

STUDY THE ROLE OF ERYTHROPOIETIN IN HIV-RELATED ANEMIA

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
RENU DEVI OMPRAKASH SINGH

UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH

DEPARTMENT OF VIROLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
MUMBAI

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**UNDER THE GUIDANCE OF
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STATEMENT BY THE CANDIDATE

As required by the University Regulation No R.2190, I wish to state that the work embodied in this thesis titled "*Study the role of Erythropoietin in HIV-related Anemia*" forms my contribution to the research work under the guidance of **Dr (Mrs.) Ranjana A. Deshmukh** at the **Haffkine Institute for Training, Research and Testing Mumbai-400012**. This work has not been submitted for any other degree of this or any other University. Whenever references have been made to previous work of others, it has been clearly indicated as such included in the "Bibliography".



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*Dedicated to my
loving and caring
parents*

|| Acknowledgement ||

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With all the flaws, I'm your own,

Renu Singh

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Introduction

&

Review of literature

INTRODUCTION

The first case of Acquired Immunodeficiency Syndrome (AIDS) was described in homosexual men in United States in 1981, and the etiologic agent, Human Immunodeficiency Virus (HIV) was identified by 1983. The extensive spread of HIV appears to have begun in the late 1970s or early 1980s in America, Australia and Western Europe (1). The first case of HIV infection in India was diagnosed among commercial sex workers in Chennai, Tamil Nadu in 1986. AIDS has since reached epidemic proportions worldwide, within less than two decades it has affected over 190 countries (2).

The origin of HIV:

It has been hypothesized that HIV has its roots in Africa. This is believed because certain Simian Immunodeficiency Viruses (SIVs) are closely related to HIV-1 and HIV-2. In *Sooty-mangabey*, a type of African monkey, a counterpart of HIV-2 has been demonstrated to have gene homology with SIVs found in these monkeys. (3) The HIV-2's connection to the *Sooty mangabey* is probably the most compelling evidence for animal to man transfer of HIV. A likely source of HIV-1 has been more difficult to pin down. The closest simian virus to HIV-1 discovered to date exists in certain chimpanzees. (4). Scientists have long recognized the ability of certain viruses and other pathogens to pass from animals to humans. Once an animal pathogen has infected people, it may then be passed from human to human. Although it has not been proven that HIV came from primates, scientific literature shows that SIV has been known to infect laboratory workers working with the virus. (5).

The earliest and most compelling evidence of HIV infection in humans has been shown in a plasma sample taken from a man, living in Democratic Republic of Congo, in 1959. Researchers believe that the ancestor of this strain may date to the 1940s or 50s and were introduced into humans a decade or earlier (6). In June and July of 1981, cases of an extremely uncommon pneumonia caused by *Pneumocystis carinii* pneumonia, and a very rare skin tumor, Kaposi's sarcoma, were first reported in previously healthy young homosexual and bisexual men (7). With the rapidly increasing number of cases, it was soon recognized that other life threatening infections and neoplastic diseases were also observed and found to be associated with an unexplained defect in cell mediated immunity, common to each of these patients (7).

By early 1982 the group of diseases was named as Acquired Immune Deficiency Syndrome (AIDS) by the Center for Disease Control and Prevention (CDC). (7) The term "syndrome" has been used because AIDS does not constitute a single illness, but rather encompasses a wide range of clinical diseases including specific life threatening infections and neoplasm's associated with a profound and irreversible, unexplained acquired disorder of cell mediated immunity. Since the appearance of the original definition in September of 1982, the CDC has subsequently revised this definition to accommodate other opportunistic infections, and decrease in CD4+ T cell counts. These manifestations of advanced HIV disease were termed as AIDS (8).

When the first cases of AIDS were reported, many hypotheses were proposed to explain the possible cause(s) of the newly recognized syndrome, but it is now widely accepted that AIDS is caused by a previously unknown human retrovirus, which was initially discovered and isolated in 1983 from patients with persistent generalized lymphadenopathy at the "Institute Pasteur" in Paris(7).

In initial studies the virus was called human T-lymphotropic virus type III (HTLV III), or lymphadenopathy-associated virus (LAV), or AIDS-related virus (ARV), it was then named as human immunodeficiency virus (HIV) by the International Committee on the Taxonomy of Viruses in 1986. (7)

Transmission of HIV (9):

1. Among adults, HIV spreads most commonly during sexual intercourse with an infected partner. During sex, the virus can enter the body through the mucosal linings of the vagina, vulva, penis, or rectum or rarely, via the mouth and possibly the upper gastrointestinal tract after oral sex. The likelihood of transmission is increased by factors that may damage these linings, especially other sexually transmitted diseases that cause ulcers or inflammation of the genital mucosa. Research suggests that dendritic cells of immune system, which reside in the local mucosa, may get infected with the virus and carry the virus to the lymph node; here other immune cells may get infected.

2. HIV also can be transmitted by contact with infected blood, most often by the sharing of needles or syringes contaminated with minute quantities of blood containing the virus. The risk of acquiring HIV from blood transfusions is now extremely small as all blood products in the country are screened routinely for evidence of the virus.
3. Almost all HIV-infected children acquire the virus from their mothers by prenatally, perinatally or postnatally.

EPIDEMIOLOGY (10):

According to estimate from the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), 37 million adults and 2.5 million children are living with HIV at the end of 2003. This is 50% higher than the projected figures of WHO in 1991. During 2003, approximately 5 million people were infected with HIV. Despite antiretroviral therapy 3 million people died because of HIV/AIDS in the year 2003. Deaths among those already infected will continue to increase for some more years, inspite of the prevention programs which may lessen the numbers of newly infected cases.

Around half of the younger people who were exposed to HIV are infected before they turn 25, and typically die of the life-threatening illnesses by the age of 35 years. As this disease is found in sexually active younger populations, the brunt of this life threatening disease is borne by younger generation leading to many orphans to be looked after by the community. By the end of 2001, the epidemic has left behind a cumulative total of 14 million

AIDS-orphans, defined as those who lost one or both parents due to AIDS before reaching the age of 15 years. In 2003 alone, an estimated 70, 0000 children aged 14 or younger were infected with HIV. Over 90% were babies born to HIV- positive women, who acquire the virus at birth or through breast-feeding.

India showed a sharp increase in the estimated numbers of HIV infections, from a few thousands in the early 1990s to a working estimate of between 3.8 million children and 4.6 million adults living with HIV/AIDS at the end of 2002 (11). With a population of over one billion, the HIV epidemic in India will have a major impact on the overall spread of HIV in Asia and the Pacific and indeed worldwide.

The spread of HIV within the country is as diverse as the societal patterns between its different regions and metropolitan areas. In fact India's epidemic is made up of a number of epidemics, and in some places they occur within the same state. The epidemic varies from states with mainly heterosexual transmission of HIV, to some states where injecting drug use is the main route of HIV transmission. Both tracking the epidemic and implementing effective programs for its prevention pose a serious challenge to the health authorities and communities in India. It would be easy to underestimate the challenge of HIV epidemic in India as the country has a large population, poverty, low literacy rate and consequently low levels of awareness. All these factors make HIV/AIDS as one of the most challenging public health problems ever faced by the country (11).

From the moment scientists identified HIV/AIDS, social responses of fear, denial, stigma and discrimination have accompanied the epidemic. Discrimination has spread rapidly, fuelling anxiety and prejudices against the groups most affected, as well as those living with HIV or AIDS. It goes without saying that HIV and AIDS are as much about social and psychological phenomenon as they are about biological and medical concerns. HIV is strongly associated with stigma, repression and discrimination, as individuals affected by HIV have been rejected by their families, their loved ones and their communities. This rejection holds as true in the richer and developed countries as to the developing countries like ours. This gives a different dimension to this grave and ever-growing problem of HIV/AIDS (12).

Some important factors, which contribute to HIV/AIDS – related stigma are,

- HIV/AIDS is life threatening disease
- People are scared of contracting HIV
- The disease's association with behaviors (such as sex between men, extramarital-sex, injecting drug use etc.) that are already stigmatized in many societies.
- People living with HIV/AIDS are often thought to be responsible for their immoral behaviors.
- Religious or moral beliefs.

The impact of HIV/AIDS on women is particularly very grave and serious. In many developing countries, including India, women are often economically,

culturally, and socially disadvantaged and lack equal access to treatment, financial support and education. In a number of societies, women are mistakenly perceived as the main transmitter of sexually transmitted diseases (STDs). HIV-positive women are treated very differently from men in many developing countries. Men are likely to be 'excused' for their behavior that resulted in their infection, whereas women are not. In India for example the husbands who infect them may abandon women living with HIV or AIDS. Rejection by wider family members is also common (12).

Government and national authorities sometimes cover-up and hide cases, or fail to maintain reliable reporting systems. Ignoring the existence of HIV and AIDS, neglecting to respond to the needs of those living with HIV infection and failing to recognize growing epidemics in the belief that HIV/AIDS 'can never happen to us' are some of the most common forms of denial. These denial fuels AIDS stigmas by making those individuals who are infected appear abnormal and exceptional.

The ever-growing infection in the main productive age group (between 15-35 years) has created a huge burden on the economy of the family and in turn to the country. The HIV/AIDS related death in this group has wiped out a whole generation in the countries like Africa, leaving a gap between the children who are orphaned because of parental deaths, and the older generation who are economically non-productive. Loss of a substantial proportion of the active work force is likely to have a serious socioeconomic impact, potentially impeding development. The economic impact of HIV is likely to be more

important for other diseases, as treatment and health care for this chronic disease will take its toll on the health budget. The costs of production loss are thought to be 10 to 20 times greater than direct medical costs for treatment. Together with the loss of income-earning opportunities and of productive labor time, the diversion of cash to medical expenses has been the main problem in the developing countries (13). The cronicity of the infection is also an added factor for the increased cost on the treatment of the HIV/AIDS infection, which once given to patients should be given life-long like retroviral therapy and treatments for concurrent opportunistic infections.

VIROLOGY (14):

HIV belongs to family *Retroviridae* (which has RNA as genetic material) and sub-family of *Lentivirinae*, or “slow growing” viruses. The course of infection with these viruses is characterized by a long interval between initial infection and the onset of serious symptoms. HIV has a reverse transcriptase enzyme (RNA-dependent DNA polymerase) within the virion. It is 100 nm in diameter, with glycoprotein surface projections approximately 8 nm in diameter. Within the envelope is an inner shell or capsid, which is icosahedral in shape, containing helical nucleoprotein. The genome is an inverted dimer of linear, single stranded, positive RNA that dissociates readily into two or three pieces. *Infectivity* is sensitive to ether, acid and heat. Replication of the viral RNA involves a DNA *provirus* that is integrated into host cellular DNA.

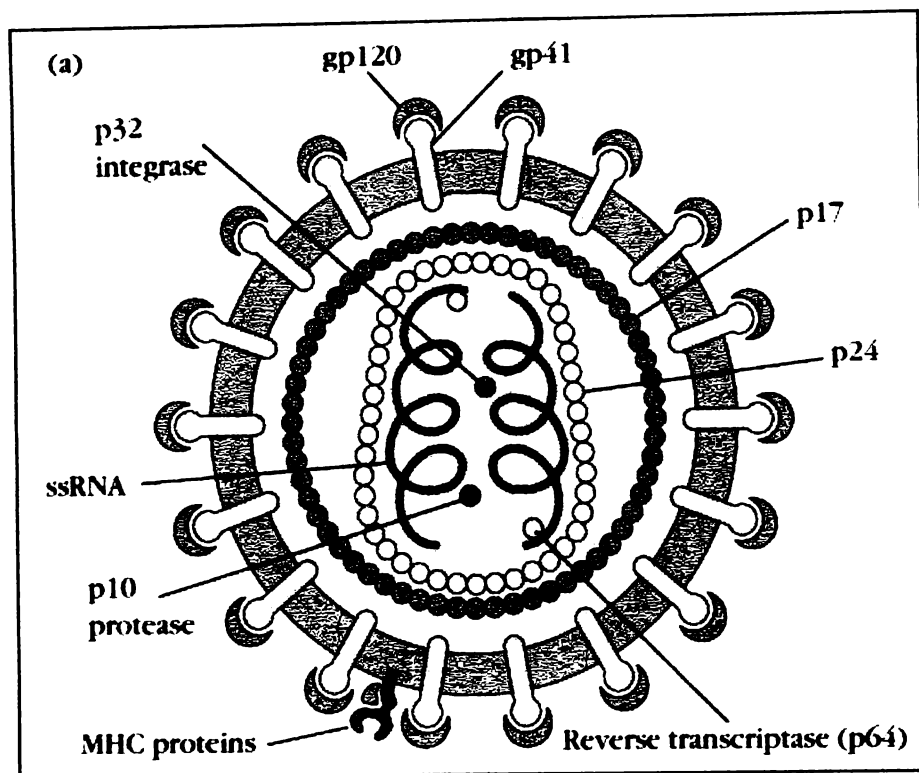


Figure 1: Diagrammatic representation of HIV virus

HIV characteristically shows, by electron microscopy, a cylindrical core within the viral envelope in contrast to the oncogenic retroviruses, which show spherical cores. The HIV genome contains, in addition to the three known structural genes *gag*, *pol*, and *env*, genes for at least six additional proteins, which induce antibodies in infected persons. Of these genes, *tat* and *rev* are of special interest since viral replication without the products of these genes apparently is not possible or at very low levels only.

Of the structural genes, *gag* codes for the core proteins p24, p17, and p15, which are cleared from a p55 precursor protein by the viral protease. The

RNA-dependent DNA polymerase (reverse transcriptase) is encoded by the **pol** gene, which also codes for the protease (p16) and the **integrase/endonuclease** (p31). The viral polymerase exists in two forms, p66 and p51. Associated with the polymerase is RNase H, which specifically **degrades RNA** in RNA-DNA hybrids.

The **envelope** proteins are responsible for virus adsorption and seem to be important for the cytopathic effects of HIV. They are encoded by the **env** gene and consist of gp160 and its two sub units, gp120 and gp41. gp120 is the **outer membrane** part of the envelope protein which forms the knobs on the virus surface, gp41 the transmembrane part by which gp120 is fixed to the viral membrane. Gp120 binds to the CD4 receptors of the T helper lymphocytes and other cells and mediates adsorption of HIV to the cell surface (14).

There are two types of HIV, HIV-1, the predominant AIDS- causing virus strain in the world, and HIV-2, which is biologically distinct second type of the virus. It was identified in 1986 and generally restricted to West Africa (15). The structure of HIV-2 is similar to that of HIV-1. However, HIV-2 has a distinct genes and different configuration of the envelope proteins particularly that of **gp120** (16).

HIV receptors and co-receptors:

HIV-1 coreceptor usage is believed to play a critical role in pathogenesis. To initiate infection, HIV-1 interacts with two cell surface receptors, CD4 is the

primary receptor and the β -chemokine receptors i.e. CCR5 and CXCR4, usually serve as secondary receptors. HIV-1 strains transmitted in vivo, generally use CCR5. Viruses that use CCR5 (R5 viruses) appear to be associated with relatively stable infection. Years after chronic infection is established, CXCR4 receptor utilizing strains emerge in 50% of infected individuals. Viruses that use the coreceptor CXCR4 (X4 viruses) are associated with rapid CD4⁺ cell decline and disease progression. However, the mechanism by which X4 viruses are associated with accelerated disease progression has never been properly elucidated, e.g., the association between X4 virus and acceleration of HIV-1 disease progression has been ascribed to the expanded spectrum of CXCR4⁺ precursor cells susceptible to infection by X4 strains. It has also been postulated that the decline of the host immune system associated with clinical AIDS may allow X4 viruses to evolve and replicate freely in late-stage infection. Discriminating between these and other alternatives is important to understand the HIV pathogenesis (17).

REPLICATION CYCLE (9)

The first step of infection is the binding of gp 120 to the CD4⁺ receptor of the cell. The binding is mediated, as shown in experiments with minus mutants, by three highly conserved regions of the carboxyl end of the gp120 molecule; it is a binding reaction of high affinity. The next important step is the fusion of the virus and cell membranes, which is mediated by the amino end of the gp41 molecule. This end of gp41 is freed probably by a conformational change of gp120 after its binding to CD4. The free amino end of gp41 can then intrude into the lipid layer of the cell membrane and thereby mediate

penetration of the virus into the cell. Uncoating, reverse transcription, provirus synthesis and integration, and subsequent synthesis and maturation of virus progeny as shown in the above figure. HIV-infected cells, like other retrovirus-infected cells, express retroviral envelope proteins on their cell surfaces (14).

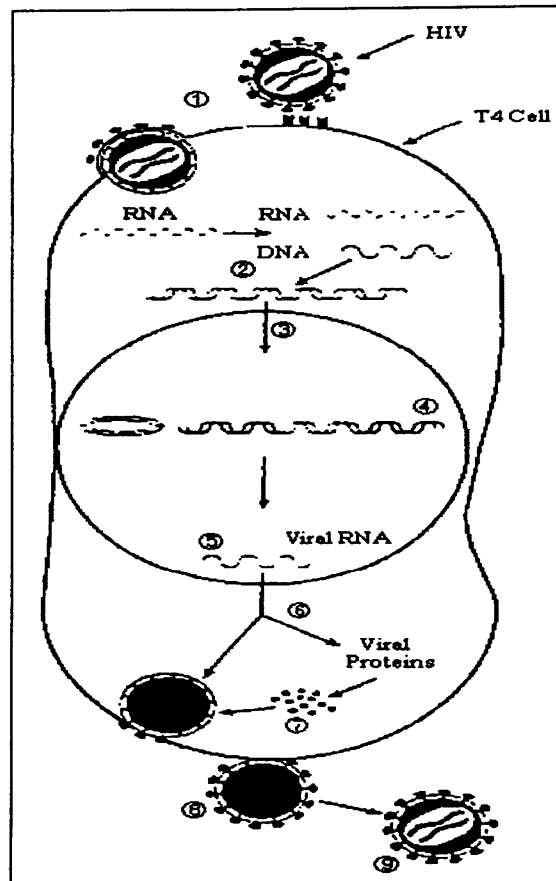


Figure 2: Replication cycle of HIV virus

PATHOGENESIS:

Natural history of disease:

In brief the course of HIV infection varies among individual patients. However a common pattern of disease progression has been recognized. Primary infection with HIV is followed by the development of detectable humoral and

cell mediated immune (CMI) responses. This is followed by a prolonged period (median 10 years) of clinical latency during which patient is usually asymptomatic which is followed by the appearance of continuous signs and symptoms (18).

Acute HIV infection:

Acute HIV infection is represented by an infectious, mononucleosis- like syndrome that develops within 3 weeks to 3 months time after initial exposure to HIV (19, 20, 21). It is characterized by vague symptoms such as the sudden onset of fever, sweat, myalgias, orthralgias, headache, sore throat, diarrhea, fatigue, malaise, a generalized lymphadenopathy and maculoerythematous trunkle rash which may last from 3 to 14 days.

After the acute phase has passed, other sequelae may occur, such as aphthous ulceration, extracerbalion of seborrhea or psoriasis recurrence of herpes simplex infection, and rarely rhabdomyolysis or acute renal failure or mesangioproliferative glomerulonephritis. Most patients are seronegative at the time of acute illness, but usually seroconvert within 2 months.

Early symptomatic diseases:

Early symptomatic HIV diseases, which was previously termed AIDS Related Complex (ARC), includes patients with symptoms suggestive of impaired cellular immunity but not meet CDC's criteria for AIDS (22, 23). Such conditions may include persistent generalized lymphadenopathy, recurrent fever, night sweat, malaise, oral or vaginal candidiasis, oral hairy leukoplakia,

recurrent herpes zoster infections (i.e. shingles), dermatitis, seborrhea, and immune thrombocytopenia.

Late symptomatic HIV disease:

During late disease, the risk of developing AIDS defining conditions rises substantially with CD4 cell counts dropping below 200 (24). Patients at late stage of disease are at risk of developing opportunistic infections with *Pneumocystis carinii*, *Toxoplasma gondii*, esophageal candidiasis, encephalitis, cryptosporidiosis, isosporiasis, tuberculosis, lymphoma and Kaposi's sarcoma are also some of the tumors commonly seen in these patients.

Neurological manifestations of the disease occur at this stage of infection. Some patients develop primary nervous system lymphoma, progressive multifocal leucoencephalopathy; HIV-associated dementia characterized by cerebral atrophy and diminished cognitive functions. HIV wasting syndrome defined as involuntary weight loss (> 10 pounds of usual body weight) is also seen (25).

The course and rate of HIV disease progression is quite variable. Although, the median time between initial infection and the development of AIDS defining illness is 10 - 12 years (slow- progressors), few individuals may progress to AIDS within 18 months (rapid progressors) while some individuals may not progress to the disease at all (long term non- progressors) (26).

IMMUNOPATHOGENESIS:

HIV outsmarts the host with an ingenious strategy of injecting a cell, which is essential for its elimination. The CD4+ lymphocytes play an important role in the immune network (27). The CD4+ T lymphocytes are crucial for the maintenance of appropriate immunological responses against a wide range of pathogens as well as malignant tumors. The interaction of CD4+ T cells with B cells, natural killer cells (NK cells), cytotoxic T cells and monocytes/macrophages is achieved primarily by the release of cytokines or other soluble factors and to a lesser extent, by the direct cell to cell contact. CD4+ T cell secretes factors that affect the growth and differentiation of non-lymphoid cells as well. As a result, it is clear that either quantitative or qualitative abnormalities in the CD4+ T cell population can have profound effects on immune system functions (28).

The precise mechanism whereby HIV brings about destruction of its target cells is not completely understood, but a number of probable mechanisms have been put forth:

1. High levels of viral RNA and aberrant RNAs accumulate in the cytoplasm of HIV infected cells in-vitro (29, 30). These RNA species may interrupt the normal cellular RNA processing, leading to cell destruction and death.
2. Massive virion budding from cell membrane of infected cell producing HIV may weaken the integrity of the cell membrane and causes lysis of the cell (31, 32).

3. Several studies have shown that intracellular complexing of CD4 and gp120 during assembly of virion plays an important role of HIV mediated cytopathicity (33, 34, 35).
4. Multinucleated giant cells and syncytia are formed from fusion of uninfected CD4+ T cells with infected cells. Syncytia formation has been implicated as a potential pathway for CD4+ T cell destruction, even in the presence of relatively few HIV infected cells (36, 37). HIV infected monocytes derived macrophages express gp120 and can fuse with uninfected CD4+ T cells in vitro (38). However, the role of syncytia formation in the immunopathogenesis of HIV infection in vivo remains unknown.

HIV replicates poorly and mechanisms other than direct HIV- mediated cytopathicity must be responsible for the slow but persistent CD4+ T cell decline (39). In addition to the direct HIV- mediated cell killing, indirect mechanisms may play a role;

AUTO IMMUNE REACTIONS:

Non-polymorphic determinants of MHC Class II particularly HLA- DR and HLA- DQ share homology with gp120 and gp41 proteins of HIV (40). And antibodies to gp120 and gp41 may cross- react with MHC class II molecules. Such antibodies have been found in HIV patients. The antibodies could prevent interaction between CD4 and class II molecules. Thus impairing

cellular interactions required for efficient antigen presentation and inhibiting antigen specific functions mediated by helper CD4+ T cells (40, 41).

PRESENCE OF AUTO ANTIBODIES (42):

The basis for an HIV-induced autoimmune response lies in the relationship between the HIV envelope glycoprotein and CD4. Because both HIV and MHC class II molecules bind to CD4, it has been hypothesized that an anti-HIV envelope response will also react with MHC class II molecule on the surface of uninfected cells. In this regard, it has been shown that a gp120 peptide with homology to MHC class II β -chains can enhance antigen-specific and auto reactive T cell activation. Numerous studies of AIDS patients have demonstrated the presence of antibodies that have the potential to react with the following: normal cells including lymphocytes, platelets, and Neutrophils; CD4+ molecules; and CD43+ molecules on the surface of normal thymic lymphocytes and IL-2. In addition, antibodies that react to peptides derived from regions of homology between HIV gp41 and MHC class II have been reported in HIV-infected individuals. There have also been reports of autologous antibodies bound to T cells in HIV infected individuals and cytotoxic T lymphocytes (CTL) that can lyse uninfected CD4+ T cells.

Autoimmune destruction of uninfected CD4+ T cells may also develop as a result of the binding of soluble HIV envelope glycoprotein to the surface of CD4+ T cells. The binding of free HIV gp120 molecules produced by infected cells to CD4 receptors on uninfected cells may inadvertently render these latter cells targets for HIV-specific CTLs or antibody-dependent cell-mediated

Cytotoxicity (ADCC). It has also been shown that in-vitro CD4⁺ gp120-specific CTL can kill uninfected CD4⁺ T cells that have taken up and processed bound gp120 molecules during the process of antigen presentation. It has also been shown that natural killer (NK) cells can mediate ADCC against uninfected cells that are coated with HIV antigens.

SUPER ANTIGENS (43):

One of the most recent hypotheses for HIV-mediated immunosuppression involves the role of HIV as superantigen in the deletion or modulation of specific subpopulation of CD4⁺ T cells. Superantigens differ from typical antigens in their interactions with T cells. During traditional antigen presentation, the class II MHC molecule bearing within its groove interacts with both the α and β subunits of the T cell receptors (TCR). The variable (V) regions of the α and β subunits are encoded by 100 and 60 different genes, respectively. The requirement for any given conventional antigen to interact with specific TCR α and β subunits will result in the stimulation of only a small percentage of T cells. Superantigens on the other hand do not require presentation within the groove of the class II MHC and need only interact with the $v\beta$ subunits of the TCR. As a result, superantigens can stimulate a much higher proportion of T cells than conventional antigens. After superantigen exposure, the stimulated cells undergo expansion and may be either deleted or rendered anergic.

To determine whether HIV-encoded or HIV-associated proteins may act as superantigens in vivo, several studies have analyzed the repertoire of $v\beta$ T cell subsets in HIV-infected individuals. In HIV disease, it is possible that the major role that the superantigens play in HIV disease is the activation of specific $v\beta$ subsets of CD4⁺ T cells, resulting in enhanced HIV infection and replication and subsequent cytopathicity.

APOPTOSIS (44):

It has been demonstrated that a larger proportion of CD4⁺ T lymphocytes from HIV-infected individuals undergo apoptosis after invitro stimulation, and it has been hypothesized that apoptosis represents the primary mechanisms of CD4⁺ T cell death in HIV infection. It has been shown that apoptosis of CD8⁺ T lymphocytes, which are not the targets of HIV infection, also occurs. Although apoptosis is greatly increased in HIV infection, recent studies performed in both peripheral blood and lymphoid tissue have failed to show a relation between the levels of apoptosis and clinical stage of HIV disease.

A number of molecular mechanisms that may be responsible for apoptosis in HIV infection are currently being proposed, including involvement of BCL-2 gene expression, increased sensitivity to reactive oxygen species, the role of cell-cycle and mitotic arrest and Fas/Apo1 antigen expression (44). In human cells, transduction of apoptotic signals has been variably ascribed to TNFR-55 to TNFR-75 or to both TNF-receptors. The kinetics of TNF-mediated apoptosis indicates that TNF-requires a larger period (48 hours) than Fas (24 hours) to induce apoptosis (45).

With regard to the physiological role of apoptosis in the immune system, It is the primary mechanism leading to the elimination of auto-reactive T cells (negative selection) and it may be involved in the regulation of the termination of antigen specific immune responses by causing death of those cells that have been stimulated repetitively with a specific antigen. The phenomenon of apoptosis is directly dependent on the state of cellular activation, which would explain why in addition to CD4+ T cells different effector cells involved in the normal immune responses, including those not permissive to HIV infection (i.e. B-cells and CD8+ T cells) may undergo apoptosis in HIV infected individuals. The increased apoptosis observed in HIV infection may reflect at least in part an amplification of the normal mechanism of elimination of activated effector cells that have been involved in specific immune responses to HIV and other pathogens. Apoptosis can also occur in several other conditions such as abnormal signaling after gp120- CD4+ interaction, inappropriate stimulation in both CD4+ and CD8+ T cells caused by abnormal antigen presenting cell function and deficiencies in certain growth factors such as IL-2 or abnormal patterns of cytokines expression (high IFN γ or low IL-2) (44).

MARROW ABNORMALITIES WITH HIV INFECTION:

The majority of patients with HIV infection exhibit morphological abnormalities of the bone marrow. The incidence of marrow abnormalities increases in frequency with progression of the HIV infection (46, 47). Although marrow abnormalities are common, none are specific for this disease (48, 49, 50).

The most common morphological abnormality is hypercellularity of the bone marrow (51, 52, 53), this occurs in 50- 60-% of cases (51, 54) and is due to **absolute hyperplasia** of one or more of the nonlymphoid cell lines (47). Marrow cellularity correlates neither with peripheral blood counts nor with the **stage** of the HIV infection (49, 50, 52,53). Hypocellularity of the bone marrow occurs in approximately 5% of cases (46), usually as a manifestation of **advanced** HIV infection (55). However, this may be due to hypoplasia of one **cell line** such as absolute erythroid cell-lines as a result of parvovirus or **mycobacterial** infections (48). In the end stages of HIV infection bone marrow **atrophy** and necrosis may occur (50). Dysplasia of atleast one cell line occurs in approximately 70% of HIV infected patients (51, 55, 56).

In general the degree and frequency of dysplastic changes in the marrow of HIV infected patients increases with concurrent opportunistic infections (54).

HIV INFECTION AND HEMATOPOIESIS:

Hematopoietic suppression is observed in HIV disease in the form of thrombocytopenia, granulocytopenia, **anemia**, lymphopenia and pancytopenia (57, 58). There is a significant loss or decrease in colony forming potential of **granulocytes**, erythrocytes, monocytes, megakaryocytes and erythroid cells (59). Some of the hematological manifestations may be the result of opportunistic diseases. But a certain proportion appears to be the direct result of the HIV- related effects. HIV can infect purified bonemarrow cells in vitro (60, 61). Infection of precursor cells in thymus is proposed to be one possible

explanation for the lack of regeneration of mature CD4⁺ T- lymphocytes during HIV infection.

IMMUNOPATHOLOGICAL DEFECTS IN THE OTHER TYPES OF IMMUNE CELLS:

Various deficiencies in chemotaxis, monocyte dependent T- cell proliferation, Fc receptor function, C3 receptor mediated clearance and oxidative burst responses have been reported in HIV infection. Infection of monocytes/ macrophages has been shown in vitro as well as in vivo which may cause impairment of antigen dependant cell cytotoxicity (ATCC), intracellular antimicrobial activity (62), and induction of IFN- α secretion (63).

Elevated number of B-cells spontaneously secreting antibodies and their refractoriness to signals for T-cell independent B-cell activation are characteristic of HIV infection (64). Circulating B-cell levels decrease early in the course of disease and the decrease becomes more pronounced as the disease progresses (65). B-cells from HIV infected individuals secrete higher levels of cytokines in response to gp120 and HIV (66, 67).

Since HIV is not completely eliminated by the immune responses, its persistence over time induces a chronic activation of immune system. This chronic activation of immune system reflects continuous activation of different effector cells involved in the ongoing immune response to HIV and/ or other pathogens. However certain components of this general state of immune

activation may be detrimental to the body and contribute to HIV pathogenesis and disease progression.

The chronic immune activation may potentiate virus replication either by stimulating secretion of various cytokines, which in turn activate various immune cells, which induce a large pool of activated target cells for HIV that may efficiently support virus replication. The progressive responses favour virus dissemination. Clearance of newly produced HIV will be less efficient and the vicious cycle of pathogenic events will be perpetuated.

After prolonged period, the balance tilts towards the detrimental counterpart of the chronic state of immune activation and the outcome is the severe deterioration of the immune system.

HIV AND CYTOKINES:

Activation of T- cells enhances HIV entry in to the cell, triggering the completion of reverse transcription and viral DNA integration or by stimulating viral transcription from proviral DNA (68). The fact that activated CD4+ T- cells are major contributors to the production of cytokines and cytokines exhibit either activation or suppression of the cell that produced them. On the basis of their ability to modulate virus expression and replication, cytokines are divided into;

1. Inducer of viruses
2. Suppressor of virus expression
3. Bifunctional modulators

Tumor Necrosis Factor- alpha (TNF- α):

Tumor necrosis factor alpha (TNF- α) i.e. cachectin is a pleiotropic pro-inflammatory cytokine. It was first isolated by Carswell *et al* in 1975 in an attempt to identify tumor necrosis factors responsible for necrosis of the sarcoma (69). TNF is produced by stimulated monocytes and macrophages, T-lymphocytes, stimulated B cells and vascular endothelial cells (70). Most organs of the body appear to be affected by TNF- α and the cytokine serves a variety of functions, many of which are not yet fully understood. TNF- α possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self-regulatory properties as well. In 1990, Tracey and Cerami suggested two beneficial functions of TNF- α , which have lead to its continuous expression,

1. Low levels of TNF- α may help in maintaining homeostasis by regulating the body's circadian rhythm.
2. Low levels of TNF- α promote the remodeling or replacement of injured and senescent tissue by enhancing fibroblast growth

Additional beneficial role of TNF- α is in the immune responses to bacterial, certain fungal, viral and parasitic infections as well as its important role in the necrosis of specific tumors. It also acts as a key mediator in the local inflammatory immune responses. It helps in increasing vascular permeability, thereby recruiting macrophages and neutrophils to a site of infection. TNF- α secreted by the macrophages causes blood clotting which serves to contain the infection (69).

On the other hand high levels of TNF- α exhibit chronic effects as well as resulting in acute pathogenesis. Prolonged overproduction of TNF- α also results in a condition known as cachexia, which is characterized by anorexia, increased catabolism, weight loss and anemia which is common phenomenon in illnesses like cancer and AIDS.

Structure:

TNF- α is a trimeric protein encoded within the MHC complex. It was first identified as its 17 KD secreted form, but further researchers showed that a noncleaved 27 KD precursor form also exist in transmembrane form. Stimulated macrophages produce 27 KD TNF- α , which can either bind directly to TNFR-55, and TNFR-75 receptors through cell to cell contact or undergo cleavage and bind in its soluble form. Due to its jelly roll like structure, which it shares in common with viral core protein, it has been hypothesized that TNF- α and virus originated from a common ancestor cell (69). TNF- α shares only 36.10% amino acid sequence homology with TNF- β , also called lymphotoxin (LT). Yet the tertiary structures of the two proteins are remarkably similar and both bind to TNF receptors TNFR-55 and TNFR-75. These receptors are expressed on all somatic cells (69).

Although it is not produced constitutively by HIV- infected cells, it can be produced in response to diverse stimuli concurrent with the HIV infection. The Tat protein of HIV-1 may activate TNF- α genes in vitro. In particular, cytotoxic T lymphocytes are capable of producing TNF- α on contact with targeted cells

presenting HIV-1 antigens. Monocytes and macrophages are capable of producing TNF- α after contact with gp120, and this does not require the presence of live virus.

TNF- α may act with ceramide as a second messenger. HIV-1 binding to surface receptors stimulates the hydrolysis of plasma membrane sphingomyelin to ceramide, initiating a molecular cascade that results in transcriptional activation of HIV-1 provirus. TNF- α levels are elevated in the sera of HIV- infected patients, and rise progressively with the disease. In vitro, TNF- α can maintain HIV expression in chronically infected cells. In vitro studies reveal that TNF- α also functions in an autocrine and paracrine manner to upregulate HIV expression. TNF- α has been shown to upregulate HIV expression in a variety of acutely and chronically infected T and monocytic cell lines and primary macrophages. TNF- α potentiates viral replication via the induction of NF- κ B protein, which acts as transcriptional activators of HIV. Abnormal cytokine secretion by T cells, B cells and macrophages in lymphoid organs of HIV infected individual play an important role in the propagation of HIV infection. Recent data showed that TNF- α preferentially induces lysis of HIV infected cells.

TNF- α can act as a positive or negative regulator of hematopoiesis. It can alter the production of growth factors by marrow stromal cells and modulate the expression of cell surface receptors for growth factors on cells, which are targets of HIV infection. The concentration of TNF- α is important to the

quantitative effects on hematopoiesis. Low concentrations stimulate IL-3 and GM-CSF-induced colony formation but high levels inhibit the action of these growth factors. TNF- α also has differential effects on various hematopoietic cell lines; the same concentrations of TNF- α that stimulates growth of committed granulocytes and monocyte progenitor cells inhibit erythroid growth. TNF- α indirectly suppresses hematopoiesis by inducing the production of IL-1 by monocytes (70).

Th1 AND Th2 SHIFT IN HIV INFECTION:

Loss of helper T-cell function in asymptomatic individuals is seen even before the fall in number of CD4⁺ T cells. This is evident from in vivo and in- vitro studies shown by Clerici *et al* (71). Progression of HIV infection from asymptomatic to symptomatic stage and then to AIDS involves shift from Th1 (mainly cell mediated immune responses) to Th2 (mainly humoral responses). The main cytokines which play an important role and modulate these responses are IFN- γ , IL-2, IL-12, IL-15 (Th1 type cytokines) and IL-4, IL-5, IL-10, IL-13 (Th2 type cytokines).

Cytokine induced immune dis-regulation is a central component in progression to AIDS (71). This shift in type of immune response and consequent progression to AIDS may be on account of the fact that HIV infects T cells primarily as it enters the host body. This shift in immune responses is associated with shift in cytokine profile also, and this change in cytokine profile results in inability of host system to combat with the opportunistic

pathogens and hence to the opportunistic infections and neoplasms which ultimately prove fatal.

ANEMIA:

Anemia comes from Greek word "Anemia" meaning blood deficiency or lack of **Red Blood Cells (RBCs)** (72). Anemia can be defined as a reduced oxygen carrying capacity of the blood (73). Whatever is the etiology, the reduction in the oxygen carrying capacity results into multisystem involvement.

The basic pathophysiological categories of anemia are;

1. **Blood loss**
2. **Impaired blood cell production (ineffective erythropoiesis)**
3. **Excessive red cell destruction**

It is estimated that about 30% of the world's population are anemic. More than half of these cases are of iron deficiency anemia (74).

Anemia in HIV:

Anemia is the commonest co-morbid condition of HIV infection with incidences ranging between 10 - 90% among HIV/ AIDS patients (75). Though being the commonest underlying causes of ill health in HIV/ AIDS patients, anemia is multifactorial (75). Etiology of anemia can include:

1. **Nutritional deficiency**
2. **HIV infection itself (infection of stem cell)**
3. **Medication**
4. **Malabsorption**

Table 1: Causes of anemia and mechanism in HIV affected individuals
(76,77)

CAUSES OF ANEMIA	MECHANISM
Decreased RBC production	A) Neoplasm Infiltration Bone Marrow
Retic count low indirect	Lymphoma
Bilirubin low or normal	Kaposi's sarcoma
	Hodkins Disease
	Others
	B) Infection
	Mycobacterium tuberculosis
	Fungal infections
	Cytomegalo virus
	Atypical mycobacterium
	B19 parvovirus
	Others
	C) Drugs
	Zidovudine
	Trimethoprim/ sulphamethoxazole
	Pyrimethamine
	Amphotericin B
	Dapsone
	Acyclovir
	Interferon- alpha
	Pentamidine
	Ganciclovir
	5- flucytosin
	Trimetrexate
	D) HIV
	Abnormal growth of burst forming units
	Erythroid (BFU-E)
	ANEMIA OF CHRONIC DISEASES
	Blunted erythropoietin production/ response
	E) Iron deficiency anemia secondary to blood loss
	Blood loss
Ineffective production	A) Folic acid deficiency
(Retic count low, indirect	Dietary
bilirubin high)	Jejunal pathology: malabsorption
	B) B12 deficiency
	Malabsorption of ileum
	Gastric pathology with decreased production of intrinsic factor (I.F.)
	PRODUCTION OF ANTIBODIES TO INTRINSIC FACTOR, IE. PERNICIOUS ANEMIA
Increased red cell	A) Coombs positive hemolytic anemia
Distruption- hemolysis.	B) Thrombotic thrombocytopenia
(Retic count high, indirect	C) Disseminated intravascular coagulation
Bilirubin high)	D) Hemophagocytic syndrome
	E) Drugs
	Sulphonamides
	Dapsone
	Oxidant drug in G6PD deficiency

Other main causes leading to anemia are, anemia caused due to chronic diseases (ACD) or can be due to presence of various opportunistic infections in HIV/ AIDS patients, or it can be due to autoimmune diseases e.g. Coomb's positive autoimmune hemolytic anemia (78).

Patient of chronic HIV disease complains of fatiguability, breathlessness, weakness, palpitations and inability to look after him/herself, with a reducing work capacity day by day (79). With advancing immunodeficiency, the severity of anemia also increases adding to the morbidity and mortality. With advanced HIV disease, the anemia does not respond to hematinic therapy. Anemia of chronic infections or anemia of chronic diseases (ACD), which is characterized by normochromic and normocytic anemia with a normal TIBC, improves with the treatment of infection.

Serum ferritin:

Iron dependent anemia is the most common diet or nutrition related anemia found in HIV/AIDS patients. When the iron is absorbed from the small intestine, it immediately combines in the blood plasma with a beta globulin apotransferrin to form transferritin, which is then transported in the plasma. The iron is loosely combined with the globulin molecule and consequently can be released to any of the tissue cells at any point in the body. Excess of iron in the blood is deposited in all cells of the body but especially in the reticuloendothelial cells and liver hepatocytes. In the cell cytoplasm, it combines mainly with a protein, *Apo ferritin* to form *Ferritin*, but ferritin can be incorporated by phagolysosomes to *Hemosiderin*. There are about 2 gm of

iron in the adult female and up to 6 gm in the adult male, out of which 1.5 to 2 gm of this total iron is found in red blood cells as heme in hemoglobin and 0.5 to 1 gm occurs as storage iron (80).

The ferritin molecule consists of 24 protein sub-units, each with molecular weight of 20 KD. The primary ferritin function consists in accumulating intracellular iron thereby protecting the cells from toxic effects of unbound iron; it also represents a reservoir of easily released iron. Ferritin is mostly found in liver cells and in the reticuloendothelial system of liver, spleen and bone marrow. Minor ferritin amounts are also found in heart, pancreas and kidneys. Small but significant amounts are found in human serum.

The exact role of serum ferritin is unknown; however there is a close correlation between serum concentration of this protein and the status of body iron stores. Measuring serum ferritin levels represents therefore a simple non-invasive method to evaluate iron metabolism. Serum ferritin concentration trends reflect iron balance variations due to age and sex. In normal subjects the mean levels are slightly higher at birth after which they decrease during childhood until puberty is reached.

Subsequently a progressive increase in iron storage is observed in the male body with consequent rise of ferritin levels. In females during their child bearing years, lower and more stable value are observed until menopause, when ferritinemia tends to rise. Ferritin levels below normal ranges clearly

indicate iron deficiency, thereby allowing a differential diagnostic between iron and non iron deficiency anemia (81, 82).

Laboratory testing for iron may include tests of;

- Serum iron
- Serum iron-binding capacity
- Serum ferritin
- Complete Blood Count (CBC)
- Bone marrow biopsy
- Liver biopsy

The simplest test that indirectly give an indication of iron stores are the serum iron and iron binding capacity, with calculation of the percentage of transferrin saturation. The serum ferritin correlates well with iron store, but it can also be elevated with liver diseases, inflammatory conditions and malignant neoplasms. The CBC also gives an indirect measure of iron stores because of MCV can be decreased with iron deficiency (83).

PATHOLOGY OF ANEMIA IN HIV:

Anemia is a common manifestation of HIV infection and occurs at all stages of HIV-associated disease (84). The epidemiology of HIV-associated anemia was determined in a longitudinal study of 32,867 persons with HIV infection who received medical care in hospitals or HIV clinics between January 1990 and August 1996 (76). The one-year incidence of anemia (defined as a hemoglobin level <10 g/dL or a physician diagnosis of anemia) was 36.9

percent in persons with one or more AIDS-defining opportunistic illnesses, 12.1 percent in those with a CD4+ count <200 cells/mm³ or CD4+ cells <14 percent without clinical AIDS, and 3.2 percent in those without clinical or immunologic features of AIDS.

HIV-related anemia remains a significant problem despite recent advances in antiretroviral therapy including the introduction of Highly Active Anti Retroviral Therapy (HAART) in routine clinical practice (85). The prevalence of anemia was recently determined in 4,183 persons with HIV infection who obtained medical care between January and December 2000 (86). The majority of patients in this study were treated with 3 antiretroviral drugs. Anemia (defined as a hemoglobin level <12 g/dL in women and <13 g/dL in men) was documented in 12-41 percent of the study group, with the highest prevalence occurring in women. Overall, women had an 80 percent increased risk for both anemia and severe anemia (hemoglobin level <10 g/dL in women and <11 g/dL in men) when compared with men.

The prevalence and correlates of anemia were determined in 2056 HIV-infected women and 569 HIV-negative women who were studied as part of the US National Institutes of Health-sponsored Women's Interagency HIV Study (87). Anemia (defined as a hemoglobin level <12 g/dL) was documented in 37 percent of the HIV-infected women and 17 percent of the HIV-negative controls. Among the HIV-infected women, anemia was significantly more common in those with CD4+ counts <200 cells/mm³,

increased plasma HIV RNA levels, history of a clinical AIDS-defining condition, and in those currently treated with zidovudine.

Abnormalities of hematopoiesis in HIV infection:

Basis for normal hematopoiesis. The hematopoietic stem (progenitor) cell, termed Colony-Forming-Unit-Granulocyte/Erythroid/Monocyte/Megakaryocyte (CFU GEMM) is necessary for normal blood cell production and provides the source from which all hematopoietic cells are produced. CFU-GEMMs are found in the bone marrow and in those organs in which hematopoiesis occurs in the embryo, including the spleen, liver, and peripheral blood, although CFU-GEMMs in these latter sites do not function except under conditions of extreme stress in the adult. The vast majority of CFU-GEMMs in the marrow are quiescent, with only about 5 percent dividing at any given time. CFU-GEMMs are difficult to distinguish morphologically, with an appearance similar to that of small lymphocytes, but they may be identified by the presence of various markers on the cell surface including the CD34 antigen (88).

Under normal conditions, the CFU-GEMM differentiates, giving rise to committed progenitor cells with a more limited capacity for self-renewal and restricted range of cell lineage differentiation. In the environment of various growth factors such as Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), the CFU-GEMM will eventually give rise to the Colony-Forming-Unit-Granulocyte-Macrophage (CFU-GM), which in turn, gives rise to granulocytes and monocytes. In the environment of **erythropoietin**, the CFU-GEMM gives rise to the Burst-Forming Unit-Erythroid (BFU-E) followed by the

Colony-Forming Unit-Erythroid (CFU-E) and then the pronormoblast, which is the first recognizable erythrocyte precursor in the marrow. The CFU-E is capable of differentiating specifically into the mature erythrocyte, with a life span of about 120 days. In the presence of thrombopoietin, the CFU-GEMM eventually differentiates into recognizable megakaryocytes and platelets (88).

Normal hematopoiesis also requires an appropriate microenvironment within the marrow. The normal marrow microenvironment includes the presence of T lymphocytes, macrophages, endothelial cells, and fibroblasts. The cells comprising the marrow microenvironment produce the various cytokines and hematopoietic growth factors required for the proliferation and differentiation of CFU-GEMM, including IL-3, stem cell factor, and hematopoietic colony-stimulating factors.

HIV infection of CFU-GEMM:

There is great controversy regarding whether HIV can infect CFU-GEMM. A mechanism for HIV infection of CD34+ progenitor cells is present, as these cells have been shown to express both the CD4 receptor and the CXCR4 (fusin) coreceptor needed for HIV-1 entry (89, 90). Some degree of CFU-GEMM infection by HIV may occur, creating the potential for abnormal hematopoiesis (61, 91). The degree of infection of CFU-GEMM by HIV is probably minor, however, since consistent evidence of such infection has simply not been demonstrated (91, 92, 93).

Numerous defects in the growth of committed progenitor cells may occur in the setting of HIV infection. Thus, while infection of CD34+ progenitor cells by HIV is controversial, it is clear that stromal elements of the marrow microenvironment including T lymphocytes, fibroblasts, endothelial cells, and macrophages are all readily infectable by HIV (94, 95, 96, 97). HIV-infected stromal cells have been shown to produce decreased amounts of granulocyte colony-stimulating factor and interleukin-3, leading to a decreased ability to support normal hematopoiesis. Exposure of progenitor cells to HIV may also promote apoptosis in these cells with marked decreases in colony growth. Further, cytokine dysregulation, with increased production of inhibitory cytokines such as TNF- α and interferon-gamma by HIV-infected stromal cells in the marrow, is another potential cause of decreased growth of hematopoietic colonies.

Causes and mechanisms of HIV-associated anemia:

Multiple interacting factors contribute to the development of anemia in persons with HIV infection (98). Direct infection of hematopoietic progenitors by HIV is a possible underlying mechanism as well as decreased production of hematopoietic growth factors and increased production of inhibitory cytokines (99). Intercurrent opportunistic infections may cause bone marrow suppression or induce specific cytopenias in persons with HIV infection. Therapies used to treat HIV infection and its complications are also frequently implicated as causes of myelosuppression and hematologic dysfunction. Deficiencies of vitamin B12, folate, and iron are frequently reported in persons with HIV infection. Abnormally low levels of endogenous erythropoietin may

also be a contributing factor in the pathogenesis of HIV-related anemia, related to impaired erythropoietin production as well as antibodies against erythropoietin (100, 101).

Decreased erythrocyte production:

HIV-associated hypoproliferative anemia may result from suppression of CFU-GEMM by inflammatory cytokines or HIV itself (94, 102). Blunted production of erythropoietin has been documented in anemic HIV-infected patients, similar to the suppression seen in other states of chronic infection or inflammation (100). Infiltration of the marrow by tumor, such as lymphoma, or by infection such as *Mycobacterium avium* complex (MAC), may also lead to decreased erythrocyte production (103). MAC may also be associated with cytokine-induced marrow suppression. Involvement of the gastrointestinal (GI) tract by various infections or tumors may lead to chronic blood loss, with eventual iron deficiency anemia.

Another prominent cause of hypoproliferative anemia in-patients with HIV infection is the use of multiple medications, many of which cause marrow suppression. Zidovudine (ZDV) is uniformly associated with macrocytosis (mean corpuscular volume [MCV] >100 fl), a condition that can be a useful objective marker of compliance with this medication (104). Of note, transfusion-dependent anemia (hemoglobin <8.5 g/dL) has been reported in approximately 30 percent of patients with AIDS who are receiving 600 mg/d of ZDV. However, the incidence of severe anemia is only 1 percent when the same dose of ZDV is used in-patients with asymptomatic HIV infection (105).

Stavudine (d4T) has also been associated with erythrocyte macrocytosis, although anemia is uncommon with this drug (106).

Infection of the marrow by parvovirus B19 is another possible cause of hypoproliferative anemia in HIV-infected patients, resulting in specific infection of the pronormoblast (107, 108). While marrow failure has been described in association with parvovirus B19 infection, a pure erythrocyte aplasia is the usual consequence, with resultant anemia and minimal or absent neutropenia or thrombocytopenia. Parvovirus infection is usually acquired during childhood, leading to Fifth Disease, one of the common childhood exanthems. Exposure to parvovirus leads to an antibody response with subsequent resistance to further infection. Approximately 85 percent of adults have serologic evidence of prior parvovirus infection. However, the seroprevalence of such antibodies among HIV-infected patients is only 64 percent. This observation would suggest that HIV-infected persons may be unable to maintain an adequate humoral response to parvovirus; leading to reactivation of preexisting latent viral infection, or those persons may have an inadequate immune response against newly acquired infection.

Increased erythrocyte destruction:

Hemolytic anemia may develop in HIV-infected patients with glucose-6-dehydrogenase (G6PD) deficiency, when exposed to oxidant drugs, as well as in those with disseminated intravascular coagulation (DIC) or thrombotic thrombocytopenic purpura (TTP) (109). Heinz bodies are seen in blood smears in association with G6PD deficiency, and the presence of fragmented

erythrocytes and thrombocytopenia is observed in the latter conditions. Hemophagocytic syndrome has been described in association with HIV infection, with phagocytosis of erythrocytes by macrophages within the marrow.

An additional cause of erythrocyte destruction and resulting anemia in patients with HIV infection is the development of autoantibodies with resultant decreased erythrocyte survival (110). Of interest, the presence of antibody coating the erythrocyte surface (positive Coombs' test) has been reported in 18 percent to 77 percent of HIV-infected patients, although the incidence of hemolysis is extremely low. The presence of anti-i antibody and antibody against auto-U antigens has been described, occurring in 64 percent and 32 percent of HIV-infected patients, respectively (111, 112, 78). A positive direct Coombs' test may also be seen as a nonspecific finding secondary to the polyclonal hypergammaglobulinemia as known to occur in patients with HIV infection (113).

Ineffective erythrocyte production:

Deficiencies in vitamin B12 and/or folic acid may result in anemia due to ineffective erythrocyte production. Folic acid is absorbed in the jejunum and plays a key role in DNA synthesis. A deficiency of folic acid leads to a reduction in red blood cells, platelets, and neutrophils. In the peripheral blood, one sees a megaloblastic anemia, characterized by the presence of large, oval erythrocytes, hypersegmented neutrophils, and decreased platelets.

HIV-infected patients with inadequate nutritional intake, as well as those with underlying disease of the jejunum, may be unable to absorb sufficient amounts of folic acid and thereby develop anemia, neutropenia, and thrombocytopenia.

Malabsorption of vitamin B12 can occur relatively easily in various disorders of the stomach (achlorhydria), with the production of antibodies to intrinsic factor or parietal cells (pernicious anemia), or in various disorders of the small bowel or ileum (infection, Crohn's disease). Patients with HIV infection appear to have an increased susceptibility to B12 malabsorption, possibly due to the myriad of infections and other disorders that may occur in the small intestine of these patients. Negative vitamin B12 balance has been documented in approximately one-third of patients with AIDS, the majority of whom demonstrate defective vitamin B12 absorption (114).

Consequences of HIV-related anemia: survival and disease progression:

The impact of anemia on survival in persons with HIV infection was examined in the Multistate Adult and Adolescent Spectrum of HIV Disease Surveillance Project, in which the medical records of over 32,000 persons with HIV infection were reviewed (75). Anemia (defined as a hemoglobin level <10 g/dL or a physician diagnosis of anemia) was associated with an increased risk of death in the study group. Among persons with CD4+ counts >200 cells/mm³ at study entry, the risk of death was 148 percent greater in those with anemia than in those without. Among persons with CD4+ counts < 200 cells/mm³ at the time of enrollment, the risk of death was 58 percent greater

in those with anemia than in those without anemia. The risk of death decreased in- patients, who recovered from anemia, whereas the *risk of death was*, increased 170 percent in persons who did not recover from anemia.

A similar relationship between anemia and mortality in persons with HIV infection was observed in a study of 2,348 patients treated in a large urban HIV clinical practice between 1989 and 1996 (115). Importantly, the use of erythropoietin and antiretroviral therapy were associated with a decreased risk of death in anemic patients. An analysis of data from the EuroSIDA study, performed in 6,725 persons with HIV infection, revealed that severe anemia (defined as a hemoglobin level < 8 g/dL) was a strong independent risk factor for death after controlling for CD4+ cell count and plasma HIV RNA level (116). In the Women's Interagency HIV Study, a study of 2,056 women with HIV infection, anemia was a significant independent predictor of decreased survival (87, 117). Thus, several large cohort studies have demonstrated that anemia is a significant independent risk factor for decreased survival in persons with HIV infection.

Anemia may also be associated with increased risk of clinical and immunologic disease progression in persons with HIV infection treated with HAART. In a recent case-control study of 501 patients with HIV infection who were treated in a large urban infectious diseases clinic between 1996 and 1998, the odds ratio for the development of any AIDS-related opportunistic infection or death was 5.17 in patients with anemia (defined as >2 hemoglobin test results <11 g/dL) as compared with controls (118). The odd ratio for HIV

disease progression and death was 3.3 and 8.7 in women and men with anemia, respectively, as compared with their non-anemic counterparts.

ERYTHROPOIETIN (EPO):

The development of anemia usually results in increased production of erythropoietin in an attempt to correct the low hemoglobin level. In the setting of HIV infection, the normal compensatory erythropoietin response to anemia is impaired, resulting in a decreased ability of the marrow to respond to the anemic state. The mechanism of decreased erythropoietin production in HIV infection is a post-transcriptional defect in production, with normal levels of messenger RNA for erythropoietin but decreased production of the erythropoietin protein. The development of auto-antibodies to erythropoietin has been described in HIV-infected patients and may also contribute to the pathogenesis of HIV-related anemia (103).

Our understanding of the biology of erythropoietin is based on a series of observations made over the past 150 years. Many famous scientists and physicians of earlier times refuted the concept that red blood cell production was influenced by a humoral factor, erythropoietin. Despite this pessimism, continued perseverance to understand hematopoiesis ultimately led to the isolation and molecular cloning of erythropoietin (119). IN 1863, Dennis Jordanet (120) observed that the blood of high altitude-dwellers in Mexico was more viscous than the blood of individuals who live at lower altitudes. He also found that patients who suffer altitude sickness also suffer with the same

symptoms as patients with blood loss, mainly shortness of breath, dizziness, rapid pulse and fainting.

In 1890, Viault (121, 122) demonstrated during a trip from Lima, Peru to the mining town of Morococha (15,000 feet) that his red blood cell count increased from 5 million to 8 million cubic millimeters (cc^3). This was paralleled by erythrocyte counts obtained from other travelers, a lively dog, and a rooster. Thus, exposure to high altitude over a short period of time led to an adaptive altitude polycythemia.

In 1893, Friedrich Meischer (123), realized that the hypoxia accounted for this effect. He postulated that the low oxygen pressure directly stimulate bone marrow to increase red cell production. This became the predominant view of Erythropoiesis for more than 50 years. In 1906, Paul Carnot (124) from university of Paris injected normal rabbits with serum obtained from anemic rabbits. The results were startling. The recipient rabbits increased their red blood cell counts. Based on this experiment Carnot suggested that a "hemopoietine" present in the anemic serum was responsible for the increase in red blood cell production. Banting and Starling (125) coined the term "Erythropoietin" in their article entitled, "a humoral mechanism in anoxic erythrocytosis", rather than hematopoietin because the effect of the factor appeared on red blood cells and not on other hematopoietic lines. In 1953, Allan Ersly (126) injected larger quantities of plasma of anemic rabbits in to normal rabbits.

The number of nucleated red blood cells in the marrow, the no of peripheral reticulocytes and the hematocrits of the normal rabbits increased dramatically. This proved that red cell production was mediated by a humoral factor.

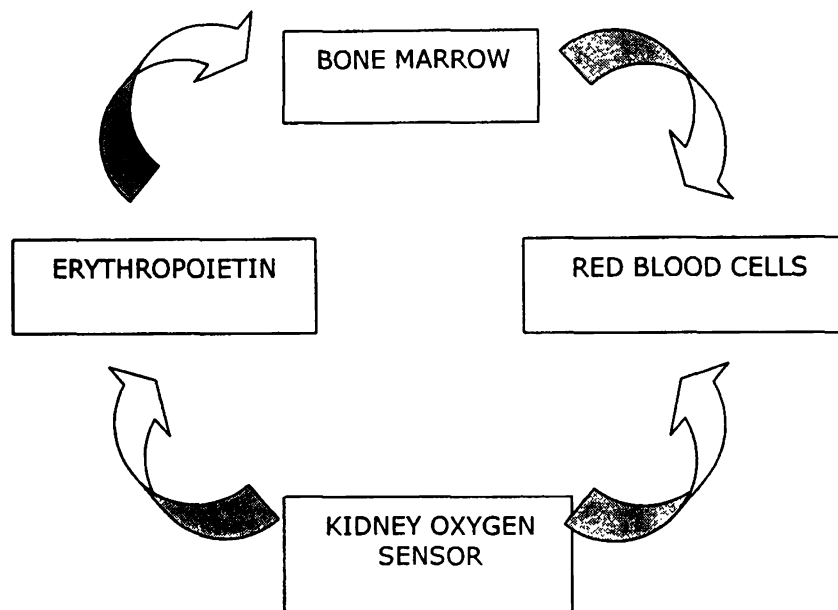
Site of synthesis:

In mid 1950s investigators began to search for the site of erythropoietin production. In 1957 Jacobson (127), demonstrated that erythropoietin production in response to bleeding was significantly decreased in bilaterally nephrectomized rats. Thus the kidney had an important role for *in-vivo* EPO production.

Northern blot analysis showed that EPO m-RNA transcripts are not present in normal kidneys, but are detected in kidneys from anemic animals (128, 129). The conclusion that the tubular/ interstitial cells are important in EPO production is supported by the findings that renal cell carcinoma a tumor that may originate from tubular epithelium of the kidney, has been associated with a polycythemic paraneoplastic syndrome (130). Using *in-situ* hybridization analysis investigators have recently determined the site of EPO production to be peritubular interstitial cells, perhaps of capillary endothelial origin (128, 129, 131). Levels of EPO m-RNA appears to be related to the degree of anemia and in response to severe anemia the no of EPO-producing cells increased 500 to 1000 folds (132).

Erythropoietin levels and diseases:

The basic feedback regulation of erythropoietin is as shown in the fig 3 (133) a red cell mass is produced from the bone marrow stem cell and the progenitor pool. These red blood cells are responsible for carrying oxygen to peripheral tissue.



Oxygen sensors are present within the kidney, which signal the kidney to produce erythropoietin. Through circulation it travels to the bone marrow. In marrow it acts on the receptors present on the cell surface of erythroid progenitors. Due to feedback mechanism it acts as a Hormone.

Under the conditions of hypoxia the kidneys sense the reduced levels of oxygen and signal to produce more amount of erythropoietin, which in turn leads to increase in the production of red cell mass (134).

Molecular biology of Erythropoietin:

Structural information (135):

The circulating form of human EPO is a 165 amino acid (aa) glycoprotein with a molecular weight of approximately 30,000. Although the cDNA for EPO predicts a molecule with 166 amino acid residues, the carboxy-terminal arginine is removed in a post- translational modification. There are three potential sites for N-linked glycosylation and all are filled. One O-linked carbohydrate moiety is also present. The effects of glycosylation are complex. Although unglycosylated *E. coli* derived EPO shows full biological activity *in-vitro*, glycosylation is apparently necessary for full activity *in vivo*. Thus *E.coli* produced and deglycosylated, naturally derived EPO shows very low activity in animal studies. The branching pattern of fully sialyated EPO also makes a difference in biological activity. Predominantly tetra- antinnary branched EPO shows activity equivalent to "standard" EPO, while predominantly bi- antinnary branched shows three fold more activity *in-vitro* but only 15% of normal activity *in-vivo*.

Finally recent studies indicate that only N linked and not O linked sugars are important in EPO functioning. Amino acid identity is 92% homology has been reported between Human and monkey EPO and 82% in human and marine EPO. The oxygen carrying capacity of blood is mainly governed by the concentration of erythrocytes in the blood hence the appropriate regulation of Erythropoiesis is very crucial (136). Predominantly EPO in adults is synthesized in the kidney (137). Although some amount of the hormone synthesis has also been reported from the liver.

Regulation of EPO gene expression:

Experiment with RNA-blot analysis from hypoxic and non-hypoxic kidney tissue has shown that EPO synthesis in the mouse kidney is regulated at the molecular level (129, 138). EPO synthesis in the human hepatoma cell line Hep3B is also regulated at RNA level, with a greater than 100 fold increase in erythropoietin mRNA levels when the cells have been made hypoxic or exposed to cobalt chloride for 24 hours (139). Where as Goldberg *et al.* (140) in their study found only 5 to 10 fold increase in the rate of erythropoietin gene transcription in Hep3B cells exposed to hypoxia or cobalt chloride and have presented data suggesting a significant post transcriptional component to the regulation of erythropoietin mRNA levels as well.

Mechanism of action of Erythropoietin:**Target cells:**

Unlike other hematopoietic growth factors, erythropoietin is a lineage- specific hemopoietin, influencing the growth of erythroid lineage cells. When EPO is secreted by the erythropoietin producing cells as a response to tissue hypoxia, it travels through blood to its target organ. The hematopoietic tissue in man before birth is within the liver while after birth it is in the bone marrow. Here erythropoietin binds to the receptors present on the erythroid progenitor cells called **Burst Forming Unit-Erythroid (BFU-E)** and **Colony Forming Unit-Erythroid (CFU-E)** and stimulate them to proliferate and differentiate (141). BFU-E is the earliest erythroid progenitors and constitutes approximately 0.01% of the nucleated bone marrow cells. They are erythropoietin dependent but also require other hematopoietic growth factors

for their viability. Where as CFU-E, which are derived from BFU-E account for about 0.1% of marrow cells and are much more responsive to erythropoietin (141, 142). They can be grown in a defined serum free medium containing erythropoietin and physiological concentrations of insulin like growth factor-I (without a requirement of any other hematopoietic growth factors), with a plating efficiency equal to that of serum containing medium (143).

In developed nations anemia as such is not such serious problem, as the nutritional deficiency is almost non-existent as well the opportunistic infections are prevented with adequate chemoprophylaxis/ medication. So as a result most of the western literature describes anemia secondary to AZT therapy which is being treated with blood transfusion (144). On the contrary anemia in developing countries like ours is very serious and multifactorial, and most importantly is the commonest feature or complication of all HIV cases that are drug naive (145). Hence in this study we plan to evaluate HIV related anemia.

Aims

&

Objectives

AIMS AND OBJECTIVES

1. To detect the type of anemia seen in the HIV seropositive patients.
 - a) Complete Blood Cell (CBC) counting with RBC indices.
 - b) Estimation of erythropoietin levels in serum (by ELISA)
 - c) Estimation of Total Iron Binding Capacity (TIBC) of blood
 - d) Estimation of Serum Ferritin levels in serum (by ELISA).
2. To evaluate the status of disease (CDC- staging) by CD4/CD8 counts.
3. Estimation of TNF- α in serum (by ELISA).
4. Viral Load Testing (by PCR).

Materials

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Methods

MATERIALS AND METHODS

PATIENTS:

The patients cohort comprises 80 age matched HIV-seropositive patients from both the sexes, from Lokmanya Tilak Memorial Hospital, Sion. A detailed clinical history of patients was taken and elaborate medical history forms were filled. Ethical committee's clearance was obtained from hospital authorities and Haffkine Institute. Written consent was taken from each patient before collecting the blood samples and clinical data.

CONTROLS:

Control group comprises 10 apparently healthy, HIV- seronegative individuals from student community of Haffkine Institute.

Mortality rate and morbidity data were collected by keeping a regular record of the patients in terms of their regular visit to the HIV/ AIDS clinic for a period of 18 months i.e. study period.

Following inclusion and exclusion criteria were used for selection of the patients for this study;

Inclusion Criteria:

1. Adult Male/ Female HIV seropositive patients
2. Asymptomatic/ symptomatic AIDS patients
3. Clinically significant anemia, Hb <10 gm % in these patients.

Exclusion Criteria:

1. Patients less than 18 yrs. of age
2. Patients with a hemoglobin greater than 10 gms %
3. Pregnant women
4. Lactating women
5. Patients on anti-retroviral therapy

BLOOD SAMPLES:

Ten-ml. blood samples from HIV seropositive patients with significant anemia were collected in EDTA and plane bulbs (5 ml each). Sera and plasma were separated and aliquoted and kept at -20°C till the use. Blood smears were made for all the patients enrolled for this study.

REAGENT KITS:

1. EPO- ELISA kit (Roche Diagnostics)
2. TNF- α ELISA kit (BD Biosciences)
3. Serum ferritin ELISA kit (RADIM)
4. CD4+/ CD8+ counting FACS kit (Becton and Dickinson)
5. High Pure Viral RNA kit (Roche Diagnostics)

METHODS:

1. To detect the type of anemia seen in the HIV seropositive patients, the following investigations were carried out;

A. Complete Blood Cell (CBC) counting with RBC indices:

RBC counting: was carried by using conventional method (146)

Requirements:

- i. RBC diluting reagent
- ii. Whole blood
- iii. Improved Neubour's chamber

Procedure:

- i. Take 0.5 (up to the mark) blood in the capillary tube.
- ii. Dilute it up to 1.01 mark with RBC counting fluid.
- iii. Mix the solution (with the help of red-bead) in the bulb.
- iv. Mount it on the Neubour's chamber and count the cell in the RBC-chamber.

Calculation:

Total no. of RBCs (cells/ cumm) = (No. of cells counted) x (dilution factor)

$$\begin{aligned} & \frac{\text{No. of cells counted} \times \text{dilution factor}}{(\text{Area of squares}) \times (\text{depth of squares})} \\ &= \frac{N \times 200}{\frac{1 \times 1}{5 \times 10}} \\ &= N \times 10,000 \end{aligned}$$

i) **Hemoglobin (Hb) concentration:** by cyanomethemoglobin technique
(147)

Principle: The principle of cyanomethemoglobin technique is the use of single reagent, Drabkin's solution that reacts with all forms of hemoglobin except sulphahemoglobin. The latter pigment normally occurs only in minute quantities in blood. According to this technique total Hb at alkaline pH is rapidly converted to cyanoderivative by the initial oxidation of ferrous ions of Hb to ferric state by potassium-ferricyanide. Methemoglobin is subsequently converted to cyanomethemoglobin. Absorbance of cyanomethemoglobin is measured at 540nm. at which it is maximum. The colour intensity is proportional to total Hb content.

Requirements:

1. Drabkin's solution
2. Hemoglobin (human) standard (15 g/dl)
3. Whole blood sample
4. Distilled water

Procedure: The various reagents were mixed in the following proportions

Reagents	Standard (S)	Blank (B)	Test (T)
Hb reagent	5ml.	5ml.	5ml.
Distilled water	-	0.02ml.	-
Whole blood	-	-	0.02
Standard	0.02ml.	-	-

Mix well all the reagents and incubate at room temperature (R.T) for 10-min. measure Optical Density (OD) of all tubes and standard against blank adjusted to zero at 540 nm.

$$\text{Calculations: g/dl of Hb in blood} = \frac{\text{OD test} \times 15}{\text{OD standard}}$$

ii) **Packed Cell Volume (PCV):** calculated by macrohematocrit method (148)

Principle: when anti-coagulated blood is centrifuged in a hematocrit tube at high speed, the erythrocytes sediment at the bottom. The red cell column is called as packed cell volume (PCV) or hematocrit (cell volume present).

Requirements:

- i. EDTA or double oxalated blood sample
- ii. Wintrobe hematocrit tube

Procedure:

- i. Mix the blood sample carefully.
- ii. Label and fill a Wintrobe by using pasture pipette or a syringe up to 100 mark. Avoid tapping of air bubbles.
- iii. Place the tube in a centrifuge and spin it at 3000rpm for 30 minutes.
Note the reading.
- iv. Multiply by 100 for volume percent. If the blood is above 10 mark, calculate the cell pack by deviding the height of the column of the packed

erythrocytes by the total height of the column and plasma then multiply by 100.

Calculations:

The standard calculations for **RBC- indices** were used as follows:

The erythrocyte indices were calculated from

- i. Hemoglobin concentration
- ii. PCV
- iii. Total erythrocyte count

Three indices commonly calculated are;

1. Determination of Mean Cell Volume (MCV):

$$\text{MCV} = \frac{\text{PCV} \times 10}{\text{RBC in Millions}}$$

Determination of Mean Cell Hemoglobin (MCH):

$$\text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC in Millions}}$$

Determination of Mean Cell Hemoglobin Concentration (MCHC):

$$\text{MCHC} = \frac{\text{Hb} \times 100}{\text{PCV}}$$

4. Determination of Colour Index (MCHC):

$$\text{MCHC} = \frac{\text{Hb} \%}{\text{RBC\%}}$$

2. Estimation of erythropoietin levels in serum (by ELISA):

Levels of erythropoietin were estimated in the serum of patients by using commercially available ELISA- kit (Roche Diagnostics) according to the instructions given along with the kit. EPO-ELISA marketed by Roche Diagnostics is a solid phase immuno-assay, the wells being coated with anti-EPO antibodies.

Principle: In the first step EPO is simultaneously bound to anti-EPO-coated surface of the microtitre plate and the peroxidase-conjugated detection antibody. Following the washing step, the peroxidase (HRP) bound in the complex is developed by the substrate tetramethyl benzidine (TMB) and determined photometrically at 450/690 nm. The color intensity is proportional to the concentration of EPO.

Estimation of Total Iron Binding Capacity (TIBC) of blood:

TIBC was estimated using Magnesium Carbonate adsorption method (149)

Requirements:

- i. FeCl_3 solution
- ii. MgCO_3
- iii. Protein precipitate
- iv. Coloring reagent

Procedure:

- i. Take 1ml. serum + 2ml. FeCl_3 solution. Mix it well and keep for 5 minutes.

- ii. After incubation add 200 mg. Of MgCO_3 (powder) and mix on magnetic stirrer. Wait for 30 min.
- iii. Mix it 3 times (i.e. after 10 min each).
- iv. Separate the supernatant by centrifugation.
- v. Take 2 ml. supernatant + 2 ml. protein precipitate. Mix it well with a glass rod. Keep it for 30 min.
- vi. Centrifuge for 20-30 min. until clear.
- vii. 2 ml. supernatant + 2 ml. color reagent. Mix and stand for 5 min.
- viii. Read at 535 nm (green filter).

Calculation:

$$\text{TIBC } \mu\text{g}/100 \text{ ml.} = \frac{\text{OD test} - \text{OD blank} \times 223 \times 3}{\text{OD std.} - \text{OD blank}}$$

D Estimation of Serum Ferritin levels (by ELISA):

Serum ferritin was estimated by using commercially available ELISA- kit (RADIM), the test was performed as per the kit insert.

Principle: Serum ferritin marketed by RADIM is based on immunoenzymometric assay (IEMA). Two different anti-ferritin monoclonal antibodies are used, one adsorbed on the wells and the other conjugated to horseradish peroxidase (HRP). During the first incubation, the ferritin present in the standards and the samples is bound to both monoclonals at once, by forming a "sandwich". Following this incubation, the unbound material is removed by a washing cycle.

The residual enzyme activity found in the wells will thus be directly proportional to ferritin concentration in the standards and samples and evidenced by incubating the solid phase with a chromogen solution (TMB), determined photometrically at 450/405 nm. The color intensity is proportional to the concentration of serum ferritin.

2. To evaluate the status of disease (CDC- staging) by CD4/CD8 counts:

CD4+/ CD8+ lymphocyte counting was performed on fully automated FACS- count (Becton and Dickinson), procedure was performed as per the manual instructions.

Principle: when whole blood is added to the reagent, fluorochrome labeled antibodies in the reagent bind specifically to Lymphocyte surface antigens. After fixative solution is added to the reagent tube, the sample is run on the FACS counter (Becton & Dickinson). Here the cells come in contact with the laser light, which causes the fluorochrome labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells.

In addition to containing the antibody reagent the reagent tube also contain a known number fluorochrome integrated reference bead. These beads function as fluorescence standard for locating the lymphocyte and also as a quantitation standard for enumerating the cells.

3. Estimation of TNF- α in serum (by ELISA):

Levels of TNF- α were estimated by using commercially available ELISA-kit (BD Biosciences), according to the instructions given along with the kit.

Principle: the BD OptEIA™ test is a solid phase sandwich ELISA. It utilizes a monoclonal antibody specific for TNF- α coated on a 96-well plate. Standards and samples are added to the wells, and TNF- α present binds to the immobilized antibody. The wells are washed and an avidine-horseradish peroxidase conjugate mixed with a biotinylated anti-human TNF- α antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and TMB substrate solution is added, which produces a blue color in direct proportion to the amount of TNF- α present in the initial sample. Stop solution changes the colour from blue to yellow, and the microwell absorbances are read at 450 nm.

4. Viral Load Testing (by PCR) (150, 151, 152, 153):

Isolation of viral RNA:

The RNA was isolated from the serum/plasma samples using the High Pure Viral RNA kit (Roche Diagnostics). One milliliter of serum/plasma was centrifuged at 25,000g at 40C for 1 hour. The supernatant was discarded, retaining 20 μ l of the pellet. To this, 180 μ l of the wash buffer was added and mixed using centrifugation. Four hundred microliters of the binding buffer, supplemented with poly [A] were added to this solution. This was then overlaid on a High Pure Filter column installed on a

collection tube. The sample assembly was then centrifuged at 8000g at 40°C for 15 seconds. The collection tube containing the flowthrough was discarded. The Filter column was inserted onto a fresh collection tube. Five hundred microliters of the inhibitor removal buffer were added to the column, followed by centrifugation at 8000g at 40°C for 1 minute. The column was then washed twice with 450 µl of wash buffer and the flowthrough was discarded. Residual wash buffer was removed by centrifugation at 13,000g at 40°C for 10 seconds. The filter tube was then inserted into a nuclease-free collection tube. Fifty microliters of the elution buffer were added onto the column and the assembly was centrifuged at 8000g at 40°C for 1 minute. The viral RNA thus isolated was used for PCR.

Real Time RT PCR for viral load estimation:

The viral load estimation by Real Time RT-PCR was carried out at Department of virology, University of Bordeaux, France. TaqMan™ One Step RT-PCR kit (Applied Biosystems) was employed for the viral load estimation in patient's plasma samples. The Long Terminal Repeat (LTR) region of HIV was selected for the designing of the primers.

Primer5' (NEC152): 5' GCC TCA ATA CTT GCC TTG A 3'

Primer3' (NEC131): 5' GGC GCC ACT GCT AGA GAT TTT 3'

The following probe was used for the detection of amplification:

5' AAG TAG TGT GTG CCC CTC TGT TRT KTG ACT 3'

The probe was labeled at 5' with a reporter dye, viz. 6-carboxyfluorescein-aminoethyl amide (6-FAM), and at 3' end with a quencher, viz. Tetramethylrhodamine (TAMRA).

Each reaction mix consisted of 30 μ l, comprising of 25 μ l of 2X Master mix (without UNG*), 1.25 μ l of Multiscribe RNA mix in 40X RNase inhibitor, 1 μ l each of 25 μ M sense and anti-sense primer, 1 μ l of 10 μ M probe, and 0.75 μ l of DNase/ RNase free water. To this, 20 μ l of the sample RNA were added.

The amplification was carried out using the following programme:

Reverse transcription	48 ⁰ C	30 min	1 cycle
Initialization	95 ⁰ C	10 min	1 cycle
Denaturation	95 ⁰ C	15 sec	50-60
Annealing & Extension	60 ⁰ C	1 min	cycles

The external standard comprised of culture-amplified virus or strong positive plasma. This was then diluted until a dilution of 1 copy by PCR was obtained. The accumulation of the PCR products was detected by the increase in the fluorescence of the reporter dye.

Results

RESULTS:

Patients coming to out patients department (OPD) of LTMH and indoor patients admitted to medicine and skin wards were taken.

Out of 80 sero positive patients, 52 (65%) male and 28 (35%) female were enrolled for the present study.

Table 2: Sex distribution

Sex	No. of patients
Male	52
Female	28
Total	80

As per table number 2 males comprised (65%) and females (35%) of the total cases. The higher incidences of male population are due to the increased incidences of HIV/AIDS and prevalence of the infection in the general population. For female population the case reporting may not be the true no as most of the cases go unreported.

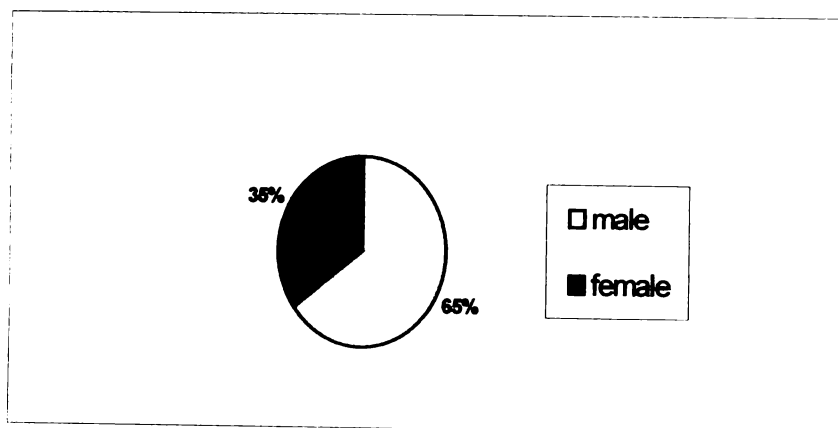


Figure 4: Age and sex distribution among the patients:

Table 3: Age and Sex Distribution (n= 80)

Age group (years)	Total no. Of patients (n=80)	Males (n=52)	Females (n=28)
18-28	28	13	15
29-39	39	27	12
40-50	12	12	0
>50	01	0	1
Total	80	52	28

For this study population majority of the patients fall in the age-group of 18 - 40 years (%), which is the most productive and sexually- active age group in our country.

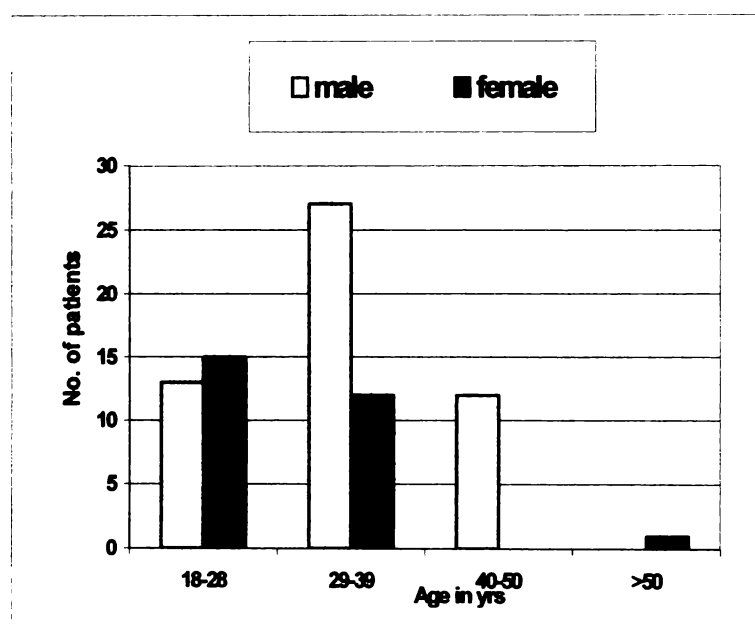


Figure 5: Age and Sex-Distribution

Probable risk behaviors and mode of transmission amongst the patients:

Table 4: Risk behaviors of patients

Probable Mode of transmission	Total No. Of Patients	Male	Female
Heterosexual- unprotected sex multiple sex partners	57	49	08
Infection from spouse	19	Nil	19
Homosexual	01	01	Nil
Mode of infection unknown	03	02	01
Blood transfusion	Nil	Nil	Nil
Total	80	52	28

Our observation showed that heterosexual mode of transmission of the HIV infection being the largest (71.25%), as seen overall trend of HIV-transmission in the country.

3. Red blood cell indices:

Red blood cell indices, which included Packed Cell Volume (PCV), Mean Cell Volume (MCV), Mean Cell hemoglobin (MCH), and Mean Cell Hemoglobin concentration (MCHC), and Colour Index were performed for all the patients.

The normal values for above parameters are as shown in table no 4.

Table 5: Normal values

Parameters	Normal-values
PCV	36 - 52%
MCV	82 - 92 cu mm.
MCH	27 - 32 pg.
MCHC	32 - 36%
Colour-Index	0.85 - 1.15

For all patients the values for PCV MCV, MCH, and MCHC parameters fall below normal range. The colour index for all the patients was < 1. This is a typical observation in hypochromic and normocytic anemia.

4. Based on Hemoglobin concentration the patients were graded in grade I to grade IV. The following table shows exact no. of patients.

Table 6: Severity of Anemia (n = 80)

	Grade I	Grade II	Grade III	Grade IV
Hemoglobin (gms %)	8.0 - 9.4	7.0 - 7.9	6.5 - 6.9	< 6.5
Males	22	13	06	11
Females	09	04	06	09
Total	31	17	12	20

Recorded mortality in various grades of anemia amongst patients:

Table 7: Mortality rate recorded in different grades of anemia (n = 80)

Grade	Total no. Of Patients	No. of patients Expired	%
I	31	05	16.12
II	17	06	35.29
III	12	06	50.00
IV	20	12	60.00
Total	80	29	36.25

All the patients including controls were tested for endogenous serum erythropoietin levels:

Table 8: Distribution of endogenous serum erythropoietin levels in HIV/AIDS patients: (n = 80)

EPO levels (MU/ml.)	Total no. Of Patients	Males	Females
0-20	46	27	19
20- 40	05	04	01
40- 60	09	06	03
60- 80	06	06	-
80- 100	03	01	02
100- 120	03	02	01
120- 140	02	01	01
140- 160	02	01	01
160- 180	-	-	-
180- 200	03	03	-
<200	01	01	-
Total	80	52	28

8. Serum ferritin levels: (n=80)

Ferritin levels in serum of HIV/ AIDS patients were done. Out of 80 patients, 18 (22.5%) patients showed high ferritin and low TIBC (between 182- 270 ug/100ml.), which is a typical indicator of ACD or may be the indicator of active secondary infection. Remaining 62 (77.5%) had their ferritin values in the normal range.

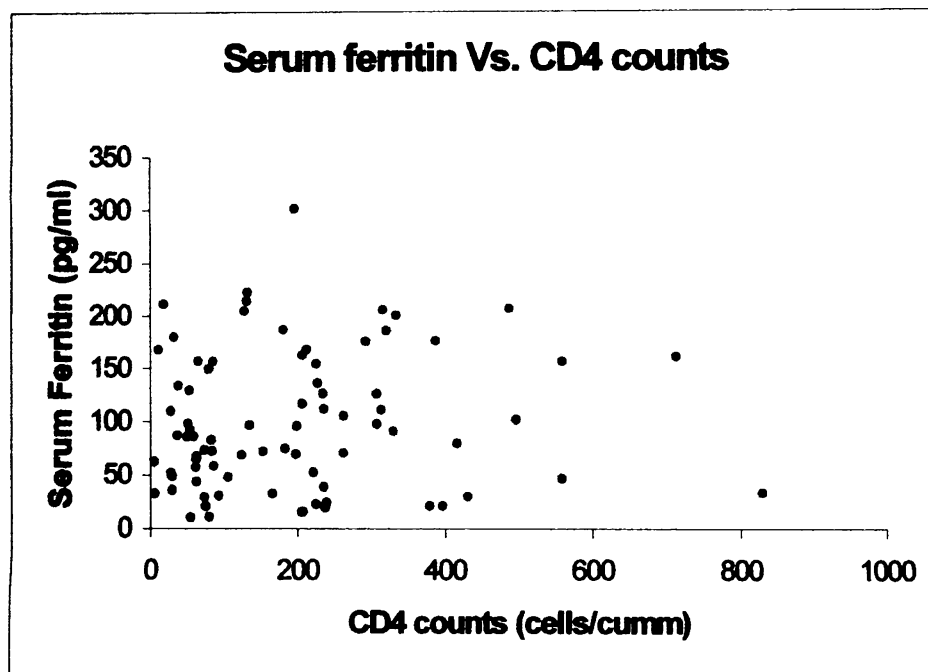


Figure 6: Serum ferritin values in the patients with respect to their CD4+ counts.

When we plotted CD4+ values to Serum Ferritin, most of the patients 64 (77.5%) showed ferritin in normal range (i.e. 98 - 251 pg/ml) only 18 (22.5%) patients showed elevated S. Ferritin levels when compared to the normal range. The main purpose to couple these two factors i.e. CD4+ counts and S. ferritin is, it gives a clear picture of patient's immune status or pathological condition.

In our study 18 patients who showed high S. ferritin and low TIBC (between-), is a typical indicator of ACD, or may also indicate active secondary infection in these patients.

8: TIBC against CD4+ counts:

We found negative correlation when TIBC was plotted against the CD4+ counts in the patients.

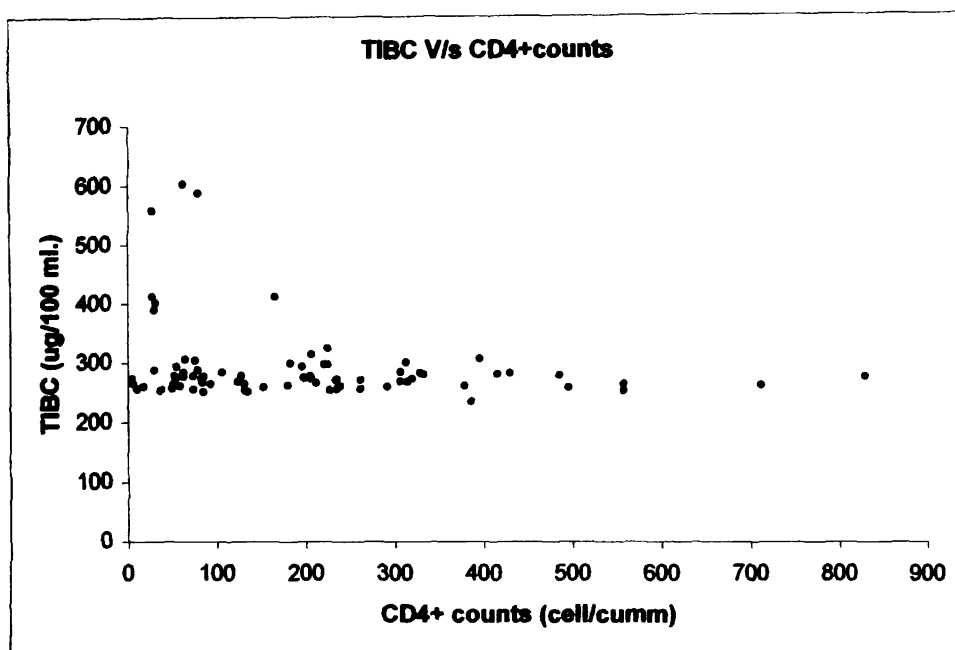


Figure 7: TIBC values seen in the patients with respect to their CD4+ counts.

The normal value for TIBC in iron-deficiency anemia is in the range of 304 – 705 for both the sexes. When we observed the corresponding graph between TIBC and CD4+ counts, our most of the patients are having CD4+ counts below 500 cells/cumm. But most of the patients are showing TIBC values near 300, which exclude the possibility of iron-deficiency anemia.

The four patients showing high TIBC values represent active opportunistic (secondary infections).

9. Staging of the patients was done according to CDC guidelines depending on their CD4+ / CD8+ counts:

Table 9: CD4⁺ counts of 80 patients

CD4+ COUNTS	No. of patients
0- 100	32
100- 200	13
200- 300	17
300- 400	10
400- 500	04
> 500	04
Total	80
Total	80

10. Levels of TNF- α in HIV-patients:

Table 10: Observed TNF- α values in HIV/AIDS patients

TNF-α levels (pg/ml.)	Total No. of Patients
0 – 50	63
50 – 100	07
100 – 150	04
150 – 200	05
200 – 250	01
TOTAL	80

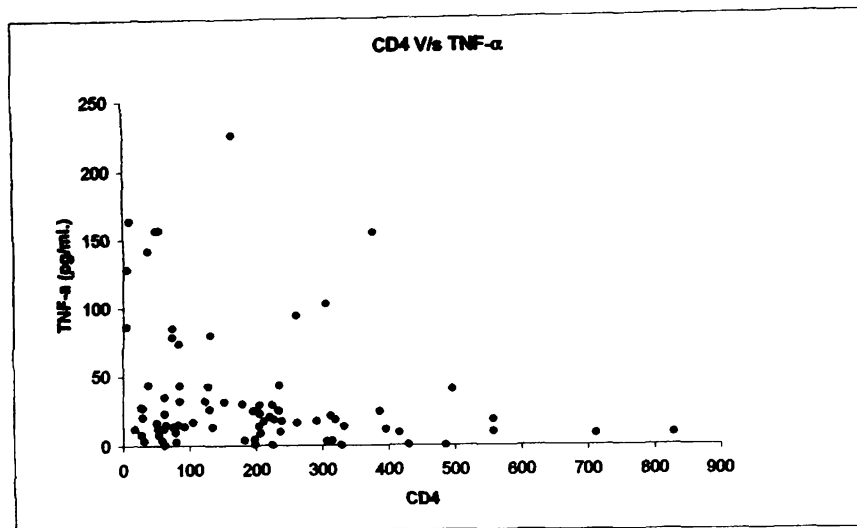


Figure 8: TNF- α values in the patients with respect to their CD4+ counts.

11. Control- Group:

Table 11: Estimated parameters in the control group population

Patient's no.	Hb gm%	CD4+ counts	S. EPO (mU/ml)	S. Ferritin (ng/ml)	TNF- α (pg/ml)	TIBC (μ g/100ml)
1	11.5	645	13.0	153	<2	296
2	11.8	702	12.1	126	2.3	354
3	12.2	930	8.5	98	3.6	268
4	11.6	825	11.3	109	3.8	376
5	12.6	965	9.1	149	4.5	325
6	14.8	957	16.2	205	4.2	392
7	14.2	1005	12.3	251	4.7	356
8	14.9	876	14.2	189	6.0	378
9	15.1	980	17.0	222	3.4	380
10	14.3	756	15.2	125	4.3	405

Ten apparently healthy, non HIV-infected, non anemic, not having any known infection at the time of blood collection was comprised of Control Group

population. The values obtained for various parameters were further used to set a cut-off value for this set of experiments.

All the patients were compared to this control group reading by taking the mean value for the above shown readings.

12. VIRAL LOAD ESTIMATION:

Viral load (for selective number of patients) was performed by using Real-Time PCR technique on Long Terminal Repeats (LTR) of HIV-1 virus. Experiments were carried out at Virology Department, University of Bordeaux, France.

Table 12: Estimated viral load in HIV/AIDS patients with their other parameters.

Serial No.	Patients code	Hb gms %	CD4+ counts (Cells/cumm)	TNF- α pg/ml.	Viral load
1	A	9.3 (grade I)	062	12.50	7.59 e+ 5
2	B	7.8	050	156.66	4.05 e+ 6
3	C	7.4	396	11.597	2.73 e+ 4
4	D	7.1	262	16.402	<500
5	E	6.9	128	42.636	<500
6	F	6.5	028	8.22	<500
7	G	6.2	206	29.129	3.3 e+ 5
8	H	5.9	063	34.974	2.48 e+ 5
9	I	5.8	135	13.675	1.09 e+ 6
10	J	4.0	053	12.376	<500

Due to limited resources, the viral load detection for all the patients was not possible. We calculated the viral load for 10 patients. The categorization of

Discussion

the patients was based on their Hb concentrations. We categorized patients in 4 groups as per their Hb- concentrations. And taken few representative patients from all the 4 groups. The sensitivity of the assay was 500 copies/ ml.

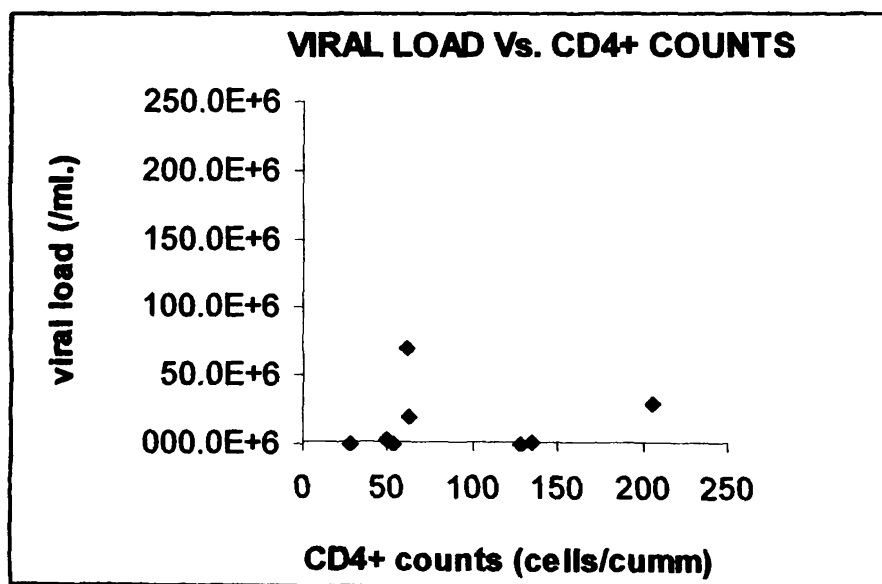


Figure 9: Estimated values of viral load in the HIV/AIDS patients plotted against their CD4+ counts

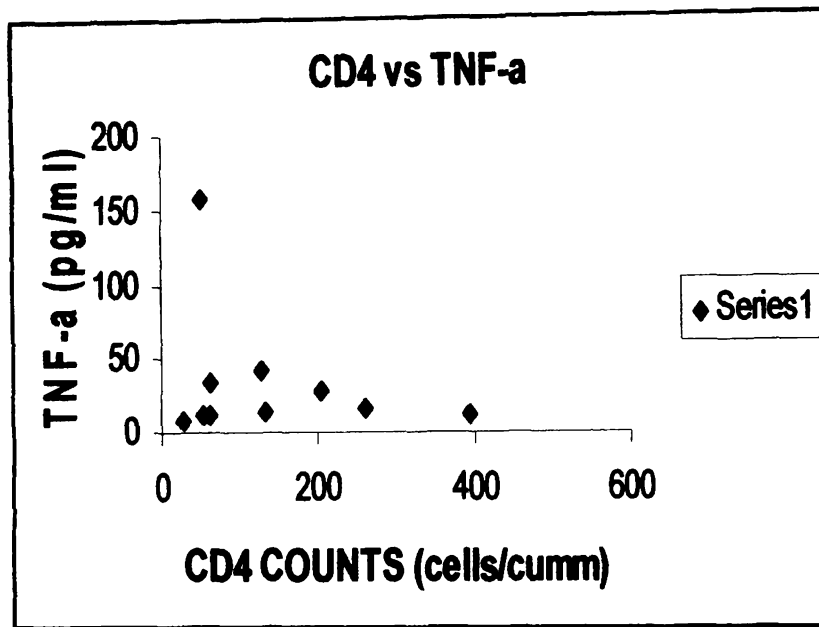


Figure 10: Estimated values of TNF- α levels in HIV/AIDS patients plotted against their respective CD4+ counts.

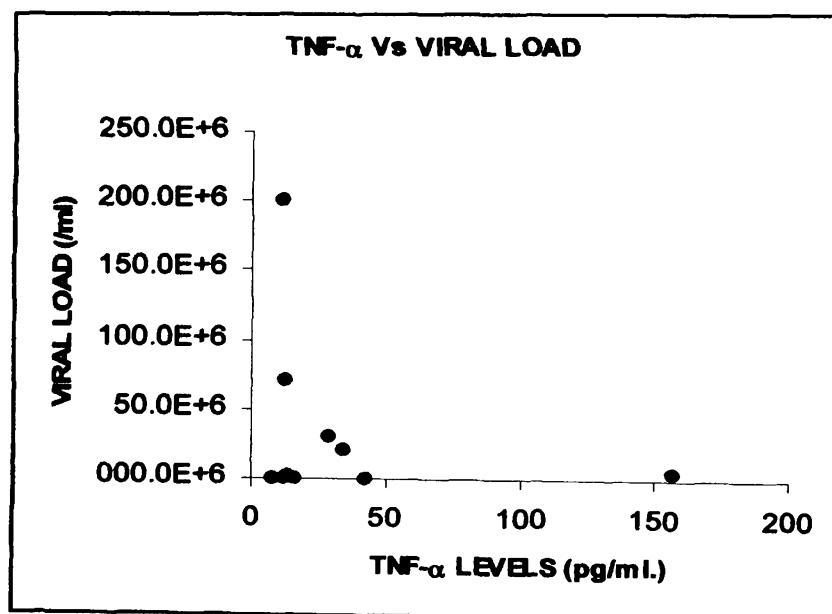


Figure 11: Viral Load plotted against TNF- α levels in the patients.

DISCUSSION:

HIV/AIDS is considered as "young age" disease. The main reason for which is the occurrence of the disease in a particular (i.e. adult) age group, which is sexually active and main productive age group in all the communities. Adults are defined as men and women aged 15-49. This age range captures those in their most sexually active years. While the risk of HIV infection continues beyond the age of 50, the vast majority of people with substantial risk behaviors are likely to have become infected by this age. Since population structures differ greatly from one country to another, especially for children and the upper adult ages, the restriction of 'adults' to 15-49 has the advantage of making different populations more comparable (154).

At the end of 2002, the Center for Disease Control and Prevention (CDC) reported approximately 384, 906 persons in the **United States** living with AIDS. Out of which 46% were whites, 34% blacks, 18% Hispanics, <1% Asians and Pacific Islanders, and <1% in American Indians and Alaskan Natives. Out Of total 298,248 infected men (as over 13 years) suffering from AIDS, 57% were men who had sex with men (MSM), 23% were intravenous drug users (IDU), 10% were exposed through heterosexual contact, 8% were both MSM and IDU. For 82,764 adult and adolescent females, the scenario was different. Sixty one % were exposed through heterosexual contact, 36% were exposed through intravenous drug use. By the end of 2002 year 3893 children were living with AIDS (155).

An estimated 25 million adults and children are living with HIV in Sub-Saharan Africa, and an estimated twelve million children have been orphaned because of AIDS related deaths in their parents.

In **South East Asia**, the main countries, which are spotted with the gross and grave problem of HIV/AIDS, are Thailand, Myanmar, Cambodia, Vietnam, China and India (156). **Thailand** was the first country in Asia to document the HIV epidemic amongst intravenous drug users, female sex workers and their clients. HIV prevalence among female sex workers appears to have peaked in the mid-1990's and has been slowly declining since, while amongst IDU's, prevalence is between 20% and 50% both in and outside Bangkok (156). The true data for **Myanmar** is difficult to obtain, and the Ministry of Health has confirmed about 26,000 cases of HIV since 1988. HIV prevalence amongst IDU's in the capital Yangon had reached 73% by 1989 and since then has been found to be between 50% and 85%. The prevalence rate amongst female sex workers had increased to about 26% in 1997. Outside the main urban areas, HIV prevalence at antenatal clinic attendees reached about 5% in 2000. It is estimated that AIDS deaths will be a major (if not the main) cause of death among young adults during the next 10 years (156). The estimated reporting rate of HIV in **Cambodia** is low, at 8%, indicating that the problem is likely to be much greater. Although main route being heterosexual, homosexuality and IDU's do exist (156).

Since the first HIV case in **Vietnam** was reported in 1990, the number of infections has grown rapidly in all provinces. Vietnam's transition to a market economy was accompanied by an increase in injecting drug use and commercial sex. In 2000, HIV prevalence was highest amongst IDU's (65% prevalence), and there is evidence that as much as 81% of IDU's share injecting equipment, despite the fact that clean injecting equipment has been made legally and cheaply available (156).

China has a larger population of 1.3 billion than any other country in the world. Seven hundred and twenty million of whom are aged between 15 and 49 years. China has become more of a market-lead economy in the last decade, and these economic changes have had social repercussions. Premarital sex is now more socially acceptable, especially amongst young people, and there has been a rapid rise in commercial sex. As a result of the increase in sexual freedom, STDs have risen at a rate of about 30% each year for the past ten years. HIV was first reported in China in 1985, and the epidemic became established in 1989 amongst IDUs. Unregulated sales of blood products also lead to an increase in infections. HIV had been reported in 28,133 cases by September 2001, although the actual number of cases is likely to be much higher. The information available so far indicates an intense explosion of infection amongst IDU's, with some areas having prevalence rates of 44% to 85%. It is estimated that 69% of all new infections are due to injecting drug use and the epidemic is concentrated in poor rural areas. No significant spread in the non-IDU population has been reported, although HIV

infections due to heterosexual contact are now being reported more frequently (156).

India has a population of more than one billion and around half the number comprises adults in their prime youth. The first AIDS case in India was detected in 1986, and since then, HIV infection has been reported in all States and Union Territories. The spread of HIV in India has been diverse, with much of India having a low rate of infection and the epidemic being most extreme in the southern States. 96% of the total numbers of nationally reported AIDS cases were found in 10 of the 28 States and 7 Union Territories. The worst affected states are being Maharashtra in the west, Tamil Nadu and Pondicherry in the south, and Manipur in the north-east. In Maharashtra and Tamil Nadu the infections are mostly due to heterosexual contact, while infections are mainly found amongst injecting drug users (IDU) and their sexual partners in Manipur (157).

In the present study, out of 80 seropositive patients, 52 (65%) were male and 28 (35%) were females. The higher incidences of HIV infection in male population are well documented. For female population the number of reported cases may not be true as most of the cases may go unreported. In our study population, out of 80 patients 57 (71.25%), got infected via heterosexual mode. Out of these 57, 49 were male and 8 were female patients. Off the total 28 females, 19 (67.87%) were monogamous and got

infected via their HIV positive husbands. One male patient was homosexual. The age group of the patient ranged between 18 to 50 years, with a mean age group of 30 years. Thirty-nine patients were in the age group of 29 to 30 years, which is the main productive and sexually active age group.

According to the Joint United Nations Programme on AIDS (UNAIDS) (158), report of 2001, in India, the estimated prevalence of HIV infection rate amongst persons aged 15 to 49 years is 0.7%, and hence total estimated prevalence from the country is around 3.5 million. It is estimated that 89% of patients are in the age group of 15 to 44 years. Heterosexual transmission is a major mode of HIV transmission all over India except for Manipur, where intravenous drug abuse or sharing the infected needles is the major mode of HIV transmission (159).

The incidence of anemia in the setting of HIV infection is dependent on the severity of HIV disease as well as the levels of hemoglobin and hematocrit used in defining anemia. In a published study by the CDC from the Adult & Adolescent Spectrum of HIV disease surveillance project involving over 3,200 HIV-infected persons receiving medical care throughout the United States from 1990-1996, showed a one year incidence of severe anemia (Hb <10g/dl.) In 31.6% of patients with CD4+ count > 200/mm³ (75). Same study showed that 72% men and 69% women who had been HIV infected but without AIDS-defining illness, had hemoglobin concentration within the normal reference range (>14 or 12 g/dl, respectively). However for patients with clinical AIDS

only 13% of men and 23% of women had hemoglobin concentration within the reference range.

The prevalence of severe anemia was 28.3% in patients with AIDS and 2.3% in those without an AIDS diagnosis. On the other hand the prevalence of moderate anemia, defined as Hb 10-14 g/dl for men or 10-12 g/dl for women, was 31.3% and 26.6% for those with and without an AIDS diagnosis. This suggests that while anemia is very common in those with a clinical or immunological diagnosis of AIDS, moderate anemia is also very common in those in earlier stages of disease. It has been observed in this study that only 25% of anemia was drug related, while the rest was due to a variety of other causes including HIV-induced chronic anemia.

This study also showed that the risk of death was substantially greater for those who developed anemia compared to those who did not and this higher risk of death was seen regardless of the patient's CD4+ counts.

Recovery from anemia, by whatever means reduced the risk of death substantially, indicating the independent prognostic significance of anemia in patients with HIV infection. Several other studies have also shown the importance of anemia on the prognosis in HIV outcomes (115,160,161). Numerous causes of anemia exist in HIV-infected patients. This mainly include, neoplasm infiltrating bone marrow (mainly because of various AIDS-related lymphomas), infections (due to bacteria, fungi and viral pathogens), drug related (mainly due to antiretroviral therapy), HIV itself and diet related

(which include, iron deficiency, folic acid deficiency, B-12 deficiency), etc. (162).

Out of all these possible underlying causes, the diet related causes are most prevalent in HIV/AIDS patients in developing nations like ours. Iron dependent anemia is the most prevalent in our patients. To exclude the possibility of iron dependent anemia, we carried out, TIBC and serum ferritin in patients showing significant anemia (Hb < 10 gm/dl). As shown in fig. 6, sixty-four (77.5%) of patients showed ferritin in normal range (17-430 ng/ml), while 18 (22.5%) showed elevated levels of serum ferritin. Serum ferritin is the true indicator to differentiate between iron deficiency anemia and ACD. In a study reported by Kis and Carnes (163) it was found that serum ferritin concentrations of 100 ng/ml or less were the most sensitive and specific marker for identifying iron deficiency anemia. Hence serum ferritin concentration over 100 ng/ml usually rule out iron deficiency anemia and high serum ferritin concentrations in the presence of low total iron binding capacity (TIBC) suggests anemia of chronic diseases. To eliminate the possibility of iron deficiency anemia it is necessary to detect serum ferritin levels in the serum of the HIV / AIDS patients. In most clinical circumstances, the serum ferritin concentrations are proportional to total body iron stores. Serum Total Iron Binding Capacity (TIBC) is an indirect measure of serum transferrin, a specific iron binding β_2 - globulin. The elevated levels of total TIBC cannot be well correlated to decreased serum iron since in ACD both the serum iron and total iron binding capacity levels are decreased (164,165).

We categorize the patients in to four grades based on WHO guidelines for anemia (166). In our study 9 out of 28 (32.14%) of the female population falls in grade IV with a very low hemoglobin concentration. In India, the majority of women have low hemoglobin due to multiple deliveries, poor nutrition and neglected health care; HIV infection is an additional factor to aggravate anemia.

HIV disease progression in the patients was staged on the basis of CDC guidelines considering their CD4+ counts (167). The chances of developing an AIDS-defining condition and dying within 18-24 months is less than 5%. Patients with early HIV infections having CD4+ counts $>500 \text{ cell/mm}^3$ having (24). Patients showing CD4+ counts between $200\text{-}500 \text{ cells/mm}^3$, when left untreated these patients with middle-stage HIV disease have 20-30% chances of developing an AIDS-defining condition, dying within the next 18-24 months. With therapy the risk can be reduced by 2-3 folds (24,168). Patients in late stage of the disease i.e. CD4+ between $50\text{-}200 \text{ cell/mm}^3$, with no treatment have a 50-70% likelihood of developing a new AIDS-related conditions or dying within an 18-24 months period (24). As shown in table no 9, forty five (56.25%) patients had a CD4+ count of less than 200-cells/mm^3 , and were classified to have developed full-blown AIDS.

Relative to their degree of anemia, patients with HIV have inappropriately low endogenous EPO levels (100). High levels of circulating inflammatory cytokine such as $\text{TNF-}\alpha$ and IL-1 can blunt the normal EPO response to anemia in patients with HIV (169). The blunted response to EPO is common

1. There are 22 (27.5%) patients showing levels less than the normal range (<48 mU/ml.) of EPO. These levels suggest that there is an inappropriate erythropoietin response in the body as compared to that of healthy controls, shown in table no. 10, where all the values were between 3-17 mU/ml.
2. Out of 80 patients, 51 (63.75%) show the normal erythropoietin levels and are still suffering with severe degree of anemia. Such scenario may arise due to faulty synthesis of EPO or improper post-translational modifications, as a result the circulating EPO may not be in its active form.
3. There is possibility of bone marrow not responding to the normal levels of EPO, in a proper fashion.
4. There is possibility of bone marrow microenvironment getting infected by HIV virus itself, so it may not produce the required number of erythrocytes, which in-turn leads to anemia and pancytopenia.

TNF is a cytokine best known for its role in mediating inflammatory and immune responses in acute infection. TNF, also known as cachectin, is associated with wasting in persons with malignancies or AIDS. It has been documented that HIV replication requires activation of Nf- κ B, a viral binding site. High levels of TNF- α accelerate this binding. TNF- α induces nuclear translocation of the transcriptional factor Nf- κ B leading to increased activity

from the HIV-1 LTR and stimulation of viral expression. Hence it appears that TNF- α is responsible for an enhanced viral replication. In fact HIV/ AIDS infection has been called a "TNF- α disease" (172).

HIV wasting syndrome and other HIV related weight loss is a major problem in HIV infected patients. The available data strongly suggests that wasting is associated with decreased survival. It may also further impair immune function. A variety of etiologies probably contribute to this wasting, including hyper-metabolism, lessened oral intake, Malabsorption, **cytokine effects** and endocrine dysfunction. The relative contributions of each etiology to wasting probably vary considerably from patients to patients (173).

However several authors have reported elevations of TNF, particularly in symptomatic patients, other studies has reported that TNF is not consistently elevated in HIV infected patients where as Dworkine *et al* noted that in a group of 33 HIV positive outpatients, elevated TNF levels did not correlated with weight loss and increased metabolic rate (173). Some investigators have suggested it may be the interaction of TNF with other cytokines, such as IL-1 or with other inflammatory mediators that induces anorexia, accelerated protein breakdown, or wasteful energy expenditure (174). Therefore, TNF is an interesting cytokine to be studied along with erythropoietin and HIV viral load to asses the correlation if any between these three factors. High levels of TNF- α have been found in plasma samples from HIV-1 infected individuals.

Seventy seven (96.25%) out of total 80-patients in the study population suffering with HIV/ AIDS showed elevated levels of TNF- α . There is direct correlation between the CD4+ counts and the TNF- α level (175). From fig.8 we can observe that as the CD4+ counts go down TNF- α levels increase in the blood of HIV/AIDS patients. After applying *Pearson' correlation Coefficient*, $r = - 0.23$ and $p = 0.04$ was calculated. As shown in fig: 8 when we correlated the findings with their CD4+ counts we found a significant negative correlation between the two factors. When we compared these values with those seen in the control population (values between 2-6 pg/ml.) we found > 6 fold increase.

As shown in the table no. 12 when the TNF- α levels were plotted with viral load values (as shown in the fig: 10), the values of viral load were well correlated with high TNF- α values (between 10- 50 pg/ml.), but as the TNF- α values increased further, the viral load was undetectable. This particular observation shows that;

- i) There is possibility of reduced viral replication at extremely high (compared to that of normal values) levels of this cytokine.
- ii) Elevated levels of TNF- α lead to apoptosis of viral infected cells, and the extent of apoptosis happen to such an extent that there are no more cells to support the viral replication in the body, especially in the cases of full-blown AIDS cases.

iii) It may happen that active viral replication occurs in cells like dendritic cells and lymph nodes, which acts as reservoirs for the virus, but the circulating copies of the virus in the plasma are undetectable, even though the true copy number of the virus may be very high/large in the body.

When we correlated the mortality, seen in the study population with their TNF- α levels we found that a total of 29 (36.25%) patients died out of 80, all of them showed a elevated level of TNF- α levels compared to normal control population. Sixteen (55.172%) out of total 29 dead patients were having their CD4+ counts <200 cells/cumm, followed by 11 (37.931%) patients showing their CD4+ counts between 200-499 cells/cumm. Out of total 29 dead patients 23 (79.31%) had their TNF- α values between 0-50 pg/ml. Out of which 19 (65.31%) had their values above the normal range of 0-7.8 pg/ml. When we compared these values with the control group we found a significant increase of >6 folds. This particular observation points out the prognostic importance of this cytokine, i.e. TNF- α can be used as a prognostic marker to evaluate the morbidity and the mortality in HIV/AIDS infected patients.

As the viral load increases in the plasma, the immune status deteriorates much faster leading to the compromised state of full-blown AIDS. Due to this increase in the viral load depletion of the CD4+ cells happen at an alarming rate, which increases the chances of getting opportunistic infections in the patients making the situation worst.

Due to limited resources, viral load was estimated only in 10 patients. These patients were categorized in to 4 different groups depending on their hemoglobin concentrations and few representative samples from each of these groups were taken for further analysis. As shown in the table number 12, 4 patients had undetectable levels of viral load even though their CD4+ counts were < 100 cells/cumm. There can be 3 possible reasons for this condition:

1. Sensitivity of the assay was > 500 viral copies/ml. The copies below 500 could not be detected by this assay.
2. RNA-transport hypothesis: which states that even in the best of transporting conditions there is slow and steady degradation of RNA molecules in the sample, and repeated freeze-thaw also add-up to this problem.
3. As observed in cases of severe bacteremia, there is sudden drop in the levels of bacterial counts in patients before the terminal-stage. Same conditions may occur in severally immuno-compromised AIDS patients too. The levels of anergy, apoptosis, protein malabsorption and severe deterioration of the immune system are increased to such a high extent that no further replication or perpetuation of the viral particles is possible in the patient's body.

As shown by researchers, this can also be explained by explaining the exponential growth by the virus as it enters the body after initial stages of latency, which then attain a plateau, a sudden drop is seen in the levels of viral particles, after an initial increase in the plasma samples of patients whose CD4+ counts deplete suddenly.

In the study population out of total 10 patients, one patient showing grade I anemia had 7.59×10^5 viral copies. Two patients showing grade II anemia had 4.05×10^6 and 2.73×10^4 viral copies respectively. Three patients showing grade IV anemia had 3.3×10^5 , 2.48×10^5 and 1.09×10^6 viral copies. When we correlated these viral loads to their TNF- α levels, we found that most of the patients who had their TNF- α levels between 0 to 50 pg/ml showed detectable viral copy numbers. As the TNF- α levels go high (i.e. above the normal limit) the viral copy numbers also increased, which supports the classical belief that TNF- α helps in HIV replication.

There is no correlation found between levels of EPO and viral load of these patients, which shows that EPO does not help either in replication or elimination of HIV virus particles (176).

HIV/AIDS related mortality has been well documented and came out as an alarming signal and probably the main reason for deaths in adults and children all over the world. By the end of the year 2003, a global total of 2.9 million deaths were reported with a geographical distribution as 2.2 million in

Sub Saharan Africa, 0.04 million in East Asia, 0.46 million in South and South East Asia, 0.049 million in East Europe and Central Asia, 0.016 million in North America.

HIV/AIDS related mortality has many dimensions and various etiological causes, which mainly include deaths due to opportunistic infections, **anemia**, **malnutrition**, lack of proper medication and on a whole the HIV infection itself. The degree of anemia exhibits a proportional relationship with morbidity and **mortality** conditions amongst the patients. For patients with severe anemia, $Hb < 10 \text{ gm/dl}$, corrections of the anemia results in improvement in survival as demonstrated in the CDC's Adult Spectrum of Disease (75), the authors have also showed that the incidences of anemia was strongly and consistently associated with the progression of HIV disease as measured by diagnosis of an AIDS-defining opportunistic illness and measurement of a CD4 count of $<200 \text{ cell}/\mu\text{L}$. According to them this association is most likely explained by the increasing viral burden as HIV disease progresses, which could cause anemia by increasing cytokine-mediated myelosuppression. Alternatively, anemia may be a surrogate marker for some aspect of disease progression not captured by controlling for CD4 counts and clinical AIDS diagnosis.

During the study period of 18 months, deaths were reported in-patients of all grades of anemia. The highest being in grade IV (60%) i. e. 12 patients out of 20 followed by grade III (50%) i. e. 6 patients out of 12. Incidences of deaths were well correlated with their elevated levels of $\text{TNF-}\alpha$ and decreased CD4+ counts i.e. $<200 \text{ cells}/\text{mm}^3$.

Summary

&

Conclusion

SUMMARY AND CONCLUSION:

The present study was designed to evaluate the role of erythropoietin in HIV/AIDS patients. Eighty seropositive male and female patients from the Mumbai city were evaluated; following are the observations of the present study,

1. Anemia is a co- morbid condition of Human Immunodeficiency Virus disease.
2. Irrespective of high or low levels of serum erythropoietin patients suffered a severe degree of anemia. The endogenous serum erythropoietin levels were low in 63.75% patients, while high in 25 % patients.
3. Cytokine dysregulation is seen in HIV/AIDS patients. Seventy seven (96.25%) patients showed elevated levels of TNF- α . These levels showed significant correlation with morbidity and mortality (Pearson's correlation coefficient = $r = - 0.23$ and $p = 0.04$).
4. Fifty six point twenty five % patients had full-blown AIDS (CD4 counts <200 cells/cumm), while 33.75% were in second stage of the disease (CD4 counts between 200-499 cells/cumm).
5. Viral load was done by RT-PCR. A high viral load (between 2.73×10^4 to 4.05×10^6 viral particles/ml.) was observed as the HIV disease progressed.

6. High mortality rate of 55.172 % was observed in the study group. The mortality was correlated to elevated levels of TNF- α and decreased CD4 counts in HIV/AIDS patients.

Hence it appears that estimation of Th1 cytokine viz. TNF- α , CD4 cell counts and viral load, are important parameters to predict morbidity and mortality in HIV/AIDS patients. However anemia though a common symptom is not dependent on endogenous serum erythropoietin levels.

Appendix

APPENDIX:

Total Iron Binding Capacity (TIBC):

1. FeCl_3 solution- 5 $\mu\text{gm/ ml.}$ of HCl - 5-m mol/ lit.

Stock Solution:

1.47 mg FeCl_3 /100 ml of HCl 500 m mol/ lit. Dilute this solution 1 to 100 with distilled water to give concentration of 5-mg/lit HCl 5-m mol/lit.

2. MgCO_3 (powder)

3. Protein Precipitate:

100 gm. of Trichloro acetic acid in 700 ml. of water + 30 ml. Thioglycolic acid + 180 ml. Concentrated HCl . Make the volume of 1 liter.

4. Coloring Reagent:

272 gm of Sodium acetate trihydrate in 400 ml. of water + 250 mg. of Batho-phenanthroline disulph acid disodium salt (GR) ($\text{C}_{24}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_6\text{S}_2\text{3H}_2\text{O}$). Add Distilled water to make the volume 1lit.

Drabkin's Reagent:

It contains in 1000 ml of Distilled water

- | | |
|------------------------------------|---------|
| a) Potassium ferricyanide: | 400 mg. |
| b) Potassium dihydrogen phosphate: | 280 mg. |
| c) Potassium cyanide: | 100 mg. |
| d) Nonidet: | 1 ml. |

List

Of

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Synopsis

**SYNOPSIS OF THE THESIS
TO BE SUBMITTED
TO
THE UNIVERSITY OF MUMBAI
FOR
THE DEGREE OF
MASTER OF SCIENCE
IN MICROBIOLOGY**

TITLE OF THE THESIS : Study the role of erythropoietin in
HIV- related anemia.

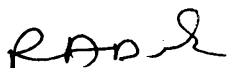
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INTRODUCTION:

The discovery of Human immunodeficiency virus (HIV) in the beginning of 80s (1) has heralded a new chapter in the history of medicine and mankind's ongoing battle against pathogenic microorganisms. The morbidity, mortality, social stigmatization and the economic devastation caused by this pathogen affects not only the infected individuals, but indeed the entire society (2). In developing countries like India, it redefined the terms of social and sexual relationships among people (3). In some of the African nations where large parts of the population have already been infected or are being infected by this virus has had devastating effect on the entire future of these nations and the continent (3). Our ability to effectively understand and combat it in its myriad manifestations will help us in tackling the grave problems posed by this infection (3). India has had a sharp increase in the estimated number of HIV infections, from a few thousands in the early 1990s to a working estimate of between 3.8 to 4.6 million children and adults living with HIV / Acquired Immunodeficiency Syndrome (AIDS) in 2002 (4). With a population of over one billion, the HIV epidemic in India will have a major impact on the overall spread of HIV in Asia/ Asia- Pacific/ South Asia and indeed worldwide (5).

HIV/AIDS infection is characterized by a profound acquired and irreversible immunosuppression (6) that predisposes patients to opportunistic infections, malignancies (7) and progressive dysfunction of multiple organ systems.

Anemia being the most common manifestation of HIV infection and the

recorded incidences of which vary from 10 % to 90 % (7), it contributes to the morbidity caused by the disease. It is normally seen that HIV seropositive patients (or patients with full-blown AIDS) suffer from anemia (8), though the exact cause of this is unknown (idiopathic) (8). The etiology of anemia in HIV infected individuals is multifactorial and may include the following,

1. The HIV virus itself (causing infection of pluripotent blood cells)
2. Anemia of Chronic Diseases (ACD) (7)
3. Opportunistic infections with other viruses, bacteria and fungi etc.
4. Iron deficiency or vitamin B₁₂ deficiency/ protein malabsorption (9).
5. Medications, especially Zidovudine (10)

To assess the possibility of involvement of erythropoietin, which may be responsible for the anemic conditions in HIV/AIDS patients, it is necessary to consider the involvement of above-mentioned factors

Studies have consistently found anemias to be associated with reduced survival, even when confounding factors were controlled (11). Several clinical studies have suggested that anemia is an independent risk factor for death in patients with HIV disease (12). Due to multifactorial and idiopathic origin of anemia, it complicates the differential diagnosis and adequate treatment of HIV infected patients.

Direct infection of erythroid progenitors as a cause to elucidate the HIV associated anemia has been discussed, but could not be confirmed (13). HIV infection of the bone marrow stromal fibroblasts has been demonstrated, but the extent to which hematopoietic progenitors are susceptible to HIV infection remains unclear. Soluble factors like HIV proteins and cytokines have been

suggested to inhibit the growth of hematopoietic cells in the bone marrow of HIV infected patients, which may also lead to development of anemia (14)

HIV infection is associated with increased expression of inflammatory cytokines, including transforming growth factor (TGF) and Tumor Necrosis Factor- alpha (TNF- α), which may play a major role in suppression of hematopoiesis. The HIV protein p24 and antibodies directed against p24 have also been implicated in hematopoietic suppression (15, 16).

Opportunistic infections represent the underlying cause for anemia in a large number of HIV infected patients. Amongst which bacterial infections of bone marrow being the most important. Atypical mycobacteria are the most likely bacterial pathogens to infect the bone marrow in HIV - infected patients, and such patients exhibit elevated serum levels of TNF- α (10). Intestinal infection with microsporidia and other pathogens may contribute to the pathogenesis of HIV- related anemia by leading to iron or vitamin B₁₂ malabsorption.

Serum ferritin is the true indicator to differentiate between iron deficiency anemia and ACD. In a study reported by Kis and Carnes (17) it was found that serum ferritin concentrations of 100 ng/ml or less were the most sensitive and specific marker for identifying iron deficiency anemia. Hence serum ferritin concentration over 100 ng/ml usually rule out iron deficiency anemia and high serum ferritin concentrations in the presence of low total iron binding capacity (TIBC) suggests anemia of chronic diseases. To eliminate the possibility of iron deficiency anemia it is necessary to detect serum ferritin levels in the

serum of the HIV / AIDS patients. In most clinical circumstances, the serum ferritin concentrations are proportional to total body iron stores. Serum Total Iron Binding Capacity (TIBC) is an indirect measure of serum transferrin, a specific iron binding β_2 - globulin. The elevated levels of total TIBC cannot be well correlated to decreased serum iron since in ACD both the serum iron and total iron binding capacity levels are decreased (18, 19).

It is well known that erythropoietin (EPO); a hormone is responsible for normal red blood cell (RBC) production and maturation in healthy individuals. Low levels of this hormone can lead to an anemic condition (20). It is therefore important to study the levels of erythropoietin in the serum of HIV positive patients suffering from anemia. As per CDC guidelines for HIV/ AIDS patients anemia becomes profound and a consistent complication in terminal stages of the disease ($CD4^+$ counts < 200 cells/ mm^3).

It has been documented that HIV replication requires activation of $Nf- \kappa B$, a viral binding site. High levels of $TNF-\alpha$ accelerate this binding. Hence it appears that $TNF-\alpha$ is responsible for an enhanced viral replication. In fact HIV/ AIDS infection has been called a " $TNF-\alpha$ disease" (21). It is therefore an interesting cytokine to be studied along with erythropoietin and HIV viral load to asses the correlation if any between these three factors.

AIMS AND OBJECTIVES

Aims and Objectives: -

1. To detect the type of anemia seen in the HIV seropositive patients on the basis of;
 - a) Complete Blood Cell (CBC) counting with RBC indices.
 - b) Estimation of erythropoietin levels in serum (by ELISA)
 - c) Estimation of Total Iron Binding Capacity (TIBC) of blood
 - d) Estimation of Serum Ferritin levels in serum (by ELISA).
2. To evaluate the status of disease (CDC- staging) by CD4/CD8 counts.
3. To estimate TNF- α in serum (by ELISA) of these patients.
4. To test the Viral Load (by PCR) of these patients.

MATERIALS AND METHODS

PATIENTS:

The patient cohort comprised of 80 age matched HIV-seropositive patients from both the sexes, from Lokmanya Tilak Memorial Municipal Hospital, Sion, Mumbai. A detailed clinical history of patients was taken and elaborate medical history forms were filled. Ethical Committee's clearance was obtained from the concerned hospital authorities and Haffkine Institute. Informed written consent was taken from each patient before collecting the blood samples and clinical data.

CONTROLS:

Control group comprised of 10 apparently healthy, HIV seronegative individuals from student community at the Haffkine Institute, Mumbai.

Mortality rate and morbidity data were collected by keeping a regular record of the patients at the time of their regular visit to the HIV / AIDS clinic over a period of 18 months i.e. during the study period (March 2002 to November2003).

Following inclusion and exclusion criteria were used for selection of the patients for this study;

Inclusion Criteria:



1. Adult Male/ Female HIV seropositive patients
2. Asymptomatic/ symptomatic AIDS patients
3. Clinically significant anemia, Hb< 10 g % in these patients.

Exclusion Criteria:

1. Patients less than 18 yrs. of age
2. Patients with a hemoglobin greater than 10 g %
3. Pregnant women
4. Lactating women
5. Patients on anti-retroviral therapy

BLOOD SAMPLES:

10-ml. blood samples from HIV seropositive patients with significant anemia were collected in EDTA and plain bulbs (5 ml. each). Serum and plasma were separated and aliquoted and stored at -20°C till the time of use. Blood smears were prepared for all the patients enrolled for this study.

REAGENT KITS:

1. EPO- ELISA kit (Roche Diagnostics)
2. TNF- α ELISA kit (BD Biosciences)
3. Serum ferritin ELISA kit (RADIM)
4. CD4+/ CD8+ counting FACS kit (Becton and Dickinson)

Methods:

1. To detect the type of anemia seen in the HIV seropositive patients, the following investigations were carried out:

- A. Complete Blood Cell (CBC) count with RBC indices.

- i) Hemoglobin concentration: by Drabkin's method
- ii) Packed Cell Volume (PCV): calculated by macrohematocrit method.

The standard calculations used for RBC indices were as follows;

1. Determination of Mean Cell Volume (MCV):

$$\text{MCV} = \frac{\text{PCV} \times 10}{\text{RBC in Millions}}$$

2. Determination of Mean Cell Hemoglobin (MCH):

$$\text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC in Millions}}$$

3. Determination of Mean Cell Hemoglobin Concentration (MCHC):

$$\text{MCHC} = \frac{\text{Hb} \times 100}{\text{PCV}}$$

4. Determination of Colour Index:

$$\text{MCHC} = \frac{\text{Hb \%}}{\text{RBC\%}}$$

- B. Estimation of erythropoietin levels in serum (by ELISA):

Levels of erythropoietin were estimated in the serum of patients by using a commercially available ELISA kit (Roche Diagnostics) following the manufacturer's instructions.

C. Estimation of Total Iron Binding Capacity (TIBC) of blood:

TIBC was estimated biochemically, by following the method described by Varley.

D. Estimation of Serum Ferritin levels (by ELISA):

Serum ferritin was estimated by using a commercially available ELISA kit (RADIM), following the instructions provided with it.

2. Evaluation of the status of disease (CDC- staging) by CD4/CD8 counts:

CD4+/ CD8+ lymphocyte counts was performed on fully automated FACS- count (Becton and Dickinson), as per the procedure specified in the instruction manual.

3. Estimation of TNF- α in serum (by ELISA):

Levels of TNF- α were estimated by using a commercially available ELISA kit (BD Biosciences), following the manufacturer's instructions.

4. Viral Load Testing (by PCR):

The HIV viral load will be estimated by the RT- PCR technique.

RESULTS:

Patients reporting at the out patients department (OPD) of LTMMH and indoor patients admitted to the medicine and skin wards were screened. Out of 80 sero-positive patients, 52 (65%) males and 28 (35%) females were enrolled for the present study.

1. Age and sex distribution among the patients:

Table 1: Age and Sex Distribution (n= 80)

Age group (years)	Total no. Of patients (n=80)	Males (n=52)	Females (n=28)
18-28	28	13	15
29-39	39	27	12
40-50	12	12	0
>50	01	0	1
Total	80	52	28

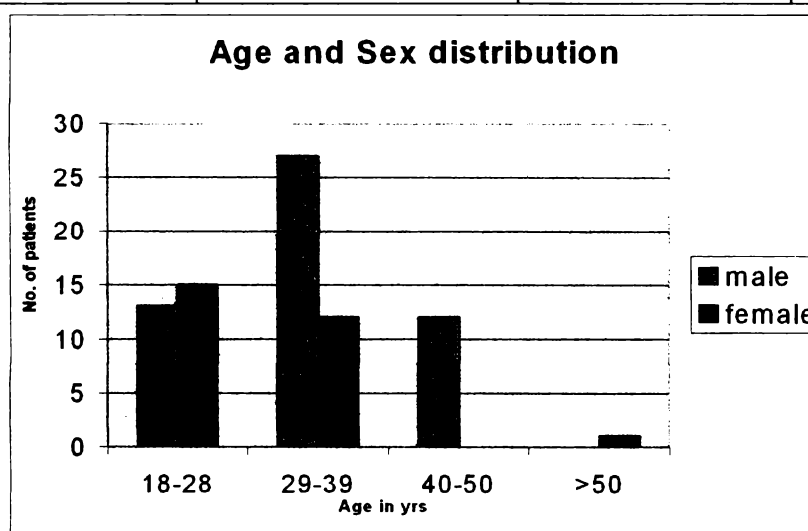


Fig: 1 Age and Sex Distribution among the Patients studied.

2. Probable risk behaviors and mode of transmission amongst the patients:

Table 2: Risk behaviors of patients:

Probable Mode of transmission	Total No. Of Patients	Male	Female
Heterosexual- unprotected sex multiple sex partners	57	49	08
Infection from spouse	19	Nil	19
Homosexual	01	01	Nil
Mode of infection unknown	03	02	01
Blood transfusion	Nil	Nil	Nil
Total	80	52	28

3. Red blood cell indices:

Red blood cell indices, which included Packed Cell Volume (PCV), Mean Cell Volume (MCV), Mean Cell hemoglobin (MCH), and Mean Cell Hemoglobin concentration (MCHC), and Colour Index were performed in all the patients. In all patients studied the values for PCV MCV, MCH, and MCHC parameters were below the normal range. The colour index for all the patients was < 1 . This is a typical observation in hypochromic and normocytic anemia.

4. Categorization of patients in to 4 grades based on the degree of severity of anemia:

Table 3: Severity of Anemia (n = 80)

	Grade I	Grade II	Grade III	Grade IV
Hemoglobin (gms %)	8.0 - 9.4	7.0 - 7.9	6.5 - 6.9	< 6.5
Males	22	13	06	11
Females	09	04	06	09
Total	31	17	12	20

5. Observed mortality in various grades of anemia amongst patients:

**Table 4: Mortality rate recorded in different grades of anemia
(n=80)**

Grade	Total no. Of Patients	No. of patients Expired	%
I	31	05	16.12
II	17	06	35.29
III	12	06	50.00
IV	20	12	60.00
Total	80	29	36.25

6. Estimated levels of endogenous serum erythropoietin levels amongst patients:

Table 5: Distribution of endogenous serum erythropoietin levels in HIV/AIDS patients: (n = 80)

EPO levels (MU/ml.)	Total no. Of Patients	Males	Females
0-20	46	27	19
20- 40	05	04	01
40- 60	09	06	03
60- 80	06	06	-
80- 100	03	01	02
100- 120	03	02	01
120- 140	02	01	01
140- 160	02	01	01
160- 180	-	-	-
180- 200	03	03	-
<200	01	01	-
Total	80	52	28

7. Serum ferritin levels: (n=80)

Ferritin levels in serum of HIV / AIDS patients were estimated. Out of 80 patients, 18 (22.5%) patients showed high ferritin and low TIBC (between 182- 270 ug/100ml), which is a typical indicator of ACD or may be the indicator of active secondary infection. Remaining 62 (77.5%) had their ferritin values in the normal range.

8. Staging of the patients based on their CD4+ / CD8+ counts (cells/mm³):

Table 6: CD4⁺ counts of 80 patients

CD4+ COUNTS	No. of patients
0- 100	32
100- 200	13
200- 300	17
300- 400	10
400- 500	04
> 500	04
Total	80

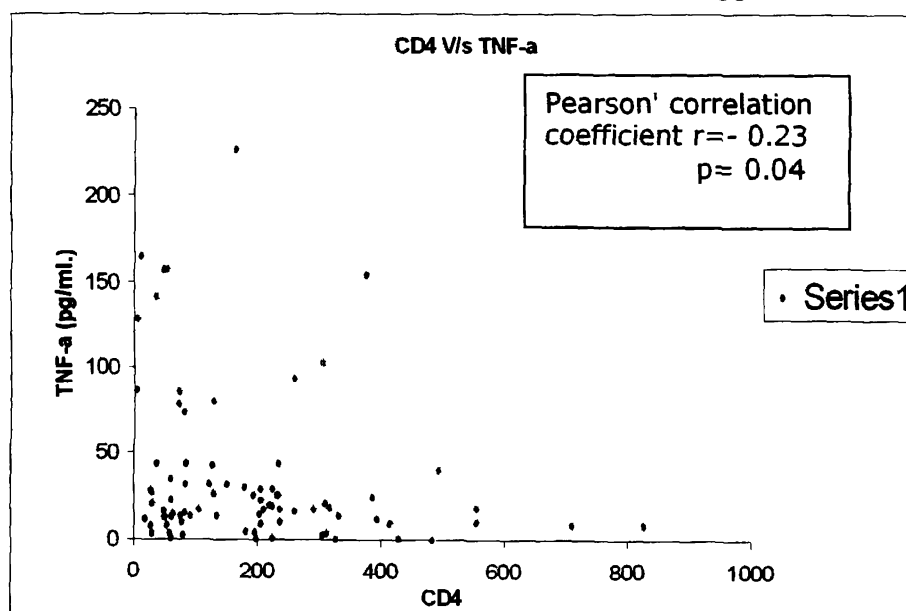


Fig: 2 TNF- α Vs CD4+ Distribution

9. VIRAL LOAD ESTIMATION:

Viral load estimations (for selective number of patients) are presently being carried out and the results will be discussed in the thesis.

DISCUSSION:

In the present study, out of 80 seropositive patients, 52 (65%) were males and 28 (35%) were females. The higher incidence of HIV infection in the male population is well documented. For the female population the case reporting may not be the true number as most of the cases may go unreported.

The age of the patients ranged from 18 to 50 years, with a mean age of 30 years. 39 patients were in the age group of 29 to 30 years, which is the main productive and sexually active age group.

According to the Joint United Nations Programme on AIDS (UNAIDS) (22) report of 2001, in India, the estimated prevalence of HIV infection amongst persons aged 15 to 49 years is 0.7%, with an estimated prevalence of HIV infections is around 3.5 million. According to AIDS case data, 89% of patients are in the age group of 15 to 44 years.

Heterosexual transmission is a major mode of HIV transmission all over India except for Manipur, where intravenous drug abuse or sharing of the needles is the major mode of acquisition of the HIV infection (23). In the present study we found that 57 (71.25%) out of 80 patients acquire the infection via the heterosexual route. Among 28 females 19 (67.87%) were monogamous and contracted the disease from their spouses.

We categorized the patients into four grades based on the WHO guidelines for anemia (24). In our study, most of the female population studied had grade IV anemia with a very low hemoglobin concentration. In India, majority of women have low hemoglobin due to multiple deliveries, poor nutrition and neglected health care. HIV infection is an additional factor for aggravation of anemia.

HIV disease progression of the patients was staged on the basis of CDC guidelines based on their CD4+ counts (25). As shown in table 6, 45 (56.25%) patients had a CD4+ count of less than 200-cells/mm³, and were classified to have developed Immunological AIDS.

It was observed that, considering the normal range for erythropoietin between 3-48 mU/ml, 60 patients (75%) had a normal endogenous serum erythropoietin level i.e. <48mU/ml. despite suffering from anemia, 46 (57.5%) patients had levels below 20 mU/ml. Additionally, a total of 69 (86.25%) patients had EPO levels <100 mU/ml. And 11 (13%) had levels >100 mU/ ml. The group of patients with high EPO levels i.e. >100-mU/ ml also had severe anemia thereby indicating that the bone marrow was not responsive to endogenous erythropoietin. The reason of this incomplete response has been supported by a study by Spivak et al (26) in 152 anemic patients. They showed that a "blunted response" to erythropoietin is extremely common in the setting of HIV disease, leading to anemia that can be quite significant and requires therapy (27, 28). The normal range for endogenous serum erythropoietin (EPO) is between 3-48 mU/ml.

1. There were 22 (27.5%) patients in the present study, showing levels less than the normal range. These levels suggest that there is an inappropriate erythropoietin response in the body.
2. 51 (63.75%) patients showed the normal erythropoietin levels and still showed severe degree of anemia. Such scenario may arise due to faulty synthesis of EPO or improper post-translational modifications. As a result circulating EPO may not be in its active form.
3. There is a possibility of bone marrow not responding to the EPO, which may be in normal levels.
4. There is also a possibility of bone marrow microenvironment getting infected by HIV virus itself, thereby affecting the production of the required number of erythrocytes, which in turn leads to anemia.

Majority of patients in the study population suffering with HIV/ AIDS showed elevated levels of Tumor Necrosis Factor-alpha (TNF- α). There is direct correlation between the CD4+ counts and the TNF- α levels (29). From fig.2 we can observe that as the CD4+ counts reduce TNF- α levels showed an increase in the blood of HIV/ AIDS patients. The *Pearson' correlation Coefficient* test showed a significant Negative correlation. *Pearson' correlation Coefficient* $r = - 0.23$ and $p = 0.04$

As shown in fig. 2, when we correlated the findings with their CD4+ counts we found a significant negative correlation between the two factors. Deaths were reported in-patients of all grades of anemia. The highest being in grade IV

(60%) followed by grade III (50%). Incidences of deaths were well correlated with their elevated levels of TNF- α and decreased CD4+ counts i.e.<200 cells/mm³.

CONCLUSION:

The present study clearly points out the impact of anemia on the survival of patients with HIV/ AIDS. The degree of anemia exhibits a proportional relationship with morbidity and mortality conditions amongst the patients. Irrespective of the endogenous levels of erythropoietin, HIV / AIDS patients suffer with anemia. With a reference to above-mentioned reasons the patients with low levels of the hormone can be recommended to undergo for the erythropoietin therapy. By managing the anemic conditions in the patients, the overall quality of their life can be improved. Anemia in HIV / AIDS patients should be given special attention and should not be treated only as a co-morbid condition.

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