8 cell proportions. However, an elevated proportion of CD4 and CD8 cells expressing the activation marker HLA-DR⁺CD38⁻ was observed in the parasite-infected subjects regardless of their HIV status. The expression of the activation marker was significant in the CD8⁺ cells. Treatment of parasitic infections caused a significant reduction of the activated cells. Furthermore, increased levels of CD4 naive cells and decrease in the proportion of memory cells were seen after treatment for parasitic infections. Since activation of the immune system is known to enhance HIV infection of immune cells, parasitic infections that persistently activate the immune system seem to have positive interaction in HIV progression to AIDS. However, a much more thorough investigation needs to be conducted to substantiate the present findings.

1. INTRODUCTION

1.1 General Background

Acquired immunodeficiency syndrome (AIDS) is one of the major pandemics of our century. It continues to be an increasingly complex and dangerous global burden since its recognition (Lewis and Bailey, 1993). The fact that the disease is infectious, and all infected cases remain to serve as reservoirs of the infection for a relatively long period of time has enabled the disease to constantly and progressively expand all over the world. The AIDS epidemic has thus become an urgent public health problem, in spite of the many national and international control interventions made to prevent its spread. The epidemic has touched almost all aspects of society and its social impact is linked to the selective loss of persons in their most productive years (World Bank, 1993).

According to the provisional reports on the status and trends of the HIV/AIDS (MAP, 1997), about 30.6 million people are thought to be infected world wide with HIV. Other estimates indicate that more than 40 million will be infected by the year 2000 (Quinn, 1995; Mertens and Low-Ber, 1996). Citizens of developing countries are predicted to constitute 90% of the world's infected population by the end of the century (De Cock *et al.*, 1993).

In Africa an increasing proportion of the HIV/AIDS cases are known to exist (Wilkins, 1992). A decade after the first recognition of the epidemic in the continent, the spread of the infection has already surpassed the most pessimistic expectations (Mocroft *et al.*, 1996). By World Bank estimate, the disease is expected to increase adult mortality rate by more than double in sub-Saharan Africa (World Bank, 1993). To date, more than 20.8 million people are estimated to be infected with HIV in the continent (MAP, 1997).

In Ethiopia, although, the true number of AIDS cases is not known, estimates made until the middle of 1995 by the Ministry of Health (MOH, 1996) indicate the existence of over 350,000 AIDS cases in the country. According to the 1996 report of the Ministry of Health, about 1.7 million people are estimated to be infected with the virus (MOH, 1996).

1.2. The Human Immunodeficiency Virus

AIDS is the end-stage disease manifestation of infection with a virus called the human immunodeficiency virus (HIV), a retrovirus belonging to the lentivirus family. Two distinct types of viruses, namely, HIV-1 and HIV-2, cause the AIDS syndrome. The viruses are transmitted horizontally, by sexual contact, via contaminated blood products during blood transfusion and iv drug using and vertically, from mother to child (Gallo *et al.*, 1983; Quinn, 1996). Infection with these viruses initiates a complex series of events, which frequently span more than a decade, as the infected individuals transit from primary infection to clinical latency and to the development of AIDS (Fauci, 1993).

The HIV virion is slightly more than 100 nm in diameter and appears as a dense cylindrical core surrounded by a lipid envelope (Connor and Ho, 1992). It consists of a host derived lipid bilayer membrane and virally encoded envelope protein complexes, the "outer" envelope protein (gp 120), and the "transmembrane" protein (gp 41). These envelope complexes facilitate the entry of the virus by binding to the main cellular receptor, the CD4 molecule (Weiss, 1993), and the viral co-receptors, CCR-5 and CXCR-4 (Binley and Moore, 1997).

Like other retroviruses, HIV contains a virus capsid, consisting of the major capsid protein, (P24), the nucleocapsid protein (P7/P9), the RNA genome and the viral enzymes: protease, reverse transcriptase and endonuclease. The capsid is surrounded by a matrix protein (P17). These viral structural proteins are encoded by different genes, namely the env, gag and pol genes that encode the precursor protein, gp160; the matrix and core proteins; and the viral enzymes, respectively (Modrow *et al.*, 1987).

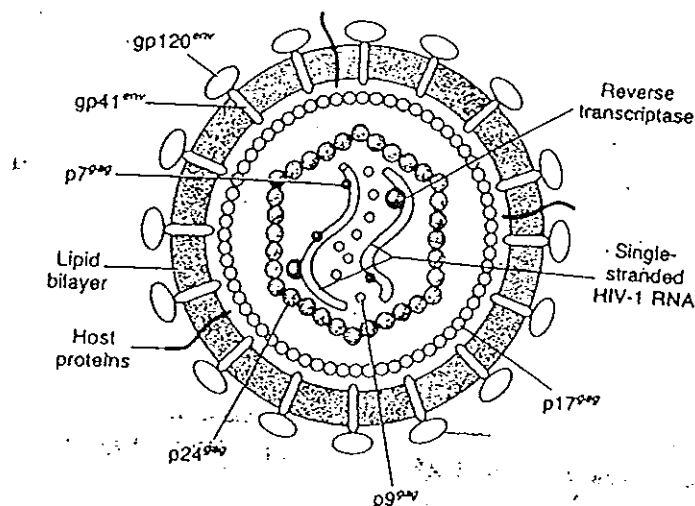


Fig. 1. HIV-1 structural components (source: de Jong, 1994)

Like any other virus, HIV is an obligate intracellular parasite, and hence needs the biochemical machinery of the host cell to be able to synthesise proteins and metabolize sugars (Nash, 1996).

The binding of the virus envelope to the cellular receptor molecules, which initiates its entry, is a critical step in the immunopathogenesis of the infection (Fauci, 1988; Chirmule and Pahawa, 1996).

Once inside the host cell, the virus replicates using the cell's machinery, during which the genomic RNA is reverse transcribed into double-stranded complementary DNA copy (cDNA), called a "provirus". The "provirus" is transported via the matrix protein into the host cell genome and can remain latent for a long period of time (Barre-Sinoussi, 1996). When HIV infected cell is activated, the viral DNA starts to serve as a template for the generation of new viral RNA genomes and subsequently, the HIV structural and regulatory proteins are formed to produce infectious viral particles (Barre-Sinoussi, 1996).

Thus HIV infection is an example of a slow disease in man, with a prolonged asymptomatic phase (Clements and Zink, 1996). During this latent period, the virus can exist as a provirus, integrated into the host's genomic DNA without any transcription process and starts to replicate when the host cells are immunologically activated (Nash, 1996).

1.3. Features of AIDS Epidemic In Developing Countries.

The United Nations expert group on AIDS (UNAIDS, 1997) observed the HIV pandemic to be highly heterogeneous in nature, where it comprises numerous subepidemics in distinct geographical locations and population groups. Several researchers (de Perre, *et al.*, 1984; Quinn, 1996) have also indicated that many different HIV epidemics, with their own specific dynamics and each influenced by several factors, exist within the global pandemic of the HIV infection.

HIV virus displays extensive genetic variation in geographically distinct locations, with multiple subtypes co circulating in many areas of the world (Artenstein *et al.*, 1995). HIV-1 of subtype B is predominating mostly in industrialised countries whereas multiple subtypes, mainly A, D, E and C are found circulating in large parts of the developing regions of the world (Louwagie *et al.*, 1993; Dumitrescus *et al.*, 1994; Louwagie *et al.*, 1995).

Furthermore, the pattern of AIDS in the developing regions, where there is poor hygiene and high rates of intercurrent infections, is observed to be different from that seen in the industrialised countries both in its clinical manifestations and course of the disease (Fleming, 1990; Bentwich *et al.*, 1995). In addition, and partly because of intercurrent infections, like STDs, the risk of seroconversion after exposure is believed to be higher, and a faster rate of progression to AIDS and death are reported from developing regions of the world (Quinn *et al.*, 1987; Berkley, 1993; Weissman *et al.*, 1996).

Even though the environmental factors prevalent in such locations are suggested to be favourable for the dissemination of the virus (Amat-Roze, 1993), the deriving forces behind this epidemic are very complex and are not well understood (UNAIDS, 1997). Despite the fact that the syndrome is the same globally, Fleming (1990) has illustrated the pattern of progression to AIDS to be governed by the type of pathogenic micro-organisms (parasitic, viral, bacterial etc.) prevalent in the area. This suggests the interaction between HIV infection and other endemic diseases to be of a major concern (de Perre, 1995).

Repeated episodes of immune activation occurring in the third world situation may contribute to the suggested different rate of disease progression in this set up (Marrow et al., 1989). Infectious diseases which are endemic to this part of the world, might activate the immune system and alter its balance in such a way that makes the host receptive to HIV and more vulnerable to its effects (Marrow *et al.*, 1989; Bentwitch *et al.*, 1995).

1.4. HIV and Parasite Interactions with the Immune System

Both humoral and cellular arms of the immune system are known to be involved during HIV infection (Mc Michael, 1996). HIV virions infect macrophages and efficient transmission of the virus is demonstrated during antigen presentation to activated T cells, making macrophages to be the main producers of HIV at the early stage of infection passing the virus to circulating T cells (Miedema, 1992).

T lymphocytes, the principal components of cell-mediated immunity, exhibit a variety of functions in antiviral immunity during HIV infection (Nash, 1996; Taverne and Bradley, 1996). CD4⁺ T helper cells further proliferate and differentiate into Th1 and Th2 cells depending on the type of cytokines they produce. Helper CD4⁺ (Th1) cells help in the induction of CD8⁺ cytotoxic T lymphocytes (CTL), the principal surveillance system operating against viruses by cytotoxic killing and by secreting factors that influence viral growth and replication, such as TNF- α (Watret *et al.*, 1993; Nash, 1996). An elevated number of these CTLs have been demonstrated in HIV infection, which is thought to be a result of immune activation (Watret *et al.*, 1993; Miedema *et al.*, 1994).

Th2 cells induce the B cell mediated antibody responses (Nash, 1996). Activated B lymphocytes secrete antibodies that recognise specific peptides on the viral surface. The antibodies bind free viral particles, those not yet sequestered in cells, for destruction (Nowak and Mc Michael, 1995). Cytotoxic T lymphocytes and B lymphocytes mount a strong defence and kill many virus infected cells and viral particles (Miedema and Klein, 1996). Nevertheless, in HIV infection, the virus persists, using different mechanisms such as: high rate of replication, continuous mutation of the virus leading to the emergence of new viral variants that can evade recognition by the immune system, direct impairment of the immune function, and by sequestration of virus in immunologically privileged sites of the body (Bermerman, 1995; Pantaleo and Fauci, 1996). The viral load which initially is controlled at low levels by the immune system rises gradually in parallel with the decline in the CD4 T lymphocyte population (Pantaleo *et al.*, 1990; Turke, 1995). The tropism of the virus towards the CD4⁺ T cells has presented a formidable challenge to the immune system, resulting in the CD4 depletion being the hallmark of immunopathogenesis

of HIV infected individuals (Shearer *et al.*, 1995; Zanuders *et al.*, 1995; Daniel *et al.*, 1996). Intestinal parasites elicit quite distinct immune responses which are different from the responses to bacteria and viruses. The increased size and complexity, together with the often drastic morphological changes at different stages of the life cycle leads to a much greater variety in the antigenic profiles that the immune system encounters. T cells are the major elements responsible for parasite specific and non-specific immunity during the complex immune response of the host (Fraser, 1994; Taverne and Bradley, 1996). The type of T cell responsible for controlling the infection varies with the parasite and stage of infection (Hyde, 1990). The CD4+ cells can be divided into Th₁ and Th₂ subsets during parasitic infection. Th₁ subsets develop preferentially during infections by intracellular parasites and trigger phagocyte-mediated host defence (Rook, 1996). In contrast, human Th₂ cells predominate during helminthic (extracellular) infestations (Romagnani, 1995), and provide help for IgE and IgG₁ synthesis and mediate eosinophilia. A shift from Th₁ to Th₂ type cells is also documented to occur in HIV infection (Clerici *et al.*, 1993; Meyaard *et al.*, 1996).

1.5. Immunological Activation and Differentiation Markers

The activation status of immune cells can be assessed by measuring activation markers such as HLA-DR and/or CD38, expressed on the surface of lymphocyte subsets (Prince and Jensen, 1991; Levacher *et al.*, 1992; Ho *et al.*, 1993; Bofill *et al.*, 1996; Mocroft *et al.*, 1997).

The human leukocyte antigen (HLA-DR), is a major histocompatibility complex (MHC) class-II antigen responsible for antigen presentation, and its expression is reported to increase on T cells in association with cellular activation (Prince and Jensen, 1991; Levacher *et al.*, 1992; Ho *et al.*, 1993; Peakman *et al.*, 1995; Bofill *et al.*, 1996; Mocroft *et al.*, 1997). CD38 is a cell membrane marker expressed in most immature haematopoietic cells. It is decreased during cellular maturation. However in T lymphocytes, CD38 expression can be up-regulated upon activation (Levacher *et al.*, 1992). CD38 is known to induce activation and proliferation signals in lymphocytes (Cesano *et al.*, 1998). A dramatic increase in the expression of HLA-DR and CD38, specially in the CD8⁺ compartment, has been shown when comparing HIV seropositive patients with healthy controls (Levacher *et al.*, 1992; Kestens *et al.*, 1994; Zaunders *et al.*, 1995).

The proportion of CD4⁺ and CD8⁺ cells expressing DR and CD38 increases during progression of the disease (Levacher *et al.*, 1992). Ramzaoui *et al.*, (1995) proposed the increased expression of CD38 and DR on circulating CD4⁺ and CD8⁺ T cells of HIV infected patients to be a feature of two different mechanisms: immunological activation state responsible for the increase of HLA-DR and CD38 expression, and immune cell immaturity, due to an increased turnover, responsible for additional CD38 expression.

Many studies have focused on changes in the representation of differentiation markers in subsets of CD4⁺ and CD8⁺ T cells in HIV infected subjects (Prince and Jensen, 1991; Zimmerman *et al.*, 1996). For example, Hamann *et al.*, (1997) have proposed a subdivision of T cells based on the differential expression of CD45RA (a transmembrane molecule found in all leukocytes which is

used in T cell signalling) and CD27 (a member of the TNF family used as a co-stimulatory molecule in the appropriate stimulation of T cells). CD45RA and CD27 double positive phenotypic expression show characteristics of unprimed cells. They have also documented that, the CD45RA⁺CD27⁻ phenotype sub-populations to have cytolytic activity. These cells were found to express abundant amounts of FAS-ligand mRNA and cytolytic enzymes, and hence designated as effector cells.

Other discrete primed subpopulation found in the circulation are the memory cells, which respond more and appropriately after renewed antigen is encountered. Such potent cytolytic activity have been observed in the CD45RA⁻CD27⁺ phenotype (Hamann *et al.*, 1997). These CD8⁺ cells were most closely found to resemble memory type cells (Zimmerman *et al.*, 1996).

1.6. Negative Consequences of Persistent Immune Activation

HIV infection is known to be accompanied by profound changes in the immune system of the infected host. This can be demonstrated by changes in the cell surface marker phenotype (Landay, *et al.*, 1993; Kestens *et al.*, 1994). These unique changes observed in the different T cell subsets are induced by the sustained immune activation or the rapid turnover of T cells (Jouen-Beads *et al.*, 1996).

The generalised immune activation observed shortly after HIV infection and its persistence throughout the course of the disease may contribute to the onset of symptoms (Brinch *et al.*, 1989; Fahay *et al.*, 1990; Levacher *et al.*, 1992). Unlike other viral infections, activation of the immune system is conducive for HIV replication. Immune activation stimulates cells harbouring dormant HIV genomes and the secretion of HIV stimulating factors (such as TNF α), as a result of which, the viral titers increase (De Boer and Bonche, 1996). Persistent exposure of the immune system to an antigen ultimately lead to immune system dysfunction and loss of the ability to maintain an adequate response (Pantaleo, *et al.*, 1990; Meyaard *et al.*, 1994). This has been proposed to be the result of the enormous turnover of CD4⁺ T cells, which eventually exhausts the lymphopoietic system (Miedema and Klein, 1996). Chronic activation also induce viral expression on the infected cells, leading to Cytotoxic killing (Meyaard *et al.*, 1992). Enhancement of programmed cell death of both CD4⁺ and CD8⁺ T cells in early stages of HIV infection has been demonstrated through *in vitro* activation tests (Ameisen and Capron, 1991; Gougeon *et al.*, 1993; Oyaizu *et al.*, 1993).

Activated T cells have been shown to be more susceptible to productive infection upon exposure to HIV (Wachter, *et al.*, 1986; Mahalingam, *et al.*, 1993). Study by Weissman *et al.*, (1996) has shown the ability of an enhanced initiation of productive infection with much less amount of the virus during antigen-specific immune activation. This shows that immune system activation could be a central part of HIV pathogenesis (Wachter, *et al.*, 1986; Ascher *et al.*, 1995; Bofill *et al.*, 1996). Monitoring the level of activated immune cells is therefore critical in assessing progression of HIV infection (Mahalingam *et al.*, 1993; Mocroft *et al.*, 1997).

In light of these observations and findings of enhanced viral replication following immune activation, efforts to assess the effect of co-infection with some diseases that appear to have association with HIV infection are continuing. Opportunistic infections are suggested to increase progression of HIV disease because of the general increase of the immune system activity (Bentwich, *et al.*, 1995). Goletti *et al.*, (1996) have studied the influence of antigen specific T cell response to *Mycobacterium tuberculosis* (MTB) on HIV replication, and have found an increased replication of the virus during active MTB infection. Bush and co-workers (1996) have also observed increased viral replication in Pneumonia.

Studies on the influence of co-infection with intestinal parasites in enhancing immune activation, and hence viral replication, are scarce. Arellano *et al.*, (1996) have reported immune activation due to amoebic liver abscess, where a significant increase in the activation marker HLA-DR was found.

Intestinal parasitic infections are the most common infestations of man in the developing world, presenting major medical problems (Bundy *et al.*, 1992; Zumla and Croft, 1992; Alonso-Sanz *et al.*, 1995; Bentwich *et al.*, 1996). In addition to the fact that the immune system is chronically activated in HIV infection, co-infection with parasitic infections is known to typically stimulate additional immunological responses (Taverne and Bradley, 1996).

In Ethiopia, like any other developing country, intestinal parasitism is of a wide distribution (Dagneu *et al.*, 1993; Erko and Tedla, 1993; Yenenh, 1994; Haile *et al.*, 1994; Bentwich *et al.*, 1996). The prevalence of HIV and AIDS is also reported to be rapidly increasing in the country (MOH, 1994 and 1996).

In spite of the fact that the number of new AIDS cases is continuing to increase, little has been done to prevent the development of AIDS and improve the quality of life of the infected patients in Ethiopia. Interventions focusing on controlling factors that might aggravate disease progression are of crucial importance. The role of chronic intestinal parasitic infections and the effect of their prevention in the clinical management of HIV disease has not been addressed.

2. Objectives

The study therefore had the following objectives:

- i. To assess the immune status of HIV seropositive and seronegative individuals with and without intestinal parasitic infections.
- ii. To evaluate the immunological effect of treating intestinal parasitic infections.

3. Materials and Methods

3.1. The Study Population

A subset of the Akaki cohort study participants of the Ethiopian-Netherlands AIDS Research Project (ENARP) was studied after informed consent. The cohort study on HIV-1 infection progression, established in a factory at the suburb of Addis Abeba in early 1997, includes factory workers aged of 18-44 years. Four groups were identified based on serological and parasitological findings. These were: HIV seropositives with intestinal parasite infection (n=20); HIV seropositive individuals negative for parasites (n=18); HIV seronegative individuals positive for parasites (n=22), and healthy controls (n=20) diagnosed negative for both infections. In addition, dually infected individuals with HIV and parasite (n=5) and HIV seronegative controls with parasitic infections (n=6) were analysed immunologically for the cell surface markers of circulating cells before and after treatment for parasites.

3.2. Laboratory Investigations

3.2.1. Parasitological Determinations

Stool specimens were collected from the study participants in sterile stool cups during the first visit and analysed immediately. Individuals diagnosed positive for intestinal parasites were

a) Plasma Isolation

Whole blood sample in the EDTA coated vacutainer tubes were centrifuged at 300 g for 10 minutes. The upper level of the tube content was marked for later peripheral blood mononuclear cell (PBMC) isolation. Plasma was transferred with a sterile pasture pipette into labelled 15 ml tubes. Plasma tubes were centrifuged at 300 g for another 10 minutes. Cell free plasma was transferred into labelled freezing vials, and immediately stored at -80 °C for further serological assays.

b) HIV Antibody Assays

Plasma specimens were tested for the presence of antibodies for HIV using the HIV SPOT Rapid assay, the enzyme-linked immunosorbent assay (ELISA), and the Western blot assay, for confirmation.

i) HIV-SPOT Test

Three drops of reconstituted buffer (Tween 20, bovine serum albumin and heat treated goat serum) were added into the HIV-SPOT test device (Genelabs Diagnostics, Singapore) and allowed to soak. One drop of each plasma sample was added into the labelled devices, which was followed by the addition of 2 drops of the reconstituted buffer and 2 drops of wash buffer (Deionized water). After allowing to soak in, 2 drops of conjugate (lyophilised protein A-gold

reagent) and 3 drops of wash buffer were added. Results were read within 10 minutes. Clear membranes were considered to be negative for HIV antibodies, and the appearance of a distinct red spot was interpreted as positive for HIV antibodies (Genlabs Diagnostics, Singapore).

ii) *Enzyme-linked Immunosorbent Assay (ELISA)*

100 μ l of specimen diluent (stabilizing protein) was pipetted into HIV antigen coated wells contained in microelisa strip plates. 50 μ l of each plasma sample and controls were added into the assigned wells, mixed by taping the sides of the strip holder and incubated at 37°C for 1 hour. Wells were washed by soaking in phosphate buffer. 100 μ l TMB (Tetramethylbenzidine) substrate was pipetted into each well and incubated at room temperature for 30 minutes. Reaction was stopped by adding 100 μ l sulphuric acid and the absorbance of the solution was read at 450 nm (Reader 230 Microwell System, Organon Teknika).

Validity of the test procedure was checked (by checking the quality of the negative control results, where, negative control reading should be < 0.25 ; and the remainder of the mean absorbance of the positive controls minus the mean absorbance of their negative controls should be ≥ 0.4). Cut-off value for each test procedure was determined (mean of the negative controls plus 0.1) and results were interpreted based on the cut-off value. A test sample was considered reactive when the sample absorbance reading was found to be greater than or equal to the cut-off value, and non-reactive, if the sample absorbance was less than the cut-off value (Organon Teknika, NL).

iii) Western blot Test

Quantitative enzyme immunoassay (HIV-BLOT 2.2, Genelabs Diagnostics, Singapore) kits were used for the *in vitro* detection of antibodies to HIV-1 and HIV-2 in plasma. Required number of strips incorporated with HIV antigenic proteins were placed into wells. Two ml of diluted wash buffer (Tris with Tween-20) was added into each well, and strips were incubated for 5 minutes at room temperature on a platform shaker (STUART Scientific, UK). Buffer was removed by aspiration and 2ml of blotting buffer (Diluted non-fat dry milk) was added to each well. This was followed by the addition of 20 μ l of each test plasma sample and controls to the appropriate wells. The test strips were incubated at room temperature on a platform shaker. After 1 hr, test material was aspirated from the wells. Strips were washed three times with 2 ml diluted wash buffer allowing them to soak for 5 minutes on the platform between each wash step. 2 ml of working conjugate (goat anti-human IgG conjugated with alkaline phosphatase) was added to each well after washing, and the tray was incubated for another hour at room temperature on rocking platform. Conjugate was aspirated from the wells, washed, and 2 ml of substrate (5-bromo 4-chloro 3-indolyl phosphate, BCIP, and nitroblue tetrazolium, NBT) was added, covered and incubated for 10-15 minutes on the rocking platform. Substrate was aspirated and strips were rinsed several times with distilled water to stop the reaction. By using forceps, strips were removed onto paper towels covered and left to dry. Developed bands were observed and interpretation of the test result was based on the detection of specific band patterns as recommended by Genelabs Diagnostics HIV BLOT2.2. Samples were considered positive for HIV-1 antibodies, if they appear to develop at least two env bands with or without gag or pol.

3.2.3. Lymphocyte Phenotype Analysis

a) PBMC Isolation and Freezing

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA treated venous blood by using density-gradient centrifugation on ficoll-hypaque (Pharmacia Biotech). Tubes from which plasma was removed were filled with EHN (475 ml Earles, 25 ml New Born Calf Serum (NBCS), 2 ml heparine (20 $\mu\text{g/l}$) and 1 ml penicillin/streptomycin (100 $\mu\text{g/ml}$; 100 $\mu\text{g/ml}$), Life Technologies, Scotland) up to the level which was contained by the plasma, mixed with pipette, and pooled into a corresponding 50 ml tube. The remaining blood sample in the vacutainer tubes was rinsed with additional 5 ml EHN medium and collected into the corresponding tube. EHN medium was added to the 25 ml mark and mixed carefully. Diluted blood samples were carefully loaded on the top of 12.5 ml Ficoll in 50 ml tubes and centrifuged at 2200 rpm for 15 minutes, with out brake. Supernatant was removed and the cell band on top of the Ficoll was collected. The harvested cells were washed by adding EHN medium to 50 ml level and by centrifuging at 1500 rpm for 10 minutes. The supernatant was discarded and the pellet was loosened by gentle tapping, and washed with 30 ml EHN by centrifugation. After aspiration of the supernatant, 0.9 ml IF20 (200 ml ISCOVES, 50 ml 20% Foetal Calf Serum (FCS) and 0.5 ml penicillin/streptomycin) medium was added. The cell suspension was counted by a Coulter counter (COULTER, Electronics Ltd., NY), and stored on ice for about 30 minutes. Freezing medium (IF20 and dimethyl sulfoxide, DMSO) was added to the PBMC, cells were transferred into freezing vials and were frozen by using a freezing machine (KRYO 10 Planer, Biomedical Series II). The frozen PBMCs were stored in liquid nitrogen.

b) Cytofluorometric Analysis

A two and three-colour immunofluorescence analysis of whole blood and PBMCs was carried out to enumerate the proportions of mature human leukocyte subsets and the phenotypic expression of activation and maturation molecules. Fresh whole blood samples were analysed directly, while cell samples frozen from subjects before and after treatment for intestinal parasitic infections were thawed and analysed for cell fluorescence analysis to assess the phenotype of the immune cells.

Cells were simultaneously stained with either CD4 or CD8 monoclonal antibodies in combination with different activation and differentiation state monoclonal antibodies. Panels of monoclonal antibodies used for immune cell phenotyping with their appropriate CD designations are listed in Table 1.

In brief, 20 μ l of monoclonal antibody was pipeted into FACS tubes. Using fresh micropipettor tips, 100 μ l of well-mixed, anticoagulated whole blood or PBMC (250000 cells) were added into the bottom of the tubes containing the monoclonal antibodies. The mixture was vortexed and incubated for 15-30 minutes at room temperature in the dark. 2 ml of diluted lysing solution (50% diethylene glycol and 15% formaldehyde) was added to each tube, vortexed and incubated for a maximum of 10 minutes at room temperature in the dark. Tubes were centrifuged at 300 g for 5 minutes immediately after incubation. The supernatant was discarded leaving approximately 50 μ l of residual fluid in the tube. 2 ml of isotone (Azide-free balanced electrolyte solution) was added to the residue, mixed thoroughly, and centrifuged at the same speed and

time interval. Supernatant was removed and 0.5 ml isotone was added to each tube. The cells were mixed by vortexing and analysed by flow cytometry (Becton Dickinson FACScan, San Jose, CA). The percentage of fluorescent cells were acquired on a log scale on gated lymphocytes defined in forward versus side scattering.

Table.1. Panels of monoclonal antibodies used.

Reagent	CD designation	Utility / target cells
Hle-IVLeu-M3	CD45 CD14	Quality assured lymphocyte gating
Isotype control	IgG1 IgG2a	Control for antigen non specific staining
Leu - 4	CD3	Human T lymphocyte cells
Leu - 4 3a	CD3/ CD4	CD4+ lymphocytes
Leu - 4 2a	CD3 / CD8	CD8+ T cells
Leu - 18	CD45RA	Human leukocytes (for naive versus memory)
Anti- HLA - DR	HLA-DR	Activated MHC class II antigen.
Leu - 17	CD 38	Activated \ immature (CD38+) lymphocytes
Anti - leu - 28 / 27	CD28/ CD27	Co-stimulatory molecules expressed by T lymphocytes

3.3. Data Analysis

Two sample t test was used to test the difference in the cell phenotypes between the studied populations. Paired t test was performed to evaluate the pre\post treatment data. P-value of 0.05 and less was considered as a significant difference.

4. RESULTS

4.1. Parasitological Investigations

The study population was seen to be highly infested with intestinal parasitic infections. More than half (53.2%) of the analysed individuals were found to harbour one or more intestinal parasites. *Ascaris* and *E. histolytica* were observed to be the most commonest infections (33.3% both) followed by *T. trichiura*.

Table 2. Helminths and protozoa distribution (in percentage) in HIV seropositive and seronegative individuals.

	Helminths	Protozoa
HIV +	73.6	10.6
HIV -	52.1	27.4

Table 3. Types of Intestinal Parasites Diagnosed'

Type of parasites	Percentage distribution
<i>A. lumbricoides</i>	33.3
<i>E. histolytica</i>	33.3
<i>T. trichiura</i>	23.8
<i>S. stercoralis</i>	21.4
<i>S. mansoni</i>	7.1
<i>G. lamblia</i>	4.7
<i>H. nana</i> and <i>Taenia</i> spp.	2.3 each

4.2. CD4⁺ and CD8⁺ T Cell Quantification

Helper (CD4⁺) and cytotoxic (CD8⁺) T cells were detected and quantified based on the lymphocyte gate (fig.2). CD4⁺ lymphocyte counts were significantly lower in HIV seropositive individuals compared to the seronegative controls ($P < 0.05$) (fig.3). No significant difference was found in the CD4⁺ T cell counts between parasite infected and non-infected individuals ($P > 0.05$, at 95% CI) (fig.4). Treatment of intestinal parasitic infections had no effect in the T helper cell levels in both HIV infected and non-infected cases.

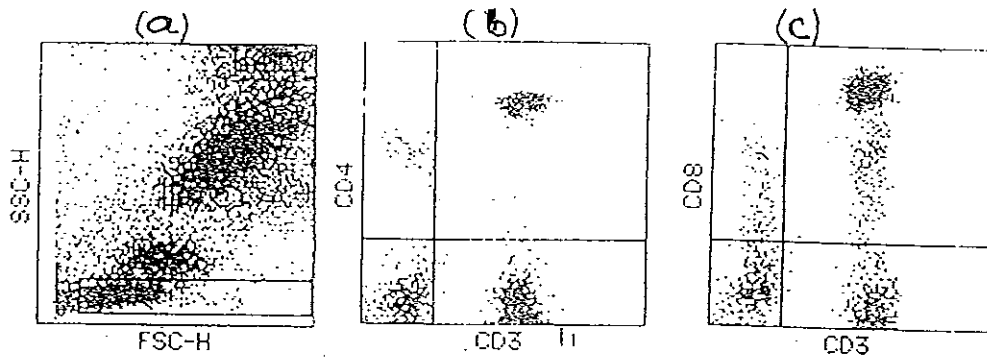


Fig. 2. Representative dot plots of CD4⁺ and CD8⁺ quantification: Optimal lymphocyte gating (a); plots of CD4⁺ and CD8⁺ cells (b & c).

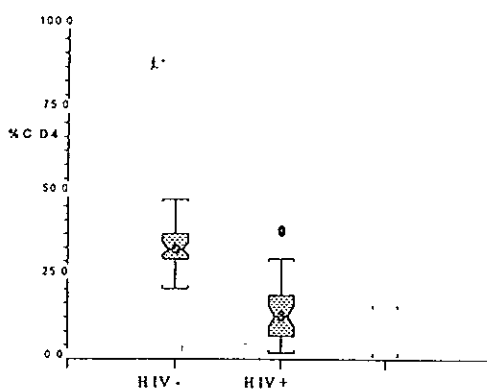


Fig.3. % CD4⁺ cells in HIV seropositive & Seronegative individuals.

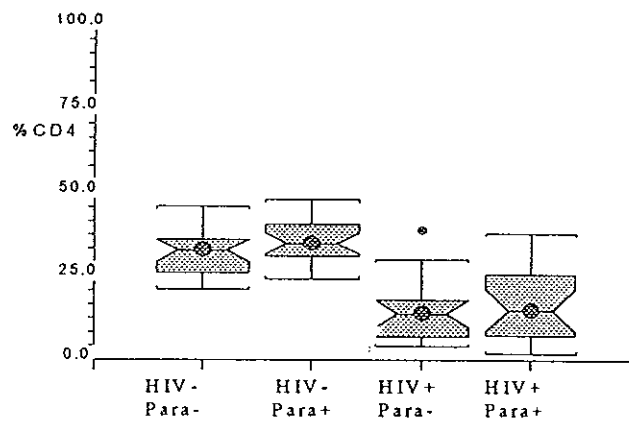


Fig.4. % CD4⁺ cells in HIV Vs parasite infection.

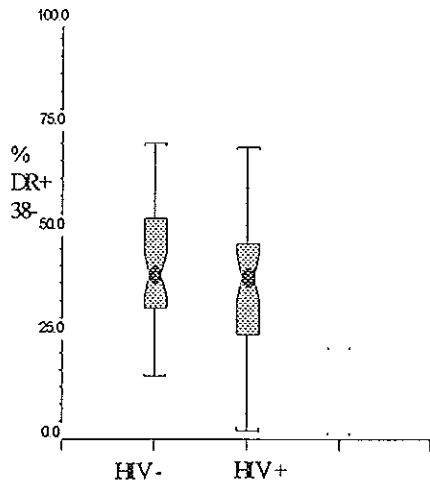


Fig.8. %CD8+ DR+38-cells in HIV seropositive & seronegative individuals

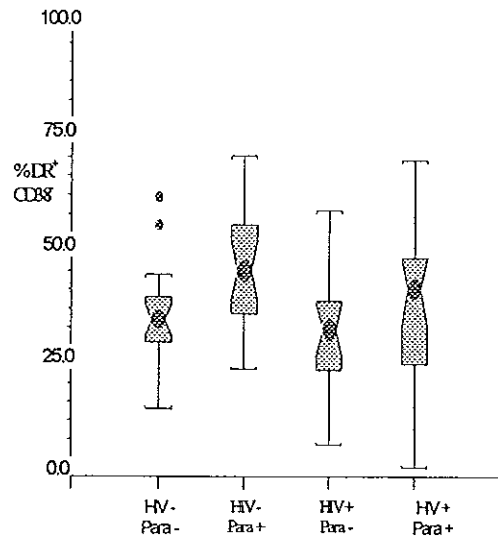


Fig.9. DR⁺CD38⁺ expression on CD8⁺ Cells in HIV VS parasitic infections.

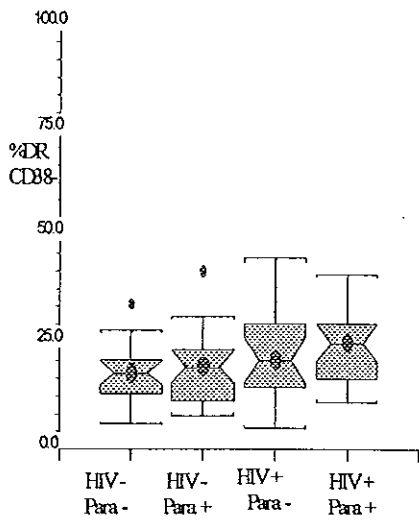


Fig.10. Expression of DR⁺CD38⁺ on CD4⁺ cells in HIV Vs parasitic infections.

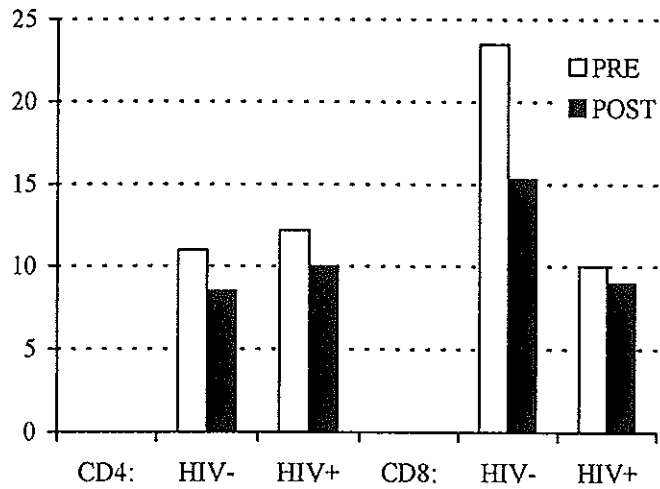


Fig.11. Pre/Post parasite treatment on DR+ 38- expression on CD4+ & CD8+ T cells.

4.4. The DR⁺CD38⁺ Expression Results

Helper (CD4⁺) and cytotoxic T cells (CD8⁺) with CD38⁺DR⁺ phenotype were increased significantly with HIV infection (P<0.05) (fig.12 & 13). CD38 and DR expressing CD8⁺ T cells were found to decrease with parasitic infections in the HIV infected subjects (P<0.05) (fig.12). Age and gender had no effect on both CD4⁺ and CD8⁺ cells expressing DR and CD38. Treatment of parasitic infections was not observed to have significant effect in the proportion of CD4⁺ and CD8⁺ T cells co-expressing CD38 and DR (fig.14).

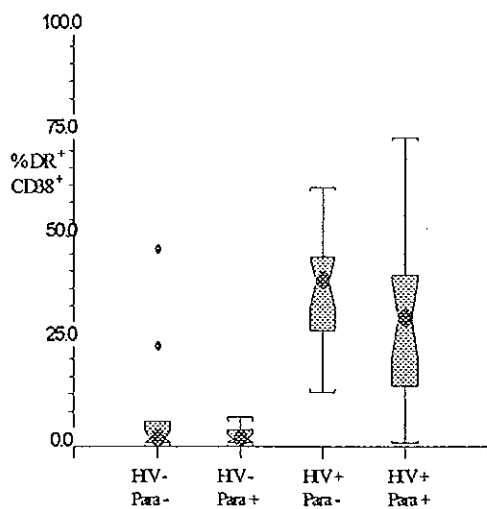


Fig.12. DR⁺ and CD38⁺ CD8⁺ cells
Vs HIV and parasitic infections.

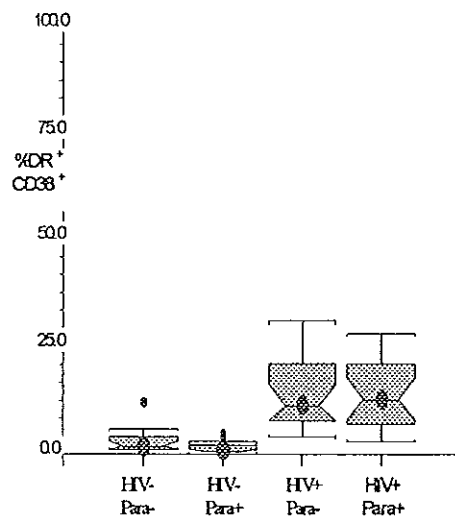


Fig.13. DR⁺ and CD38⁺ CD4⁺ cells
Vs HIV and parasitic infections.

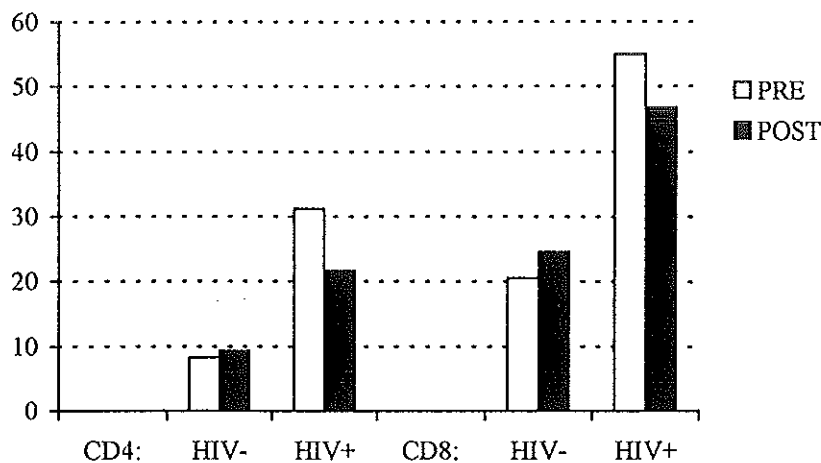


Fig.14. Pre/Post parasite treatment on DR+ 38+ expression on CD4+ & CD8+ T cells

4.5. Naive (unprimed) T lymphocytes

CD8⁺ cells with naive phenotype (CD8⁺CD45RA⁺CD27⁺) were not observed to be significantly affected in the HIV infected subjects ($P > 0.05$). Intestinal parasitic infections had no significant effect on CD8⁺ and CD4⁺ naive cells (fig.15 & 16). Removal of parasitic infections by treatment also appeared to have no significant effect on the level of unprimed CD8⁺ cells in both HIV positive and negative cases (fig.17). An increase of naive CD4⁺ cells was observed after treatment for parasitic infections and this increase was highly significant in the HIV seronegative controls ($P < 0.05$) (Fig.17).

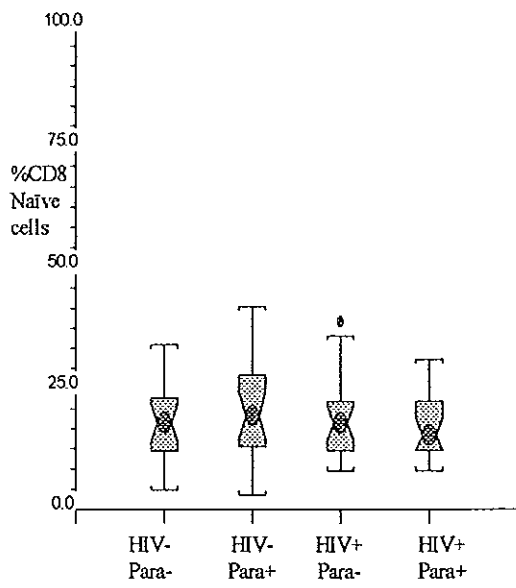


Fig.15 . Naïve CD8 cells Vs HIV and parasite infections.

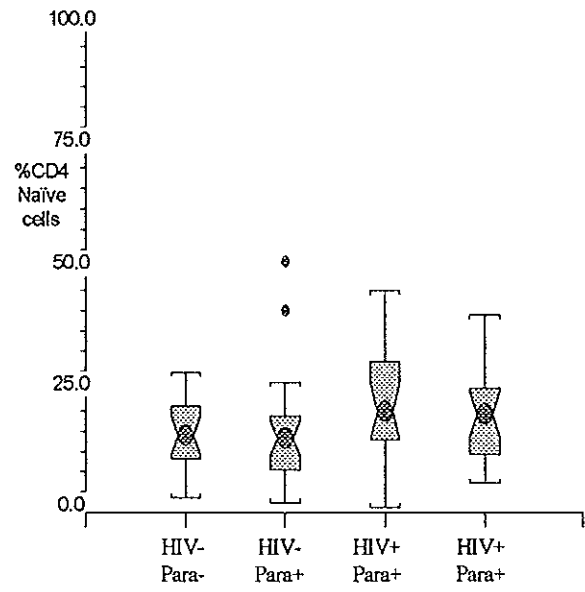


Fig.16. Naïve CD4+ cells Vs HIV and parasite infections.

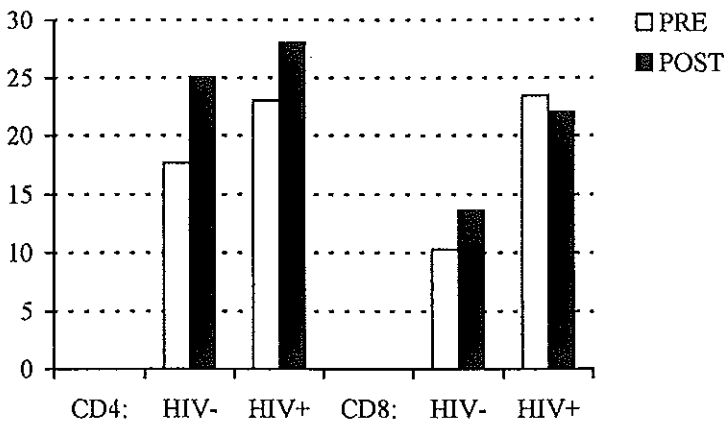


Fig.17. Pre/Post parasite treatment on naive (CD45RA+27+) CD4+ & CD8+ T cells

4.6. Effector CD8+ T lymphocytes

CD4⁺ cells expressing effector phenotype (CD45RA⁺27⁺) were observed to be very few (fig.18). Effector CD8⁺ T cells were significantly reduced with HIV infection (P<0.05) (fig.19). Parasitic infections and their removal following treatment had no statistically significant effect on the level of these cell subsets (P>0.05) (fig.20), in both HIV seropositive and seronegative individuals. No effect of age and gender was observed.

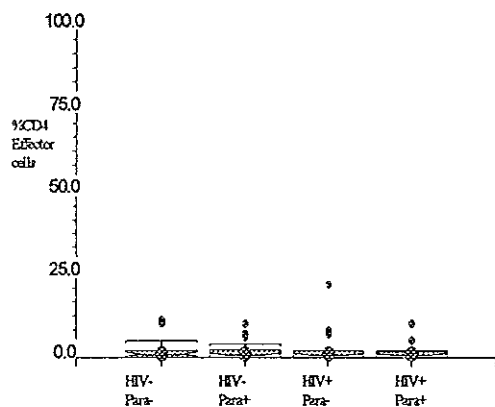


Fig.18. CD4⁺ effector cells Vs HIV and parasite.

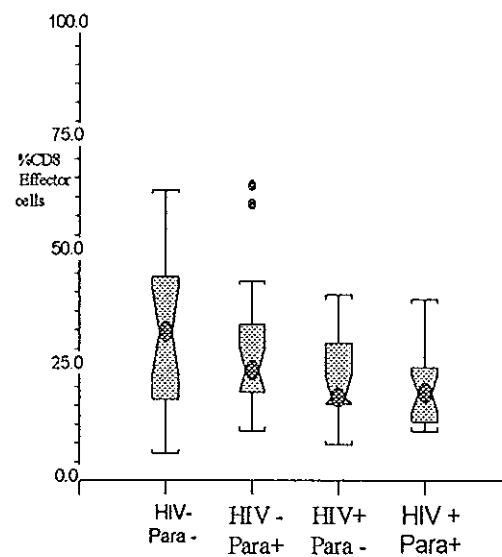


Fig.19. CD8⁺ effector cells Vs HIV and Parasite

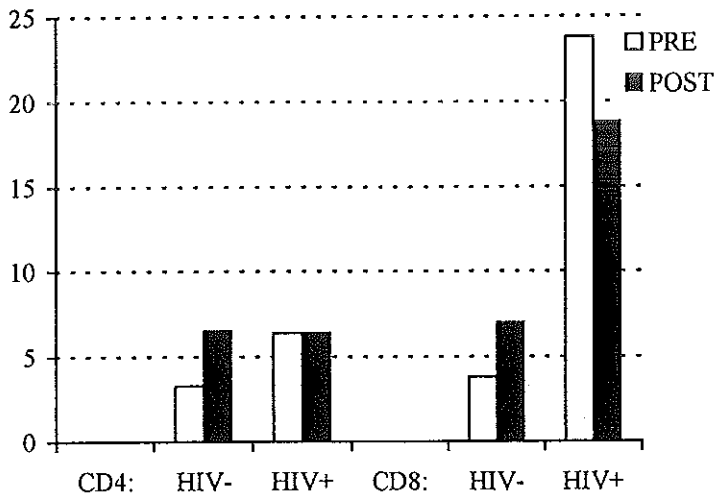


Fig.20. Pre/Post parasite treatment on effector CD4+ & CD8+ T cells

4.7. Memory T cells

Significant increase of memory CD8⁺ cells ($P < 0.05$) and decrease of memory CD4⁺ cells ($P < 0.05$) was observed in HIV infected cases (Fig. 21 & 22). Parasitic infections showed a trend of increase in the memory CD4⁺ and CD8⁺ cells, although this was not statistically significant ($P > 0.05$). Treating parasitic infections had no significant effect on the level of CD8⁺ memory cells in both HIV+ and HIV- individuals. Marginally significant decrease of memory CD4⁺ cells was observed after treatment in the HIV seronegative participants ($P = 0.06$) (Fig.23).

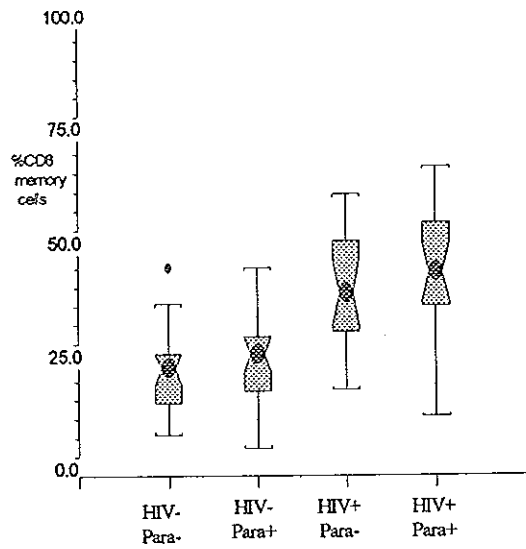


Fig.21. Memory CD8+ cells Vs HIV & parasite.

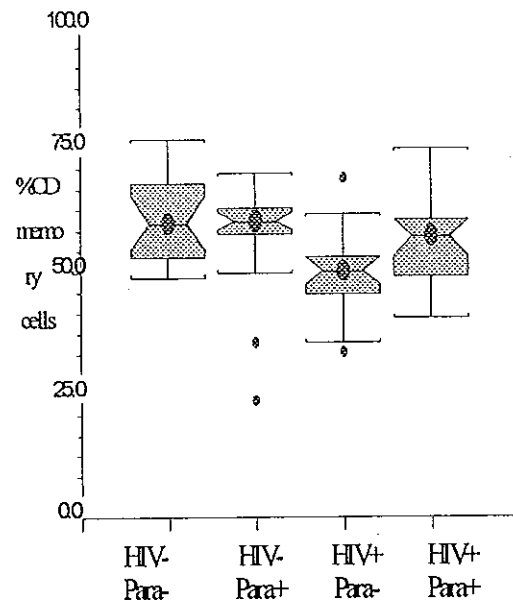


Fig.22. Memory CD4+ cells Vs HIV & parasite.

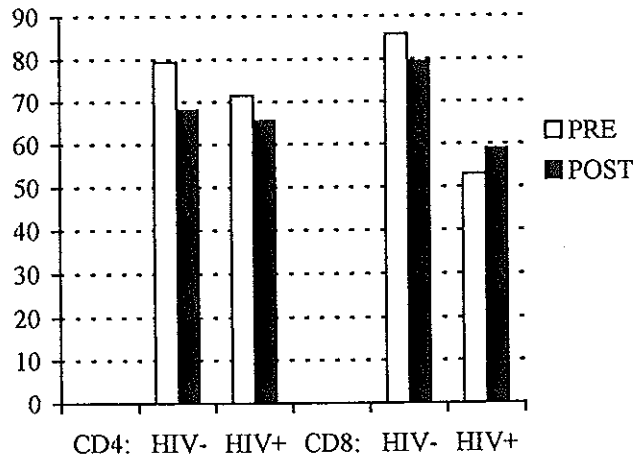


Fig.23. Pre/Post parasite treatment on memory CD4+ & CD8+ T cells.

5. Discussion

Studies of the natural history of infection with HIV in general focus on the progression of the disease and the assessment of co-factors related to the pattern of disease progression. As already established by previous studies (Zaunders *et al.*, 1995; Margolic *et al.*, 1995; Chirmule and Pahwa, 1996; Daniel *et al.*, 1996; Mittler *et al.*, 1996), the over all CD4⁺ T lymphocyte counts were found to be highly reduced in the HIV infected subjects. Low CD4 cell counts (CD4<200/mm³) were observed in the clinically healthy non-AIDS HIV seropositive subjects irrespective of parasitic infections. Our observation is consistent with the findings of Pollac (1993), where he had a considerable proportion of the Ethiopian immigrants in Israel to have low CD4⁺ cell counts (CD4<200/mm³) in spite of their being recently infected with HIV. Although no concrete explanation has been given for these observations, the atypically low CD4⁺ counts might indicate the difficulty in relying solely on this parameter as a prognostic marker, at least at an individual level, in Ethiopia.

Although the observed increase was not statistically significant, intestinal parasitic infections showed an increasing pattern in the CD4⁺ T cells irrespective of HIV infection. This implies that intestinal parasitic infections might increase activated CD4⁺ cells resulting in a higher number of host cells for HIV infection and replication. This may be a phenomenon that might be evident in the early phase of HIV co-infection with intestinal parasites, followed by reduction in CD4⁺ cells as the virus multiplies.

The dramatic increase of the over all total cytotoxic cells (CD8⁺ CTL) count observed with HIV infection is consistent with other studies (Ho *et al.*, 1993; Watret *et al.*, 1993; Lewis *et al.*, 1994; Zaunders *et al.*, 1995; Mittler *et al.*, 1996). This finding indicates the general immune stimulation that occurs early in HIV infection. The observed increase of total CD8⁺ lymphocytes was confined to HIV infection. Parasitic infections had no association with the level of CD8⁺ cells. This implies that parasitic infections would not influence the cytotoxic mechanism operating against HIV.

HIV associated disruption of the natural proportion of the CD4⁺ and CD8⁺ T cells population has resulted in the observed reduced CD4/CD8 ratio in the HIV infected individuals. The CD4/CD8 ratios in both the HIV seropositives and HIV seronegative subjects, was lower compared to observations in the industrialised countries (Zaunders *et al.*, 1995). This lower CD4/CD8 ratio may partly account for the relatively shorter period of conversion into AIDS in Africa by lowering the threshold for immunodeficiency, since this lowering of CD4/CD8 ratio is the result of the loss of CD4⁺ T cells (Fahey *et al.*, 1990; Bofill *et al.*, 1996).

The trend of increased expression of activation marker HLA-DR⁺CD38⁺ on both CD4⁺ and CD8⁺ T lymphocytes, was associated with intestinal parasitic infections. Increased expression of this marker was statistically significant in the CD8⁺ subset. In contrast to other studies (Levacher *et al.*, 1992; Watret *et al.*, 1993; Zaunders *et al.*, 1995; Peakman *et al.*, 1995), increased CD8⁺DR⁺CD38⁺ levels do not seem to be an exclusive feature of HIV seropositive patients (Fig. 10). No significant difference in the expression of this marker between the HIV

seropositive and their seronegative counterparts was observed. The increase expression in the HIV seronegative individuals probably is due to exposure to other infections, making the difference in the immune activation marker not much significantly different from the HIV seropositives.

Although there are some studies (Giorgi *et al.*, 1994) associating expression of these membrane molecules on the CD8⁺ cells with protection and long term survival, other reports show that DR is an activation-associated molecule and human T cells express this molecule upon activation (Prince and Jensen, 1991; Levacher *et al.*, 1992; Ho *et al.*, 1993; Bofill *et al.*, 1996; Mocroft *et al.*, 1997). The observed increased levels of CD8⁺DR⁺CD38⁻ expression may indicate an enhanced immune activation due to parasitic infections. This has been proved by its remarkable reduction following the removal of parasitic infections. Our observation corresponds to the increased activation markers observed in helminth-infected Ethiopian immigrants to Israel by Bentwich and his group (1996), although they worked on soluble activation markers.

Several studies have related immune activation of the host with the pathogenesis of HIV/AIDS (Daar *et al.*, 1991; Piatak *et al.*, 1993; Connor *et al.*, 1993; Mahalingan *et al.*, 1993; Ascher *et al.*, 1995; Bentwich *et al.*, 1995). Cellular activation is conducive for HIV replication and spread. There are indications that immune activation is associated with higher susceptibility to HIV infection.

Studies by Livingstone and colleagues (1996) have further shown a broader tropism of HIV than previously described, where they found widespread infection of lymphocytes of the CD8⁺ phenotype. From the observed increase of the activated immune cells during parasitic infections, the possibility that intestinal parasitic infections trigger HIV production in the latently infected individuals thereby exacerbating the course of the disease may be suggested. The long term effect of high parasite infection-associated DR⁺CD38⁺ expression needs to be further elucidated.

The increase in the proportion of CD8⁺ T cells, co-expressing CD38 and DR, observed in the HIV seropositive subjects, has been reported by other workers (Meidema, 1992; Ho *et al.*, 1993; Jouen-Beads *et al.*, 1996). This shows the existence of continuous HIV specific CD8⁺ cytotoxic response against the persistent antigenic stimulation in the HIV positive subjects. The observed increase of the proportion of CD8⁺ T cells co-expressing CD38 and DR was found to be confined to HIV infection. The effect of parasitic infections became significant only when HIV was introduced.

As HLA-DR⁺CD38⁺CD8⁺ lymphocyte population contained most of the circulating CD8⁺ cells with higher HIV-specific CTL activity in HIV infected subjects at early stages of the infection (Ho *et al.*, 1993; Giorgi *et al.*, 1994), co-infection with parasitic infections may down regulate the HIV specific activity of the CD8⁺ T lymphocytes. This could result in a reduction of a fraction of the CD8⁺ lymphocyte population. On the other hand, the observed pattern of reduction(not statistically significant) of CD4⁺DR⁺38⁺ cells after treatment in the HIV seropositive individuals, could be due to the reduction of the CD4⁺ cells as a result of HIV infection.

Similar to the observations of Watret and colleagues (1993), the naive CD8⁺ T cell proportions remained unchanged in the HIV positive individuals. On the other hand, although not statistically significant, parasitic infections have shown a pattern of decrease in both CD4⁺ and CD8⁺ naive cells. This observation together with the significant increase in the naive CD4⁺ cells following the removal of parasitic infections, may indicate the effect of parasitic infections in enhancing the activation of these cells.

Effector CD8⁺ cells (CD8⁺CD45RA⁺CD27⁻) were found to be reduced significantly with HIV infection. The observed reduction of the cytotoxic effector cells might be due to reduced levels of T helper cell function during HIV infection, since cells in this population subset depend on exogenous growth factor. That is, they require T helper cell derived signals for proliferation (Hamann *et al.*, 1997). According to Hamann *et al.*, (1997), such a quantitative reduction of effector cells (CD8⁺RA⁺CD27⁻) observed in HIV infection does not mean reduced effector activity because lysing of target cells can be performed by the CD28⁻ cytotoxic cells at the early stage of the disease. The immunological consequences of reduced CD8⁺ effector cell proportions during HIV infection needs further investigation, since parasitic infections and their removal by treatment was not shown to have any effect on these cells.

Even though it was not statistically significant, both CD4⁺ and CD8⁺ memory cells were found to show an increasing pattern with parasitic infections. The decrease of memory CD4⁺ cells observed in the HIV seronegative individuals following treatment for parasitic infections, shows reduced differentiation of the CD4⁺ immune cells as a result of the removal of persistent

antigenic stimulation by parasitic infections. This supports the above observation where by naive CD4⁺ cells were shown to increase with the removal of the intestinal parasitic infections which might indicate reduced activation and proliferation of these cells. Thus it can be assumed that the activation of CD4⁺ cells by persistent parasitic stimulation, that would precede further functional differentiation, could provide an ideal environment for HIV infection upon exposure.

6. CONCLUSION

Most of the major changes with HIV and intestinal parasite co-infection were seen in the CD8⁺ subset. That is, there was a significant increase of activated CD8⁺ cells and an increase in the level of CD8⁺ memory cells.

Increased levels of CD8 cells expressing the activation marker HLA-DR⁺CD38⁻ were seen with intestinal parasitic infections. This observation of high expression of the HLA-DR⁺CD38⁻ activation marker might indicate that intestinal parasitic infections may enhance the activation of the immune system that is known to culminate in exacerbating disease progression in HIV infection. The significance of this finding with regard to disease progression in HIV co-infection with intestinal parasites needs to be further examined in prospective studies.

The study has shaded some light on the understanding of the cellular immunology of HIV infection in patients co-infected with intestinal parasites. It is hoped that the preliminary information obtained through the present study would provoke more thorough studies that deal with the interaction of endemic infectious diseases and HIV infections versus the rate of disease progression to AIDS in the developing countries.

The present findings suggest an exasperating role of intestinal parasitic infections in HIV patients. This implies that proper elimination of intestinal parasites would contribute to the longevity and quality of life of HIV/AIDS patients.

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