

STUDY OF IMMUNE RESPONSES IN SPOUSES OF HIV SEROPOSITIVE MIGRANT WORKERS

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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***Study of Immune Responses In Spouses Of
HIV Seropositive Migrant Workers.***

A Thesis Submitted to the

University of Mumbai

For the

**MSc. (Science) Degree,
In Microbiology**

Submitted by

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STATEMENT BY THE CANDIDATE

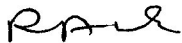
As required by the University Regulation No.2190, I wish to state the work embodied in this thesis titled "Study Of Immune Responses In Spouses Of HIV Seropositive Migrant Workers", forms my own contribution to the research work carried out under the guidance of Dr. (Mrs.) R. A. Deshmukh at Department of Virology, Haffkine Institute Parel, Mumbai 400 012. This work has not been submitted for any other degree of this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.



Signature of the Candidate

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Dedicated Lovingly to my

Family....

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Shweta Tawde



Contagious Beauty

Hunter O'Reilly, Ph.D.

2001

The painting, Contagious Beauty, features an abstraction of a woman infected with HIV. The oval structure surrounding her face is an abstraction based on scientific research of the structure of an HIV particle. The painting has both positive and negative connotations. In a negative light, the woman can be seen as carrying a deadly contagious disease. In a positive light, this woman infected with HIV can also be seen as a beautiful person filled with life.

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Chapter 1

Introduction

*"Research is to see what everybody else has seen, and to think
what nobody else has thought."*

— Albert Szent-Gyorgi

Human immunodeficiency virus (HIV) is a retrovirus that infects cells of the human immune system. Infection with HIV has been established as the underlying cause of Acquired immunodeficiency syndrome (AIDS). HIV has been a subject of intense scientific research and basic information about HIV and AIDS keeps changing constantly.

HIV has been sweeping the world for more than two decades, causing a disease which has killed millions of people and which looks likely to kill millions more. India stands at the edge of a public health disaster HIV/AIDS. HIV primarily infects vital cells in the human immune system such as helper T cells (specifically CD4⁺ T cells), macrophages and dendritic cells. HIV infection leads to depletion of CD4⁺ T cells through three mechanisms (1) Direct viral killing of infected cells; (2) increased rates of apoptosis in infected cells; and (3) killing of infected CD4⁺ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. When CD4⁺ T cell numbers decline below critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections. If untreated eventually most HIV infected individuals develop AIDS and die; however about one in ten remain healthy for many years, with no noticeable symptoms. ¹

HIV infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that AIDS has killed more than 25 million people since it was first recognized on December 1, 1981,

making it one of the most destructive pandemics in recorded history. Despite recent, improved access to antiretroviral treatment and care in many regions of the world the AIDS pandemic claimed 3.1 million [2.8-3.6 million] lives in 2005. The total number of people living with the HIV reached its highest level; an estimated 39.5 million [34.1 million-47.1 million] people are now living with HIV. ² Approximately 4.3 million [3.6 million-6.6 million] adults and children were newly infected with HIV in 2006. HIV/AIDS disproportionately affects women. In 2006 globally there were 17.7 million women [15.1-20.9 million] living with HIV/AIDS. Since 1985 the percentage of adult women with HIV/AIDS has risen from 35% to 48%. Young women make up over 60 % of the 15-24 year olds living with HIV/AIDS. Women are now becoming infected faster than men are. Increasingly there is feminization of HIV/AIDS. Currently there are 2 million [1.3 -3 million] women living with HIV in Asia. ²

In 1981 the 1st HIV infected case was seen in USA. At that time it was considered a disease of males. Now 23 yrs later the rapid increase in the number of women infected with HIV worldwide forecasts an inevitably greater number of women who will develop and die from AIDS. Currently almost half of all new HIV infections are being reported in women; of these less than 50% are aware of their infection and 84% are in their reproductive age group that is 15 to 45 years. Hence worldwide the HIV risk for women is rising. ³

Women's status has an important and direct influence on their vulnerability to HIV infection particularly in a country like India where the status of women is still much below par. They provide active support to HIV infected men in overall management of family life, but in return they do not get due recognition and status. In view of their low status, women are exposed to various factors, which increase their vulnerability to HIV infection and HIV related diseases.⁴

Women are often unable to discuss sexual matters with their male partners, as socially it is not acceptable. Even if a woman knows how to protect her from infection by use of condom or other safe sexual practices, she fears violence or rejection from her male partner. Wife sharing by men results in women having multiple sex partners thereby increasing their vulnerability to infection. Women are financially dependent on men. They fear of being thrown out and disowned if they talk about the use of safe sex practice to reduce the risk of infection. Sometimes unemployment, poverty added with the responsibilities of taking care of her children or siblings drives her to become a commercial sex worker.⁴

Women's burdens are intensified when families are affected by HIV because they have to provide care in addition to maintaining household incomes. Women who have lost partners to AIDS or who have been abandoned because they are HIV seropositive are often deprived of financial security and economic opportunities. Many experience sexual

and economic subordination in their personal relationships and at work and so cannot negotiate safe sex or refuse unsafe sex, making them especially vulnerable to HIV infection.⁴ Thus women especially spouses of High-risk group individuals (Intravenous drug users- IDU's Truck drivers and migrant workers) are at greater risk due to lack of knowledge regarding HIV/AIDS and its route of transmission.

Theoretically, migration can be seen as a simple process that consists of three phases: where a person comes from, where they are going and where they end up. Migration is also about the how a person travels; the length of time they are away; why they left in the first place; the relationships they maintain with home while they are away and how far they are from home. Migration is often cyclical or seasonal, as people return home for periods of time.

Globalization processes have profoundly affected the character of population mobility and migration and posed serious challenges to the protection of mobile and migrant workers. The International Labour Office (ILO) has estimated that over 25 million are workers. The AIDS pandemic is having a serious impact on employment, especially in sectors with a highly mobile workforce such as the transport industry the armed forces, construction workers and seasonal farm workers.

HIV/AIDS is likely to affect mobile populations in certain regions more intensively than others due to patterns of migration, development strategies, legal provisions, cultural norms and levels of poverty. Africa

has among the largest number of migrant workers in the world, with approximately 20 million or one-fifth of the global total. In Abidjan, Côte d'Ivoire, which is one of the main stops along a major migration route, HIV prevalence rates are thought to be as high as 10-15%. Labour mobility and a range of changes associated with modernization have contributed to the spread of HIV/AIDS in India. The transport sector is particularly at risk. Some estimates suggest that the number of truck drivers contracting HIV increases at the rate of 1,000 truckers every week. ⁵

Being mobile itself is not a risk factor for HIV infection. However, the situations encountered and the behavioral patterns during mobility or migration lead to increased vulnerability and risk of acquiring HIV/AIDS. ⁶ The ILO Code of Practice on HIV/AIDS and the World of Work identifies work situations, which cause the worker to be more susceptible to the risk of HIV infection. Most of which apply to many mobile workers: traveling regularly; living away from spouses and partners; working in geographically isolated environments with limited social interaction and health facilities; single-sex working and living arrangements among men; and work that is dominated by men, where women are in a small minority. ⁷

Most migrant workers are highly mobile and often live in unhygienic conditions in urban slums. Long working hours, relative isolation from the family and geographical mobility may foster casual sexual

relationships and make them highly vulnerable to STDs and HIV/AIDS. Migrant workers tend to have little access to HIV/STD information, voluntary counseling and testing and health services. Cultural and language barriers worsen their lack of access to such services. Returning or visiting migrants, many of who do not know their status, infect their wives or other sex partners in the home community.⁸ Poor status of women as seen in India makes her vulnerable to HIV infection.

India is rapidly urbanizing with above 28 % of the population living in urban areas. Most of these are migrant workers with families in rural areas. A study of migrant workers in Mumbai (where 46% of workers are migrants) found that men living with families were half as likely to report visiting commercial sex workers as those not staying with their families.^{9, 10}

Research on population mobility and HIV/AIDS risk among migrant population is quite limited, and little or no data is available on spouses of the migrant workers.

Exposure to HIV does not have a single common outcome in all individuals. Even in the absence of antiretroviral drug treatment, several classes of clinical progression have been defined like rapid progressors, typical progressors, long-term non-progressors and highly exposed seronegative individuals (ESN's). These ESN's have been a subject of special interest as it is not clear why these people do not

develop HIV disease even after multiple exposures to the virus. Data indicating immune protection against HIV by this group is quite limited. Both arms of the immune system (humoral and cell mediated) are thought to be important elements for protection hence it is required to know the type of response that protects these ESN's due to which they remain seronegative even after multiple exposures to the virus.

The pathogenesis of HIV infection is a complex, multifactorial process involving viral and host factors. The virus utilizes molecules of human immune system to infect the very cells that should be responsible for orchestrating the immune counterattack. The immunologic hallmark of successful infection with the Human Immunodeficiency Virus (HIV) is a progressive decline in CD4⁺ T cell count, which ultimately results in the appearance of infections characteristic of Acquired Immune Deficiency Syndrome (AIDS). However, prior to this decline in CD4⁺ T cell number, impairment of T helper cell (T_H) function is observed. ⁽¹¹⁾ Because a major function of T_H is to produce cytokines (IL-2, IL-12 and IFN- γ) that activate different effector components, analysis of the changes in cytokine production may permit identification of multiple Th defects, which result from HIV infection.

Progression of HIV infection has been clearly associated with the occurrence of oral candidiasis, which may be a sentinel event presenting months or years before more severe opportunistic diseases

set in. Oral Candidiasis has been clearly shown to be an independent prognostic marker of the risk of progression to overt AIDS.

Almost all HIV infected individuals mount HIV-specific immune response. However, only few can control the viraemia successfully for prolonged period. The adaptive immune responses use multiple effector mechanisms to control the virus multiplication. HIV specific neutralizing antibodies, T helper cell responses and (Cytotoxic T lymphocyte) CTL responses are the major players in HIV-specific adaptive immune response.

Humoral response is evidenced by formation of antibodies of different classes against different structural proteins (**gag**: p15, p17, p24, p55; **env**: gp41, gp120, gp160; and **pol**: p31, p51, p66), regulatory proteins (nef, rev, tat) and accessory proteins (vif and vpu). The antibodies to gag protein (p24 and p55) are first to appear usually, though antibodies to env proteins and pol proteins may also be produced simultaneously. As infection progresses to AIDS, antibody to p24 usually declines as p24 antigen levels rise concomitant with progression of disease to AIDS. However antibodies to env proteins persist throughout the infection. Anti-HIV antibodies may be IgA, IgM and IgG. The IgA and IgM responses are inconsistent. IgM response appears earlier than IgG but it is detectable for a short period. ⁽¹²⁾

Humoral immunity appears to be less effective in controlling viraemia than cellular responses, as HIV is remarkably effective at evading host

antibody responses.^{13,14} The role of neutralizing antibodies in protection against HIV in ESN's is not clear. Cytotoxic T lymphocytes (CTL's) are believed to play critical roles in the control of many viral infections. HIV-1 specific CD8+ CTL's act to eliminate HIV infected cells and suppress further replication by secretion of antiviral cytokines. (e.g. interferon γ). Cytotoxic T lymphocyte responses have said to be associated with protection from HIV-1 infection in people with high degree of exposure to HIV and who show no serological evidence of HIV infection (ESN's, exposed but seronegative). However, it remains unclear how protective CTL responses could apparently develop in a minority of people, whilst majority of HIV-infected people show CTL responses yet progress to develop AIDS and death. Hence the current study has been carried out to study the demographic and clinical factors influencing the vulnerability of spouses of HIV infected male migrants.

Review
of
Literature

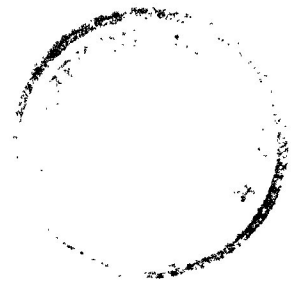
History

On June 5, 1981, the U.S. Centers for Disease Control and Prevention reported a cluster of *Pneumocystis carinii* pneumonia (now classified as *Pneumocystis jiroveci* pneumonia) in five homosexual men in Los Angeles.¹⁵

The disease was originally dubbed GRID, or Gay-Related Immune Deficiency, but health authorities soon realized that nearly half of the people identified with the syndrome were not homosexual men. In 1982, the CDC introduced the term AIDS to describe the newly recognized syndrome. In 1983, scientists led by Luc Montagnier at the Pasteur Institute in France first discovered the virus that causes AIDS.¹⁶

They called it lymphadenopathy-associated virus (LAV). A year later a team led by Robert Gallo of the United States confirmed the discovery of the virus, but they renamed it human T lymphotropic virus type III (HTLV-III).¹⁷

In 1986, both the French and the US names for the virus were dropped in favour of the new term, Human immunodeficiency virus (HIV).¹⁸



Epidemiology:

Promising developments have been seen in recent years in global efforts to address the AIDS epidemic, including increased access to effective treatment and prevention programmes. However, the number of people living with HIV continues to grow, as does the number of deaths due to AIDS. ²

Global scenario

A total of 39.5 million [34.1 million–47.1 million] people were living with HIV in 2006. An estimated 4.3 million [3.6 million–6.6 million] adults and children were newly infected with HIV in 2006. ² Globally approximately 2.1 million children under age 15 have HIV/AIDS. There were 2.9 [2.5 million–3.5 million] million deaths in 2006 out of which 2.6 million [2.2 million–3.0 million] were adults and 380,000 were children [290,000–500,000] under 15 years of age.

In many regions of the world, new HIV infections are heavily concentrated among young people (15–24 years of age). Among adults 15 years and older, young people accounted for 40% of new HIV infections in 2006. Sub-Saharan Africa continues to bear the brunt of the global epidemic. Two thirds (63%) of all adults and children with HIV globally live in sub-Saharan Africa, with its epicentre in southern Africa. One third (32%) of all people with HIV live in southern Africa and there were 34% of deaths due to AIDS in 2006. Globally, and in

every region, more adult women (15 years or older) are now living with HIV. Approximately 17.7 million [15.1 million–20.9 million] women were living with HIV in 2006. Across all age groups, 59% of people living with HIV in sub-Saharan Africa in 2006 were women.²

Asian scenario

An estimated 8.6 million [6.0 million–13.0 million] people were living with HIV in Asia in 2006, including the 960,000 [640,000–1,280,000] people who became newly infected in the past year. Approximately 630,000 [430,000–900,000] died from AIDS-related illnesses in 2006. The number of people receiving antiretroviral therapy has increased more than threefold since 2003, and reached an estimated 235,000 [180,000–290,000] by June 2006. This represents about 16% of the total number of people in need of antiretroviral treatment in Asia.²

An estimated 2.2 million [1.3 million–3.6 million] of women above 15 years of age are living with HIV in South and South East Asia. Approximately 270,000 [170,000–820,000] adults and children were newly infected with HIV in Eastern Europe and Central Asia in 2006. An increase of almost 70% was found in 2006 over the 160,000 [110,000–470,000] people who acquired HIV in 2004. In South and South-East Asia, the number of new HIV infections rose by 15% in 2004–2006.²

Indian scenario

The world's second-most populous country, India, is experiencing a highly varied HIV pandemic, which appears to be stable or diminishing in some parts while growing at a modest rate in others. Approximately 5.7 million [3.4 million–9.4 million] people, of which, 5.2 million were adults aged 15–49 years, were living with HIV in 2005 with adult prevalence of 0.91%. A total of 124,995 new AIDS cases were estimated in 2006, of which, 88,245 were male and 36,750 were female. The highest prevalence rates are found in the Mumbai Karnataka corridor, the Nagpur area of Maharashtra, the Nammakka district of Tamil Nadu, coastal Andhra Pradesh, and parts of Manipur and Nagaland (in the north-east of India). Recent analysis of HIV data from 216 antenatal clinics and 132 sexually transmitted infection clinics for 2000–2004 suggest that HIV prevalence among women aged 15–24 years in southern states declined from 1.7% in 2000 to 1.1% in 2004. Consequently, women account for a growing proportion of people living with HIV (some 38% in 2005), especially in rural areas. HIV infection levels of over 1% have been found among pregnant women in Andhra Pradesh, Maharashtra and Karnataka. ²

Human Immunodeficiency Virus

HIV the etiological agent for causing AIDS is classified as a member of the genus lentivirus.^{19, 20} HIV is transmitted as single-stranded, positive-sense, enveloped RNA virus. Upon entry in the target cell, the viral RNA genome is converted to double-stranded DNA by a virally encoded reverse transcriptase that is present in the virus particle. This viral DNA is then integrated into the cellular DNA by a virally encoded integrase so that the genome can be transcribed. Once the virus has infected the cell, two pathways are possible: either the virus becomes latent or the infected cell continues to function, or the virus becomes active and replicates, and a large number of virus particles are liberated that can then infect other cells.

Two species of HIV infect humans: HIV-1 and HIV-2. HIV-1 is thought to have originated in southern Cameroon after jumping from wild chimpanzees (*Pan troglodytes troglodytes*) to humans during the twentieth century.^{21, 22}

HIV-2 is believed to be originated from the Sooty Mangabey (*Cercocebus atys*'), an Old World monkey of Guinea-Bissau, Gabon, and Cameroon. HIV-1 is the most virulent. It is easily transmitted and is the cause of the majority of HIV infections globally. HIV-2 is less transmittable and is largely confined to West Africa.²³

Transmission

Since the beginning of the pandemic, three main transmission routes for HIV have been identified:

(i) Sexual route:

Transmission as result of sexual intercourse accounts for about three fourths of all HIV infections worldwide. Initially, men in developed countries were more exposed to HIV than women, primarily as a result of sexual intercourse between men or from injecting drug use.

The majority of HIV infections are acquired through unprotected sexual relations. In India transmission of HIV through sexual route in 2005 was found to be 85 %. HIV prevalence of 6.8% and 9.6% were found among men who have sex with men in Chennai and Mumbai, respectively. ²⁴. More recently, HIV prevalence of 12% was found among men who have sex with men seeking voluntary counselling and testing services in Mumbai, and 18% prevalence was found at 10 clinics in Andhra Pradesh. ^{25, 26}

(ii) Transmission through Blood or blood products:

This transmission route can account for infections in intravenous drug users, hemophiliacs and recipients of blood transfusions. People who give and receive tattoos, piercings and scarification procedures are considered to be at risk of infection. A major problem in the developed

and developing world is HIV transmission resulting from sharing contaminated injection equipment by drug users.

Injecting drug use is the main risk factor for HIV infection in the Northeast especially in the states of Manipur, Mizoram and Nagaland, and features increasingly in the epidemics of major cities, including in Chennai, Mumbai and New Delhi. Products injected include pharmaceuticals that are not illegal (such as buprenorphine, pentazocine and diazepam), in addition to heroin. An estimated 6.2 % of transmission of HIV was found in Injecting drug users in India in 2006. In Chennai, 31% of injecting drug users were found to be HIV-infected in a recent study.²⁷

A recent study among men seeking treatment for sexually transmitted infections in Mumbai clinics showed that among those patients who injected drugs, 12% were HIV positive.⁽²⁸⁾

(iii) Mother-to-child transmission (MTCT):

The transmission of the virus from the mother to the child can occur in utero during the last weeks of pregnancy and at childbirth. In the absence of treatment, the transmission rate between the mother and child is 25%-40%. However, where drug treatment and Cæsarian section are available, this can be reduced to 1%. Breast-feeding also presents a risk of infection for the baby.²⁹

In 2005, around 7,00,000 children under 15 became infected with HIV, mainly through mother-to-child transmission. About 90% of these

MTCT infections occurred in Africa. According to World Health Organisation (WHO) guidelines, the regimen currently recommended for preventing mother-to-child transmission in resource-limited settings uses a combination of AZT and single dose nevirapine. This approach is much more difficult to administer than single dose nevirapine on its own, but it is also significantly more effective, and is less likely to lead to drug resistance. AZT was first shown to reduce MTCT rates in 1994, and is the best-studied drug for this purpose. ²

The woman should begin taking AZT after 28 weeks of pregnancy (or as soon as possible thereafter). During labour she should take AZT and 3TC, as well as a single dose of nevirapine. Her baby should receive a single dose of nevirapine immediately after birth, followed by a seven-day course of AZT. The mother should continue taking AZT and 3TC for seven days after delivery, to cut the risk of drug resistance still further. MTCT was found to be approximately 4% in 2005 in India. ² HIV-2 is transmitted much less frequently by the MTCT and sexual route than HIV-1.

HIV has been found at low concentrations in the saliva, tears and urine of infected individuals, but the risk of transmission by these secretions is negligible. The use of physical barriers such as the latex condom is widely advocated to reduce the sexual transmission of HIV. ³⁰

Structure and genome of HIV:

The genome and proteins of HIV have been the subject of extensive research since the discovery of virus in 1983. It is a well-known fact that no two HIV genomes are the same, not even from the same person causing some to speculate that HIV is “quasispecies” of a virus.³¹

A. Structure of HIV

HIV is around 120 nm in diameter and roughly spherical. HIV-1 is composed of two copies of single-stranded RNA enclosed by a conical capsid comprising the viral protein p24, typical of lentiviruses. This is in turn surrounded by a plasma membrane of host-cell origin. The single-strand RNA is tightly bound to the nucleocapsid proteins, p7 and enzymes that are indispensable for the development of the virion, such as reverse transcriptase and integrase. The nucleocapsid (p7 and p6) is associated with the genomic RNA (one molecule per hexamer) and protects the RNA from digestion by nucleases. A matrix composed of an association of the viral protein p17 surrounds the capsid, ensuring the integrity of the virion particle. Also enclosed within the virion particle are Vif, Vpr, Nef, p7 and viral protease. The envelope is formed when the capsid buds from the host protein, taking some of the host-cell membrane with it. The envelope includes the glycoproteins gp120 and gp41.

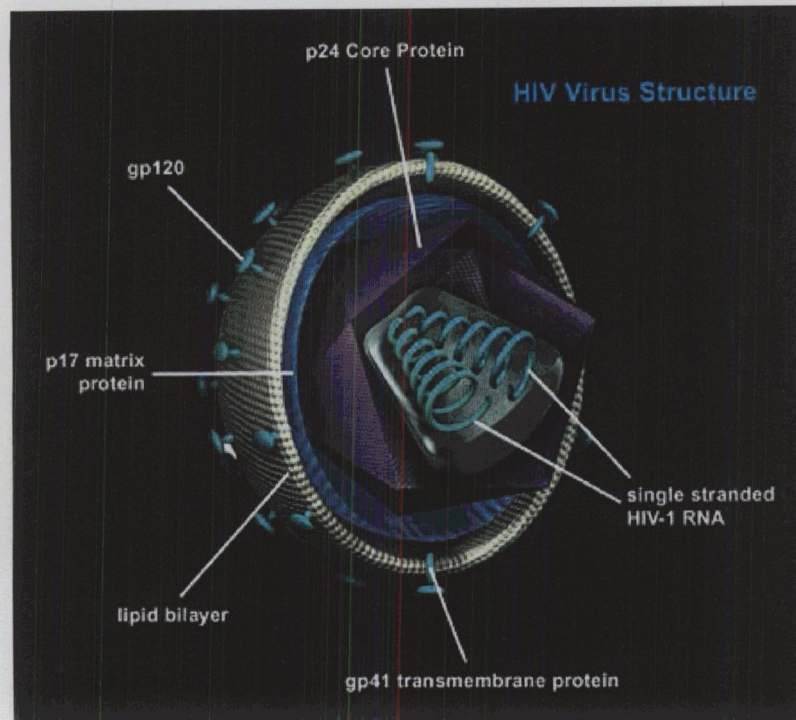


Figure 1: Human Immunodeficiency Virus

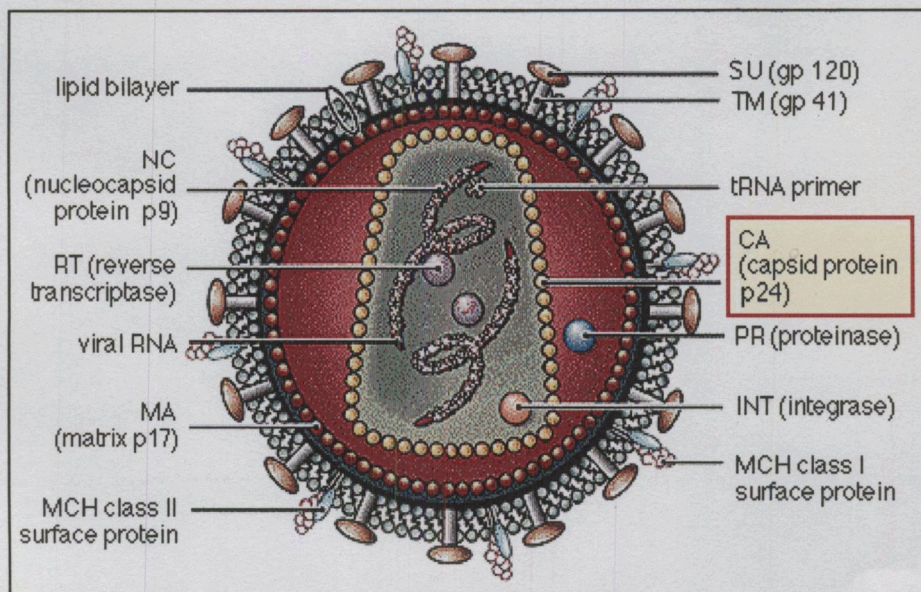


Figure 2: Structure of HIV

B. Genome Organization and Protein Function

HIV has several major genes coding for structural proteins that are found in all retroviruses, and several nonstructural ("accessory") genes that are unique to HIV. The "gag" gene provides the basic physical infrastructure of the virus, and "pol" provides the basic mechanism by which retroviruses reproduce, while the others help HIV to enter the host cell and enhance its reproduction. Though they may be altered by *mutation*, all of these genes except "rev" exist in all known variants of HIV.³³

(I) *Gag (Group-specific Antigen):*

Codes for p24, the viral capsid; p6 and p7, the nucleocapsid proteins and p17, a matrix protein. These proteins are encoded by the "gag" gene, and provide structural elements of the virus: p24 makes up the viral capsid, p6 and p7 provide the nucleocapsid, and p17 provides a protective matrix. When a Western blot test is used to detect HIV infection, p24 is one of the three major proteins tested for, along with gp120/gp160 and gp41.³³

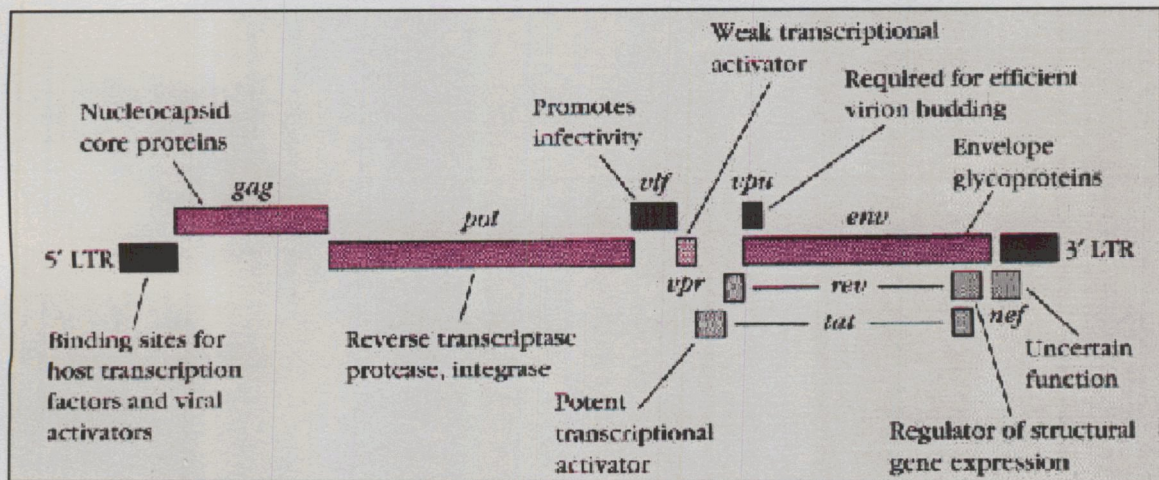


Figure 3 : HIV Genome

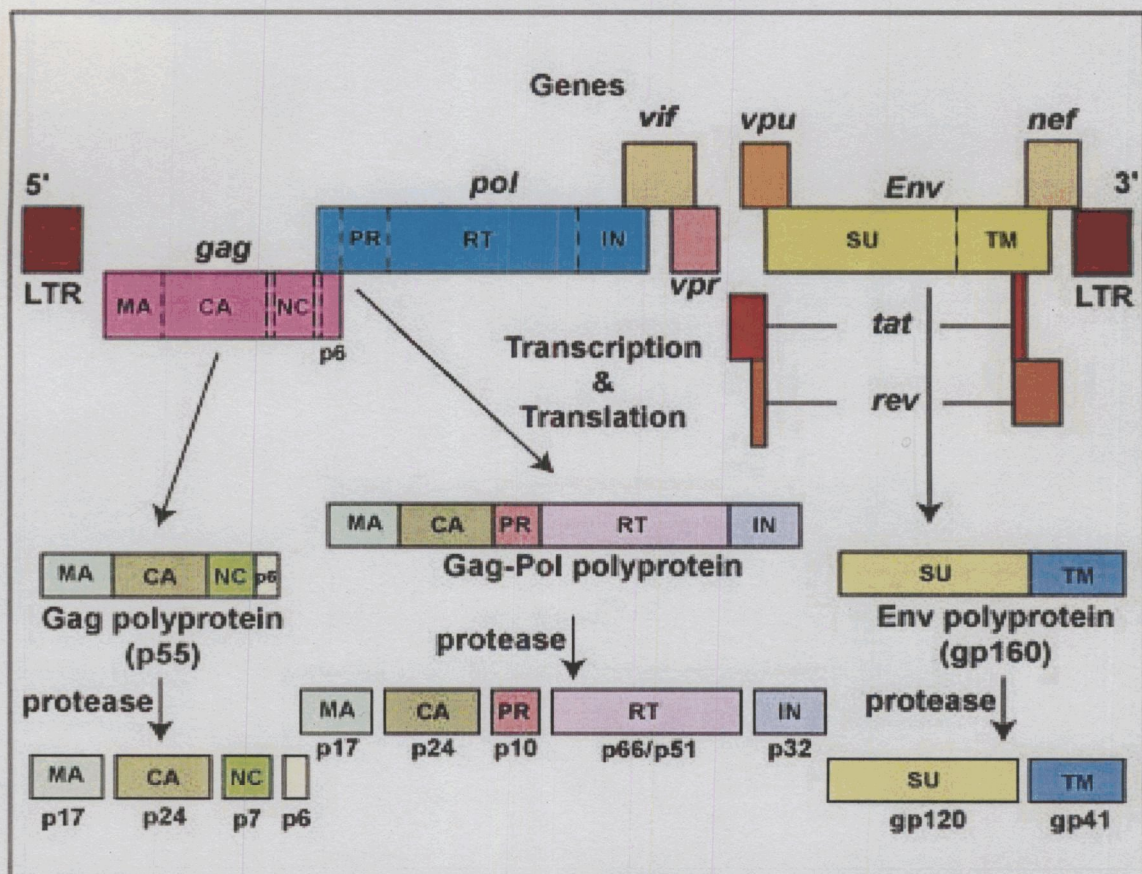


Figure 4: Genome organization of HIV

(II) *Pol*

Codes for viral enzymes, the most important of which are reverse transcriptase, integrase, and protease, which cleaves the proteins derived from "gag" and "pol" into functional proteins.

(a) Reverse transcriptase

Common to all retroviruses, this enzyme transcribes the viral RNA into double-stranded DNA. Reverse transcriptase enzymes include an RNA-dependent DNA polymerase and a DNA-dependent DNA polymerase, which work together to perform transcription. In addition to the transcription function, retroviral reverse transcriptases have a domain belonging to the RNase H family, which is vital to their replication. Reverse transcriptase has a high error rate when transcribing RNA into DNA, as unlike DNA polymerases it has no proof reading ability. This high error rate allows mutations to accumulate at an accelerated rate relative to proofread forms of replication. ³³

(b) Integrase

HIV integrase is a 32-kDa protein produced from the C-terminal portion of the pol gene product. Integrase, therefore, is an attractive potential target for new anti-HIV therapeutics.

(c) Protease

A protease is any enzyme that cuts proteins into segments. HIV's "gag" and "pol" genes do not produce their proteins in their final form, but as larger combination proteins; the specific protease used by HIV cleaves these into separate functional units. Protease inhibitors (PIs) drugs block this step.

(III) *Env* (for "envelope"):

Codes for the precursor to gp120 and gp41, proteins embedded in the viral envelope that enables the virus to attach to and fuse with target cells. ⁽³³⁾

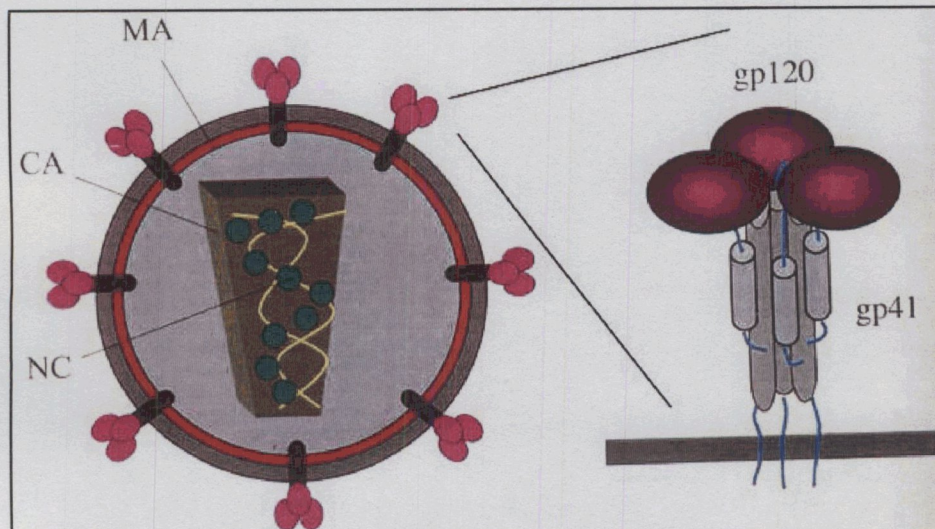


Figure 5 : HIV envelope glycoprotein

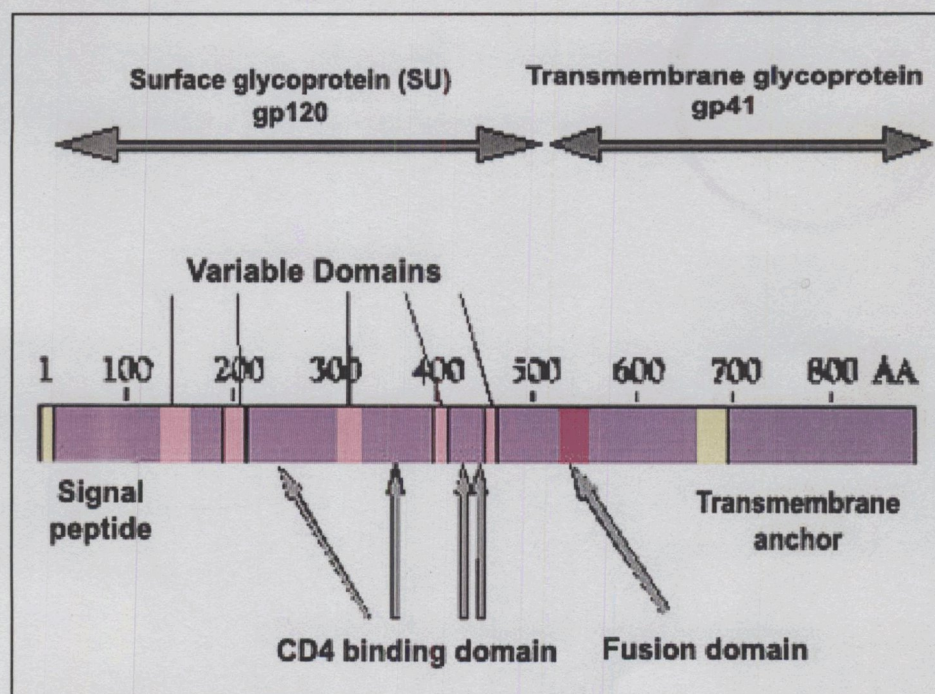


Figure 6 : Envelope glycoprotein genome

(a) gp120

gp120 is a glycoprotein expressed on the surface of human immunodeficiency virus envelope. The structure of gp120 is important for virus-receptor interaction and virus entry. Env is a highly glycosylated protein. Comparison of the sequences of divergent HIV-1 isolates show significant diversity concentrated primarily in the gp120-coding region of env, in five discrete regions named hypervariable regions (V1 to V5). The regions between the variable regions are more conserved and are named constant (C1 through C5). Immunization with purified Env proteins leads to neutralizing antibodies binding to the V3 hypervariable region, a loop of 33 to 35 amino acids confined by a conserved disulfide bond (V3 loop). This region has been named the principal neutralizing determinant of HIV-1. The region of Env glycoprotein gp120 is responsible for the difference in cell tropism in many cases.³²

HIV can mutate frequently to stay ahead of the immune system. There is however a highly conserved region in the virus genome that codes for the structures to allow the virus to bind to a human cell and enter. The glycoprotein gp120 is anchored to the viral membrane through non-covalent bonds along with gp41, both coming from cleaved protein, gp160. It infects any target cell with a CD4 receptor, particularly the helper T-cell, by binding to that receptor. Binding to

CD4 is mainly electrostatic although there are van der Waals interactions and hydrogen bonds.

The structure of gp120 involves an outer domain, an inner domain and a bridging sheet. The genome is around 1.5Kb coding for about 500 amino acids. gp120 forms a sort of cap over the end of gp41 to make a gp120/gp41 subunit. The functional Env protein is a multimer (trimer?). Multimerization of Env glycoprotein is mediated by the ectodomain of the transmembrane glycoprotein gp41. ³³

(b) Gp41

The glycoprotein gp41 is non-covalently bound to gp120, and provides the second step by which HIV enters the cell. It is originally buried within the viral envelope, but when gp120 binds to a CD4 receptor, gp120 changes its conformation causing gp41 to become exposed, where it can assist in fusion with the host cell.

Fusion inhibitor drugs such as enfuvirtide block the fusion process by binding to gp41.

The "env" gene does not actually code for gp120 and gp41, but for a precursor to both, gp160. During HIV reproduction, the host cells own enzymes cleave gp160 into gp120 and gp41. ³³

(IV) *Tat, Rev, Nef, Vif, Vpr, and Vpu:*

Each of these genes codes for a single protein with the same names.

(a) Tat

Stands for "Trans-Activator of Transcription". Tat consists of between 86 and 101 amino acids depending on the subtype.³⁴

Tat helps HIV reproduce by compensating for a defect in its genome: the HIV RNA initially has a hairpin-structured portion, which prevents full transcription occurring. However, a small number of RNA transcripts are made, which allow the Tat protein to be produced. Tat then binds to and phosphorylates cellular factors, eliminating the effect of the hairpin RNA structure and allowing transcription of the HIV DNA.³⁵ This itself increases the rate of transcription, providing a positive feedback cycle. This in turn allows HIV to have an explosive response once a threshold amount of Tat is produced, a useful tool for defeating the body's response.

Tat also appears to play a more direct role in the HIV disease process. The protein is released by infected cells in culture, and is found in the blood of HIV-1 infected patients.³⁶ It can be absorbed by cells that are not infected with HIV, and can act directly as a toxin producing cell death via apoptosis in uninfected "bystander" T cells, assisting in progression toward AIDS.³⁷ By interacting with the CXCR4 receptor Tat also appears to encourage the reproduction of less virulent

M-tropic strains of HIV early in the course of infection, allowing the more rapidly pathogenic T-tropic strains to emerge later.³⁶

(b) Rev

Stands for "Regulator of Virion". This protein allows fragments of HIV mRNA that contain a Rev Response Unit (RRE) to be exported from the nucleus to the cytoplasm. In the absence of the "rev" gene, RNA splicing machinery in the nucleus quickly splices the RNA so that only the smaller, regulatory proteins can be produced. In the presence of "rev", RNA is exported from the nucleus before it can be spliced, so that the structural proteins and RNA genome can be produced. Again, this mechanism allows a positive feedback loop to allow HIV to overwhelm the host's defenses, and provides time-dependent regulation of replication (a common process in viral infections).³⁸

(c) Nef

Stands for "Negative Regulatory Factor". The expression of Nef early in the viral life cycle ensures T cell activation and the establishment of a persistent state of infection, two basic attributes of HIV infection. Nef also promotes the survival of infected cells by downmodulating the expression of several surface molecules important in host immune function. These include major histocompatibility complex-I (MHC I) and

MHC II present on antigen presenting cells (APCs) and target cells, CD4 and CD28 present on CD4+ T cells.

(d) Vif

Stands for "Viral infectivity factor". Vif is a 23-kilodalton protein that is essential for viral replication.³⁸ Vif inhibits the cellular protein, APOBEC3G, from entering the virion during budding from a host cell by targeting it for proteasomal degradation. Vif hijacks the cellular Cullin5 E3 ubiquitin ligase in order to target APOBEC3G for degradation. In the absence of Vif, APOBEC3G causes hypermutation of the viral genome, rendering it dead on arrival at the next host cell. APOBEC3G is thus a host defence to retroviral infection, which HIV-1 overcomes by the acquisition of Vif.

(e) Vpr

Stands for "Viral Protein R". Vpr, a 96 amino acid 14-kDa protein, plays an important role in regulating nuclear import of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells such as macrophages. Vpr also induces cell cycle arrest and apoptosis in proliferating cells, which can result in immune dysfunction.^{39, 40} Vpr is also immunosuppressive due to its ability to sequester a proinflammatory transcriptional activator in the cytoplasm. HIV-2 contains both a Vpr protein and a related (by sequence

homology) Vpx protein (Viral Protein X). Two functions of Vpr in HIV-1 are split between Vpr and Vpx in HIV-2, with the HIV-2 Vpr protein inducing cell cycle arrest and the Vpx protein required for nuclear import.

(f) Vpu

Stands for "Viral Protein U". Vpu is involved in viral budding, enhancing *virion release* from the cell.

(g) *Tev*:

This gene is only present in a few HIV-1 isolates. It is a fusion of parts of the "tat", "env", and "rev" genes, and codes for a protein with some of the properties of, but little or none of the properties of Rev.

Genetic variability

HIV differs from many other viruses as it has very high genetic variability. This diversity is a result of its fast replication cycle, with the generation of 10^9 to 10^{10} virions every day, coupled with a high mutation rate of approximately 3×10^{-5} per nucleotide base per cycle of replication and recombinogenic properties of reverse transcriptase. This complex scenario leads to the generation of many variants of HIV in a single infected patient in the course of one day. ⁴¹

This variability is compounded when a single cell is simultaneously infected by two or more different strains of HIV. When simultaneous infection occurs, the genome of progeny virions may be composed of RNA strands from two different strains. This hybrid virion then infects a new cell where it undergoes replication. As this happens, the reverse transcriptase, by jumping back and forth between the two different RNA templates, generates a newly synthesized retroviral DNA sequence that is a recombinant between the two parental genomes. This recombination is most obvious when it occurs between subtypes. ⁴¹

The relationship, if any, between genetic diversification, immune response, and disease progression is unknown. Three groups of HIV-1 have been identified on the basis of differences in "env": M, N, and O. ⁴² Group M is the most prevalent and is subdivided into eight subtypes (or clades), based on the whole genome, which are geographically distinct. ⁴³

The most prevalent are subtypes B (found mainly in North America and Europe), A and D (found mainly in Africa), and C (found mainly in Africa and Asia); these subtypes form branches in the phylogenetic tree representing the lineage of the M group of HIV-1. Coinfection with distinct subtypes gives rise to circulating recombinant forms (CRFs). In 2000, 47.2% of infections worldwide were of subtype C, 26.7% were of subtype A/CRF02_AG, 12.3% were of subtype B, 5.3% were of subtype D, 3.2% were of CRF_AE, and the remaining 5.3% were composed of other subtypes and CRFs. ^{44, 45}

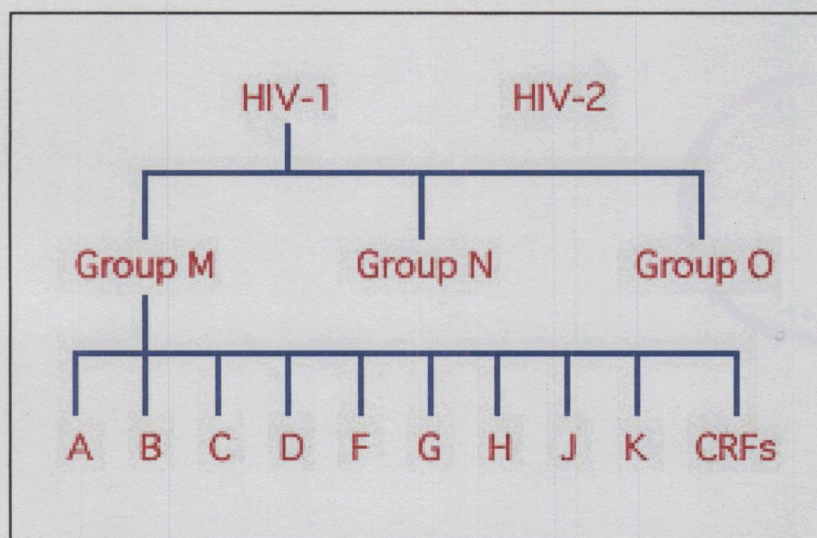


Figure 7 : HIV Subtypes

Tropism:

The term "viral tropism" refers to which cell types HIV infects. HIV can infect a variety of immune cells such as CD4⁺ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and CD4⁺ T cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine co-receptors. ⁴⁶

Macrophage (M-tropic) strains of HIV-1, or non-syncytia-inducing strains (NSI) use the "β"-chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and CD4⁺ T cells. ⁴⁷

This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Indeed, macrophages play a key role in several critical aspects of HIV infection. They appear to be the first cells infected by HIV and perhaps the source of HIV production when CD4⁺ T cells become depleted in the patient. Macrophages and microglial cells are the cells infected by HIV in the central nervous system. In tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleated giant cells that produce huge amounts of virus. T-tropic isolates, or syncytia-inducing (SI) strains replicate in primary CD4⁺ T cells as well as in macrophages and use the "α"-chemokine receptor, CXCR4, for entry. ^{47, 48, 49}

The "α"-chemokine, SDF-1, a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates. It does this by down-regulating

the expression of CXCR4 on the surface of these cells. HIV that use only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. However, the use of coreceptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection and HIV can also infect a subtype of myeloid dendritic cells which probably constitute a reservoir that maintains infection when CD4+T cell numbers have declined to extremely low levels.^{47, 50}

Some people are resistant to certain strains of HIV.⁵¹ People with the CCR5-Δ32 mutation are resistant to infection with R5 virus as the mutation stops HIV from binding to this coreceptor, reducing its ability to infect target cells. Heterosexual intercourse is the major mode of HIV transmission. Both X4 and R5 HIV are present in the seminal fluid which is passed from partner to partner. The virions can then infect numerous cellular targets and disseminate into the whole organism. However, a selection process leads to a predominant transmission of the R5 virus through this pathway.^{52, 53, 54}

How this selective process works is still under investigation, but one model is that spermatozoa may selectively carry R5 HIV as they possess both CCR3 and CCR5 but not CXCR4 on their surface and that genital epithelial cells preferentially sequester X4 virus.^{55, 56}

In patients infected with subtype B HIV-1, there is often a co-receptor switch in late-stage disease and T-tropic variants appear that can

infect a variety of T cells through CXCR4.⁵⁷ These variants then replicate more aggressively with heightened virulence that causes rapid T cell depletion, immune system collapse, and opportunistic infections that mark the advent of AIDS.⁵⁸

Thus, during the course of infection, viral adaptation to the use of CXCR4 instead of CCR5 may be a key step in the progression to AIDS. A number of studies with subtype B-infected individuals have determined that between 40 and 50% of AIDS patients can harbour viruses of the SI, and presumably the X4, phenotype.^{59, 60}

Replication cycle

a. Entry to the cell

HIV enters macrophages and CD4⁺ T cells by the adsorption of glycoproteins on its surface to receptors on the target cell. This is followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell. The interactions of the trimeric envelope complex and both CD4 and a chemokine receptor (generally either CCR5 or CXCR4 but others are known to interact) on the cell surface.

The gp160 spike contains binding domains for both CD4 and chemokine receptors. The first step in fusion involves the high-affinity attachment of the CD4 binding domains of gp120 to CD4. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor. This allows for a more stable two-pronged attachment, which allows the N terminal fusion peptide gp41 to penetrate the cell membrane.

Repeat sequences in gp41, HR1 and HR2 then interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid.^{61, 62}

Once HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase and protease, are injected into the cell.⁶¹ HIV can infect dendritic cells (DCs) by the CD4-CCR5 route, but another route using mannose-specific C-type lectin receptors such as DC-SIGN is also used.⁶³

DCs are one of the first cells encountered by the virus during sexual transmission. They are currently thought to play an important role by transmitting HIV to T cells once the virus has been captured in the mucosa by DCs.⁶³

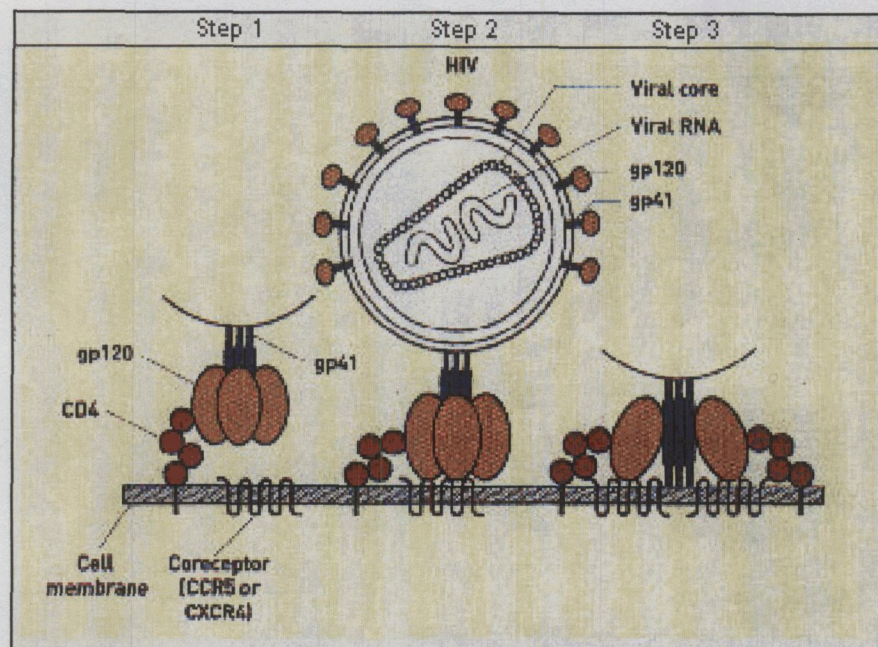


Figure 8: Attachment of gp120 to CD4

b. Replication and transcription

Once the viral capsid enters the cell, an enzyme called "reverse transcriptase" liberates the single-stranded (+) RNA from the attached viral proteins and copies it into a complementary DNA of 9 Kb size. ⁶⁴

This process of reverse transcription is extremely error-prone and it is during this step that mutations may occur. Such mutations may cause drug resistance. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). This vDNA is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by another viral enzyme called "integrase". This integrated viral DNA then lie dormant, in the latent stage of HIV infection. ⁶⁴

To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF- κ B (NF kappa B), which is upregulated when T cells become activated. ⁶⁵ In this replication process, the integrated provirus is copied to mRNA, which is then spliced into smaller pieces. These small pieces produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates it gradually starts to inhibit mRNA splicing. ⁶⁶

At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 binds to any appropriate RNA whereas HIV-2 preferentially binds to the mRNA, which was used to create the Gag protein itself. This means that HIV-1 is better able to mutate (HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections).

c. Assembly and release

The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion.⁶ Protease inhibitors can inhibit this cleavage step. The mature virus is then able to infect another cell.

Life Cycle of HIV

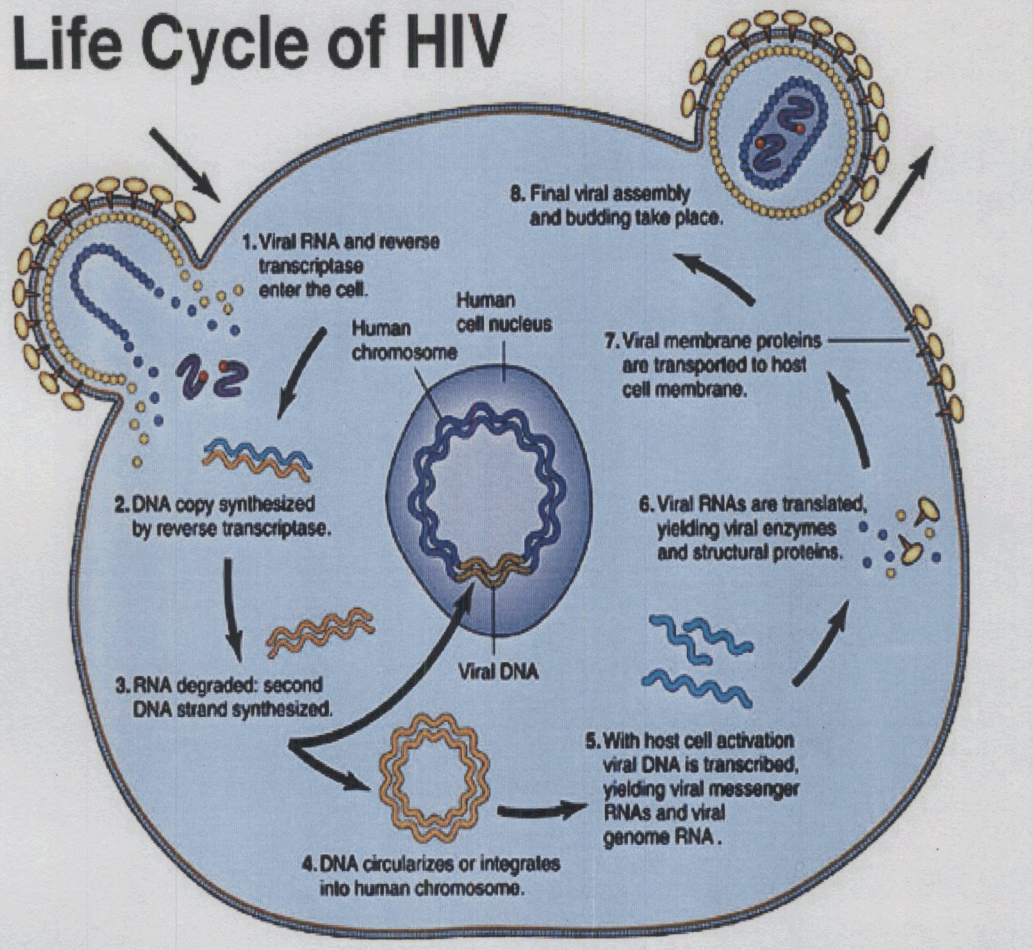


Figure 9 Replication cycle of HIV

(Bver/Shainberg/Galliano Dimensions of Human Sexualitv. 5e Copyright © 1999)

Clinical Course Of Infection

Infection with HIV-1 is associated with a progressive decrease of the CD4⁺ T cell count and an increase in viral load. The stage of infection can be determined by measuring the patient's CD4⁺ T cell count, and the level of HIV in the blood. The initial infection with HIV generally occurs after transfer of body fluids from an infected person to an uninfected one.

(A) Primary (Acute) infection

The first stage of infection, **the primary, or acute infection**, is a period of rapid viral replication that immediately follows the individual's exposure to HIV. An abundance of virus is found in the peripheral blood with levels of HIV commonly approaching several million viruses per ml. ⁶⁸

This response is accompanied by a marked drop in the numbers of circulating CD4⁺ T cells. This acute viremia is associated in virtually all patients with the activation of CD8⁺ T cells, which kill HIV-infected cells, and subsequently with antibody production, or seroconversion. The CD8⁺ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4⁺ T cell counts rebound to around 800 cells per ml (the normal value is 1200 cells per ml). In addition to the elimination of virus expressing cells by HIV specific cytotoxic T lymphocytes (CTLs), the trapping of virus particles in the

Follicular dendritic cells (FDC) network represents an important mechanism for the decrease of virus in the circulation. A good CD8⁺ T cell response has been linked to slower disease progression and a better prognosis, though it does not eliminate the virus.⁶⁹

During this period most individuals (80 to 90%) develop an influenza-like illness with symptoms of fever, malaise, lymphadenopathy, pharyngitis, headache, myalgia, and sometimes a rash.⁷⁰ Because of the nonspecific nature of these illnesses, it is often not recognized as a sign of HIV infection. Consequently, these primary symptoms are not used to diagnose HIV infection as they do not develop in all cases and because many are caused by other more common diseases. However, recognizing the syndrome can be important because the patient is much more infectious during this period.

With regard to HIV-specific immune responses, robust cell-mediated and humoral responses are readily detected during primary infection. Usually, virus specific CTLs can be detected within 2 to 4 weeks from the onset of symptoms, and their appearance coincides with the dramatic decrease in viraemia. HIV specific CTLs have been detected against both structural (e.g., Env, Gag-Pol) and regulatory (e.g., Nef, Tat) viral proteins. CD8⁺ T cell mediated suppressor activity has been detected during primary infection. These observations strongly support the important role for CD8⁺ T cells in the primary immune response to HIV infection.

Cytokines play an important role in the initial regulation of the virus expression. High levels of expression of certain cytokines such as interferon- γ , TNF- α and IL-10, have been observed during primary infection.

With regard to the virus specific humoral immune response, neutralizing antibodies, which represent the protective component of this response, are usually detected after the phase of downregulation of viraemia, when the transition from the acute to chronic phase of infection has already occurred.³²

A strong immune defense reduces the number of viral particles in the blood stream, marking the start of the infection's incubation **clinical latency** stage.

(B) Clinical Latent Stage:

Clinical Latent stage consists of Asymptomatic stage and Symptomatic stage which further leads to AIDS stage.

(i) Asymptomatic stage

Clinical latency can vary between two weeks and 20 years. During this early phase of infection, HIV is active within lymphoid organs, where large amounts of virus become trapped in the follicular dendritic cells (FDC) network.⁷¹

The surrounding tissues that are rich in CD4⁺ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who are in this phase are still infectious. During this time, CD4⁺ CD45RO⁺ T cells carry most of the proviral load.⁷²

This stage is marked by disappearance of symptoms of acute viral disease, down regulation of viraemia; CD4 cell counts become almost normal and neutralizing and complement (C¹) fixing virus specific antibodies appear in the blood. All virological parameters in the peripheral blood (viral RNA copies/viral load, virus expressing mononuclear cells etc) are very low. However, active and continuous virus replication goes on in the lymph nodes and lymphoid organs which express virus 1-3 logs higher than the peripheral blood. As long as the CD4 counts are higher than 500-cells/ μ l, the immune response mounted by the lymphoid tissues is effective; there is follicular hyperplasia of germinal centres indicating immune activation of lymph nodes. The important paradox to note is that cellular activation seen in lymph nodes is critical for viral replication i.e. virus replication is better in activated CD4⁺ cells. There is gradual reduction in number of CD4⁺ cells and increase in virus load during the long asymptomatic stage. Increase in viral load in peripheral blood indicates failure of progressive deterioration of effective immune response. Humoral immunity is intact during the asymptomatic stage that is specific antibodies are produced against different viral proteins. But the

antibodies are not protective and are not able to interfere with cell to cell transmission and infectivity of virus on account of constant variation of virus. This period on average lasts for 8-10 years. The CD4⁺ counts range between >200 to 500 cells/ μ l in peripheral blood.

With regard to the cell-mediated immunity, both HIV-specific CD4⁺ and CD8⁺ T cell-mediated immune responses to a variety of HIV proteins are detected in early-stage disease. In addition, *in-vitro* proliferative responses to mitogens, alloantigens, and soluble antigens such as tetanus toxoid are conserved in most individuals in early stage of disease.

With regard to humoral immunity, a variety of antibodies against HIV structural and regulatory proteins are generated during HIV infection. Among these are neutralizing antibodies that are directed against the V3 loop of the gp120 envelope protein. They inhibit the infectivity of free virions, but they are less effective in blocking cell-to-cell transmission. Impairment of HIV-specific and non-specific cell mediated and humoral immune response is usually observed during the progression from early to intermediate stage of disease.³² Progressive impairment of HIV specific and non-specific cell-mediated and humoral immune responses heralds the onset of AIDS.

(ii) Symptomatic Stage

Declining CD4⁺ T cell counts accompanied by frequent opportunistic infections generally signal the onset of AIDS. Opportunistic infections of greatest concern are *Pneumocystis carinii* pneumonia, whose frequency increases significantly when the CD4⁺ cell count falls below 200/mm³, and toxoplasmosis, which in AIDS patients often affects the brain. In some patients, CD4⁺T cell counts decline gradually throughout the course of infection, while others experience a sharp drop after a period of relative stability. As CD4⁺ cell counts drop below 100/mm³ infections with *Mycobacterium avium-intracellulare* or cytomegalovirus (CMV), esophageal candidiasis, cryptococcal pneumonitis and meningitis, recurrent herpes simplex infection, and wasting increase in frequency. The release of TNF- α (cachectin) may participate in the wasting syndrome (known as “slim disease” in Africa) that characterizes advanced AIDS.

Longitudinal analyses demonstrate that the levels of virus present in the peripheral blood increase prior to the onset of AIDS, indicating that viral load in vivo is directly related to the immunologic and clinical deterioration of the patient. Viral isolates obtained during this period replicate rapidly in culture, and in about 50% of patients this is accompanied by a switch to a more cytopathic, syncytium inducing viral phenotype. It is assumed that disease progression is linked to the

emergence of variants with increased virulence or, alternatively variants that can escape detection by the immune system.

B cells isolated from AIDS patients are often in a state of chronic activation leading to hypergammaglobulinemia and elevations in serum β 2-microglobulin and neopterin levels. Cytokine profiles are also altered, leading to increased levels of interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) in the plasma of AIDS patients. Despite declining numbers of CD4⁺ T cells, CD8⁺ T cell counts may rise as disease progresses, leading to an inversion of the normal CD4/CD8 cell ratio. However, in the absence of CD4⁺ T cell help, HIV-1 specific cytotoxic activity mediated by CD8⁺ T cells is often significantly reduced or absent in AIDS patients. ⁽⁷³⁾

(C) AIDS stage

When CD4⁺ T cell numbers decline below a critical level, cell-mediated immunity is lost, and infections with a variety of opportunistic microbes appear. The first symptoms often include moderate and unexplained weight loss, recurring respiratory tract infections (such as sinusitis, bronchitis, otitis media, pharyngitis), and oral ulcerations. Common opportunistic infections and tumors, most of which are normally controlled by robust CD4⁺ T cell-mediated immunity then start to affect the patient. Typically, resistance is lost early on to oral *Candida species* and to "Mycobacterium tuberculosis", which leads to an

increased susceptibility to oral candidiasis (thrush) and tuberculosis. Later, reactivation of latent herpes viruses causes patients to suffer from shingles from Epstein-Barr virus-induced B-cell lymphomas, and from Kaposi's sarcoma, a tumor of endothelial cells that occurs when HIV proteins such as Tat interact with Human Herpesvirus-8. Pneumonia caused by the fungus "*Pneumocystis jiroveci*" is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus (another herpes virus) or Mycobacterium avium complex is more prominent. Not all patients with AIDS get all these infections or tumors, and there are other tumors and infections that are less prominent but still significant.³³

From a virological standpoint, the advanced stage of HIV infection (ie CD4⁺ T cell counts < 200 cells/ μ l) is characterized by a substantial increase in all virological parameters in both peripheral blood and lymph nodes. In advanced-stage disease, the levels of viral load equilibrate between peripheral blood and lymph nodes. Lymphoid tissue has for the most part been destroyed and replaced by fibrotic tissue; most of the virus is cell-associated, and virus trapping is minimal or absent. The obvious consequence of the destruction of lymphoid tissue is the severe impairment of immune function and profound immune suppression.³²

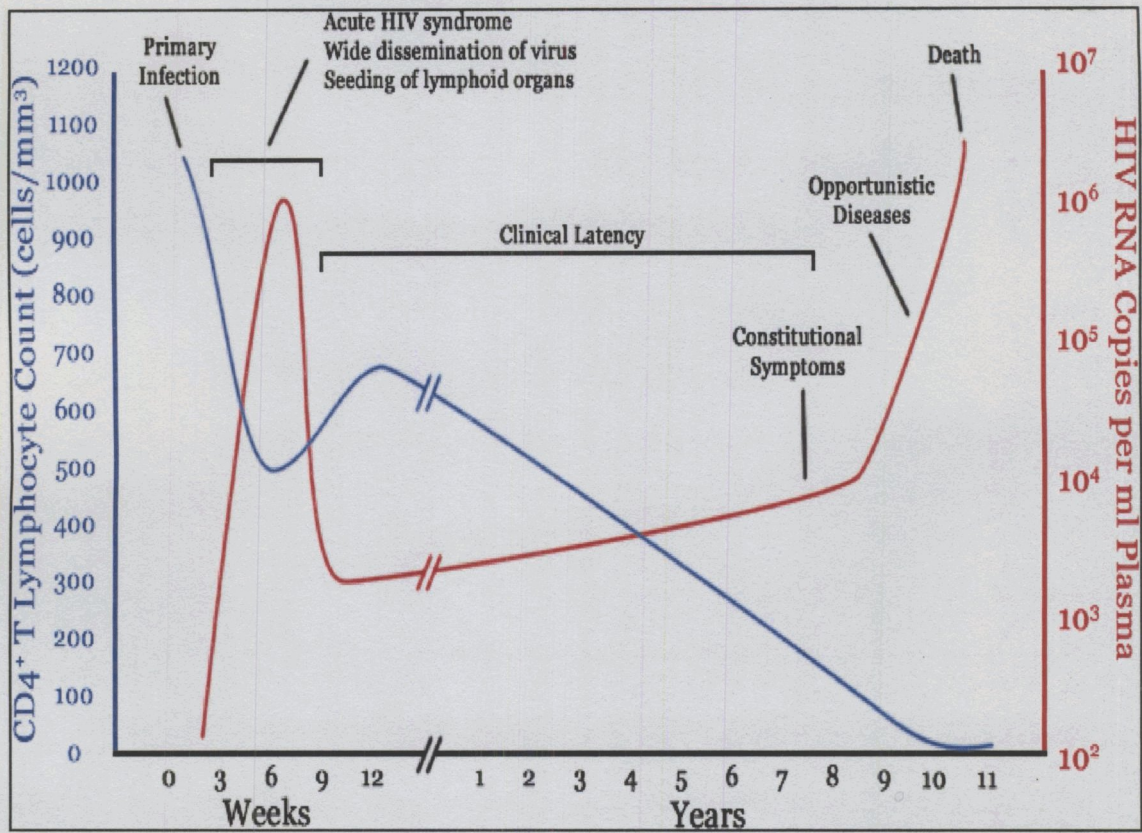


Figure 10: HIV-Timecourse

HIV Disease Progression Rates

Following infection with HIV-1, the rate of clinical disease progression varies between individuals. Factors such as host susceptibility genetics and immune function ⁷⁴, healthcare and co-infections ⁷⁵ as well as viral genetic variability ⁷⁶ may affect the rate of progression to AIDS.

Three dominant patterns of HIV disease progression have been described. These are based on kinetics of immunologic and virologic events described above. Eighty to ninety percent of HIV infected are "**Typical progressors**" with a median survival time of 10 years approximately. Five to ten percent of HIV infected individuals are "**Rapid progressors**" with a median survival time of 3-4 years approximately. About 5% of HIV infected do not experience disease progression for an extended period of time (at least 7 years) and are called "**Long term non progressors**" (LNTPs).

[A] Typical progressors

The Typical course of HIV infection includes three phases: primary infection (seroconversion), clinical latency and clinically apparent disease. The diagnosis of primary infection is made in only a minor percentage of cases. Difficulties reside in the variable severity and nonspecificity of the clinical syndrome, which is characterized by mononucleosis or flu like symptoms such as fever, lethargy, sore throat, malaise, macupapular rash

lymphadenopathy, arthralgias, myalgias, headaches, retroorbital pain, photophobia and rarely meningitis. Hospitalization is required in only a minority (10% to 15 %) of individuals, and thus this phase therefore goes unnoticed. During the initial period of primary infection HIV specific antibodies may not be detected however high levels of virus are found in circulation (viraemia).³²

Primary phase may be totally inapparent or may be associated with acute flu like mononucleosis like syndrome in 50% to 70% individuals. This occurs within 3-6 weeks of infection and may last for 9-12 weeks. Progression to AIDS and clinically apparent disease occurs within 8 to 10 years in typical progressors. The progression to AIDS results from the continuous replication of the virus in the lymphoid tissue, which is associated with the progressive destruction of the tissue and severe impairment of the immune function.

[B] Rapid Progressors

A small percentage (5% to 10 %) of HIV-infected individuals rapidly progress to AIDS within four years after primary HIV-infection and are termed Rapid Progressors (RP).⁷⁷ Some individuals have been known to progress to AIDS and death within a year after primo-infection. Rapid progression was originally thought to be continent specific, as some studies reported that disease progression is more rapid in Africa but others have contested this view.^{78, 75, 79, 80, 81}

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Immune response is defective in these Rapid progressors. Levels of neutralizing and C¹ fixing HIV specific antibodies are low and CD⁴ mediated suppression of HIV replication is 'impaired'. As a result progression to AIDS is rapid in these individuals. A series of immunologic abnormalities typical of HIV infected individuals in late stage disease are usually observed in rapid progressors, including high percentages of activated CD8⁺ T cells expressing CD38 and HLA-DR and elevated serum levels of β 2 microglobulin, neopterin, soluble CD8 and soluble IL-2 receptors. The levels of viral load are usually very high in rapid progressors.³²

[C] Long Term Non-Progressors

Another subset of individuals (5%) who are persistently infected with HIV-1, but show no signs of disease progression for over 12 years and remain asymptomatic are classified as Long Term Non-Progressors (LTNPs). In these individuals, it seems that HIV-infection has been halted with regard to disease progression over an extended period of time.⁸² However, the term LTNP is a misnomer, as it was seen that progression towards AIDS can occur even after 15 years of stable infection.⁸³ LTNP are not a homogeneous group regarding both viral load and specific immune responses against HIV-1. Some LTNPs are infected with HIV that inefficiently replicates whilst others are infected with HIV that is virally fit and replicates normally, but the infected

individual has had a strong and broad set of HIV-specific humoral and Cell-mediated responses that seems to delay the progression to AIDS.⁸⁴ In some cohorts, individuals who experience signs of progression, but whose clinical and laboratory parameters remain stable over long periods of time, are classified as **Long Term Survivors (LTS)**.⁸⁵

CD4 counts stay at around $>500\text{cells}/\mu\text{l}$, cell mediated and humoral immune responses are comparatively strong in these individuals. Absolute number of CD8^+ cells is also persistently high in these individuals. In addition the cytotoxic T lymphocytes retain their cytotoxicity activity against HIV. HIV specific CTLs against various HIV proteins (Env and Gag) are readily detected in LNTP's. The titer of neutralizing antibodies against HIV is also higher in these individuals. LNTP's exhibit preserved proliferative responses to mitogens, alloantigens and soluble antigens. With regard to the humoral immune response, higher titers of neutralizing antibodies are found in LNTP's compared with typical progressors.³² The virus specific parameters like viral load, virus replication in peripheral blood and lymph nodes are four fold to 20 fold lower in long-term nonprogressors. In addition, the virus infecting these individuals may be of low pathogenicity. Some host genetic factors may also be responsible for these LNTP's.

[D] Highly Exposed Persistently Seronegative or Exposed Seronegative

There is another, smaller percentage of individuals who have been identified. These are called Highly Exposed Persistently Seronegative (HEPS). This is a small group of individuals and were initially observed in a group of uninfected HIV-negative prostitutes in Kenya and in the Gambia. When these individuals PBMCs are stimulated with HIV-1 peptides, they have lymphoproliferative activity and have HIV-1 specific CD8+ CTL activity suggesting that transient infection may have occurred.^{86, 87, 88} This does not occur in unexposed individuals. The CTL epitope specificity differs between ESN's and HIV positive individuals. In HEPS, the maintenance of responses appears to be dependent upon persistent exposure to HIV.⁸⁹

Diagnosis

The prevention, control, and management of HIV infections depend greatly on the methods to comprehensively identify and characterize the causative agent. At present, serological assays are the cornerstone of routine blood screening for clinical and blood bank purposes. These assays identify infected patients by detecting antibodies produced by the host immune system against HIV proteins like envelope protein, reverse transcriptase polymerase protein and the core protein. Most HIV antibody screening assays use the rapid, convenient and well-known Enzyme Linked Immunosorbent assay (ELISA). Other assays in detecting HIV specific antibodies include Rapid Latex Agglutination assay, Dot blot immunobinding assays and Passive hemagglutination assay. HIV-1 antibodies can also be detected from oral fluid. For confirmation of HIV infection test like, Western Blotting, Indirect Immunofluorescence assay, Radioimmunoprecipitation assay, p24 ELISA and HIV culture is used. A number of markers like HIV p24 core antigen, HIV β 2 microglobulin, CD4+ T lymphocyte counts are used to assess the progression of HIV disease from asymptomatic phase to full blown AIDS. Assays measuring viral nucleic acids and viral load quantification using PCR technique are also used.³²

Treatment

There is currently no vaccine or cure for HIV or AIDS. The only known method of prevention is avoiding exposure to the virus. However, an antiretroviral treatment, known as post-exposure prophylaxis is believed to reduce the risk of infection if begun directly after exposure.⁹⁰ Current treatment for HIV infection consists of highly active antiretroviral therapy, or HAART.⁹¹ This has been highly beneficial to many HIV-infected individuals since its introduction in 1996, when the protease inhibitor-based HAART initially became available.⁹²

Current HAART options are combinations consisting of at least three drugs belonging to at least two types, or "classes," of anti-retroviral agents. Typically, these classes are two nucleoside analogue reverse transcriptase inhibitors (NARTIs or NRTIs) plus either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Because AIDS progression in children is more rapid and less predictable than in adults, particularly in young infants, more aggressive treatment is recommended for children than adults.⁹³

In developed countries where HAART is available, doctors assess their patients thoroughly: measuring the viral load, how fast CD4 declines, and patient readiness and when to recommend starting treatment. HAART allows the stabilization of the patient's symptoms and viremia, but it neither cures the patient, nor alleviates the symptoms, and high

levels of HIV-1, often HAART resistant, return once treatment is stopped.^{94, 95} Moreover, it would take more than a lifetime for HIV infection to be cleared using HAART.⁹⁶ Despite this, many HIV-infected individuals have experienced remarkable improvements in their general health and quality of life, which has led to a large reduction in HIV-associated morbidity and mortality in the developed world.^{92, 97, 98}

A computer based study in 2006 projected that following the 2004 United States treatment guidelines gave an average life expectancy of an HIV infected individual to be 32.1 years from the time of infection if treatment was started when the CD4 count was 350/ μ L.⁹⁹

In the absence of HAART, progression from HIV infection to AIDS has been observed to occur at a median of between nine to ten years and the median survival time after developing AIDS is only 9.2 months.¹⁰⁰

However, HAART sometimes achieves far less than optimal results, in some circumstances being effective in less than fifty percent of patients. This is due to a variety of reasons such as medication intolerance/side effects, prior ineffective antiretroviral therapy and infection with a drug-resistant strain of HIV. However, non-adherence and non-persistence with antiretroviral therapy is the major reason most individuals fail to benefit from HAART.¹⁰¹

The reasons for non-adherence and non-persistence with HAART are varied and overlapping. Major psychosocial issues, such as poor access to medical care, inadequate social supports, psychiatric

disease and drug abuse contribute to non-adherence. The complexity of these HAART regimens, whether due to pill number, dosing frequency, meal restrictions or other issues along with side effects that create intentional non-adherence also contribute to this problem.^{102, 103, 104} The side effects include lipodystrophy, dyslipidaemia, insulin resistance, an increase in cardiovascular risks and birth defects.^{105, 106} Anti-retroviral drugs are expensive, and the majority of the world's infected individuals do not have access to medications and treatments for HIV and AIDS.¹⁰⁷

Research to improve current treatments includes decreasing side effects of current drugs, further simplifying drug regimens to improve adherence, and determining the best sequence of regimens to manage drug resistance. Unfortunately, only a vaccine is thought to be able to halt the pandemic. This is because a vaccine would cost less, thus being affordable for developing countries, and would not require daily treatment. However, after over 20 years of research, HIV-1 remains a difficult target for a vaccine.¹⁰⁷

Opportunistic infections in HIV

(a) Herpes simplex infection

Herpes simplex virus (HSV) types 1 and 2 cause both primary and recurrent oral and genital disease. HSV usually appears as a vesicular eruption of the mucous membranes of the oral or perioral area, vulva, perianal skin, rectum, and occasionally the inguinal areas. The eruption develops into tender or painful ulcerated lesions that are frequently covered with a clear yellow crust. In some patients, however, the typical painful vesicular or ulcerative lesions may be absent. Persons with HIV disease and low CD4 counts have more frequent recurrences of HSV and more extensive ulcerations than HIV-uninfected people. Persistent HSV eruption (>1 month) is an AIDS-indicator diagnosis. ⁽¹⁰⁸⁾

(b) Cytomegalovirus infection

Chronic infection with cytomegalovirus (CMV), a member of the human herpes virus family, is common among sexually active adults. CMV is spread by sexual or other types of close personal contact, blood-to-blood contact (via transfusion or needle sharing), organ transplantation, and perinatal transmission. The estimated prevalence of CMV infection among adults ranges from 40% to 60% in resource-rich countries and from 80% to 100% in resource-poor countries. As with other herpes viruses, CMV is not cleared from the body, but is

kept in a state of latency by an intact immune system. Chronic CMV infection rarely causes disease among immunocompetent persons, but it is a major cause of morbidity and mortality in HIV-infected patients with CD4 counts of <50 cells/ μ L. CMV can cause several clinical illnesses in immunocompromised patients, including chorioretinitis, pneumonia, esophagitis, colitis, encephalitis, polyradiculopathy, adrenalitis, and hepatitis. Symptomatic disease represents either primary infection or reactivation of latent infection that has escaped immunologic control. Effective antiretroviral therapy (ART) greatly reduces the risk of CMV reactivation and disease. ⁽¹⁰⁸⁾

(c) Kaposi sarcoma

Kaposi sarcoma (KS) is an endothelial neoplasm that usually occurs as skin or oral lesions but may involve the internal organs. It is the most common AIDS-associated neoplasm and is an AIDS-defining disease. AIDS-associated KS is 1 of 4 types of KS, along with classic, endemic, and organ transplant-associated KS. Although the types vary in epidemiology and clinical presentation, all are associated with human herpesvirus type 8 (HHV-8), also known as KS-associated herpesvirus. The clinical manifestations of AIDS-associated KS (sometimes called epidemic KS) range in severity from mild to life threatening. The progression of disease may be rapid or slow, but the overall prognosis is poor in the absence of treatment. The skin lesions of KS, even when

they do not cause medical morbidity, may cause significant disfigurement and emotional distress.

AIDS-associated KS usually occurs in HIV-infected persons with advanced immunosuppression (CD4 count <200 cells/ μ L), but may occur at any CD4 count. The incidence of KS in resource-abundant countries has declined markedly since the early 1990s, in part because of the widespread availability of effective combination antiretroviral therapy (ART). ART appears to be effective in reducing the risk of AIDS-associated KS, particularly when initiated before the development of advanced immunosuppression. ⁽¹⁰⁸⁾

(d) *Pneumocystis jiroveci* pneumonia infection

Pneumocystis jiroveci pneumonia (previously called *Pneumocystis carinii* pneumonia, and still abbreviated PCP), is caused by an unusual fungus, *Pneumocystis jiroveci*. Many humans appear to be infected in childhood, but clinical illness occurs only in people with advanced immunosuppression, either through new infection or reactivation of latent infection. Cases of PCP in otherwise healthy young homosexual men were among the first recognized manifestations of AIDS, in 1981. The organism can affect many organ sites, but pneumonia is far and away the most common form of disease. In the United States, the incidence of PCP has declined sharply since the use of prophylaxis and effective antiretroviral therapy (ART), but PCP is still man

patients' initial presenting opportunistic infection and a significant cause of morbidity and mortality in HIV-infected patients. ⁽¹⁰⁸⁾

(e) *Mycobacterium avium* complex (MAC)

Mycobacterium avium complex (MAC) is an opportunistic infection caused by species of *Mycobacterium* that can cause severe illness in people with advanced AIDS but rarely affects others. The risk of disseminated MAC (DMAC) is directly related to the severity of immunosuppression. DMAC typically occurs in persons with CD4 counts of <50 cells/ μ L, and its frequency increases as the CD4 count declines. In the absence of antibiotic prophylaxis, DMAC occurs in up to 40% of AIDS patients with CD4 counts of <50 cells/ μ L. Antimicrobial therapy, especially if given in conjunction with antiretroviral therapy (ART) that achieves immune reconstitution, can be successful in treating MAC disease. Specific antimicrobial prophylaxis and effective ART may also be used to prevent MAC in patients with advanced AIDS. ⁽¹⁰⁸⁾

(f) Tuberculosis

Tuberculosis (TB) is an infection caused by organisms in the family *Mycobacteria*. These organisms grow slowly and can be identified only with special staining techniques, a trait that led to the name "acid-fast bacteria." Organisms in the *Mycobacterium tuberculosis* (MTB) group cause human disease, usually a chronic pneumonia. The destruction of MTB may produce holes or cavities in the lung containing huge numbers of organisms. MTB can also cause disease in other individual organs (eg, lymph nodes, meninges, bone, pericardium, peritoneum, intestines, urogenital tract) and can disseminate to multiple organs, often including the lungs, blood, liver, and spleen. TB is almost always transmitted by persons with active pulmonary TB who release large numbers of organisms in their sputum. The organisms remain suspended in the air for hours or days, making TB one of the most easily transmitted respiratory pathogens. Most immunologically healthy persons who are infected with MTB do not develop active TB but remain infected with inactive organisms (latent TB infection); only about 10% of infected persons develop active disease during their lifetimes. Persons with HIV infection have much higher rates of active TB and develop active disease at a rate approximating 10% per year.

HIV and TB cause more deaths than any other infectious disease worldwide, each claiming millions of lives annually. A biologic synergy exists between these infections: HIV-induced immunosuppression

increases susceptibility to TB infection, and active TB infection enhances HIV replication through immunologic stimulation. The populations infected by these 2 pathogens overlap in many respects creating epidemiologic synergy. Poverty, crowded living conditions and inadequate efforts to reduce transmission combine to enhance the transmission of both organisms.

Classic pulmonary TB, with upper-lobe infiltrates and cavitary lesions may occur in HIV-infected persons with relatively intact immunity. As the CD4 cell count decreases, TB is more likely to manifest atypically in the chest (without cavitary disease, or with lower-lobe disease adenopathy, pleural effusions, or interstitial or miliary infiltrates), as extrapulmonary disease (particularly in lymph nodes, as meningitis and as disseminated infection), or both. Bone, joint, and urogenital sites of TB are less commonly associated with HIV-induced immunosuppression. Symptoms and signs of TB in HIV-infected persons therefore can vary widely. ⁽¹⁰⁸⁾

(g) Oral Candidiasis

Oropharyngeal candidiasis ("thrush"), a fungal disease of the oral mucosa and tongue, is the most common intraoral lesion in persons infected with HIV. In the absence of other known causes of immunosuppression, oral thrush in an adult is highly suggestive of HIV infection. Three clinical presentations of thrush are common in people

with HIV: pseudomembranous, erythematous, and angular cheilitis. Thrush usually occurs with CD4 counts of <300 cells/ μ L and is not an AIDS-defining illness. *Candida* may also infect the esophagus in the form of esophageal candidiasis which causes dysphagia (difficulty with swallowing) or odynophagia (pain with swallowing). Esophageal candidiasis is an AIDS-defining condition, generally occurring in individuals with CD4 counts of <100 cells/ μ L. It is the most common cause of esophageal infection in persons with AIDS.

Oropharyngeal and esophageal candidiasis are most commonly caused by *Candida albicans*, although occasionally non-*albicans* species cause disease and may be resistant to first-line therapies. ⁽¹⁰⁸⁾

HIV and Candidiasis:

Fungal infections are extremely common as a complication of HIV disease although they vary in their severity from mild discomfort associated with mucosal thrush to life threatening fungal meningitis. In 1981 the first reports of AIDS included patients with *Pneumocystis pneumonia* and mucosal candidiasis. Yeast *Candida albicans* is the strain most commonly associated with disease in HIV positive patients. Most HIV positive patients become colonized with *Candida albicans* especially oral cavity, at rates that increase with decreasing CD4 counts. In addition to well-characterized defect in Cell mediated

immunity associated with HIV infection, local oral defense mechanisms appears to be impaired. ⁽¹⁰⁹⁾

Mucocutaneous candidiasis occurs in three forms in individuals with HIV infection: Oropharyngeal, esophageal, and vulvovaginal disease.

Candida infection has greater impact on HIV women than men. Some of the common infections caused by *Candida albicans* that are observed in HIV positive females are Chronic vulvovaginitis, Chronic vulvovaginitis with mycotic localization at skin, Chronic vulvovaginitis with localization at nail and Oral candidiasis.

Candida infection of the vulvogenital vaginal area is common complication in HIV infection and recurrent vaginitis often precedes oral candidiasis and may be the first indicator of immune dysfunction. Progression of HIV infection has been clearly associated with the occurrence of oral candidiasis, which may be a sentinel event presenting months or years before more severe opportunistic disease. Oral candidiasis has been clearly shown to be an independent prognostic marker of the risk of progression to overt AIDS. ¹⁰⁹

Candida albicans

Candida albicans is the principal fungal species responsible for oropharyngeal candidiasis, the most frequent opportunistic infection associated with immune deficiencies especially AIDS. Less frequently, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei* may be associated

with HIV/AIDS. *Candida albicans* is a polymorphic organism, which undergoes morphologic transition between yeast, pseudohyphal and hyphal forms. The ability of *C. albicans* to change from yeast to filamentous types is a major virulence determinant of this organism. Cell wall of *Candida albicans* not only maintains the shape which characterize each growth form but is also the site of the initial interaction between the organism and the environment playing an important role in colonization of tissues and presentation of antigens to host. Glucan, chitin and mannoproteins are the main constituents in cell wall of yeast. Proteins and glyco (manno) protein components are bound to the cell wall in *Candida albicans*. Mannose polymer (mannan) does not exist as such but are found in covalent association with proteins (mannoprotein) represent about 40% of cell wall polysaccharide and are the main material of cell wall matrix.

The capacity of cell wall constituents, including glucan, chitin, and mannoproteins, to modulate (activate or depress) the immune response is well documented. Mannans and mannoproteins of *Candida albicans* cell wall display the most potent immunomodulatory activity being able to regulate the action of virtually all arms of immune system (NK cells, phagocytic cells, Cell mediated and humoral response).¹¹⁰

Host responses to fungi result from a coordinate interplay between innate and adaptive immune system. Neutrophils and monocytes are involved in the non-specific clearance of yeasts (e.g. *Candida albicans*).

and *Cryptococcus neoformans*), while T helper 1 type responses are protective via release of interferon γ .¹¹¹

In particular, its production from neutrophils may support memory T helper 1-cell responses of the fungus. With respect to anti-Candida vaccines several approaches are in progress, such as use of heat killed *Candida albicans* in combination with adjuvants, purified peptides and proteins and immunogenic peptide-lipid conjugates.¹¹¹

Various reports suggested the importance of the innate and adaptive cell-mediated immune responses in the control of mycotic infections contributing to a better understanding of the overall disease pathogenesis. Firstly, the innate immunity is associated with the capacity of phagocytic cells to ingest and inhibit fungal growth. This response needs to be completed by the intervention of the adaptive immune system. In fact, fungal-acquired resistance correlates with cytokine production from Th1 cells, which further activate phagocytic cells, while cytokines secreted by Th2 cells lead to inhibition of Th1 subset and exacerbation of disease, impairing fungicidal effector cells. *Candida* leads to two types of immune response: induction of immunity or tolerance. In the last instance, this yeast may persist in infected tissues and behave as a commensal down-regulating host cell mediated adaptive immunity. The characterization of immunodominant antigens eliciting potent immune responses during candidiasis could

have important repercussions for developing novel diagnostic, prophylactic and therapeutic techniques for candidiasis. ¹¹¹

Prediction of Progression Rates

During the initial weeks after HIV infection, qualitative differences in the cell-mediated immune response are observed that correlate with different disease progression rates (i.e., rapid progression to WHO stage 4 and the rapid loss of CD4⁺ T cell levels versus normal to slow progression to WHO stage 4 and the maintenance of CD4⁺ T cell counts above 500/ μ l). The appearance of HIV-1-specific CD8⁺ cytotoxic T cells (CTLs) early after primo-infection has been correlated with the control of HIV-1 viremia. ⁽¹¹²⁾ The virus, which escapes this CTL response, has been found to have mutations in specific CTL epitopes. ¹¹³ Individuals with a broad expansion of the V-beta chain of the T cell receptor of CD8⁺ T cells during primo-infection appear seem to have low levels of virus six to twelve months later, which is predictive of relatively slow disease progression. In contrast, individuals with an expansion of only a single subset of the V-beta chain of the CD8⁺ T cells are not able to control HIV levels over time, and thus have high levels of virus six to twelve months later. ¹¹⁴ LTNP's have also been shown to have a vigorous proliferation of circulating activated HIV-1-specific CD4⁺ T cell and CTL response against multiple epitopes with no detectable broadly cross-reactive neutralizing antibodies in the

setting of an extremely low viral load.^{115, 116} However, a few reports have correlated the presence of antibodies against Tat and LTR status.

HIV Subtype Variation and Effect on Progression Rates

The HIV-1 subtype that an individual becomes infected with can be a major factor in the rate of progression from sero-conversion to AIDS. Individuals infected with subtypes C, D and G are 8 times more likely to develop AIDS than individuals infected with subtype A.¹¹⁷ In Uganda, where subtypes A and D are most prevalent, subtype D is associated with faster disease progression compared with subtype A.^{118, 119} Age has also been shown to be a major factor in determining survival and the rate of disease progression, with individuals over 40 years of age at sero-conversion being associated with rapid progression.¹²⁰

Host genetic susceptibility

The Centers for Disease Control and Prevention (CDC) has released findings that genes influence susceptibility to HIV infection and progression to AIDS. HIV enters cells through an interaction with both CD4 and a chemokine receptor of the 7 Tm family. They first reviewed the role of genes in encoding chemokine receptors (CCR5 and CCR2) and chemokines (SDF-1). While CCR5 has multiple variants in its

coding region, the deletion of a 32-bp segment results in a nonfunctional receptor, thus preventing HIV entry. Two copies of this gene provide strong protection against HIV infection, although the protection is not absolute. This gene is found in up to 20% of Europeans but is rare in Africans and Asians. Multiple studies of HIV infected persons have shown that presence of one copy of this gene delays progression to the condition of AIDS by about 2 years. And it is possible that a person with the CCR5-Δ32 receptor gene will not be infected with HIV.³³

The Effect of Co-infections on Progression Rates

Co-infections or immunizations may enhance viral replication by inducing a response and activation of the immune system. This activation facilitates the three key stages of the viral life cycle: entry to the cell; reverse transcription and proviral transcription.¹² Chemokine receptors are vital for the entry of HIV into cells. The expression of these receptors is inducible by immune activation caused through infection or immunization, thus augmenting the number of cells that can be infected by HIV-1. Both reverse transcription of the HIV-1 genome and the rate of transcription of proviral DNA rely upon the activation state of the cell and are less likely to be successful in quiescent cells. In activated cells there is an increase in the cytoplasmic concentrations of mediators required for reverse

transcription of the HIV genome. Activated cells also release IFN-alpha that acts on an autocrine and paracrine loop that up-regulates the levels of physiologically active NF-kappa B, which activates host cell genes, as well as the HIV-1 LTR. The impact of co-infections by microorganisms such as Mycobacterium tuberculosis can be important in disease progression, particularly for those who have a high prevalence of chronic and recurrent acute infections and poor access to medical care. Often, survival depends upon the initial AIDS-defining illness. Co-infection with DNA viruses such as HTLV-1, herpes simplex virus-2, varicella zoster virus and cytomegalovirus may enhance proviral DNA transcription and thus viral load as they may encode proteins that are able to trans-activate the expression of the HIV-1 pro-viral DNA. Frequent exposure to helminth infections, which are endemic in Africa, activates individual immune systems, thereby shifting the cytokine balance away from an initial Th1 cell response against viruses and bacteria which would occur in the uninfected person to a less protective T helper 0/2-type response. HIV-1 also promotes a Th1 to Th0 shift and replicates preferentially in Th2 and Th0 cells. This makes the host more susceptible to and less able to cope with infection with HIV-1, bacteria and other viruses.³³

Immune Dysfunction during HIV infection

[A] Dysfunction of CD4⁺ T cells

CD4⁺ T cell dysfunction and depletion are hallmarks of HIV disease. The proximate cause of the susceptibility to opportunistic infection observed with advancing disease is the depletion in T-cell number and defects in function that result directly or indirectly from HIV infection. In addition to the decrease in IL-2 production and IL-2 receptor expression, the percentage of CD4⁺ T cells expressing CD28 (i.e., the major costimulatory receptor that is necessary for normal activation of T cells) is reduced during HIV infection compared to cells from uninfected individuals.¹²² CD28 cells do not respond to activation signals and express markers of terminal activation, including HLA-DR, CD38 and CD45RO.¹²³ In addition, CD4⁺ T cells from HIV-infected individuals express abnormally low levels of ligand.

A variety of mechanisms, both directly and indirectly related to HIV infection of CD4⁺ T cells, are responsible for the defects in T cell function. Interference with CD4 expression by HIV gp120, Nef, and Vpr may impair the ability of the infected CD4⁺ T cell to interact with appropriate MHC class II molecules.^{124, 125} Preferential infection of CD4⁺ memory cells by HIV and/or the preferential susceptibility of these cells to the cytopathic effects of HIV may in part explain the loss of memory responses to soluble antigens and consequent increase in the risk of infection with opportunistic organisms.

Direct infection was considered as the sole mediator of the immunopathogenesis of HIV infection. CD4⁺ T cell dysfunction is evident in the early stages of HIV infection, prior to quantitative depletion of this key subset.

There are measurable levels of circulating soluble gp120 present in HIV infected subjects. There is an accumulation of high concentration of virions (both infectious and defective) in lymphoid tissue. This underscores the potential for envelope to contribute to T-cell dysfunction in a process distinct from infection of CD4⁺ T cells. HIV envelope glycoproteins bind with high affinity to the CD4 molecule and to a number of chemokine co-receptors. Intracellular aberrant signals transduced by HIV-1 envelope have been implicated in several immunopathogenic processes, including anergy, syncytium formation, apoptosis and inappropriate cell trafficking.^{126, 127} Most of these aberrant signals were presumed to result from interactions between HIV envelope glycoproteins and CD4, however, it has become clear that HIV envelope may transduce intracellular signals through coreceptor molecules such as CCR5 and CXCR4.^{128, 129}

There is evidence for a negative effect of HIV on signal transduction. *In-vitro*, the cross linking of CD4 molecules that occurs when gp120 is bound in the presence of anti gp120 antibodies causes suppression of T cell activation.¹³⁰ Anti-gp120 antibody coated CD4⁺ T cells have been detected in HIV-infected individuals suggesting that soluble

gp120 can bind CD4 molecules in vivo and HIV gp120 induced T-cell anergy could occur.¹³¹

Failure to respond to antigenic stimuli has also been seen in uninfected CD4⁺ T cells in HIV infected patients.¹³² HIV proteins such as purified Env glycoproteins (Both HIV gp120 and gp41), Env peptides, and purified Tat protein have been reported to suppress, *in-vitro* proliferative responses to mitogen, antigens and anti-CD3 antibody. Exposure of cells to HIV gp120 also inhibits both IL-2 production and IL-2 receptor expression. The mechanisms by which HIV envelope glycoproteins suppress immune functions in the absence of infection of the cell involve the binding of gp120 to membrane bound CD4, gp120-CD4 interactions at cell surface could sterically interfere with the MHC class II-CD4 mediated cell adhesion necessary for the induction of antigen specific responses.

Inappropriate binding of CD4 by anti-CD4 antibodies results in a reduction in the expression of the T-cell receptor (TCR) suggesting that gp120-CD4 interaction may have a similar effect.¹³³

Mechanisms of CD4+ T cell destruction

The collapse of the immune system could be explained by the gradual loss of CD4+ T cells, particularly because the immune system seems to be unable to regenerate these cells adequately in HIV infected individuals. It is often assumed that depletion of CD4+ T cells and cells of the monocytes/macrophage lineage is a direct result of HIV infection. It is relatively easy to demonstrate a number of virus related mechanisms of cell killing in vitro; however, the extent to which these mechanisms are responsible for the depletion of immune competent cells in vivo represents a combination of processes.

1. Direct Mechanism

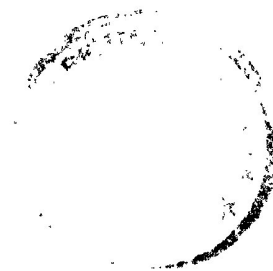
a. Direct Killing of Single cells

It has been observed that CD4+ T cells are the principle targets of HIV infection in vivo and that HIV infection of CD4 + T cells in-vitro causes cytopathicity. This led to a reasonable assumption that direct infection of CD4+ T cells in vivo results in their depletion. However, quantitative studies of the frequency of HIV-infected cells in vivo suggest that single cell killing by direct infection with HIV may not be the predominant mechanism of CD4+ T cell depletion. Viral burden and levels of virus expression are far greater in lymphoid tissue compared with peripheral blood; however these levels even in lymphoid tissue do

not appear to be sufficiently high to account for CD4⁺ T cell depletion solely by direct mechanisms.¹³⁴

A number of mechanisms have been proposed for the killing in-vitro of single HIV infected cells. These include (1) copious budding of virions from the cell surface with concomitant disruption of the integrity of the cell membrane, (2) interference with cellular RNA processing, (3) disruption of cellular protein synthesis due to high levels of viral RNA and the (4) accumulation of high levels of integrated viral DNA in the cell.⁽²⁶⁾ The intracellular interaction between HIV gp120 and CD4 has also been implicated in cytopathicity.¹²⁴

There are clear strain differences in the killing of cells by HIV-1. These are determined largely by gp120 sequences, providing strong support for the importance of env in this process. Certain strains could be less effective in inducing resistance to superinfection than others, allowing reinfection of cell by the virus it produces and a consequent building of viral replication to toxic levels.



2. Indirect Mechanism of CD4+ T cell Depletion

a. Syncytium Formation

A potential explanation of the cause of the death of the cells that are not directly infected with HIV is the formation of multi nucleated giant cells or syncytia. Some strains of HIV known as SI (Syncytium inducing strains) efficiently induce syncytia.¹³² The molecular events associated with viral entry that leads to fusion between the viral coat and cell membrane involves the interaction of the HIV envelope glycoprotein CD4 and a coreceptor molecule. Similar events may occur when an infected cell bearing HIV envelope glycoprotein molecules on its surface encounters an uninfected CD4⁺ cell with an appropriate coreceptor. Fusion between infected and uninfected cells, resulting in multi nucleated giant cells, or syncytia have long been observed *in vitro*. Syncytia have been observed only rarely in tissues obtained from HIV infected individuals; therefore it is unlikely that syncytium formation is a major pathogenic mechanism of CD4⁺T cell depletion *in vivo*.^{135 , 136}

b. Immune mediated attack on infected cells and cells coated with viral proteins

Immunologic responses directed against HIV or HIV infected cells appear to have a significant role in limiting viral replication leading to the decline in the viral load following the initial phase of primary

infection. Both humoral and cell-mediated responses likely contribute to the partial suppression of the virus and viral replication, acting both on the virus and on infected CD4⁺ T cells during the prolonged course of clinical latency. HIV specific immune responses may also target uninfected competent cells that have viral proteins bound to their surfaces. The phenomenon is known as the "Bystander Phenomena". Soluble gp120 can be shed from free virions and from HIV-infected cells and can bind to CD4 molecules on the surface of the uninfected T cells and monocytes/ macrophages. Anti HIV gp120 antibody can recognize these bound gp120 molecules and cause the elimination of these cells by antibody-dependent cell-mediated cytotoxicity (ADCC) pathway. ¹³³

In addition, gp120-specific CTL's may target uninfected cells that have bound gp120. ¹³⁷ Similarly, virus and/or viral proteins may adhere to peripheral blood or follicular dendritic cells and mark them for destruction by ADCC; CTL or other unidentified mechanisms. In this regard, effector cells from HIV-infected individuals have been shown to mediate both CTL and NK-associated ADCC against uninfected cells that were coated with HIV antigens in vitro. ¹³⁸

c. Autoimmune mechanisms

Autoimmunity may occur during the course of HIV infection as a result of molecular mimicry by viral components. Highly homologous regions exist in the carboxy terminus of the HIV-1 envelope glycoprotein and the amino terminal domains of different HLA-DR and DQ alleles. Sera from a substantial number of HIV-infected individuals react with the shared determinant of gp41 and MHC class II; these sera can inhibit normal antigen proliferative responses and also eliminate class II bearing cells by ADCC.¹³⁶

A number of clinical manifestations seen in HIV-infected individuals may be associated with autoimmunity. These include Reiter's syndrome, HIV associated arthropathy, Psoriatic arthritis, polymyositis/dermatomyositis, Sjogren's syndrome, necrotizing vasculitis, septic arthritis and other connective tissue disorders.¹³⁹ The occurrence of HIV-induced rheumatologic disease might involve opportunistic pathogens that induce a reactive arthritis; HIV antigens could also stimulate an immune reaction with subsequent tissue damage. Another pathway by which uninfected cells might be eliminated involves antigenic cross-reactivity between HIV proteins (gp120 and gp41) and MHC class II determinants.¹⁴⁰ In this method, anti HIV antibodies cause the elimination of uninfected MHC class II bearing cells via ADCC. Anti-HLA-DR antibodies have been detected in HIV infected individuals. Not only is there similarity between HIV

proteins and MHC Class II determinants, but similarity also exists between HIV envelope glycoproteins and MHC class I molecules.¹³²

A variety of antibodies that have potential to react with uninfected normal cells have been identified in the sera of HIV infected individuals. These include antibodies to lymphocytes, platelets and neutrophils, CD4 molecules, CD43 molecules, and IL-2 and autologous antibodies bound to uninfected T cells.¹³³

d. Defect in CD4⁺ T cell regeneration (HIV mediated inhibition of Haematopoiesis)

Failure of normal haematopoiesis is an obvious candidate mechanism to account for depletion of CD4⁺ T cells during HIV infection. Two major mechanisms may contribute to the failure of the CD4⁺ T cell regeneration during the course of HIV infection: (1) Destruction of lymphoid precursor cells; perhaps by infection as they become activated to divide. (2) The disruption of the microenvironment required for the efficient regeneration of immune competent cells.

A subset of CD34⁺ progenitor cells express CD4 and HIV coreceptors and are infectable in-vitro with HIV-1. Furthermore, a substantial immunity of HIV-1 infected patients with severe CD4⁺ T cell depletion have a reservoir of HIV infected CD34⁺ progenitor cells. Although the role of direct infection of CD34⁺ progenitor cells in CD4⁺ T cell depletion remains controversial, a large body of evidence suggests that

viral proteins and HIV induced cytokines can impair the survival and clonogenic potential of these cells. The subnormal mobilization of CD34⁺ cells into the peripheral blood following treatment with GM-CSF in HIV infected individuals provides further evidence of reduced haematopoietic capacity and reserve in HIV infection.

Disruption of the thymic microenvironment and HIV induced thymocyte depletion may also contribute to the failure of CD4⁺ T cell production. Thymic epithelial cells normally secrete IL-6, which can in turn increase HIV replication in infected cells. Subpopulations of thymic CD3-CD4-CD8- cells (i.e. “triple negative” cells) are susceptible to infection with HIV in-vitro, and thymic CD3-CD4⁺CD8- progenitor cells from HIV-infected patients are infected in vivo. Finally, uninfected thymocytes from HIV-infected individuals are primed for apoptotic death, suggesting that indirect mechanisms of defective thymopoiesis are operative as well. ¹³⁶

e. Superantigens

It has been hypothesized that some of the quantitative and qualitative abnormalities in CD4⁺ T cells in HIV infection may be due to the presence of superantigens ¹⁴¹. Superantigens are proteins that bind directly both to MHC molecules and to relatively constant region of the V β portion of the T cell receptor. As such, they are recognized by and stimulate a higher proportion of T cells than most conventional

antigens. Depending on the state of activation of the T cells, and the development stage of the affected individual; superantigens can cause expansion, deletion or anergy of the affected V β subset. Perturbation of the V β repertoire in HIV infected individuals has suggested a possible role for superantigens in HIV infection. In some individuals specific V β subsets of CD4⁺ T cells can be either preferentially depleted or expanded. However, since opportunistic pathogens are common in HIV-infected individuals, the V β perturbations could be caused by a superantigen expressed by an opportunistic pathogen rather than to HIV itself. ¹³³

f. Apoptosis

Apoptosis is a form of programmed cell death that normally occurs during organogenesis and during thymic development, where it is a mechanism by which autoreactive T cells are clonally deleted. ¹⁴ Apoptosis is also involved in the elimination of some activated cells during a normal immune response. The induction of apoptosis requires cellular activation, and it has been suggested that HIV infection causes apoptosis through an aberrant intracellular signalling mechanism. gp120/anti gp120 complexes are present on the surface of CD4⁺ T cells in HIV-infected individuals.¹³¹ Although it has not been demonstrated that gp120 induced CD4 cross linking results in signal transduction, extremely low concentrations of gp120 can prime cells for

apoptosis.¹⁴³ Apoptosis can be induced in CD4⁺ T cells from symptomatic HIV-infected individuals if the cells are exposed *in-vitro* to a superantigen mitogen.

High levels of anti-histone and anti-double stranded DNA antibodies are seen in HIV-infected individuals. This observation could be explained by the release of nuclear contents during apoptosis. Both CD4⁺ and CD8⁺ T cells from HIV-infected individuals can apparently undergo spontaneous apoptosis *in-vitro*.¹⁴⁴ However, most of the apoptosis in lymphoid tissue occurs in cells that are not infected with HIV, and there is no correlation between apoptosis and the stage of HIV disease. Apoptosis in lymphoid tissue of HIV infected individuals may reflect the degree of activation of the lymphoid tissue.^{145, 146}

Acute infection of T cells with HIV *in-vitro* can induce apoptosis, and T cells from HIV-infected patients undergo enhanced rates of apoptosis *in-vitro* compared with normal T cells, particularly following activation. Fas-dependent and Fas-independent pathways of apoptosis may be triggered by HIV. HIV-mediated Fas-independent apoptosis of CD4⁺ T cells has been shown *in-vitro* by several groups; enhanced susceptibility of CD4⁺ T cells to Fas-dependent apoptosis has been reported as well. Mechanisms that may contribute to this susceptibility include upregulation of Fas and Fas ligand; upregulation and activation of caspase-1, caspase 3 and caspase 8; activation of cyclin-dependent kinases; and downregulation of the anti-apoptotic Bcl-2 protein. Viral

gene products that have been associated with enhanced susceptibility to apoptosis include the viral envelope, Nef, Tat and Vpu.¹³⁶

The susceptibility of uninfected "bystander" cells to apoptosis is a possible mechanism by which large numbers of cells may be eliminated during HIV infection. Studies in lymphoid tissue from HIV infected individuals suggest that the majority of apoptosis occurs in uninfected bystander cells. Macrophages are efficient mediators of CD4⁺T cells bystander apoptosis. HIV induced upregulation of Fas ligand expression on macrophages and enhanced secretion of TNF- α from these cells appear to be responsible in part for this phenomenon. Another mechanism of bystander apoptosis may involve upregulation of CD62L on lymphocytes by HIV. These HIV exposed lymphocytes home to lymph nodes and undergo apoptosis upon signaling through homing receptors such as CD62L, CD44 and CD11a.

It remains uncertain whether HIV-induced apoptosis plays an important role in vivo in CD4⁺ T cell depletion. The frequency of apoptotic CD4⁺ and CD8⁺ T cells as well as B cells is significantly higher in lymphoid tissue from HIV infected individuals compared with uninfected controls. Some data indicate a positive correlation between the stage of HIV disease and susceptibility of peripheral blood T cells to apoptosis, and another study demonstrated very low frequencies of apoptotic cells in HIV infected long term nonprogressors.¹³⁶

[B] Dysfunction of CD8⁺ T cells

During symptomatic primary infection with HIV, the level of CD8⁺ T cells generally declines. However, following this acute phase, CD8⁺ T cell numbers rebound to higher than normal levels, sometimes remaining at these elevated levels throughout the clinically latent stage of disease.¹⁴⁷ Relatively high levels of HIV-specific CD8⁺ CTLs can be demonstrated in many HIV infected individuals during the early stages of HIV infection.¹⁴⁸ This observation suggests that the observed CD8⁺ T lymphocytosis may be due, at least in part, to expansion of HIV specific CTLs.

The rise in CD8⁺ T cells may also reflect an attempt by the immune system to maintain a normal number of lymphocytes. It has been hypothesized that as CD4⁺ T cells are selectively depleted in HIV infection, the homeostatic mechanisms cause the lost CD4⁺ T cells to be replaced with CD8⁺ as well as CD4⁺ T cells.¹⁴⁹

As HIV disease progresses, anti HIV CTL activity is lost. This decline in HIV specific CTLs may be explained by defects in the clonogenic potential.¹³² CD8⁺ T cells may also be depleted by apoptosis as "innocent bystanders". It has been demonstrated that a significant proportion of circulating CD8⁺ T cells in HIV-infected individuals has an unusual phenotype. They are HLA-DR positive (activated), but they do not express the CD25 marker (the receptor for IL-2), which is required for optimal clonogenic potential. HIV-specific CTL activity reside

almost exclusively within the CD8⁺Dr⁺CD25⁻ subset of CD8⁺ T cells. As these cells lose clonogenic potential, HIV specific CTLs are also lost.¹³³

The loss of CD4⁺ T cells as well as the defect in IL-2 production is characteristic of HIV infection and probably compounds the defect in CD8⁺ CTL's. The integrity of CD8⁺ T cell function depends in part on adequate inductive signals from CD4⁺ helper (inducer T cells). During the late stages of HIV disease when CD4⁺ T cell levels are dramatically reduced and opportunistic infections are common, there is a significant reduction in the number of CD8⁺ T cells.¹⁵⁰ One potential explanation for the decline in CD8⁺ T cells late in the course of disease is a defect in the ability of progenitor cells to regenerate mature T cells.¹⁵¹

Since cells in the thymus are infected during HIV infection, the loss of immature thymocytes may result in a failure to regenerate adequate levels of all types of mature T cells. The rapid rise in CD4 count following treatment with antiviral drugs even in patients with very low CD4⁺ T cell levels implies that there is significant restoration capacity even in advanced disease. However, these replacement CD4 cells are primarily memory cells, suggesting that they are created by division of existing cells, rather than by de novo generation.

Even with combination drug therapy that leads to long-term suppression of viral replication, CD4 cell numbers do not return to normal levels, despite the initial rapid rise, and there is some question

as to whether the immune response can completely recover in such patients.¹³³

Dysfunction of other Immune Competent Cells

(i) B Lymphocytes

Aberrant polyclonal activation of B cells is one of the first immunologic abnormalities to appear following HIV infection. B cells from HIV-infected individuals proliferate spontaneously in-vitro. Hypergammaglobulinemia of IgG, IgA, and IgD is commonly observed in vivo and may result from the lymph node hyperplasia associated with HIV infection.¹⁵² Although spontaneous B-cell proliferation and antibody production do occur in HIV infection, B cells from HIV-infected individuals are defective in their response to additional activation signals. Defects in the production of immunoglobulins in response to specific antigens or pokeweed mitogen can be seen early in the course of infection.¹³² In addition, B-cell abnormalities also cause poor responses to primary and secondary immunizations to both protein and polysaccharide antigens. Studies of primary HIV infection indicate that the absolute numbers of circulating B cells may be depressed; however, this is usually a transient phenomenon. In certain patients the number of B cells may decrease late in the disease.¹⁵³

Some of the loss of B-cell function is presumably due to loss of CD4⁺ T-helper cells that are critical to proper B-cell function.

However, HIV may directly cause functional B-cell aberrations via direct B-cell-stimulating effects. In this regard, exposure of B cells to intact or disrupted HIV in-vitro can cause many functional abnormalities similar to the abnormalities seen in B cells in vivo.¹³³ Portions of the HIV-gp41 envelope glycoprotein have been shown to induce polyclonal B cell activation. The receptor for HIV on the surface of human B cells has been demonstrated to be a product of the VH3 genes.¹⁵⁴

HIV gp120 has been observed to directly bind to an immunoglobulin chain (VH3) and activate these B cells in much the same manner as a superantigen. This antigen dependent polyclonal activation leads in part to hypergammaglobulinemia and B lymphocyte hyperactivation on HIV infection. Other portions of HIV, including gp41, directly activate B cells in a nonsuperantigen-mediated manner. Correlates of B-cell dysfunction observed in HIV infected individuals include an increase in spontaneous EBV transformations in vitro and may contribute to the observed increased frequency of EBV-induced lymphomas. In addition the increased susceptibility to bacterial infections during the course of HIV infection likely results in part from B-cell dysregulation.¹³⁶

B cells may also serve as a viral reservoir during the course of HIV infection. In this regard, stimulation of B cells with CD40L and IL-4 upregulates expression of activation and costimulatory molecules, a

well as CD4 and CXCR4. B cells stimulated in this manner are susceptible to infection with dual- and T-tropic strains of HIV. ¹⁵⁵

In patients with advanced stage HIV infection, B cells fail to proliferate and differentiate in response to ligation of the B cell antigen receptor and CD40, suggesting a defect in signal transduction. Further evidence of HIV related B cell dysfunction comes from an in-vitro lymphoid tissue culture model of HIV infection. In this model, infection of lymphoid tissue with strains of HIV that utilize CCR5 as an entry coreceptor results in enhanced antibody responses to recall antigens upon challenge. In contrast, HIV strains that utilize CXCR4 result in profound suppression of these antibody responses. This effect appears to be dependent on signal transduction through CXCR4 and may be irreversible. ¹³⁶

(ii) Monocytes / Macrophages

Cells of the monocyte / macrophage lineage play key roles in the immunopathogenesis of HIV disease. These cells serve as reservoirs of viral infection and are responsible for a variety of tissue-specific pathologic processes. Although there have been conflicting reports regarding the number of HIV infected monocytes in the peripheral blood, most studies have demonstrated that the major reservoir of HIV in the peripheral blood is the CD4⁺ T cell. The number of infected monocytes in the blood are quite low as compared to both CD4⁺ T cells in the blood and macrophages in the tissues. ¹⁵⁶

Dysfunction of monocytes / macrophages contribute to CD4⁺ T cell dysfunction and also to impaired host defence against intracellular pathogens. Monocytic cells express CD4 and numerous HIV co-receptors including CCR5, CXCR4, and CCR3 on their surface and serve as target cells of HIV infection. Unlike infection of CD4⁺ T cells HIV is relatively noncytopathic for cells of the monocyte / macrophage lineage, and HIV can replicate extensively in these cells in intracellular compartments. Chronically infected macrophages may be long-lived and currently available antiretroviral agents are generally not capable of targeting chronically infected cells. These cells therefore represent a formidable challenge to eradication of HIV. Circulating monocytes are rarely found to be infected in vivo and are difficult to infect in-vitro. However, infection can readily be demonstrated in tissue macrophages, including resident microglial cells in the brain, pulmonary alveolar macrophages, and mature macrophages derived from blood monocytes in-vitro. Infection of monocytic precursors in bone marrow may be directly or indirectly responsible for certain of the hematological abnormalities observed in HIV-infected individuals. Lymphoid tissue macrophages can be prolific producers of HIV in the setting of opportunistic infections, during which the cytokine milieu in the tissue favours a high productive state of HIV infection.¹³⁶

It may be possible that monocyte / macrophage dysfunction is caused by exposure to HIV proteins; since exposure of peripheral blood

monocytes to non-infectious HIV, to gp120 alone, or to a synthetic peptide homologous to gp41 can cause the down regulation of chemotactic ligand receptors followed by suppression of chemotactic function as well as the respiratory burst.¹⁵⁷ Exposure of monocytes to HIV gp120 leads to cellular activation and the secretion of certain cytokines, which contribute to abnormalities.

A large number of abnormalities observed in the peripheral blood monocyte / macrophages, like defects in chemotaxis, monocyte dependent T cell proliferation, Fc receptor function, C3 receptor mediated clearance, and oxidative burst responses. These can be attributed to direct infection of monocyte / macrophage by HIV.¹³²

Impaired accessory cell function may result from decreased MHC class II expression, decreased IL-12 secretion and increased IL-10 secretion. This HIV induced dysregulation of antigen presentation may in turn be a significant cause of hyporesponsiveness of CD4⁺ T cells. Defects in ADCC function of monocytes / macrophages, possibly related to low levels of expression of Fc and complement receptors have also been observed in HIV infection.¹³⁶

(iii) Dendritic Cells (DC's)

DC's are among the first cells to encounter HIV after mucosal exposure. Virions bind to the DC surface by virtue of interactions between the envelope glycoprotein gp120 and DC surface protein DC-SIGN. DC's are thus able to efficiently transport HIV to lymphoid organs, facilitating infection of CD4⁺ T cells and viral dissemination. DC also expresses several different chemokine receptors that can be used as HIV coreceptors for entry. Although the frequency of HIV infected DC in vivo may be low, these cells nevertheless may represent an important viral reservoir.

It has been reported that infectability of Langerhans cells (i.e., DC resident in the epidermis) may be dependent on viral subtype. An HIV subtype that can replicate well in Langerhans cells, likely a major cell type involved in the initiation of viral infection through mucosal contact, may be more efficiently transmitted heterosexually. The degree of DC dysfunction and depletion that occurs during HIV infection remains controversial. Emerging data suggest that a subset of DC expressing CD11c are depleted during the course of infection. These cells were not restored during highly active antiretroviral therapy.¹³⁶

(iv) Neutrophils

Neutrophils appear to be hyperactivated during the course of HIV infection, and many functional defects have been observed in these cells. Neutrophils from HIV-infected individuals produce more TNF- α and IL-6 in response to lipopolysaccharide (LPS) or *Candida* antigen compared to neutrophils from normal donors. These cells undergo apoptosis at an increased rate compared to those from normal controls. When stimulated through Fc receptors or protein kinase C, neutrophils from HIV-infected individuals produce less superoxide compared with the controls; this defect could be recapitulated by treatment of normal neutrophils with HIV envelope protein. A defect has also been observed in neutrophil complement receptor function resulting in subnormal release of IL-8 in response to cryptococcal challenge by these cells in patients with advanced HIV disease.¹³⁶

(v) Natural Killer Cells

Functional abnormalities of NK cells are observed throughout the course of HIV disease, and these abnormalities increase with disease progression. Most studies report that NK cells are normal in numbers and phenotype in HIV-infected individuals. However, decrease in numbers of the CD16⁺ / CD56⁺ subpopulation of NK cells with an associated increase in activation markers has been reported.¹⁵⁸ NK cell activity is reduced in both peripheral blood and bronchoalveolar

lavage fluids from AIDS patients. The defects in NK cells in HIV infection might involve the production or release of cytotoxic factors; neither NK cell binding nor antibody-dependent cellular cytotoxicity is impaired. NK cells from HIV-infected individuals are defective in their ability to kill typical NK target cells as well as gp-160-expressing cells. The abnormality in NK cell lysis is thought to occur after binding of the NK cell to its target. A possible mechanism for defective NK activity includes a lack of cytokines necessary for optimal function. Addition of either IL-2, IL-12, IL-15 or IFN- α to cultures enhances the defective in-vitro NK cell function of HIV-infected individuals. This suggests that the reduction in CD4⁺ T cells, which results in lower amounts of IL-2, is required for the production of NK cell cytotoxins. Enhanced expression of cytolytic inhibitory receptors during the course of HIV infection may also be responsible for depressed NK cell function; in addition selective HIV-mediated down-regulation of HLA-A and HLA-B but not HLA-C and HLA-E molecules may prevent NK cell killing of HIV infected target cells.

NK cells are an important source of HIV-inhibitory CC-chemokines in HIV infected individual.¹⁵⁹ NK cells isolated from HIV-infected individuals produce high constitutive levels of MIP-1 α , MIP-1 β , and RANTES; high levels of chemokine production are also seen when these cells are stimulated by IL-2, IL-15 or CD16 cross-linking, or during lytic killing. Thus, NK cells, like CD8⁺ T cells, may inhibit HIV

replication by cell-mediated killing as well as by secretion of soluble HIV-inhibitory factors.¹³⁶

Role of Lymphoid Organs in Immunopathogenesis

Lymphoid organs are sites of immune activation, reservoirs of viral burden, and centers of viral replication. The majority of the body's lymphocyte pool resides in lymphoid organs; lymph nodes, spleen, and gut-associated lymphoid tissue and not in the peripheral blood.^{134,141} Lymphocytes and macrophages in tissues may differ from those in peripheral blood in important ways, such as the level of activation, differentiation, cytokine milieu, and their exposure to virus. As such, studies of HIV infection in the peripheral blood may not accurately reflect events in the lymphoid organs. It is possible that the initial depletion of CD4⁺ T cells in the peripheral blood that occurs during the acute phase of HIV infection is a result of a redistribution of cells in the lymphoid organs as the host begins to mount an anti-HIV immune response. Since the function of the lymphoid organs is to filter and trap invading pathogens and present them to immune competent cells, the high levels of viremia seen following primary infection presumably lead to an efficient infection of the lymphoid tissue with HIV. Virus is trapped within the germinal centers of the lymphoid organs, which causes cellular recruitment, cellular activation, and germinal center hyperplasia that are part of the normal immune

response to invading pathogen. The process of cellular activation makes the resident and recruited CD4⁺ T cells susceptible to infection with HIV and permissive for viral replication.

Infection of CD4⁺ T cells in the lymphoid tissue is associated with rapid viral turnover and cell death.¹⁶⁰ Infected cells accumulate within lymphoid organs; however, the ratio of infected cells that express detectable viral mRNA to infected cells in which viral mRNA is not detectable is quite low. The latter population includes both cells that contain defective proviruses and any latently infected cells. Despite the fact that virus infected cells can be readily demonstrated in PBMC's by DNA-based PCR, cells that are actively expressing virus are only rarely detectable in PBMC's. In contrast, the lymphoid organs contain much larger percentage of infected cells compared to PBMC's. The viral replication (although present in variable amounts) can be detected in the lymphoid organs at all stages of HIV disease, including the period of prolonged clinical latency. As disease progresses, the relative numbers of cells in lymphoid organs that express viral mRNA increase concomitant with a decrease in the antiviral immune response.¹³⁴

The large majority of HIV genomes present in lymphoid tissues are in extracellular virions that are trapped within the germinal centers of the lymph node. The germinal center is a network of follicular dendritic cells (FDC) which contain long, interdigitating processes and in so doing present antigens to the B cells. Extracellular HIV particles

adhere to processes of the FDC's and, at the early stage of disease do not appear to cause any obvious deleterious effects to them. Initially, the structure of the lymphoid tissue, with discrete, functional germinal centers, remains intact; however, there is hyperplasia because of both in situ proliferation and activated immune cells from the peripheral blood and /or lymphatics. ¹⁶¹

Deterioration of Lymphoid organs

"In early stage HIV disease, the virus is trapped onto the follicular dendritic cell (FDC) network of the germinal centers of lymphoid tissue. CD4⁺ T cells that migrate into the area to provide help to B cells in the germinal center that are responding to the virus are susceptible to infection by these virions. At this stage of disease, the FDC network is relatively intact. Over time, the lymphoid tissue begins to involute and early degeneration of FDC's is observed. The trapping ability of these FDC's is compromised. In advanced stage disease, there is dissolution of the FDC network; virus can no longer be effectively trapped and spills over readily into the circulation. At this stage, there is loss of HIV-specific immune responses as well as loss of the ability to respond to other pathogens. ¹⁶¹

Lymph nodes from patients at an intermediate stage of disease (CD4 T-cell levels between 200 and 500 cells/ μ l) show damage. The node begins to disintegrate and the trapping efficiency of the FDC begins to

decline.¹⁶² At the cellular level, electron microscopy shows that organelles within the FDC become swollen and cell death occurs. At a more advanced stage of disease (below 200 cells/ μ l), there is an increase in both viral load and HIV expression in PBMCs. This change may reflect either increasing viral replication or inability of the lymphoid system to limit the amount of virus or infected cells reaching the peripheral blood, allowing the two compartments to equilibrate.¹⁶ During advanced stages of disease, the architecture of lymphoid tissue is virtually completely destroyed ("burnt out") with a concomitant disruption of the FDC network and the disappearance of FDC. As a result of the dissolution of the germinal center, the lymph node is no longer able to trap pathogens. The virus that was previously trapped in the node is now able to enter the peripheral blood and lymphoid organs to approach equilibrium. Because the lymphoid tissue is unable to mount an effective immune response, there is increased risk of opportunistic infections.^{161, 162} Depletion of CD4⁺ T cells occurs throughout the course of disease and can be at least partially explained by the effects of HIV infection on lymphoid tissue. Susceptible, activated CD4⁺ T cells that are continually recruited into lymphoid tissue during the course of infection are readily infected by cell-to-cell transmission of HIV. The progressive destruction of the lymph node architecture and diminution of function contribute to the

escalating immunosuppression, followed by the complete collapse of the immune system.^{161, 162}

Immune Responses to HIV

HIV induces a broad array of host immune responses in an infected individual. It is still not well known how HIV is able to *continuously* replicate and cause the inexorable decline in immune system function in the presence of these immune responses.

[A] Innate immune response

The innate responses play a major role in initial containment of infection and hence are considered to be crucial in acute primary HIV infection. Two cell populations, plasmacytoid dendritic cells (PDC) and natural killer (NK) cells play predominant role in innate cellular immune response. PDCs were first detected in lymph nodes in 1975 and were described as T-like plasmacytoid cell, which initially had no known function. Recently this cell type has been recognized as major producer of type I Interferons. Loss of these cells is reflected by decrease in interferon - α production, and is associated with higher HIV RNA levels and progression to AIDS. Normal or high PDCs were observed in HIV infected patients, who remain healthy without receiving any therapy, despite their low CD4⁺ cell count. PDCs not only participate in the innate immune response through interferon production, they become mature dendritic cells and participate in the

adaptive immune response. The presence of PDCs in advanced disease may indicate that PDCs may operate innate immune mechanisms even when adaptive immune response is lost.¹⁶³

Mature dendritic cells (DC) are potent presenters and play role in inducing adaptive immune response. DC also acts as a conduit for HIV transmission. The DC-specific lectin DC-SIGN (Dendritic Cell-Specific ICAM-3 non-integrin), which has been shown to bind HIV virions, is expressed by DCs located in mucosal sub-epithelia and may 'shuttle' viruses to susceptible T cells. NK cells form a critical component of host innate immune response against viruses, fungi parasites, and bacteria. NK cells have the natural capacity to kill virus-infected cells in the absence of prior sensitization and without MHC restriction. NK cells kill target cells either by direct contact in the absence of antibody or antibody-dependent cellular cytotoxicity (ADCC). NK cells display a decreased lytic activity in HIV-1 infected patients as compared to uninfected controls. This effect is associated with the very low expression of natural cytotoxicity receptors (NCR) on HLA-DR+ NK cells and possibly, with a differential content in perforin molecules. The finding of an enhanced NK-cell function (production of the cytokines IFN- γ and TNF- α and the β chemokines CCL3, CCL4, and CCL5) in exposed but uninfected IDUs, especially in comparison to those who became HIV-1 infected strongly supports the protective role of NK cells. NK cells also cause suppression of viraemia through CC

chemokine production. Suppression of HIV replication by NK cell culture supernatant was predominantly mediated by CC-chemokine secretion and was considerably greater in-patients with viraemia. ¹⁶³

[B] Adaptive Immune Response

(i) Humoral immune responses

Antibodies against the viral core protein p24 develop within weeks of acute HIV infection. They play a role in the decline of plasma viremia associated with primary infection. Loss of anti-p24 antibodies is associated with progression of HIV disease. ¹⁶⁴

Neutralizing antibodies are defined by their ability to block, or neutralize the infectivity of virus. They act by preventing the virus from binding to its receptor or by blocking entry events subsequent to receptor binding. HIV variants which are resistant to neutralization are observed in HIV infected individuals. This shows that neutralizing antibodies serve to limit the replication of HIV, although the extent of this limitation cannot be determined. Definition of the action and targets of neutralizing antibodies is of considerable importance for understanding the mechanisms of clinical latency and disease progression and for the development of prophylactic vaccines and immune based therapies. ¹³³

Neutralizing antibodies are type -specific (i.e., specific for one viral isolate) or group-specific (i.e., specific for a broad range of viral

isolates.). Most type-specific neutralizing antibodies recognize the V3 region of the HIV envelope gp120 protein. Neutralizing anti-V3 loop antibodies prevents conformational changes within gp120 necessary for HIV entry or for cell-cell fusion. Group-specific neutralizing antibodies recognize epitopes within the HIV envelope gp41 protein discontinuous conformational epitopes around the CD4 binding site on gp120, or carbohydrate determinants.¹⁶⁵ Both type- and group-specific neutralizing antibodies are more efficient in neutralizing laboratory strains of HIV grown in T-cell lines compared to primary isolates grown in peripheral blood mononuclear cells (PBMCs). This is likely due to differential exposure of the V3 loop and CD4 binding domain epitopes among these isolates. Some neutralizing antibodies interfere with the interaction between the HIV envelope and CCR5, thereby inhibiting cellular entry of R5 strains of HIV. Neutralizing antibodies appear to be prognostically relevant to the course of HIV infection. The regularity with which viral variants that resist neutralization emerge suggests that such antibodies are potent impediments to viral replication.¹³⁶

Some anti-HIV antibodies bind to IgG Fc receptor-positive cells and sensitize them to mediate antibody-dependent cellular cytotoxicity (ADCC) against HIV-infected or HIV-coated cells.¹⁶⁶ Most of these antibodies are directed against HIV envelope gp120 or gp41 proteins. CD16⁺ natural killer (NK) cells are important mediators of ADCC, and monocytes also mediate this activity. Anti-HIV ADCC antibodies

develop soon after primary infection and are detectable throughout the course of HIV disease, with some decrease in titers with the onset of AIDS. ADCC also represents an immunopathogenic immune response that is responsible for CD4⁺ T cell depletion during the course of HIV infection. A combination of high levels of plasma viremia with high HIV specific ADCC activity is correlated with rapid CD4⁺ T cell depletion. This correlation may reflect ADCC-mediated "innocent bystander" killing of gp-120 coated, but uninfected, CD4⁺ T cells.¹³⁶

The targets of neutralizing antibody to HIV-1 have been extensively studied. The first target, or epitope, to be identified (and the best defined) is variable region 3 (V3) of the surface glycoprotein gp120.¹⁶⁷ This region is contained within disulfide-linked cysteines between amino acids 300 and 330 in the linear sequence. Synthetic peptides based on this sequence can elicit antibodies in animals that neutralize laboratory strains of HIV-1 and inhibit the fusion of infected and uninfected CD4⁺ cells.¹⁶⁷

Because of the high level of V3 sequence variation, neutralizing antibodies to V3 are usually type- or strain specific i.e., they are capable of neutralizing only a limited number of isolates. However, sera with high-titer antibody to HIV-1 V3 peptides do not always neutralize HIV-1 with a homologous V3.¹⁶⁸ The ability of V3 to serve as a target for neutralization may be dependent on other sequences in the envelope.

Although anti-V3 and CD4 blocking antibodies are most extensively studied, other types of neutralizing antibodies have been described. These include neutralizing antibodies to V1, V2, V5, C2, and gp41 epitopes of HIV-1. Although the V3 loop has often been referred to as the "principal neutralizing determinant" of HIV-1 there is no clear evidence to suggest that V3 neutralization epitopes are more important than other epitopes during the natural course of HIV-1 infection.¹³³

Neutralizing antibodies represent only a small fraction of the total antibody response to gp120. The neutralization titers in human sera are generally low, hence it has been difficult to quantify different antibody responses. Neutralization assays have generally carried out with one or few strains of virus, which may or may not be representative of the complex mix of viral genotypes present in the infected individual. Primary HIV-1 isolates are much more resistant to neutralization than the laboratory strains most frequently used for these assays and may differ in their principal targets of neutralization.¹⁶⁹

Escape from Neutralization

Successive viral isolates are not susceptible to previously neutralizing antibody obtained earlier in the course of infection, but they are susceptible to antibody obtained several weeks later. Thus, the appearance of new variants that are resistant to neutralization always

precedes the appearance of antibody capable of neutralizing the variants. This suggests that antigenic variation has an important role in persistent viral replication in chronically infected individuals.

The HIV-1 gag gene behaves more like a cellular gene than does env and shows a bias towards synonymous mutations. Random mutation cause synonymous changes due to which the env genes are subjected to strong selective pressure to change, whereas this selective pressure for cellular genes remains unchanged. The synonymous mutation in gag presumably results from more stringent limitation on the amino acid changes that still allows optimal protein function. In contrast, variant Env proteins appear to be able to tolerate almost any amino acid change in the discrete variable domains. This provides a significant selective advantage in vivo. The host immune response is the major source of the selective pressure for change, but some changes reflect selection for altered tropism.

Attempts to demonstrate the appearance of neutralization-resistant HIV-1 variants in infected humans have been hampered. This is due to lack of information on the genetic sequence of the initial infecting strain and by the complex mix of genotypes present in infected humans when samples are taken for study.¹³³ Thus there is a clear evidence for selective pressure in discrete variable regions of envelope during the course of the HIV infection. The neutralizing antibody response of the host is the major selective force driving sequence variation in env. The

coincidence of neutralization domains of HIV-1 with variable regions in gp120 and gp41 suggests that there is a direct relationship between neutralization domains and the sequence variants that emerge.¹³³

(ii) Cell mediated immune response

CD4+ Helper T cell response

HIV proteins contain helper T-cell epitopes that are presented by MHC class II alleles. Recognition of these epitopes by CD4⁺ T cells results in secretion of cytokines and cellular proliferation. These responses are thought to be associated with abortive infection in individuals who are exposed to HIV but who remain uninfected, and these responses decrease with HIV disease progression.¹⁷⁰ An inverse correlation has been found between the magnitude of HIV-specific CD4⁺ T-cell response and levels of plasma viremia. The data available are consistent with observed correlation between the magnitude of HIV specific CD4⁺ T cell response and levels of plasma viremia.¹¹⁵ The fate of HIV specific CD4⁺ T cells during the course of HIV infection is controversial. Some evidence suggests that these cells are usually depleted in the early stages of disease and the initiation of potent antiretroviral therapy during acute HIV infection prevents their loss and leads to more effective immunological control of viral replication.¹¹ Data available from some studies suggest that HIV-specific CD4⁺

cells are present throughout the course of HIV disease and that their frequency declines during HAART-induced viral suppression.¹⁷¹

HIV infects CD4⁺ T lymphocytes leading to the death of not only infected CD4 cells but uninfected bystander cells also. The decrease in CD4 cell count affects the CD4 T cell responses, HIV-specific response, response to recall antigens as well as response to T cell mitogens. T-cell help is required for generation of neutralizing antibody response as well as cytotoxic T cell responses. The CD4⁺ T cells produce an array of cytokines that are very important in activating and maintaining effector cells. The impairment of lymphocyte proliferative response to HIV-1 antigens is a relatively early functional defect of cell-mediated immunity found in HIV-1 infected individuals. The magnitude of CD4⁺ T lymphocyte proliferation and quantity and pattern of cytokine production correlate with the clinical status. Individual who have controlled the viremia will show a strong proliferative response to HIV antigens and production of Th1 type of cytokines and the responses inversely correlate with plasma viral load.¹¹⁵ Strong CD4 T lymphocyte response in primary HIV infection was found to correlate with slower disease progression and better control of viremia. And those with poor CD4 response in primary HIV infection failed to control viraemia effectively.¹⁷²

In one study it was observed that loss of HIV-1 specific CD8⁺ T cell function correlates with progressive infection. In chronic infection the

loss was restored by augmenting of HIV-1 specific T helper cell function either by vaccine induction or by infusion of autologous CD4⁺ helper cells.¹⁷³ CD4 cells apparently do not appear to have significant cytotoxic activity. However their role in maintaining HIV-1 specific CD8⁺ CTL activity is well recognized. It is yet not clear how CD8⁺ CTL activity is maintained even when CD4⁺ helper activity is not evident during chronic stage of infection.

CD4⁺ T cells produce various cytokines that activate the effector cells of the immune system. A fine balance between the cytokines is the key to immune response that would ultimately equip immune system to fight the infection. In several infectious diseases including HIV, the profile of the cytokines produced by the CD4⁺ helper cells may decide the progression of the disease. Cytokines not only activate the cells of immune system, they may also influence the expression of various critical molecules on the cell surface and may up regulate or downregulate HIV multiplication in infected cells. Cytokines such as IL-2, IFN- γ , IL-12 are proregulatory cytokines (Th1 type) while IL-4, IL-10, IL-15, IL-13 are proinflammatory cytokines (Th2 type). Since the cytokines are shown to have immunomodulatory role and also have ability to modulate HIV expression the interplay between cytokines and HIV infection has been intensively studied.¹⁶³

HIV disease is believed to be associated with overproduction of type 2 cytokines and decreased production of type 1 cytokines. In a recent

study impaired production of cytokine was found to be predictor of mortality in HIV-1-infected patients and preserved capacity to produce cytokines was associated with prolonged survival.¹⁷⁴ This indicates clinical significance of impaired cytokine production in HIV-1 infection. Another study has demonstrated that balanced type1/ type2 response is associated with long-term nonprogressive HIV-1 infection.¹⁷⁵ Considering potential therapeutic and immunomodulatory role, several cytokines (IL2, IL12, IL15, IFN α , and IFN γ) have been tested or are under investigation in phase I/ II human study in infected individuals. Some cytokines are also being used as adjuvant for candidate prophylactic vaccine.¹⁷⁶

Cytotoxic T cell responses

Virus-specific cytotoxic T lymphocytes (CTLs) recognize antigen in association with the polymorphic products of the major histocompatibility complex (MHC). The presentation results from the partial degradation of virally encoded proteins within the cytosol, most probably by the proteasome complex. The TAP (Transporter associated with antigen processing) proteins, TAP1 and TAP2, are encoded for in the MHC, and transport polypeptides in to the endoplasmic reticulum where they can bind to and stabilize nascent class I molecules.¹⁷⁷

The mature MHC-peptide complexes transit through the Golgi apparatus. Each has three carbohydrate moieties added before they egress to the cell surface. The complex which consists of a viral peptide bound to a specific class I molecule is recognized by the T cell receptor of the CTL. Recognition of an infected cell by a CTL leads to the destruction of the infected cell, via a Fas-mediated pathway or by the release of a pore forming protein. In addition, CTLs release soluble factors such as interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), which have been shown to have a powerful antiviral effect.¹⁷⁷

CTLs play a crucial role in the control of HIV throughout infection and offers some degree of protection. Following initial infection with HIV there is a burst in viraemia with levels as high as 10^6 RNA copies per ml of serum. This viraemia peaks 4-8 weeks after infection, and then drops by many logs, sometimes to undetectable levels, as the patient enters the asymptomatic phase of disease. In 1992 Ariyoshi suggested that neutralizing antibodies were not responsible for the drop in viraemia.¹⁷⁸ In 1994 Koup and colleagues documented a rise in HIV-specific CTL precursors, which coincided with the fall of viraemia, and occurred long before neutralizing antibody could be observed. Five patients were described by Koup et al, four of whom were shown to have CTL precursors specific for Env, Gag, and Pol of HIV within 3 weeks of the onset of clinical symptoms of primary infection.¹⁷⁹

Similarly, Borrow et al in 1994 demonstrated that four out of five patients studied made responses to Env, Gag and Tat within 2 weeks of the onset of symptoms. Patient who failed to make a detectable CTL response exhibited a rapid course of infection with high viral loads and a fast decline in CD4⁺ cell numbers.¹⁸⁰

Since HIV-specific CTLs are important in the control of viral replication the presence or absence of such activity may have an influence on disease progression. Several groups have been quantified either CTL frequencies or CTL effector (CTLe) lytic capacity to HIV to explore whether these influence either the progression of disease or viral load.¹⁷⁷ Although anti-HIV-1 CTLe responses appear to be relatively stable in the first 6 years of infection, such responses do not correlate with either CD4⁺ or CD8⁺ T cell counts, rates of CD4⁺ T cell loss, HIV infectious viral load, use of anti-viral medications, or subsequent progression to AIDS.

A study conducted by Ferbas et al in 1995 consisted of 3 patients from Multicenter AIDS Cohort Study (MACS cohort) who remained asymptomatic for over 8 years and with stable CD4 counts over 1000 cells/mm³. These patients had low viral loads but no detectable CTLs specific for Gag, Pol, Env or Nef, from which it was inferred that CTLs are not always necessary for the control of viral replication.¹⁸¹ Coates et al in 1992 used cryopreserved PBMCs to compare CTLp frequencies in six rapid progressors and six long-term non-progressors (LNTPs)

the latter being patients who remained asymptomatic with stable CD4 counts after more than 8 years of infection. The LNTPs all had persistent Gag-specific CTL responses together with low levels of circulating HIV-1 infected CD4⁺ T cells stable and normal CD4⁺ counts and preserved T cell response. In contrast, the rapid progressors were shown to have transient CTL responses to gag, which were present at the time of an increase in the levels of circulating HIV-infected CD4⁺ cells. The decline in the gag-specific precursors in the rapid progressors was paralleled by a decline in the T cell response.¹⁸²

When correlations between disease progression or viral load and CTL activity are found, it is often difficult to determine whether this is due to the action of the CTLs or whether the CTLs are merely a marker for more generalized immune function. Very few studies have investigated the qualitative differences between CTLs that may be important factors in how well the response controls the virus. The differences include phenotypic and functional differences, T cell receptor repertoire and the selection of epitopes to which the patient responds all factors that are crucial to understand the development of effective vaccine against HIV.

Since the first reports of cell-mediated responses to HIV in the absence of detectable virus or antibody there has been interest and hope that these responses may be a marker of protection as well as previous exposure to HIV.

Failure of CTLs

A progressive decline in HIV-specific cytolytic activity occurs in parallel with the development of HIV-related disease. Such decline is said to be due to a general impairment of CD8⁺ cell cytolytic activity through lack of CD4⁺ T cell help.¹⁸³ In one study it was reported that there is no abnormality found in the general cytolytic capacity of CD8⁺ cells until very late in the course of disease. The decline in CTL activity in the late HIV disease is thought to be due to loss of HIV-specific CTL activity, with CTLs specific for other pathogens remaining at relatively normal levels. Such specific loss could be due to the loss of precursors in the presence of clonal exhaustion, perhaps due to overstimulation with the viral antigen.

Tanaka et al in 1992 suggested that failure to generate new CD8 CTLs could result from infection of CD4⁺CD8⁺CTL precursors in the thymus. Alternatively the CTLs themselves could become infected at the time they recognize and lyse infected target cells.¹⁸⁴

Pantaleo et al in 1990 observed a defect in the HIV specific CTL population. He showed that patients with AIDS had an expansion in the CD8⁺DR⁺ subset of their T cells which were severely defective in their ability to proliferate to variety of stimuli, including anti-CD2, anti-CD3 and mitogens such as PHA and PMA (phorbolmyristate acetate pokeweed mitogen).¹⁸⁵

The generation of variants of HIV which are able to 'escape' from CTL recognition has been observed in many individuals in whom CTL activity can no longer be quantified. Genetic variation is one of the hallmarks of HIV and is seen both in isolates from different individuals and amongst isolates from the same individual followed over time. The reverse transcriptase of HIV is intrinsically prone to error and lacks capacity for 'proofreading', leading to a base misincorporation rate *in vitro* of at least 10^{-4} , equivalent to one mismatch per genome per replication cycle.¹⁸⁶ Because of high replication rate of the virus (10 genomes per day) which has been reported by Ho et al, the initial infecting strain rapidly develops into a population of 'quasispecies' (sets of closely related but distinct genomic sequences). Philips et al demonstrated viruses with variant sequences that have the potential to escape HIV-1 specific CTL recognition. In each case sequencing of sequential viral isolates revealed mutations in T cell epitopes, which meant they could no longer be recognized by effector cells. The CTLs are generally directed against internal viral proteins, which are usually more conserved than surface glycoproteins recognized by the antibodies. Nevertheless mutations frequently occur which render the virus 'invisible' to specific CTLs.¹⁸⁷

Mucosal Antibody Response

The mucosal surfaces provide first contact for HIV and hence the defense mechanisms at mucosal levels are important in preventing establishment of HIV infection. Recent studies on anti-HIV immune response at mucosal surfaces have suggested that HIV immunopathogenesis at mucosal surfaces probably involves both innate and acquired immunity. The innate defense mechanisms at the mucosa primarily include the antimicrobial products derived from the epithelial cells and neutrophils such as lysozyme, lactoferrin and defensins, the bacterial flora and pH of the local mucosa microenvironment. The studies on adaptive mucosal immunity suggest a key role for mucosal antigen presenting cells (APCs), and more specifically dendritic cells (DCs) in HIV infection. DCs residing within epithelial surfaces are the initial cells infected by HIV after mucosal exposure to virus. DC-SIGN, a novel C-type lectin expressed by DCs has recently been shown to play a role in transport of HIV to the local lymph nodes. It has been observed that virus bound to DC-SIGN on DCs can remain infectious for several days, and virus pulsed DCs efficiently transmit virus when they come into contact with CD4 and co-receptor-positive cell types.¹⁶³

HIV-1-specific IgA has been detected in mucosal compartment in exposed seronegative partners of HIV-seropositive individuals and HIV-1 resistant sex workers. CD4⁺ and CD8⁺ CTLs have been

recovered from cervix of HIV infected women. These HIV-1-specific MHC restricted CD8⁺ and CD4⁺ CTL have been found to be capable of both destroying HIV-1 infected cells and releasing anti-viral cytokines. These cytokines might be locally active in women with vaginal or cervical infection. HIV-specific CD8⁺ CTLs have also been shown in the cervicovaginal secretions of exposed but uninfected commercial sex workers (CSWs) from Nairobi. The CD8⁺ CTL response in systemic compartment of HIV-1 resistant women was found to be directed against different subdominant or new CTL epitopes than the epitopes recognized by HIV infected women. It has also been postulated that continuous antigen stimulation is needed for eliciting anti-HIV CTL responses in these women. However, still there is no conclusive evidence to suggest protective role of mucosal CTL activity against HIV transmission. Though mucosal immunity may hold key as the first barrier for establishing HIV infection, the data on mucosal immune response is still very limited. ¹⁶³

Dysregulation of Cytokines

Dysregulation of host cytokine network is characteristic of all phases of HIV infection. Many of the alterations in cytokine production contribute to HIV pathogenesis by further stimulating viral replication suppressing the ability of the immune system to mount an efficient antiviral response, and inducing cytokine-mediated cytopathic effects.¹⁸⁸

HIV infection is associated with increased expression of proinflammatory cytokines. High levels of TNF- α , IL-1 β and IL-6 are secreted by PBMC and macrophages from HIV-infected subjects and are found at elevated levels in serum, cerebrospinal fluid, and tissues. High levels of these cytokines and IL-10 are particularly evident in lymphoid tissue, a major site of HIV replication throughout the course of disease. Chronically activated CD8⁺ T cells and macrophages are thought to be major contributors to the elevated cytokine level observed in HIV-infected subjects.¹³⁶

Th1 and Th2 are clonally defined cytokines produced by clones of CD4⁺ T cells. The Th1 responses are characterized by production of IFN- γ and IL-2 and enhance mainly cell-mediated immunity. In contrast Th2 responses are characterized by secretion of IL-4 and IL-5, with subsequent activation of humoral immunity and generation of antibodies. Th1 and Th2 are cross-regulatory in that IFN- γ suppresses

the activation of Th2 lymphocytes whereas IL-4 suppresses the production of Th1 cytokines.

Type 1 and Type 2 are functionally defined cytokines and are independent of the cell type or types that produce them. It was suggested that IL-2, IL-12 and IFN- γ are grouped as type1 cytokines as they mainly stimulate cell mediated immunity. In contrast, IL-4, IL-5, IL-6 and IL-10 are grouped as type 2 cytokines as they primarily enhance antibody production and humoral immunity. Thus, a cytokine shift from type1 to type2 was postulated to be the immunologic consequence of HIV.¹⁸⁹

A major disruption in the cytokine pattern seen in HIV disease is a progressive loss in the ability to produce immunoregulatory cytokines such as IL-2 and IL-12. These cytokines are critical for effective cell-mediated immune responses, as they stimulate proliferation and lytic activity of CTL and NK cells. IL-12 is essential for stimulating the production of Th1 cytokines, including IL-2 and IFN- γ , which favor the development of cell-mediated immune responses.

The Th1 limb of cellular immune responses is impaired during the course of HIV infection. This is due to defects in IL-2 production and IL-2 receptor expression. The Th1 defect enables the induction of IL-10 cytokine in monocytes and thereby inhibits IL-2 secretion. Some studies have proposed that a dominance of Th2 responses (i.e., IL-4, IL-5 and IL-10) characterizes progression of HIV disease. However the

cytokine dysregulation during the course of HIV infection is complex and cannot easily be classified in terms of Th1 and Th2 polarity.¹³⁶

The current study was carried out to determine cell mediated immune response of HIV seropositive spouses of HIV seropositive migrant workers and exposed seronegative spouses of HIV seropositive migrant workers *in-vitro*. Also, to determine the influence of cell mediated immune response on disease progression.

Aims
and
Objectives

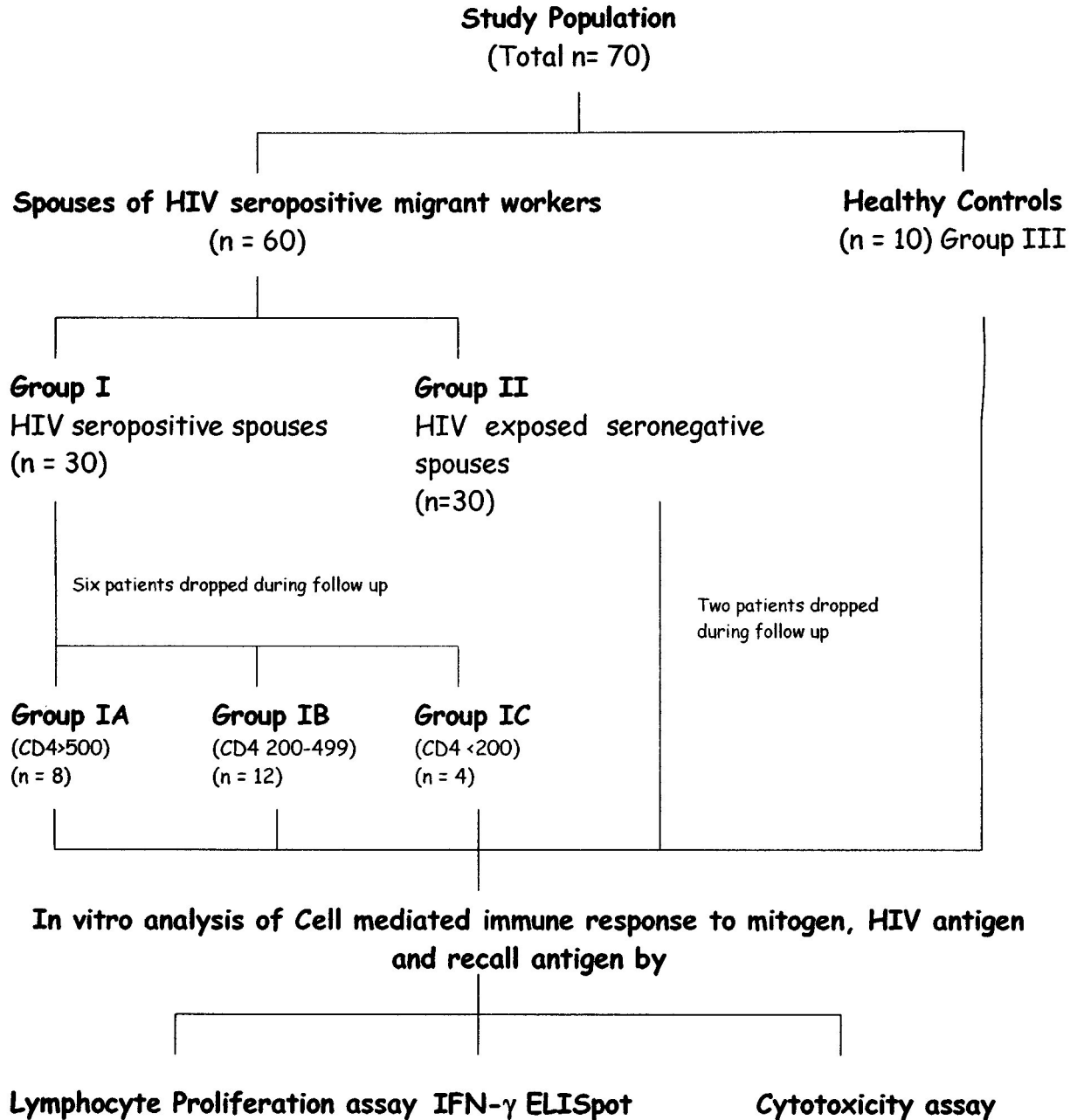
Much research should be devoted to progressors, typical progressors, and exposed but seronegative groups, as each has a potential “lesson” for the understanding and treatment of HIV. The ability to predict rapid progression to end-stage disease would be greatly helpful in designing appropriate therapeutic strategies. In addition, identification for controllable factors that may cause rapid progression would open a window for intervention to prevent such an event. A complete understanding of the mechanisms of pathogenesis is required for development of more effective preventive and therapeutic strategies. Knowledge about the migrant workers of Mumbai and the vulnerability of their spouses for HIV is limited. Hence the following study has been carried out to determine the risk factors and demographic factors influencing the spouses for acquiring HIV disease. The immune correlates of protection in ESN’s women are still unknown. The current study highlights the mechanisms responsible for CD4 cell depletion, and the role of cellular immune responses (towards HIV antigen and Recall antigen) in disease protection and progression at various stages of disease.

Aims and Objectives:

1. To study the demographic profile in spouses of confirmed HIV seropositive male migrant workers
2. To screen the serostatus and study the seroprevalence of spouses of HIV seropositive migrant workers
3. To study the *in vitro* immune responses in HIV seropositive and seronegative spouses of HIV infected migrant workers.
- 3a. To study the T cell proliferative response of study cohort to stimulants like Phytohemagglutinin, gp120 peptide (a.a seq: 254-274) and *Candida albicans* cell wall antigen.
- 3b. To estimate and study gamma interferon secreting PBMCs of the cohort when stimulated with Phytohemagglutinin, gp120 peptide (a.a seq: 254-274) and *Candida albicans* cell wall antigen.
- 3c. To study the Cytotoxic T cell activity of the cohort against target cells
 - PHA blast primed with gp120 peptide and
 - PHA blast primed with recall antigen-*Candida albicans* cell wall extract.

Materials
and
Methods

Flow Chart showing distribution of patients and controls.



(Two follow-ups were carried out at the interval of 6 months.)

Patients:

A total of 60 spouses of HIV seropositive male migrant workers were studied during the year 2003-2006.

Of the 60 women studied, 30 were HIV seropositive spouses of HIV seropositive male workers. Six women were lost to follow-up during the study hence data of 24 HIV positive women has been analysed. These patients were considered as **Group I**.

The remaining 30 women were HIV seronegative spouses of HIV seropositive migrant workers. These had no evidence of HIV infection for over 5 years even after having unsafe sexual intercourse with their partners. Two women were lost to follow-up during the study hence data of 28 HIV exposed but seronegative (**ESN's**) has been analysed. These patients were considered as **Group II**.

Blood samples from both the groups were collected from Mathad Kamgaar Hospital, Koperkhairne and Jyothis Terminal Care Center Kalamboli.

Controls

Ten age matched healthy individuals from Haffkine Institute were considered as Control group.

Detail history from all the groups with respect to demographic characteristics and clinical symptoms were recorded. Also informed written consent was obtained from all patients and control groups.

Ethical Committee Clearance

Due permission was obtained from the Ethical Committee of the Mathadi Kamgaar Hospital and Jyothis Terminal Care Center before the start of the project.

I Collection of Blood Samples

Five to ten ml of blood was drawn by vein puncture from the study population. The blood was aliquoted appropriately in K3-EDTA and plain bulbs for obtaining the plasma + cells and serum respectively. The sera was separated and stored at -20⁰ C till further testing.

II Confirmation of HIV serostatus and seroprevalence

a. HIV 1, 2-antibody detection by ELISA (As per kit insert)

Sera samples were tested for the presence of HIV 1 & 2 antibodies by commercially available ELISA kit. ("Indirect Elisa" HIV-Chex, XCyton Diagnostics Ltd)

In Indirect Elisa, the recombinant proteins representing the immunodominant epitopes (gp41, C-terminus of gp120 and gp36) of HIV 1 / 2 are coated onto the microtitre wells. Serum samples are added to these wells and incubated.

When present in the patient's serum, HIV antibodies combine with HIV peptides attached to the wells. Residual patient sample

was removed by washing and horseradish peroxidase conjugated anti-human IgG (rabbit) was added. Wells were washed and a colourless enzyme substrate (H_2O_2) and chromogen (TMB) were added. Enzyme chromogen substrate reaction was terminated with sulphuric acid (H_2SO_4). The intensity of the colour was read at 450 nm using an Elisa reader (Multiskan).

Confirmation of Seronegative status by HIV-p24 ELISA (As per Kit insert)

Individuals who were found to be HIV 1,2 antibody negative were confirmed for their seronegative status by commercially available p24 antigen detection Elisa kit (Retrotek, HIV-1 p24 antigen Elisa, Zeptometrix Corporations). In HIV-1 p24 Sandwich Elisa micro-wells are coated with a monoclonal antibody specific for the p24 gag gene product of HIV-1. Serum samples were added to these wells and incubated. When present in patients serum viral antigens (p24) specifically binds on to the immobilized antibody during specimen incubation. Residual patients sample was removed by washing and high tittered human anti-HIV-1 p24 antibody conjugated with biotin was added. Following a subsequent incubation with streptaavidin peroxidase, there was colour development as the bound enzyme reacted with substrate

(Tetramethylbenzidine (TMB) + Dimethyl sulfoxide (DMSO)). The intensity of the colour was read at 450nm using Elisa reader (Multiskan).

III Estimation of CD4 and CD8 counts by FACS Counter (As per kit insert)

Hundred microliters of blood was drawn from K3-EDTA containing bulb. Fifty microlitres each were added to the reagent pair tubes for the estimation of CD4/CD3 and CD8/CD3. After incubation in the dark for 90 minutes, the fixative solution was added to these tubes. The CD4⁺ T cells, CD8⁺ T cells and CD4 / CD8 ratio were counted using FACS count (Becton Dickinson). The sorting analysis of the cells was fully automated

V Separation of Peripheral Blood mononuclear cell ⁽¹⁹⁰⁾

PBMCs were separated from the blood sample from K3-EDTA containing bulb using the Ficoll Hypaque density gradient centrifugation. Five ml of Whole blood was overlaid on 5 ml of Ficoll Hypaque-1077 gradient (Sigma). This was then centrifuged at 1500 rpm for 15 minutes. The buffy coat layer of PBMCs was isolated and washed once with sterile RPMI-1640 medium. The cells were then cryopreserved at density of 2×10^6 cells/ml using RPMI-1640 medium, 10 % FCS and 10 % DMSO till further use.

In-vitro analysis of cell-mediated immune response

Part A: Preparation of antigens to be used in in-vitro assays.

➤ **Preparation of mitogen: - Phytohemagglutinin (PHA)**

Phytohemagglutinin [PHA] is a known mitogen of the plant origin. They are sugar-binding proteins called lectins; which specifically binds to different glycoproteins on the surface of various cells including the lymphocytes. Binding of the PHA molecules to the membrane of the cells often leads to the agglutination or clustering of the cells, which triggers cellular activation and proliferation.

PHA was procured from SIGMA Diagnostics and used for In-vitro assays (Lymphocyte Proliferation assay, ELISpot assay, Cytotoxicity Assay). PHA was prepared in RPMI-1640 medium (Hi-media). A concentration of 5µg/ml was used for the *in-vitro* assays.

➤ **Preparation of HIV-1 antigen: - gp120 fragment**

A region comprising of amino acids 254-274 that lies in the second conserved domain of gp120, takes part in a post-binding event during virus penetration and is an important target for antibody neutralization of HIV. This region is important for HIV infectivity but not HIV binding ⁽¹⁹¹⁾. gp120 peptide (a.a sequence 254-274) was procured from SIGMA Diagnostics and used for In-vitro assays (Lymphocyte Proliferation assay, ELISpot assay, Cytotoxicity Assay). gp120 was prepared in RPMI-1640 medium (Hi-media). A concentration of 1µg/ml was used for the *in-vitro* assays.

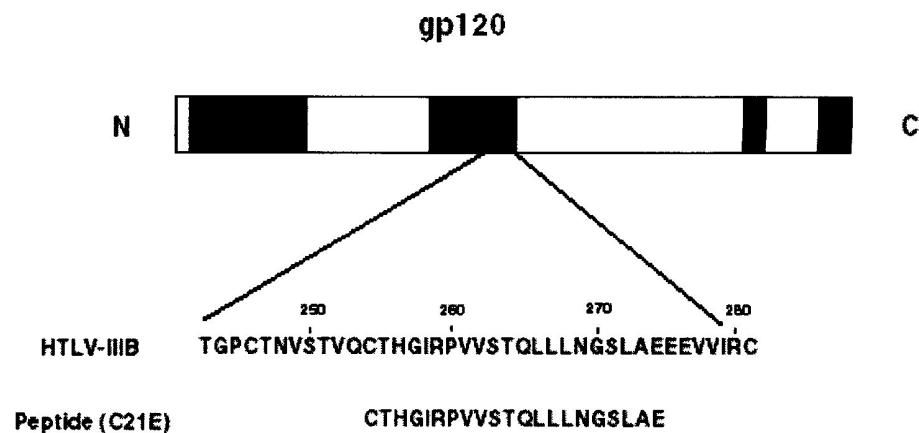


Figure No 11: Second conserved domain of gp120 (a.a seq-254-274)

➤ **Preparation of Recall antigen: - Cell Wall Extract of *Candida albicans***

Clinical isolate of *Candida albicans* was obtained from Ayurveda Research Center, K E M Hospital, Parel. The sample was streaked on Saborauds agar, colony characters were studied and compared with a standard *Candida albicans* strain. Gram staining was carried out to detect gram-positive yeast. Colonies showing characteristic similar to the standard strain were isolated on Saborauds agar and biochemical tests for *Candida albicans* were performed.

Germ Tube Test ¹⁹²

Germ Tube test was used to confirm if the isolated organism was *Candida albicans*. Loopful of culture was inoculated in suspension of 0.5ml of serum in a test tube and incubated at 37⁰ C for 2-3 hours. Wet mount of incubated culture was observed under high power lens for formation of germ tube.

Culture conditions ¹⁹²

Candida albicans strain was maintained by culturing every 2-4 weeks on slopes of Saborauds dextrose agar. Organisms were propagated in Lee medium (Appendix I) ¹⁹³ until mycelia were developed. Flask (500ml) containing 150ml of Lee medium was

inoculated with loopful of cells from 12-18h-growth slant culture and were incubated on a shaker (200rpm) at 28°C for 12-14h. After the incubation period the A_{650} was taken 0.6U (exponential growth phase) as measured with spectrophotometer. Cells were collected from the medium by centrifugation at 3000 xg, washed twice with sterile distilled water and resuspended at a concentration of about 1mg (dry wt) of cells per ml in sterile distilled water. Suspension was incubated at 28°C for 3hrs with shaking and then stored at 4°C for 72-96h (starvation period). Starved blastoconidia were inoculated in fresh medium and incubated for 6 hrs at 37°C for the formation of mycelium (germ tubes).

Cell wall extract preparation ¹⁹²

Blastoconidia or mycelium were collected by centrifugation (3000xg, 10-15min) and washed twice with chilled 0.001M phenyl methyl sulfonyl fluoride in 0.01M Tris hydrochloride buffer (pH 7.2) ----- 'Buffer A' (Appendix I). Cells were broken by suspending the cell pellet in to a final volume of 25ml with Buffer A containing Ballotini glass beads (0.45 to 0.5mm in diameter) and vortexing for 5 periods (2 min each) on cold ice. To obtain complete cell breakage 5mg of beads per mg of cell were taken. Cell walls were sedimented (1,200g for 10 min) from

cell free homogenate, washed 4 times with chilled Buffer A. Cell wall extract was boiled for 5 mins with 2% Sodium Dodecyl sulfate (SDS) in glass distilled water and finally washed four more times with chilled 0.001M phenyl methyl sulfonyl fluoride in glass distilled water. Cell breakage was assessed by examination of preparation in phase contrast microscope. Cell wall extract was dialyzed against sterile glass distilled water. Qualitative tests mentioned further were carried out to find out the presence of carbohydrate (mannan) and protein in the extract.

Qualitative tests of *Candida albicans* cell wall extract preparation.

➤ **Detection of carbohydrate by Molisch Test** ¹⁹⁴

Principle:

It is a universal test. When H₂SO₄ is added it hydrolyses polysaccharide to monosaccharide. Monosaccharides are converted to Furfurals. Furfurals act with α -Naphthanol to give purple colour.

Procedure:

Two ml of *Candida albicans* cell wall extract was taken in a glass test tube. Two drops of Molisch reagent (0.5% α -Naphthanol

solution in alcohol) was added. Two-three ml of concentrated H_2SO_4 was added slowly along the side of the test tube.

➤ **Confirmation of presence of Carbohydrate by Benedicts Test**

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Principle:

Reducing sugar reduces CuSO_4 to orange red cuprous oxide.

Procedure:

In a test tube 2.5ml of Benedict's reagent (Appendix I) was added. Then 0.5 ml of Cell wall extract was added to it. The mixture was mixed properly and boiled on burner.

➤ **Detection of Proteins by Biurette method** ¹⁹⁵

Principle: Proteins react with cupric ions in alkaline medium to form a violet coloured complex. The intensity of the colour produced is directly proportional to proteins present in the specimen and can be measured on a photometer at 530nm.

Procedure:

In a test tube 2ml of cell wall extract was added. One ml of 3N sodium hydroxide (NaOH) was added to it. The mixture was mixed well and 1ml of 2.5% of CuSO_4 solution was added. The precipitate was allowed to settle down and colour of supernatant was observed.

➤ **Confirmation of presence of proteins b Ninhydrin Test ¹⁹⁵**

Principle: Ninhydrin reacts with α -amino acids between pH 4-8 to form a purple coloured compound. Intensity of purple colour can be read at 570 nm.

Procedure:

In a test tube 5ml of cell wall extract was added. To it 0.5ml of Ninhydrin solution (0.5%) was added. The mixture was boiled and purple colour was observed.

The cell wall extract was used further for polysaccharide and protein estimation, which was carried out by Dubois et al and Miller's method respectively.

➤ **Estimation of polysaccharide by Dubois et al method ¹⁹⁶**

Principle: Simple sugars, oligosaccharides, polysaccharides and their derivatives, including the methyl ethers with free or potentially free reducing groups, give an orange colour when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the colour is stable.

Procedure: Two ml of standard mannose solution (20 μ g -120 μ g) was pipetted in a colorimetric tube. 0.05ml of 80% phenol was added. 5ml of concentrated sulfuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than the side of the test tube in order to obtain good mixing. Tube

were allowed to stand for 10 mins then they were mixed and placed for 10-20 mins in a water bath at 25⁰ C -30⁰ C before readings were taken. The absorbance of the characteristic yellow-orange colour was measured at 490nm. Blanks were prepared by substituting distilled water for the mannose solution

➤ **Estimation of protein by Millers Method** ¹⁹⁷

Principle: This method uses phenol reagent of Folin & Ciocalteu. It contains phosphotungstic-phosphomolybdic acid solution. In presence of proteins the phosphomolybdic acid forms phosphomolybdenum blue. Phosphomolybdic is reduced by phenol to molybdenum blue, which is estimated colorimetrically.

Procedure: One ml of standard Bovine serum albumin (200µg) was taken in test tube. One ml of Folin's solution (Appendix) (1% CuSO₄ + 2% Na-K tartarate + 10% Na₂CO₃) was added to each tube and Kept at room temperature for 10 min. Three ml of Folin Ciocalteu solution was added to each tube and kept at 50⁰ C for 10 mins. Readings were taken at 660nm.

The dialyzed cell wall extract (CWE) was stored at -20⁰ C then further used for in-vitro assays (Lymphocyte Proliferation assay, ELISpot assay, Cytotoxicity Assay) as recall antigen.

Part B: In-vitro Assays

(i) Antigen specific lymphocyte proliferation assay ¹⁹⁸

Principle:

Antigen specific Lymphocyte proliferation assay (LPA) measures the ability of lymphocytes placed in short term tissue culture to undergo a clonal proliferation when stimulated *in-vitro* by a foreign molecule, antigen or mitogen. CD4⁺ lymphocytes proliferate in response to antigenic peptides in association with class II major histocompatibility complex (MHC II) molecules or antigen-presenting cells (APCs). This proliferative response of lymphocytes to antigen *in-vitro* occurs only if the patient has been immunized to that antigen, either by having recovered from an infection with the microorganism containing that antigen, or by having been vaccinated.

Procedure:

PBMnC's were distributed in triplicates into wells of a 96 well round bottom plate (100µl per well= 10⁵ cells/well). Hundred microliter/well of culture medium (RPMI-1640) with appropriate concentration of antigen HIV gp120- 1 µg/ml, Candida albicans antigen- 1:100 diluted) and mitogen (Phytohemagglutinin 5 µg/ml) was added to the respective wells. Control wells with 100 µl of cells and 100 µl of RPMI-1640 medium was kept for

each individual sample. Plates were incubated at 37°C in a humidified atmosphere with 5% CO₂. Each well was pulsed with 50 µl of 1µCi of [Methyl³H thymidine] (3HTdR) in of culture medium 24 hr prior to harvesting. Plates with PHA were harvested at 96hr because of high frequency of cells that respond to this mitogen. Plates with gp120 fragment and *Candida albicans* cell wall antigen (CWE) were harvested on 6th day. The cells were harvested on to glass fiber mats, added to scintillation vial having 3 ml scintillation cocktail (Sisco Research Laboratories) and 3HTdR incorporation was estimated by scintillation counting using liquid beta scintillation counter (Wallac Beta).

Measurements were recorded as Counts per minute. Stimulation indices (SI) were calculated by the counts per minute (cpm) of radioactivity measured in the wells containing antigen divided by the cpm measured in control wells containing PBMC with RPMI 1640 medium but no antigen.

Formula used:

$$\text{Stimulation index} = \frac{\text{Counts per minute of Stimulated sample}}{\text{Counts per minute of unstimulated sample}}$$

(ii) **IFN- γ ELISpot assay** (Carried out as per kit insert)

Principle:

ELISpot assay is used to measure the ability of T cells to secrete IFN-gamma in response to a specific antigen. PBMCs from the individual being tested is added along with mitogen/ specific HIV peptide/ recall antigen to a 96-well polyvinyl difluoride (PVDF) membrane that has been coated with monoclonal antibodies to IFN-gamma. Control wells containing PBMCs and medium alone without the antigen, are assayed in parallel. During an overnight incubation at 37°C HIV-specific T cells present among the PBMCs are stimulated by the antigens to secrete IFN-gamma. The plate-bound anti-IFN-gamma antibody captures this IFN-gamma. On the next day, the PBMCs are removed and the wells are washed before addition of a second anti-IFN-gamma antibody, which is labelled, with biotin. Excess unbound antibody is removed by washing before addition of a streptavidin-enzyme conjugate. The enzyme substrate is then added, which results in the formation of a colored product, or spot, where the streptavidin/biotin complex resided. Each spot corresponds to an antigen-specific cell that has secreted IFN-gamma in response to antigen. The number of spots corresponds to the number of antigen-specific T cells present among the PBMCs.

Procedure:**Preparation and Blocking of precoated plates**

Plate was removed from the sealed package and washed with sterile PBS (4 x 200µl/well). Blocking was carried out using RPMI-1640 medium containing 10% of the fetal calf serum as used for the cell suspensions (200µl/well). Plate was incubated for 1 hour at room temperature.

Incubation of cells in plate

The blocking medium was removed and 75µl/well of PBMCs including 75 µl/well of stimulatory agents (PHA-5µg/ml, gp120-1µg/ml and *Candida albicans* cell wall antigen (CWE) 1: 100 diluted) was added (final volume of 150µl/well). Control wells containing 75µl/well of PBMCs and 75µl/well of RPMI medium were kept. The plate was covered with aluminium foil. It was then incubated in a 37⁰C humidified incubator with 5% CO₂ for 16-48h.

Detection of Spots

After incubation cells were removed from the plate and washed with PBS (5 x 200 µl). One-step detection reagent (alkaline phosphatase-conjugated detection mAb 7-B6-1) was diluted to 1.5 µg/ml (200X) in filtered PBS containing 0.5% fetal calf

serum. Hundred microliter of this reagent was added to each well and incubated for 2hrs at room temperature. Plates were washed again with PBS (5 x 200 μ l). Ready to use substrate solution (BCIP/NBT-Plus) was filtered through a 0.45 μ m filter and 100 μ l of substrate was added per well. Plate was incubated at room temperature till spots were formed. The colour development was stopped by washing in tap water (6 x 200 μ l/well). Plates were dried. Spots were counted under stereomicroscope.

(iii) **Cytotoxicity assay** ¹⁹⁹

Classically, CD8⁺ T cells have been regarded as the T-cell population that mediates cytotoxic activity. Lymphocyte mediated cytotoxic assay was used to study the cytotoxic T cell response (CTL) against target cells ((1) PHA blast preincubated with gp120 peptide and (2) PHA blast preincubated with recall antigen-CWE).

Procedure

PBMC's (Effector cells) from the above groups were placed together with appropriate target cells in-vitro, to measure the lysis of the target cells by the effector cells.

Preparation of Phytohemagglutinin blasts (PHA blasts)

Target cells

To target cells (10^6 cells/ml), 0.5µg/ml of PHA was added and incubated at 37°C in humidified atmosphere of 5 % CO₂. On the day of assay the cells were washed with RPMI-1640 medium and were labelled with ⁵¹Cr.

Labelling target cells

PHA blasts were used as target cells. These blasts were incubated with 1µg/ml of gp-120 peptide (a.a seq: 254-274) and Candida albicans cell wall extract (1:100) separately before placing it with the effector cells. The target cells were further

labeled by radioisotope Chromium (^{51}Cr - 500 μCi). The lysis of target cell by effector cell was measured by release of radiolabeled chromium. Effector cells and target cells were incubated together at effector to target ratio (100:1). Samples containing only target cells and RPMI-1640 medium were kept to measure spontaneous lysis. Samples containing target cells with detergent (Triton X-1: 100 diluted) were kept to measure maximal lysis. After 4 hours of incubation 100 μl of supernatant was removed from the mixture of effector and target cells, and the amount of radioisotope released was measured. Percentage target lysis was then calculated from the formula $(E-S) / (M-S) \times 100$; where E is the experimental lysis, S is the spontaneous release in the presence of RPMI-1640 medium with no CTLs present, and M is the maximum release from the target cells with detergent.

Experimental wells:

100 μl of Effector cells + 50 μl of Target Cells + 50 μl RPMI-1640 medium

Spontaneous Release wells:

50 μl of Target Cells + + 150 μl RPMI-1640 medium

Maximum release wells:

50 μl of Target Cells + 150 μl Triton X (1:100 detergent)

(The tests were performed in triplicates)

Formula used:

$$\text{Percent Lysis} = \frac{\text{Experimental release (E)} - \text{Spontaneous Release}}{\text{Maximum Release (M)} - \text{Spontaneous Release}} \times 100$$

Statistical analysis:

Statistical analysis for each in vitro assay was done in two parts;

Part a: The intra-group comparison of basal values of each assay was carried out using unpaired 't' test.

Part b: The inter-group comparison for each assay was carried out using ANOVA. The overall statistical analysis was carried out using SPSS package.

Observation
and
Results

An unbiased study of total **70** women was carried out. Out of which 60 were spouses of HIV seropositive migrant workers. Written informed consent was obtained from the study population prior to blood collection. Ethical Committee clearance was obtained from the Ethical Committee of the Mathadi Kamgaar Hospital and Jyothis Terminal care Center before the start of the project. Blood samples from the study groups were collected from Mathadi Kamgaar Hospital, Koperkhairnagar and Jyothis Terminal Care Center. Following groups were made:

Group I: consisted of **30** HIV seropositive spouses of HIV seropositive male migrant workers. They were confirmed as HIV seropositive by HIV-1 ELISA. This group was further divided on basis of CD4 counts into

- (i) Group I A: - CD4 counts above 500
- (ii) Group I B: - CD4 counts between 200-499
- (iii) Group I C: - CD4 counts below 200

Group II: consisted of **30** spouses of HIV seropositive migrant workers who were exposed but were persistently seronegative (**ESN's**). Their serostatus was confirmed as seronegative by HIV p24 antigen ELISA. This group had CD4+ counts above 500 hence were not divided in to groups.

Group III: consisted of **10** normal healthy employees and students of the Haffkine Institute.

Two Follow-ups of Group I (A, B, C), Group II and Group III were carried out at interval of 6 months.

During the two follow-ups 6 patients from HIV seropositive group dropped out from the study. Out of 6 patients who were lost to follow-up, 4 patients died during the study, 1 patient migrated and 1 patient went on ART. Similarly 2 women from ESN (Group II) migrated due to husband's death and hence were dropped out from the study. Therefore total number of individuals in HIV seropositive (Group I) were 24 and in ESN's (Group II) were 28. HIV seropositive groups were divided in to subgroups on basis of their CD4 count as mentioned above.

Table No. 1 : Agewise distribution

Groups	11-20 yrs	21-30 yrs	31-40yrs	41-50 yrs	51 & above
Group I (n=24)	2 (8.33 %)	12 (50 %)	10 (41.6 %)	-	2 (8.33%)
Group II (n=28)	3 (10.71 %)	14 (50 %)	8 (28.57 %)	3 (10.71 %)	-
Controls (n=10)	-	10 (100 %)	-	-	-

As depicted in Table no 1 out of 24 HIV positive women (Group I) 12 women (50 %) belonged to age group 21- 30 years and 10 (41.6 %) belonged to age group 31-40 years which are the reproductive age groups. Similarly out of 28 Exposed seronegative women (Group II) 14 women (50 %) belonged to age group 21-30 years and 8(28.57%) belonged to age group 31-40 years.

Table No. 2**Demographic Characteristics of Group I (HIV seropositive women)**

	Characteristics	Group I (n=24)
Marital status	Married	24 (100%)
	Unmarried	-
Education	Illiterate	10 (41.67 %)
	Primary	10 (41.67 %)
	SSC and above	04 (16.67%)
Employment	Employed	08 (33.33%)
	Unemployed	16 (66.67%)
Living status	With family	5 (20.83%)
	Away from family	19 (79.17%)
Addiction	No addiction	18 (75%)
	Alcohol	-
	Smoking	-
	Tobacco	06 (25 %)
	Drugs	-
Knowledge about HIV/AIDS		7(29.17 %)

(n= number of individuals in the group.)

In **Group I** (HIV seropositive) out of 24 women 10 (41.67 %) were illiterate, 10 (41.67%) received primary education and 4 (16.67%) were educated above secondary level. Amongst the 24 women 8 (33.33%) were employed. Out of 24 HIV positive women 5(20.83%) lived with their family whereas 19(79.17%) were living away from their family. Out of 24 positive women 18 (75%) of them had no addiction to alcohol, smoking, tobacco or drugs whereas 6 (25%) were addicted to tobacco. Seven (29.17%) had no knowledge about HIV/AIDS. The mode of transmission of HIV in all 24 (100%) women was heterosexual.

Table No. 3
Demographic Characteristics of Group II (Exposed seronegative women).

	Characteristics	Group B (n= 28)
Marital status	Married	28 (100%)
	Unmarried	-
Education	Illiterate	16 (57.14%)
	Primary	11 (39.28%)
	SSC and above	01 (3.57 %)
Employment	Employed	19 (50%)
	Unemployed	19 (50%)
Living Status	With family	25 (89.28%)
	Away from family	3 (10.71%)
	No addiction	18 (64.28%)
Addiction	Alcohol	-
	Smoking	-
	Tobacco	10 (35.71%)
	Drugs	-
Knowledge about HIV/AIDS		16(57.14%)

In **Group II (Exposed but seronegative –ESN's)** out of 28 exposed and persistently seronegative women 16 (57.14%) were illiterate, 11 (39.28%) received primary education and 1(3.57 %) were educated above secondary level. Amongst the 28 women 19 (50%) were employed and 19 (50%) were unemployed. Out of 28 HIV positive women 25(89.28%) lived with their family whereas 3(10.71%) were living away from their family. Out of 28 seronegative women 18 (64.28%) of them had no addiction whereas 10 (35.71%) were addicted to tobacco. Amongst the 24 ESN's 16 (57.14%) had no

knowledge about HIV/AIDS. These women despite of unsafe sexual practice had a history of seronegative status for more than 5 years.

Table No. 4
Demographic Characteristics of Group III (Healthy Controls)

	Characteristics	Controls (n=10)
Marital status	Married	01 (10%)
	Unmarried	09 (90%)
Education	Illiterate	-
	Primary	-
	SSC and above	10 (100%)
Employment	Employed	10 (100%)
	Unemployed	-
Living Status	With family	10 (100%)
	Away from family	-
Addiction	No addiction	10 (100%)
	Alcohol	-
	Smoking	-
	Tobacco	-
	Drugs	-
	Knowledge about HIV/AIDS	10(100%)

In **Group III** (Normal healthy individuals) all the 10 (100%) received higher education, 10(100%) were employed, 10 (100%) were living with their family and none were addicted to alcohol, smoking tobacco or drugs. All the control group individuals had knowledge about HIV/AIDS.

Table No 5

Region wise distribution of Study Population

Groups	North Region	South Region	East Region	West Region
Group I (n=24)	4 (16.67%)	8 (33.33%)	2 (8.33%)	10 (41.67%)
Group II (n=28)	4 (14.29%)	5 (17.86%)	4 (14.29%)	15 (53.57%)
Group III (n=10)	-	-	-	10 (100%)

Region wise distribution of the study population was carried out. Study population included women who migrated to Mumbai with their husbands from various regions of India. **Northern regions** included Punjab and Uttar pradesh. **Southern Regions** included Kerala, Chennai, and Andhra Pradesh. **Western regions** included Karad, Satara, Sangli, Pune, Kolhapur, Alibaug, Sawantwadi regions of Maharashtra state. **Eastern region** included Kolkata. As shown in Table No 5, from Group I maximum number of women (41.67%) who came to Mumbai were from Western regions followed by 33.33% from Southern regions, 16.67 % from North regions and 8.33% from Eastern regions of India. Similarly from Group II maximum number of women (53.57%) who came to Mumbai were from Western regions followed by 17.86% from Southern regions, 14.49 % from North regions and 14.49% from Eastern regions of India.

Table No. 6: CD4, CD8, counts and CD4:CD8 (Basal values):

Groups	CD4 counts / mm³	CD8 counts / mm³	CD4 : CD8
IA	730	1311	0.65
IB	294	1075	0.32
IC	153	802	0.19
II	745	540	1.42
III	901	675	1.48

Table No. 7: CD4, CD8, counts and CD4:CD8 Follow up I (after 6 months) values:

Groups	CD4 counts / mm	CD8 counts / mm	CD4 : CD8
IA	752	859	0.57
IB	264	907	0.24
IC	117	996	0.11
II	775	552	1.46
III	901	675	1.48

Table No. 8: CD4, CD8, counts and CD4:CD8 Follow up II (after 1year) values:

Groups	CD4 counts / mm	CD8 counts / mm	CD4 : CD8
IA	661	863	0.55
IB	241	824	0.20
IC	75	823	0.10
II	781	573	1.41
III	901	675	1.48

As seen in table no 6, 7 and 8 significant decrease in CD4 cell count and CD4:CD8 ratio was observed in HIV positive Group I (A, B, C) in a period of 1 year ($p < 0.05$). There was no significant change in CD4 count or CD4:CD8 ratio as observed in Exposed seronegative women in a period of one year.

Table No 9 : Opportunistic Infections among groups

Groups		n	O	CM	G	HS
Group I HIV seropositive	I A (CD4 > 500)	8	2 (25%)	-	1 (12.5%)	1 (12.5%)
	I B (CD4 200-499)	12	7(58.3%)	-	2(16.7%)	2(16.67%)
	I C (CD4 <200)	4	3 (75%)	-	1(25%)	2 (50%)
Group II (ESN's)		28	1(3.57%)	-	1 (3.57%)	-
Group III (HealthyControls)		10	-	-	-	-

- n – number of patients
O – Oral candidiasis
CM – Cryptococcal meningitis
G – Gastroenteritis
HS – Herpes Simplex

As shown in (Table no 9) 75 % of HIV seropositive women who belonged to Group I C (CD4 count below 200) had Oral candidiasis as compared to Group IA (25%) and Group IB (58.3%). Also 25% of Group IC women had Gastroenteritis and 50% had Herpes Simplex infection. Thus, opportunistic infections are seen in HIV positive individuals with low CD4 counts.

Observation and Results of

Preparation of Cell Wall extract of *Candida albicans*

(I) Colony Characteristics

Table no: 10

Characters	On Saborauds Agar
Size	Small
Shape	Round
Colour	Cream
Margin	Entire
Surface	Smooth
Opacity	Opaque
Elevation	Convex
Consistency	Moist
Grams nature	Gram +ve yeast

(II) Biochemical Tests:

Table no: 10

Test	Results
Glucose	Acid + Gas
Maltose	Acid + Gas
Sucrose	Acid
Lactose	Negative

(III) Germ Tube Test:

It was observed that the parent cell and the germ tube were one continuous cell as seen in *Candida albicans* and cell wall did not separate the two as in *Candida tropicalis*.

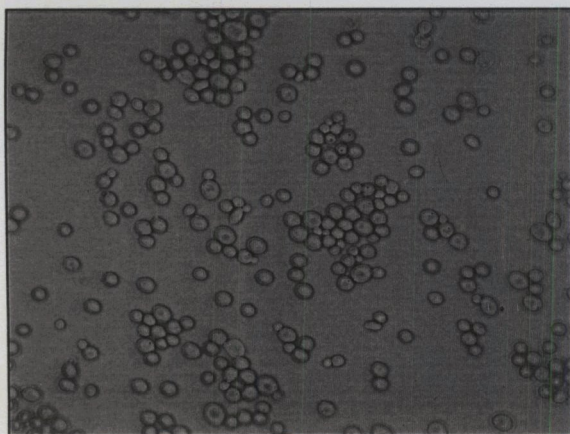


Figure1:
Candida albicans

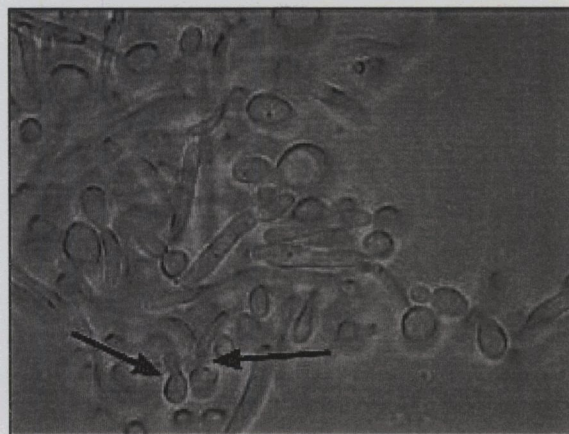


Figure 2:
Germ Tube of *Candida albicans*



Figure 3:
Gram-positive yeast (*Candida albicans*)

(IV) Qualitative tests of *Candida albicans* cell wall extract preparation.

Qualitative tests were carried out with the cell wall extract of *Candida albicans* cell wall to find out the presence of mannan and proteins.

(i) Detection of carbohydrate by Molisch Test

At junction of 2 liquids there was formation of purple ring, which suggests a positive test, and presence of carbohydrate (polysaccharide-mannan) in the cell wall of *Candida albicans*.

(ii) Confirmation of presence of Carbohydrate by Benedicts Test

There was formation of precipitate from green to orange to red which was observed which confirmed the presence of carbohydrate (mannan) in cell wall of *Candida albicans*.

(iii) Detection of Proteins by Biurette method

Purple coloured supernatant and white coloured precipitate suggested presence of proteins in Cell wall of *Candida albicans*.

(iv) Confirmation of presence of proteins by Ninhydrin Test

Formation of purple colour confirmed the presence of proteins in Cell wall of *Candida albicans*.

(IV) Estimation of Mannan in Cell wall extract of *C. albicans*

Concentration of mannan in the cell wall extract of *C.albicans* was estimated by Dubois et al method and was found to be 0.46mg/ml.

(V) Estimation of proteins in the Cell wall extract of *C. albicans*

Concentration of mannan in the cell wall extract of *C.albicans* was estimated by Miller et al method and was found to be 0.5 mg/ml.

***In- vitro* Assay Results**

- **Lymphocyte Proliferation assay results:**

Table 11: Proliferation assay (Basal values)

Groups		n	Mean Stimulation index		
			PHA	Gp120	CWE
HIV seropositive	I A (CD4> 500)	8	32.98 $\pm$ 3.35	3.21 $\pm$ 0.46	5.23 $\pm$ 0.93
	I B (CD4:200-499)	12	19.73 $\pm$ 4.03	2.32 $\pm$ 0.12	2.90 $\pm$ 0.28
	I C (CD4< 200)	4	6.13 $\pm$ 1.94	1.15 $\pm$ 0.11	1.46 $\pm$ 0.21
HIV seronegative	II	28	48.34 $\pm$ 2.34	4.80 $\pm$ 0.19	2.70 $\pm$ 0.11
Healthy Controls	III	10	76.90 $\pm$ 4.15	2.41 $\pm$ 0.16	1.26 $\pm$ 0.06

n = number of samples,

Mitogen (PHA): 5 μ g/ml Phytohemagglutinin

HIV antigen (GP120 fragment): 1 μ g/ml

Recall antigen (CWE): 1:100 diluted cell wall extract of *Candida albicans*

Stimulation index (S.I $\pm$ Standard Error) = CPM of stimulated cells / CPM of unstimulated cells).

Statistical analysis:

Part a:

Comparison of Stimulation index (S.I) (Basal values) between HIV positive Group I (A, B, C), ESN's Group II and normal controls Group III was done using unpaired t test. 'p' values less than 0.05 were considered to be significant.

i. Stimulant used: PHA

As illustrated in table (11) maximum proliferative response against mitogen PHA was observed in Controls (S.I= 76.90 ± 4.15). The proliferative response shown by subgroups of Group I or HIV seropositive women was in the order Group IA > IB > IC (S.I= $32.98 \pm 3.35 > 19.73 \pm 4.03 > 6.13 \pm 1.94$ respectively). The response towards PHA in HIV seropositive women (Group IA, IB, and IC) was significantly less than ESN's (group II) and Controls (III).

ii. Stimulant used: gp120 peptide

As illustrated in table (11) maximum proliferative response against gp120 peptide was observed in ESN's or group II (S.I= 4.80 ± 0.19). The proliferative response shown by sub-groups of Group I or HIV seropositive women was in the order Group IA > IB > IC (S.I= $3.21 \pm 0.46 > 2.32 \pm 0.12 > 1.15 \pm 0.11$ respectively). Exposed but seronegative women (Group II) showed significantly greater response

to the peptide as compared to HIV seropositive group (Group I A, IB IC) and healthy controls.

iii. Stimulant used: Cell wall extract of *Candida albicans* (CWE)

As illustrated in table (11) maximum proliferative response against recall antigen (cell wall of *Candida albicans*) was observed in HIV seropositive Group I A (S.I= 5.23 ± 0.93) followed by IB (S.I= 2.90 ± 0.28) and IC (S.I= 1.46 ± 0.21). A significantly lesser response was observed in ESN's (group II) and healthy controls group.

Part b:

Repeated measures ANOVA was applied between Basal, 6 months and 1-year readings of stimulation index with post test (Tukey-Kramer multiple comparison Test) to find out the change in response by each group. 'p' value less than 0.05 was considered to be significant.

Table No. 12: Antigen used -PHA

Groups	Mean Stimulation Index- PHA		
	Basal	6 months	1 year
IA	32.98 $\pm$ 3.35	28.38 $\pm$ 4.40	20.64 $\pm$ 2.78
IB	19.73 $\pm$ 4.03	19.01 $\pm$ 1.61	10.67 $\pm$ 1.05
IC	6.13 $\pm$ 1.94	4.80 $\pm$ 0.87	2.13 $\pm$ 0.2
II	48.34 $\pm$ 2.34	50.73 $\pm$ 2.17	50.26 $\pm$ 2.32
III	76.90 $\pm$ 4.15	73.29 $\pm$ 4.40	73.02 $\pm$ 4.47

Proliferative response of each group was studied after 6 months and 1 year to find out the change in the response. As shown in table (12 and graph I) in-group I A there was significant decrease in proliferative response towards PHA in 1 year of interval when compared to basal values ($p=0.0075$). Similarly there was significant decrease in response seen after 6 months in Group I B ($p=0.0003$). There was no significant change in the response in Groups I C, II and III (Healthy Controls).

Graph I : Proliferation assay –Ag used: PHA

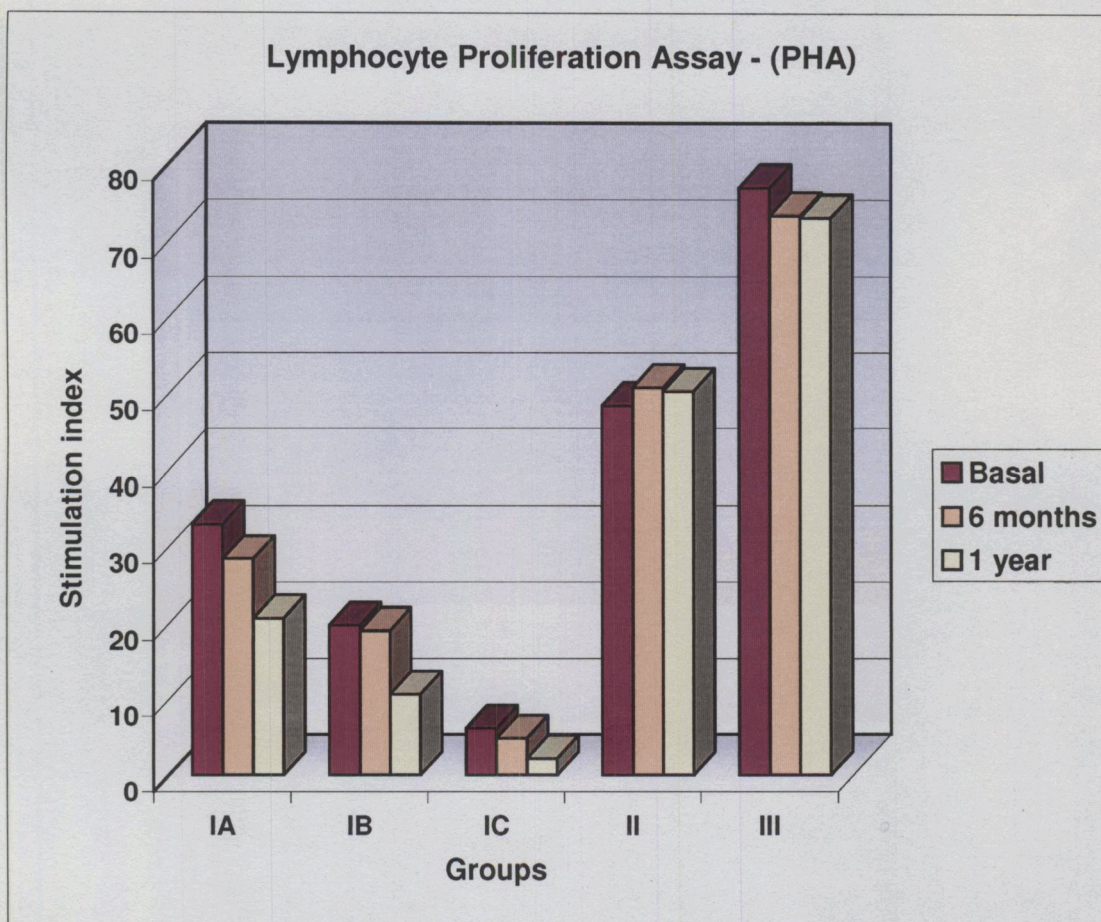


Table no.13: Antigen used-gp120 peptide

Groups	Mean Stimulation Index- gp120		
	Basal	6 months	1 year
IA	3.21 $\pm$ 0.46	3.85 $\pm$ 0.94	2.58 $\pm$ 0.61
IB	2.32 $\pm$ 0.12	2.19 $\pm$ 0.19	1.43 $\pm$ 0.14
IC	1.15 $\pm$ 0.11	0.91 $\pm$ 0.41	0.36 $\pm$ 0.19
II	4.80 $\pm$ 0.19	5.09 $\pm$ 0.17	5.22 $\pm$ 0.12
III	2.41 $\pm$ 0.16	2.13 $\pm$ 0.10	2.23 $\pm$ 0.06

As shown in table (13 and graph II) in Group I B there was significant decrease in proliferative response towards gp120 peptide when compared to basal values between 6 months and 1 year of interval ($p < 0.0001$). Similarly significant decrease in response in Group I C was seen in 1 year ($p = 0.0295$). There was no significant change in the proliferative response to gp120 peptide in groups I A, II and III (Healthy Controls).

Graph II : Proliferation assay –Ag used: gp120

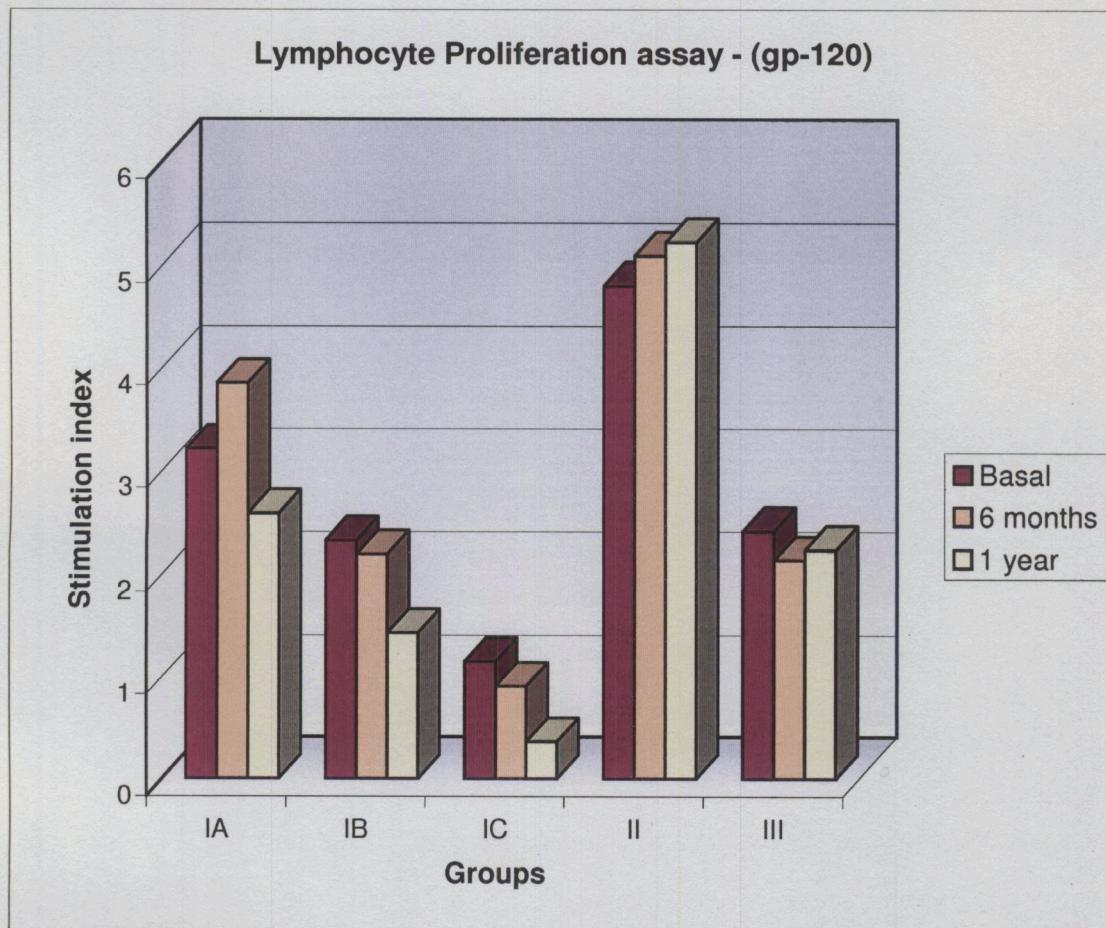
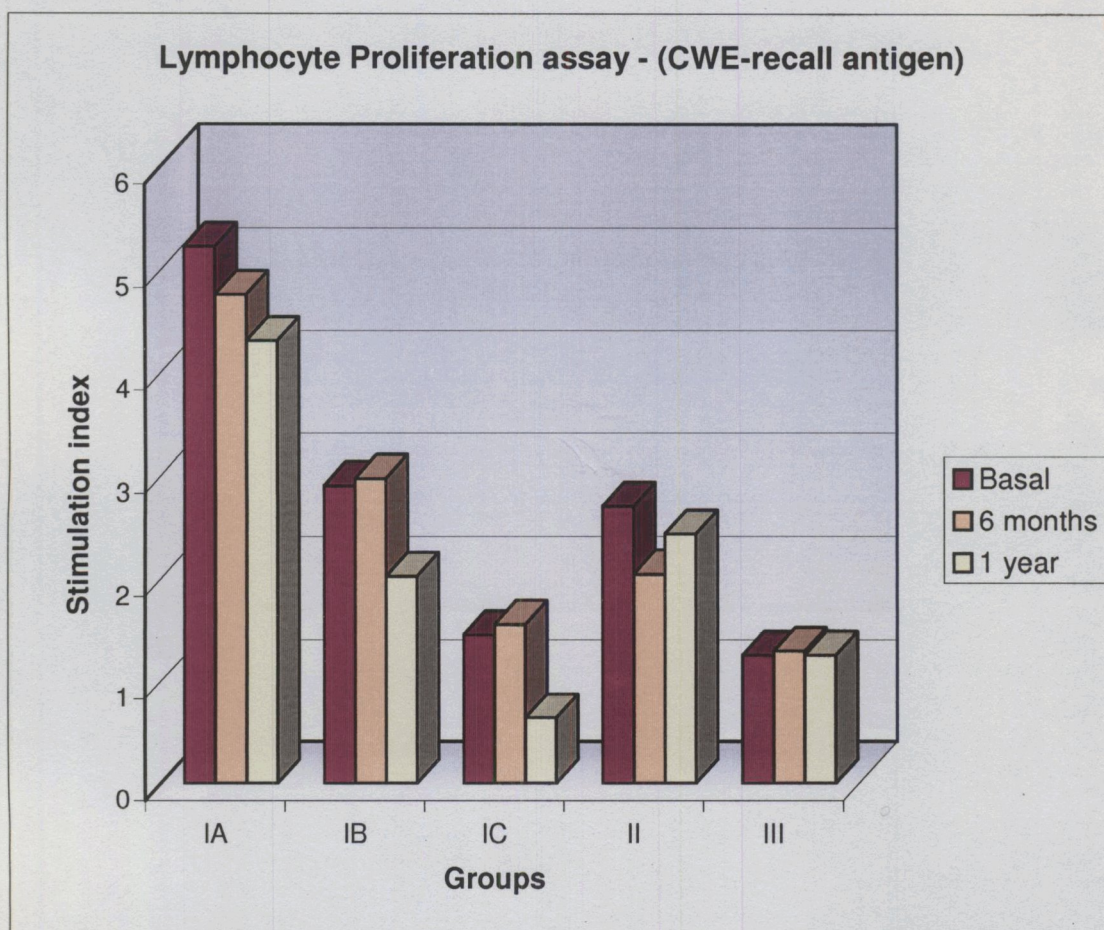


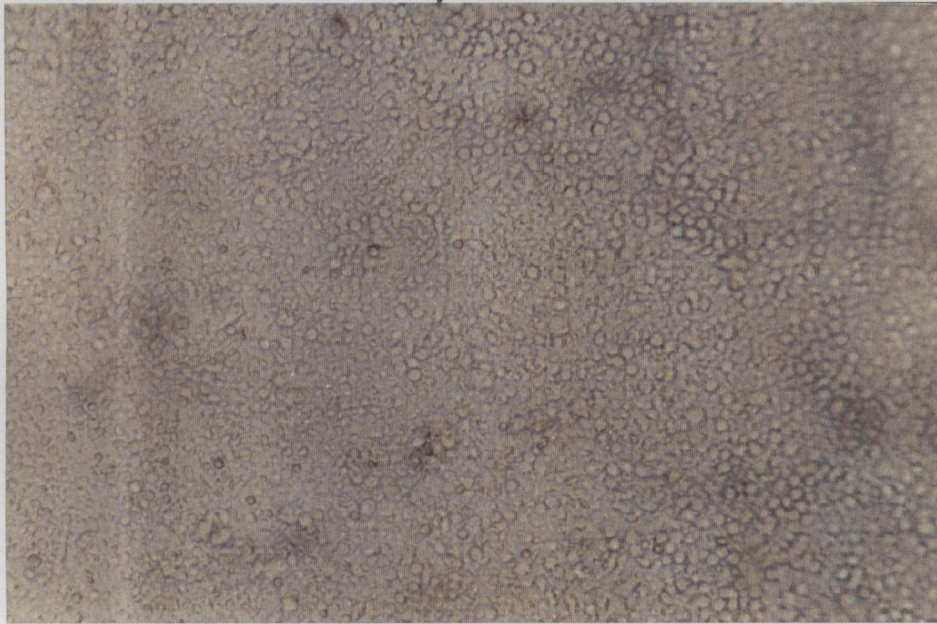
Table No 14: Antigen used- *Candida albicans* antigen

Groups	Mean Stimulation Index- CWE		
	Basal	6 months	1 year
IA	5.23 $\pm$ 0.93	4.76 $\pm$ 0.53	4.31 $\pm$ 0.63
IB	2.90 $\pm$ 0.28	2.97 $\pm$ 0.21	2.02 $\pm$ 0.26
IC	1.46 $\pm$ 0.21	1.56 $\pm$ 0.07	0.64 $\pm$ 0.11
II	2.70 $\pm$ 0.11	2.04 $\pm$ 0.07	2.43 $\pm$ 0.09
III	1.26 $\pm$ 0.06	1.30 $\pm$ 0.07	1.26 $\pm$ 0.07

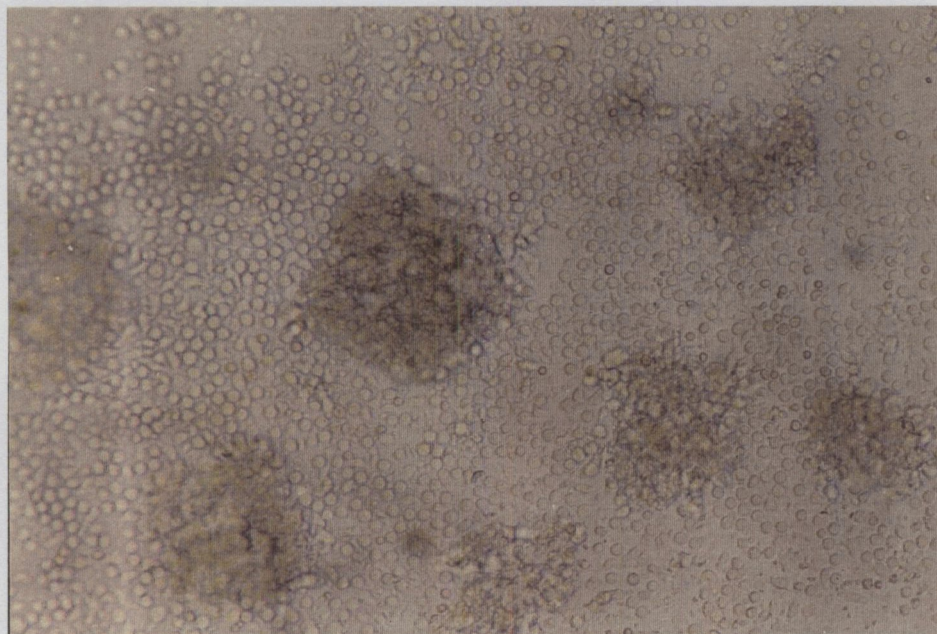
As illustrated in table (14 and graph III) in group I B and group I C after 6 months of interval there was significant decrease in proliferative response towards recall antigen (cell wall of *Candida albicans*) when compared to basal values (p=0.0059 and p=0.0189 respectively). There was no significant change in the response in groups I A, II and III (Controls).

Graph III : Proliferation assay –Ag used: CWE





Photograph 1: Light micrograph of human PBMC's cultured without stimulation (control) in complete RPMI 1640 medium in tissue culture plate, for 48 hours.



Photograph 2: Light micrograph of human PBMC's cultured with PHA (5µg/ml) stimulation in complete RPMI 1640 medium in tissue culture plate, for 48 hours.

- **Interferon γ ELISpot assay results**

Table no.15: Elispot Assay (Basal values)

Groups	SFC / 10^6 cells/ml			
	Cell Control	PHA	gp 120	CWE
IA	43 $\pm$ 5	154 $\pm$ 13	109 $\pm$ 8	90 $\pm$ 6
IB	42 $\pm$ 4	88 $\pm$ 7	54 $\pm$ 5	74 $\pm$ 8
IC	30 $\pm$ 11	29 $\pm$ 7	13 $\pm$ 7	31 $\pm$ 12
II	82 $\pm$ 10	256 $\pm$ 19	197 $\pm$ 11	155 $\pm$ 10
III	105 $\pm$ 11	228 $\pm$ 14	179 $\pm$ 10	167 $\pm$ 18

SFC/ 10^6 cells : Spots forming cells per 10^6 PBMC's.

Statistical analysis:

Part a:

Comparison of Spots forming Cells (SFC) (Basal values) between group (A, B, C), II and III using unpaired t test. 'p' values less than 0.05 were considered to be significant.

i. Cell Control (PBMC's without antigen)

As illustrated in the table (15) interferon gamma secreting cells were found to be significantly more in the Healthy individuals (Group III : SFC 105 $\pm$ 11 per 10^6 cells) and ESN's (Group II : SFC - 82 $\pm$ 10 per 10^6 cells as compared to HIV seropositive group I (IA, IB, IC) (SFC: 43 $\pm$ 5, 42 $\pm$ 4, 30 $\pm$ 11 respectively) . There was no significant difference in interferon gamma secreting cells between group II and III.

ii. Stimulant used- PHA

As illustrated in the table (15) when stimulated with PHA the number of cells secreting interferon gamma were found to be significantly more in the ESN's (Group II – SFC: 256 ± 19 per 10^6 cells) and Healthy Controls (Group III – SFC: 228 ± 14 per 10^6 cells) as compared to HIV seropositive group I (IA, IB, IC). There was no significant difference in interferon gamma secreting cells between group II and III.

iii. Stimulant used – gp120 peptide

As illustrated in the table (15) the cells secreting interferon gamma were found to be significantly more in the ESN' (Group II – SFC: 197 ± 11 per 10^6 cells) as compared to HIV seropositive group I (IA, IB, IC). The response in HIV seropositive individuals towards the gp120 antigen was in the order $IA > IB > IC$ (SFC: $109 \pm 8 > 54 \pm 5 > 13 \pm 7$ per 10^6 cells). There was no significant difference in interferon gamma secreting cells between group II and III.

iv. Stimulant used – Cell wall extract of *Candida albicans*.

As illustrated in the table (15) the interferon gamma secreting cells were found to be significantly more in the Healthy Controls (Group III: SFC- 155 ± 10 per 10^6 cells) as compared to HIV seropositive group I (IA, IB, IC). The response in HIV seropositive individuals toward the

antigen was in the order IA > IB > IC. There was no significant difference in interferon gamma secreting cells between group II and III.

Part b:

Repeated measures ANOVA was applied between Basal, 6 months and 1 year readings of Spots forming Cells (SFC) with post test (Tukey-Kramer multiple comparison Test) to find out the change in response by each group. 'p' value less than 0.05 was considered to be significant.

Table No 16: Cell Control

Groups	SFC / 10 ⁶ PBMC's –Cell control		
	Basal	6 months	1 year
IA	43 ± 5	46 ± 4	23 ± 2
IB	42 ± 4	38 ± 3	22 ± 2
IC	30 ± 11	22 ± 3	14 ± 2
II	82 ± 10	74 ± 8	76 ± 6
III	105 ± 11	101 ± 13	97 ± 10

As illustrated in table (16 and graph IV) in Group I A there was significant decrease in IFN- γ secreting cell response without antigen when compared to basal values between 6 months and 1 year of interval ($p=0.0007$). Similarly significant decrease in response was also observed in Group I B ($p<0.0001$). There was no significant change in the response in groups C, II and III (Healthy Control).

Graph IV : Elispot assay-cells without antigen

