

INTERPLAY OF SUPEROXIDE DISMUTASE, FREE RADICALS AND APOPTOSIS IN HIV/AIDS PATIENTS

THESIS SUBMITTED
UNIVERSITY OF MUMBAI

FOR THE DEGREE OF
MASTER OF SCIENCE
IN
MICROBIOLOGY

SUBMITTED BY
SANDEEPAN MUKHERJEE

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

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

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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 "**Interplay of Superoxide Dismutase, Free Radicals and Apoptosis in HIV/AIDS Patients**", forms my own contribution to the research work carried out under the guidance of **Dr. (Mrs.) R. A. Deshmukh** at the 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.



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HIV/AIDS

Nature's challenge, to the "*mighty*" mankind

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INTRODUCTION

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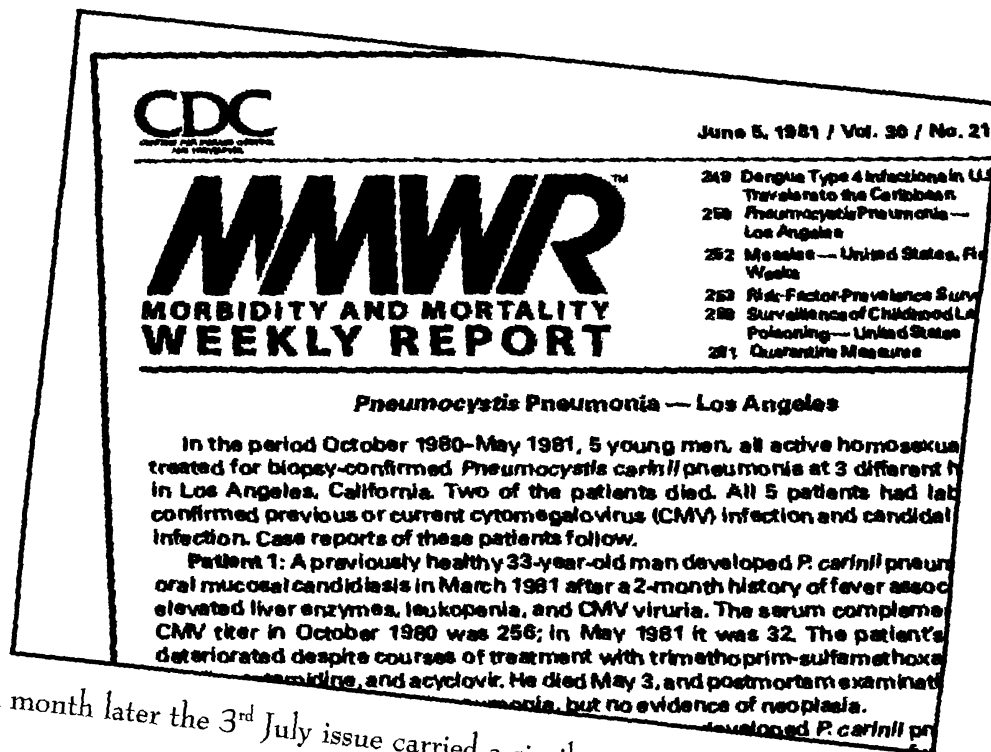
REVIEW OF LITERATURE

The killer was inexplicable and the trail looked perplexing. The first victims had infamous lifestyles and the detectives were clueless. The setting was perfect for any best selling science fiction. But this was for real... and even before most could take notice, the assassin had turned into a worldwide catastrophe. Permanently etched into the global vocabulary, HIV/AIDS continues its "Dance of Devastation," which had started in that fateful *Summer of '81*...

The Summer of '81^{1, 2}

Between October 1980 and May 1981, in the United States, 5 young men from 29 to 36 years of age were admitted to various hospitals in Los Angeles. Two of them died and the remaining were seriously ill. They were suffering from an unusual type of pneumonia, caused by a fungus *Pneumocystis carinii*. In the past *Pneumocystis carinii* pneumonia (commonly abbreviated as PCP) had been found almost exclusively in patients with severe suppression of their immune system, for example infants born with primary immune deficiency such as Severe Combined Immune Deficiency (SCID) or adults with secondary immune deficiency states caused by drugs or disease. In addition all of these patients had evidence of having been infected with a virus called cytomegalovirus (CMV) which is also common in immuno-suppressed patients. The physicians in charge of the first few cases in Los Angeles were bewildered. The five patients were all young men who had indication of a widespread immune deficiency without any obvious reason. They had evidence of other infections and coincidentally they were all homosexuals, i.e. gay men. However none of them knew each other and there did not appear to be any common sexual contact. On June 5th 1981 the Morbidity and Mortality Weekly Report (MMWR) of the Centers for Disease Control

(CDC) published the first report of these observations in a rather small and unobtrusive insert.



A month later the 3rd July issue carried a similar report of 26 homosexual men, 20 from New York and 6 from California, with a very uncommon Tumour called Kaposi's Sarcoma (KS). KS was previously associated only with patients who were immuno-compromised as a result of immunosuppressive therapy following transplantation. As with the original PCP patients, the KS patients also had evidence of infection such as CMV, thrush (Candidiasis) and PCP.

The CDC introduced a nationwide surveillance program in July, 1981. By the next year i.e. September 1982 the new disease was termed, the

Acquired Immune Deficiency Syndrome (AIDS) and it was characterized as the occurrence of unusual (opportunistic) infections and cancers in previously healthy individuals due to an acquired immunodeficiency of unknown cause. The CDC developed and published the first surveillance definition for this new disease which was used throughout the Western world and Australasia.

Probably no one really suspected the scale of the outbreak that was in the making. However evidence of the towering tempest was soon arriving.

Pandemonium of the Pandemic

Because there was so little known about the transmission of what seemed to be a new disease, there was concern about the contagion, and apprehensions whether the disease could be by passed on by people who had no apparent signs or symptoms³. Knowledge about the disease was changing so

"Dr. Curran said there was no apparent danger to non homosexuals from contagion. 'The best evidence against contagion', he said, 'is that no cases have been reported to date outside the homosexual community or in women'"

-The New York Times⁴

quickly that certain assumptions made at this time were shown to be unfounded just a few months later.

By December 1981, it was clear that the disease affected other population groups, when the first cases of PCP were reported in injecting drug users. At the same time the first case of AIDS was documented in the UK.

By the beginning of July 1982 a total of 452 cases, from 23 states of the United States, had been reported to the CDC. In December that year, a

"When it began turning up in children and transfusion recipients, that was a turning point in terms of public perception. Up until then it was entirely a gay epidemic, and it was easy for the average person to say 'So what?' Now everyone could relate."

- Harold Jaffe of the CDC for Newsweek

20-month old child who had received multiple transfusions of blood and blood products died from infections related to AIDS. This case provided clearer evidence that AIDS was caused by an infectious agent, and it also caused additional concerns about the safety of the

blood supply⁵. Also in December, the CDC reported the first cases of possible mother to child transmission of AIDS⁶. By the end of 1982 many more people were taking notice of this new disease, as it was clearer that a much wider group of people was going to be affected.

It was also becoming clear that AIDS was not a disease that just occurred in the USA. Throughout 1982 there were separate reports of the disease occurring in a number of European countries. Meanwhile in Uganda, doctors were seeing the first cases of a new, fatal wasting disease. This illness soon became known locally as 'slim'. The virus continued infecting more and more people but the investigations remained inconclusive.

In May 1983, Dr. Francoise Barré-Sinoussi together with Professor Luc Montagnier the head of the Viral Cancer Unit of the Pasteur Institute in France published a paper in the journal '*Science*' describing the isolation of a retrovirus from a patient with lymphadenopathy syndrome, one of the

disease manifestations which precedes AIDS, and called it the Lymphadenopathy Associated Virus (LAV)⁷. The following year Dr. Robert Gallo's laboratory at the National Institute of Health became the first laboratory to propagate the

"The name of the virus had itself become a political football as the French insisted on LAV (Lymphadenopathy Associated Virus), while Gallo's group used HTLV-III (Human T-cell Lymphotropic Virus, type 3)."
- *Time Magazine*⁹

virus in cell culture and called it Human T-cell Lymphotropic Virus-III (HTLV-III)⁸. After much debate and discussion, it was generally accepted that credit should be shared between the two- Montagnier for the original

isolation and Gallo for the ability to propagate the virus and to develop the critically needed diagnostic tests. In May 1986, the International Committee on the Taxonomy of Viruses ruled that both names should be dropped and the dispute solved by a new name, HIV (Human Immunodeficiency Virus)¹⁰. It was established that the virus targets and kills the T-Helper Lymphocytes (CD4+ Cells); thereby having detrimental effects on the host immune system in specific and as a corollary the entire well-being of the host.

By this time around, over 20,000 cases of AIDS had been recorded worldwide¹¹; however there were no cases of HIV or AIDS reported from India until then. Later in that year, India's first cases of HIV were diagnosed among sex workers in Chennai, Tamil Nadu¹². HIV/AIDS was now a pandemic. Since then there has only been a northward ascent in the number of HIV positive individuals globally.

Presently, as per the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), the estimated number of persons living with HIV worldwide in 2007 was 33.2 million [30.6 – 36.1 million], a reduction of 16% compared with the estimate published in 2006 (39.5 million [34.7 - 47.1 million])¹³. It is however emphasized that these differences between estimates published in 2007

and those published in 2006 result largely from refinements in methodology, rather than trends in the pandemic itself. In 2007 itself 2.5 million people were newly infected with HIV.

This prevalence however, is not uniform throughout the globe. While Oceania has 75,000 people living with HIV, with 0.4 % of its adult population (defined as those aged between 15 – 49 years of age) falling in the category, Sub-Saharan Africa has 22.5 million people testing positive for the Virus, which includes an alarming 5% of its adult population. The problem seems to be more pronounced in the developing world than the developed quarter.

India, world's second most populous country is experiencing a highly varied HIV epidemic. Approximately 2.5 million people in India were living with HIV in 2006, with national adult HIV prevalence of 0.36%. Although the proportion of people living with HIV is lower than previously estimated, India's epidemic continues to affect large numbers of people. The spread of HIV within India is as diverse as the societal patterns between its different regions, states and metropolitan areas.

The reasons for this skewed presentation however apart from being medical are also social and political.

HIV/AIDS: Medical Malady, Social Stupor or Political Perverseness

Although in terms of death and illness, it is true that AIDS falls well short of the great plague, which killed a quarter of the Europe's population in the middle ages, or even the great Spanish influenza pandemic which caused 20 million human deaths at the close of the First World War, HIV/AIDS is one of the most feared diseases we know today². The epidemic has in many ways been a humbling experience to medical science. It's advent at the closing of the twentieth century, a century which has seen technology progressively and the purposefully overcome so many human diseases, has indeed given a cause for reflection on the true limitations of what may previously have been thought to be the almost limitless achievements and accomplishments of humans. AIDS has four cardinal features which together make it a distinctively formidable disease. Firstly, it is infectious and transmissible from person to person. Secondly, once infection occurs it follows an inexorable course to disease and eventually to death in most if not all cases. Thirdly, all persons infected with HIV remain infectious; that is, they're able to transfer the virus to others to a greater or lesser extent for the rest of their lives and fourthly, the reservoir of infection; that is the total sum of

people infected, who can also act as a source of infectious virus to others, is constantly and progressively escalating as the pandemic spreads. AIDS demonstrates a capricious progression pattern. Disease progression

From the point of view of the pharmaceutical industry, the AIDS problem has already been solved. After all, we already have a drug which can be sold at the incredible price of \$8,000 an annual dose, and which has the added virtue of not diminishing the market by actually curing anyone.

- Barbara Ehrenreich

depends upon Virus type and strain, and, host factors such as age and host genetics. The disease lacks cost-effective, specific and sensitive prognostic markers. Vaccines have remained elusive and the available drugs apart from being expensive are not curatives. They only prolong the life span of the patient. Aggravating

the medical terror generated by this disease even further, are its Social, Psychological and Political dimensions.

In the Western Hemisphere, the disease AIDS has largely affected groups who have been victimized, ostracized and marginalized by the rest of the society, namely the homosexual men and the Intra-Venous Drug Users (IVDU's). In fact, for quite some time HIV/AIDS was deemed as a gay-mans disease. Even today, there persist a number of myths concerning HIV/AIDS, causing significant challenge in the prevention and control

programs of the disease. Egos and ignorance are further fuelling the inferno. The social stigmas like isolation, being the centre of insults and gossip, exclusion from social network, loss of livelihood and poor access to care within the formal health sector are so excruciating, that there is aversion to disclose ones HIV positive status¹⁴. The misery

"If men are still young and they indulge in play and get infected [with HIV], that's the general story of society. If a girl gets this disease, no one would like to get close to her, because it is a problem of her conduct and her morality. It is not tolerated in females compared to males."

- Female community counselor in Vietnam¹⁴

seems to be more prominent with women in most parts of the globe, and they experience more severe social stigmas. There is a vicious cycle at work- Ignorance leading to stigmas, fear of stigma leading to failure in HIV prevention, treatment and control, which in turn keeps the masses ignorant. The situation in India is no different. Traditionally, most Indian health officials and the health care establishment viewed HIV as a "foreign" disease or an "imported" infection, confined to people returning from foreign countries or port cities and to marginalized groups such as sex workers and drug users and unlikely to spread to the general population. Politicians initially regarded the spread of HIV/AIDS as a law

and order problem, that is, they believed that the spread of the disease could be managed by controlling certain high-risk groups, thus preventing the virus from spreading to the general population. Among other measures considered in the early 1990s were incarcerating infected sex workers, deporting HIV-positive foreign nationals, and mandatory testing of foreign students coming from AIDS-infected countries. Each measure carried the same hidden message: AIDS affects “them,” not “us.” As a result, infected people are ostracized while vulnerable groups—women and members of the marginal populations—are so afraid of the consequences of raising the AIDS issue that they will not take measures to protect themselves, lest they be accused of immorality or of spreading the virus themselves¹⁵. Combating set social taboos shall require political leaders to speak out against these beliefs in public forums. A strong political will is required, to combat the growing challenge posed by HIV/AIDS. HIV/AIDS has never been an issue at elections held in the recent past, and the health care is still considered a low priority in the political hierarchy. The health minister for example is not a high ranking official in the cabinet and social taboos delay the necessary policy decisions.

The menace that HIV/AIDS has caused for the past two and a half decades actually needs no introduction. However there are important

positive lessons to be learned; firstly the decent progress made by man in

Both the Moral Majority, who are recycling medieval language to explain AIDS, and those ultra-leftists who attribute AIDS to some sort of conspiracy, have a clearly political analysis of the epidemic. But even if one attributes its cause to a microorganism rather than the wrath of God, or the workings of the CIA, it is clear that the way in which AIDS has been perceived, conceptualized, imagined, researched and financed makes this the most political of diseases.

- Dennis Altman

the field of science and technology should not make him complacent, the tremendous power of mother nature must be acknowledged, secondly it's high time the society gets rid of its duplicitous system and establishes a fair and honest social order, and thirdly the

political system should get more democratic and compassionate towards the common man.

Thus, an encouraging socio-political environment along with a better understanding of the host-pathogen interactions should work in unison in controlling the spread of the disease.

Biology of the Bug: Decoding the Debacle

HIV/AIDS is one of the worst medical adversities of all time. Like a silent assassin that Human Immunodeficiency Virus remains hidden in the host system and strikes when the victim is most vulnerable. Understanding the virus, and the pathophysiological changes it brings about in the host, shall provide us with the keys to unlock the mysteries of the bug.

The Pathogen: Knowing thy Nemesis

Human Immunodeficiency Virus (HIV) is the aetiological agent of Acquired Immunodeficiency Disease Syndrome (AIDS). Its primary target is the activated CD4+ T helper lymphocyte (T_H Lymphocyte) but it can also infect several other cell types including macrophages. HIV is a lentivirus, a class of retrovirus. The name lentivirus means slow virus, so named because these viruses take a long time to cause overt disease. Most lentiviruses target cells of the immune system and thus disease is often characterized as immunodeficiency¹⁶.

There are two major types of HIV, HIV-1 and HIV-2, and each type has recognized to subtypes. Serologic surveys in Dakar, Senegal (West Africa), in 1985 identified antibody patterns in female sex workers that indicated infection in a virus closely related to HIV. In 1986, a retrovirus

similar to but this thing from HIV was isolated from patients ill with an AIDS like illness in Guinea-Bissau and the Cape Verde Islands in west Africa. This was originally referred to as LAV-2, but is now known as HIV-2. Although similar to HIV-1, HIV-2 has a different sequence of nucleotides in its genome. In addition, HIV-1 has a one gene (vpu) that is not found in HIV-2, and the latter has a gene (vpx) that is not found in HIV-1 but is found in SIV. Studies focused on the structural similarity (homology) of the viral genome of each virus, i.e. HIV-1 and HIV-2; suggest that they both evolved from SIV, but from different Simian species. HIV-1 diverged from the chimpanzee SIV and HIV-2 diverged from sooty mangabey SIV. Both HIV-1 and HIV-2 have the same method of transmission, and both target the same host cell for infection. HIV-2 is significantly less transmissible than HIV-1 and once infected with HIV-2, progression to end stage disease, i.e. AIDS, is much slower than HIV-1 infection.

The fast rate of viral replication, along with its inherent tendency to produce mutations and recombinant forms (the enzyme is error prone), results in the generation of a plethora of slightly different, i.e. genetically distinct variants (strains) of HIV-1.

HIV-1 is divided into 3 groups: Group M (for Major), Group O (for Outlier) and Group N (for Non-M, Non-O strains).

- Group M viruses are separated into 10 different subtypes (*Clades*) distinguished by capital alphabetical letters A to J.
- Group O viruses consist of a pool of highly divergent, genetically related strains with no defined subtypes.
- Those rare (but increasingly being identified) variants of HIV that cannot be assigned to either group are designated as group N viruses.¹

Individual subtypes may be associated with the different transmission potentials and differences in virulence between HIV-1 variants. In some regions of the world, a specific subtype can be identified as the dominant clade infecting most people. For example, in North America, Western Europe and East Asia, HIV-1 group M clade B is the dominant subtype; while in Thailand it is HIV-1 group M clade E and clade C in India.

Subtypes have also been recognized and described for HIV-2 (designated A to E.)

Anatomy of the Adversary:

Electron micrograph of HIV-1 virion illustrates that it measures approximately 100-150 nm in diameter.

*HIV Envelope and Core*¹⁷⁻²¹: Mature viral particles are spherical and are characterized by an electron dense cylindrical core surrounded by a lipid bilayer envelope, enriched in sphingomyelin and cholesterol. The envelope is studded with gp120 surface glycoprotein and gp41 transmembrane glycoprotein.

The major core protein structure of the virion includes the Capsid (p24), Matrix (p17) and Nucleocapsid (p7) protein. The virion nucleocapsid encloses a diploid RNA genome bound to it. Retroviral enzymes such as Protease (p11), Reverse Transcriptase / Ribonuclease H (p51/p66) and Integrase (p32) are also a part of the nucleocapsid core of the virion.

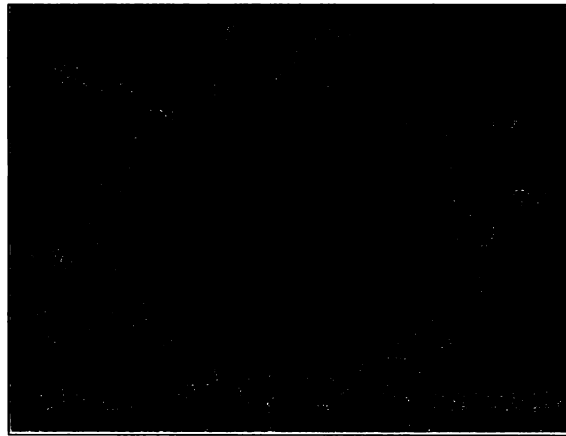


Figure 1: The Virion of Human Immunodeficiency Virus

*HIV Genome*²²⁻²³: The HIV-1 genome is approximately 10 kb in length. Flanked by two long - terminal repeats (LTRs), the 5' to 3' sequence consists of the *gag* gene which encodes the virion structural components (Capsid, Matrix and Nucleocapsid protein), the *pol* gene which encodes

several retroviral enzymes (Reverse Transcriptase, Integrase and RNAase H) and the *env* gene, which encodes for envelope glycoproteins (gp120 and gp41). The genome also contains at least six additional genes encoding for regulatory proteins: *tat*, *rev*, *nef*, *vpr*, *vpu* (*vpx* in HIV-2) and *vif*.

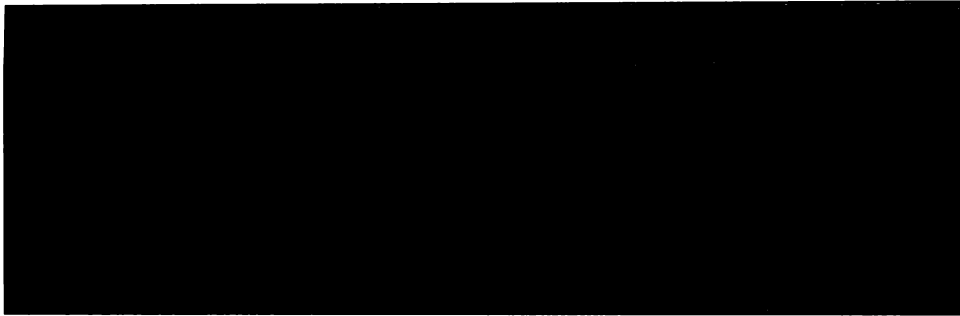


Figure 2: The Genome of HIV-1

Other important features of the HIV genome includes the U5 region, which is the first reverse transcribed, 75-250 nucleotides, non-coding region at the 5' end of viral RNA. The Primer Binding Site (PBS) follows the U5 region. It is an 18 nucleotide sequence complementary to the 3' end of the specific tRNA primer which is used by the Provirus genome. While at the 3' end of the viral genome, lies the U3 region. It is a unique non-coding region of 200-1200 nucleotides containing promoter elements responsible for transcription of the provirus to mRNA for protein synthesis.

Table 1: Genes & Proteins of HIV²⁴

Gene	Protein Product	Function of Protein
Gag	53 kDa precursor ↓ p17 p24 p9 p7	Nucleocapsid Proteins Outer Core Protein Layer Inner Core Protein Layer Component of nucleoid core Binds directly to Genomic RNA
Pol	Precursor ↓ p66 p51 p10 p32	Enzymes RT and RNase activity RT activity Protease: Cleaves gag precursor Integrase
<i>Regulatory Proteins</i>		
Rev	p19	Allows export of unspliced and singly spliced mRNAs from nucleus
<i>Auxiliary Proteins</i>		
Vif	p23	Promotes maturity and infectivity of viral particle

RT: Reverse Transcriptase; ER: Endoplasmic Reticulum

Perilous Pathways: The Virus Life Cycle

*Binding and Entry*²⁵⁻³¹

The genetic material of HIV, an RNA molecule 9 kilobases in length, contains 9 different genes encoding 15 proteins. Considerable insights have been gained into the function of these different gene products to productively infect a target cell, HIV must introduce its genetic material into the cytoplasm of this cell. The process of viral entry involves fusion of the viral envelope with the host cell membrane and requires the specific interaction of the envelope with specific cell surface receptors. The two viral envelope proteins, gp120 and gp41, are conformationally associated to form a trimeric functional unit consisting of three molecules of gp120 exposed on the virion surface and associated with three molecules of gp41 inserted into the viral lipid membrane. Trimeric gp120 on the surface of the virion binds CD4 on the surface of the target cell, inducing a conformational change in the envelope proteins that in turn allows binding of the virion to a specific subset of chemokine receptors on the cell surface. These receptors normally play a role in chemoattraction, in which hematopoietic cells move along chemokine gradients to specific sites. Although these receptors, which contain seven membrane-spanning

domains, normally transduce signals through G proteins, signaling is not required for HIV infection.

Both CD4 and chemokine coreceptors for HIV are found disproportionately in lipid rafts in the cell membrane. These cholesterol- and sphingolipid-enriched microdomains likely provide a better environment for membrane fusion, perhaps by mirroring the optimal lipid bilayer of the virus. Removing cholesterol from virions, producer cells, or target cells greatly decreases the infectivity of HIV.

The binding of surface gp120, CD4, and the chemokine coreceptors produces an additional radical conformational change in gp41. Assembled as a trimer on the virion membrane, this coiled-coil protein springs open, projecting three peptide fusion domains that "harpoon" the lipid bilayer of the target cell. The fusion domains then form hairpin-like structures that draw the virion and cell membranes together to promote fusion, leading to the release of the viral core into the cell interior.

HIV virions can also enter cells by endocytosis. Usually, productive infection does not result, presumably reflecting inactivation of these virions within endosomes. However, a special form of endocytosis has been demonstrated in submucosal dendritic cells. These cells, which normally process and present antigens to immune cells, express a specialized

attachment structure termed DC-SIGN. This C-type lectin binds HIV gp120 with high affinity but does not trigger the conformational changes required for fusion. Instead, virions bound to DC-SIGN are internalized into an acidic compartment and subsequently displayed on the cell surface after the dendritic cell has matured and migrated to regional lymph nodes, where it engages T cells. Thus, dendritic cells expressing DC-SIGN appear to act as "Trojan horses" facilitating the spread of HIV from mucosal surfaces to T cells in lymphatic organs.

*Cytoplasmic Events*³²⁻⁴⁰

Once inside the cell, the virion undergoes uncoating, likely while still associated with the plasma membrane. This poorly understood process may involve phosphorylation of viral matrix proteins by a mitogen-activated protein (MAP) kinase and additional actions of cyclophilin A and the viral proteins Nef and Vif. Nef associates with a universal proton pump, V-ATPase, which could promote uncoating by inducing local changes in pH. After the virion is uncoated, the viral reverse transcription complex is released from the plasma membrane. This complex includes the diploid viral RNA genome, lysine transfer RNA (tRNA^{Lys}) which acts as a primer for reverse transcription, viral reverse transcriptase,

integrase, matrix and nucleocapsid proteins, viral protein R (Vpr), and various host proteins. The reverse transcription complex docks with actin microfilaments. This interaction, mediated by the phosphorylated matrix, is required for efficient viral DNA synthesis. By overcoming destabilizing effects of a recently identified protein termed CEM15/APOBEC3G, Vif stabilizes the reverse transcription complex in most human cells.

Reverse transcription yields the HIV preintegration complex (PIC), composed of double-stranded viral cDNA, integrase, matrix, Vpr, reverse transcriptase, and the high mobility group DNA-binding cellular protein HMGI(Y). The PIC may move toward the nucleus by using microtubules as a conduit.

Crossing the Nuclear Pore⁴¹⁻⁵¹

Unlike most animal retroviruses, HIV can infect non-dividing cells, such as terminally differentiated macrophages. This requires an ability to cross the intact nuclear membrane. With a Stokes radius of approximately 28 nm or roughly the size of a ribosome, the PIC is roughly twice as large as the maximal diameter of the central aqueous channel in the nuclear pore. The 3 μm contour length of viral DNA must undergo significant

compaction, and the import process must involve considerable molecular gymnastics.

One of the most contentious areas of HIV research involves the identification of key viral proteins that mediate the nuclear import of the PIC. Integrase, matrix, and Vpr have been implicated. Because plus-strand synthesis is discontinuous in reverse transcription, a triple helical DNA domain or "DNA flap" results that may bind a host protein containing a nuclear targeting signal. Matrix contains a canonical nuclear localization signal that is recognized by the importins alpha and beta, which are components of the classical nuclear import pathway. However, a recent publication calls into question the contributions both of the nuclear import signal in integrase and of the DNA flap to the nuclear uptake of the PIC. The HIV Vpr gene product contains at least three noncanonical nuclear targeting signals. Vpr may bypass the importin system altogether, perhaps mediating the direct docking of the PIC with one or more components of the nuclear pore complex. The multiple nuclear targeting signals within the PIC may function in a cooperative manner or play larger roles individually in different target cells. For example, while Vpr is not needed for infection of nondividing, resting T cells, it enhances viral infection in nondividing macrophages. The finding

that both matrix and Vpr shuttle between the nucleus and cytoplasm explains their availability for incorporation into new virions.

*Integration*⁵²⁻⁵⁴

Once inside the nucleus, the viral PIC can establish a functional provirus. Integration of double-stranded viral DNA into the host chromosome is mediated by integrase, which binds the ends of the viral DNA. The host proteins HMGI(Y) and barrier to autointegration (BAF) are required for efficient integration, although their precise functions remain unknown. Integrase removes terminal nucleotides from the viral DNA, producing a two-base recess and thereby correcting the ragged ends generated by the terminal transferase activity of reverse transcriptase. Integrase also catalyzes the subsequent joining reaction that establishes the HIV provirus within the chromosome.

Not all PICs that enter the nucleus result in a functional provirus. The ends of the viral DNA may be joined to form a 2-LTR circle containing long terminal repeat sequences from both ends of the viral genome, or the viral genome may undergo homologous recombination yielding a single-LTR circle. Finally, the viral DNA may auto-integrate into itself, producing a rearranged circular structure. Although some circular forms

may direct the synthesis of the transcriptional transactivator Tat or the accessory protein Nef, none produces infectious virus. In a normal cellular response to DNA fragments, the nonhomologous end-joining (NHEJ) system may form 2-LTR circles to protect the cell. This system is responsible for rapid repair of double-strand breaks, thereby preventing an apoptotic response. A single double-strand break within the cell can induce G1 cell-cycle arrest. The ability of the free ends of the viral DNA to mimic such double-strand chromosomal breaks may contribute to the direct cytopathic effects observed with HIV.

Transcriptional Controls⁵⁵⁻⁶¹

Integration can lead to latent or transcriptionally active forms of infection. Moreover, despite a vigorous immune response early in infection, these silent proviruses are a reservoir that allows re-emergence of HIV when the body's defenses grow weaker. The chromosomal environment likely shapes the transcriptional activity of the provirus. For example, proviral integration into repressed heterochromatin might result in latency. Other causes of latency may include cell type differences in the availability of activators that bind to the transcriptional enhancer in the HIV LTR or the lack of Tat. However, of the multiple copies of provirus

that are usually integrated in a given infected cell, at least one is likely to be transcriptionally active. This fact may explain why the number of latently infected cells in infected patients is small.

In the host genome, the 5' LTR functions like other eukaryotic transcriptional units. It contains downstream and upstream promoter elements, which include the initiator (Inr), TATA-box (T), and three Sp1 sites. These regions help position the RNA polymerase II (RNAPII) at the site of initiation of transcription and to assemble the preinitiation complex. Slightly upstream of the promoter is the transcriptional enhancer, which in HIV-1 binds nuclear factor κ B (NF- κ B), nuclear factor of activated T cells (NFAT), and Ets family members. NF- κ B and NFAT relocate to the nucleus after cellular activation. NF- κ B is liberated from its cytoplasmic inhibitor, I κ B, by stimulus-coupled phosphorylation, ubiquitination, and proteosomal degradation of the inhibitor. NFAT is dephosphorylated by calcineurin (a reaction inhibited by cyclosporin A) and, after its nuclear import, assembles with AP1 to form the fully active transcriptional complex. NF- κ B, which is composed of p50 and p65 (RelA) subunits, increases the rates of initiation and elongation of viral transcription. Since NF- κ B is activated after several

antigen-specific and cytokine-mediated events, it may play a key role in rousing transcriptionally silent proviruses

When these factors engage the LTR, transcription begins, but in the absence of Tat, the polymerase fails to elongate efficiently along the viral genome. In the process, short nonpolyadenylated transcripts are synthesized, which are stable and persist in cells due to the formation of an RNA stem loop called the transactivation response (TAR) element.

Tat significantly increases the rate of viral gene expression.

Viral Transcripts^{62,63}

Transcription of the viral genome results in more than a dozen different HIV-specific transcripts. Some are processed co-transcriptionally and, in the absence of inhibitory RNA sequences (IRS), transported rapidly into the cytoplasm. These multiply spliced transcripts encode Nef, Tat, and Rev. Other singly spliced or unspliced viral transcripts remain in the nucleus and are relatively stable. These viral transcripts encode the structural, enzymatic, and accessory proteins and represent viral genomic RNAs that are needed for the assembly of fully infectious virions.

In contrast to Tat and Rev, which act directly on viral RNA structures, Nef modifies the environment of the infected cell to optimize viral replication. The absence of Nef in infected monkeys and humans is associated with much slower clinical progression to AIDS. This virulence caused by Nef appears to be associated with its ability to affect signaling cascades, including the activation of T-cell antigen receptor, and to decrease the expression of CD4 on the cell surface. Nef also promotes the production and release of virions that are more infectious. Effects of Nef on the PI3-K signaling cascade--which involves the guanine nucleotide exchange factor Vav, the small GTPases Cdc42 and Rac1, and p21-activated kinase PAK--cause marked changes in the intracellular actin network, promoting lipid raft movement and the formation of larger raft structures that have been implicated in T-cell receptor signaling. Indeed, Nef and viral structural proteins colocalize in lipid rafts. Two other HIV proteins assist Nef in downregulating expression of CD4. The envelope protein gp120 binds CD4 in the endoplasmic reticulum, slowing its export to the plasma membrane, and Vpu binds the cytoplasmic tail of CD4, promoting recruitment of TrCP and Skp1p. These events target

CD4 for ubiquitination and proteasomal degradation before it reaches the cell surface.

Nef acts by several mechanisms to impair immunological responses to HIV. In T cells, Nef activates the expression of FasL, which induces apoptosis in bystander cells that express Fas, thereby killing cytotoxic T cells that might otherwise eliminate HIV-1 infected cells. Nef also reduces the expression of MHC I determinants on the surface of the infected cell and so decreases the recognition and killing of infected cells by CD8 cytotoxic T cells. However, Nef does not decrease the expression of HLA-C, which prevents recognition and killing of these infected cells by natural killer cells.

Other viral proteins also participate in the modification of the environment in infected cells. Rev-dependent expression of Vpr induces the arrest of proliferating infected cells at the G2/M phase of the cell cycle. Since the viral LTR is more active during G2, this arrest likely enhances viral gene expression. These cell-cycle arresting properties involve localized defects in the structure of the nuclear lamina that lead to dynamic, DNA-filled herniations that project from the nuclear envelope into the cytoplasm. Intermittently, these herniations rupture, causing the mixing of soluble nuclear and cytoplasmic proteins. Either alterations in

the lamina structure or the inappropriate mixing of cell cycle regulators that are normally sequestered in specific cellular compartments could explain the G2 arresting properties of Vpr.

*Viral Assembly*⁸²⁻⁸⁵

New viral particles are assembled at the plasma membrane. Each virion consists of roughly 1500 molecules of Gag and 100 Gag-Pol polyproteins, two copies of the viral RNA genome, and Vpr. Several proteins participate in the assembly process, including Gag polyproteins and Gag-Pol, as well as Nef and Env. A human ATP-binding protein, HP68 (previously identified as an RNase L inhibitor), likely acts as a molecular chaperone, facilitating conformational changes in Gag needed for the assembly of viral capsids. In primary CD4 T lymphocytes, Vif plays a key but poorly understood role in the assembly of infectious virions. In the absence of Vif, normal levels of virus are produced, but these virions are noninfectious, displaying arrest at the level of reverse transcription in the subsequent target cell. Heterokaryon analyses of cells formed by the fusion of nonpermissive (requiring Vif for viral growth) and permissive (supporting growth of Vif-deficient viruses) cells have revealed that Vif overcomes the effects of a natural inhibitor of HIV replication.

The Gag polyproteins are subject to myristylation, and thus associate preferentially with cholesterol- and glycolipid-enriched membrane microdomains. Virion budding occurs through these specialized regions in the lipid bilayer, yielding virions with cholesterol-rich membranes. This lipid composition likely favors release, stability, and fusion of virions with the subsequent target cell.

The budding reaction involves the action of several proteins, including the "late domain" sequence (PTAP) present in the p6 portion of Gag. The p6 protein also appears to be modified by ubiquitination. The product of the tumour suppressor gene 101 (TSG101) binds the PTAP motif of p6 Gag and also recognizes ubiquitin through its ubiquitin enzyme 2 (UEV) domain. The TSG101 protein normally associates with other cellular proteins in the vacuolar protein sorting pathway to form the ESCRT-1 complex that selects cargo for incorporation into the multivesicular body (MVB). The MVB is produced when surface patches on late endosomes bud away from the cytoplasm and fuse with lysosomes, releasing their contents for degradation within this organelle. In the case of HIV, TSG101 appears to be "hijacked" to participate in the budding of virions into the extracellular space away from the cytoplasm.

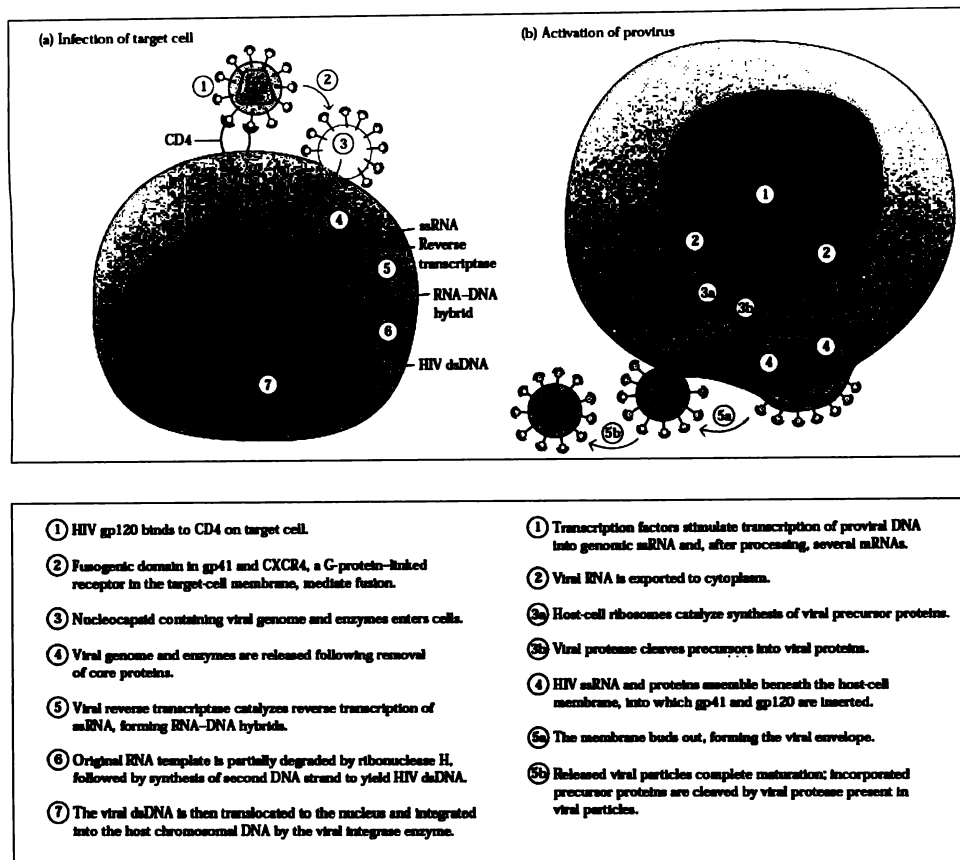


Figure 3: Life Cycle of HIV

HIV to AIDS: Clinical cul-de-sac

The spectrum of illnesses associated with HIV infection is broad and is the result of the direct HIV effects and the associated immune dysfunction. Several classification systems exist, the most widely used being the 1993 Centers for Disease Control (CDC) classification⁰³.

Stage 1: Primary Infection and Acute Sero-Conversion Syndrome –

CD4+T cell counts of more than 500 cells/mm³

Stage 2: Symptomatic HIV disease – CD4+T cell count between 200 - 499 cells/ mm³

Stage 3: AIDS Defining Condition – CD4 +T cell counts of less than 200 cells/ mm³

As immunosuppression progresses the patient is susceptible to an increasing range of opportunistic infections and Tumours, certain of which meet the criteria for the diagnosis of AIDS.

*Incubatio*⁹⁴*n*: The 2 — 4 weeks immediately following infection are usually silent but clinically and serologically.

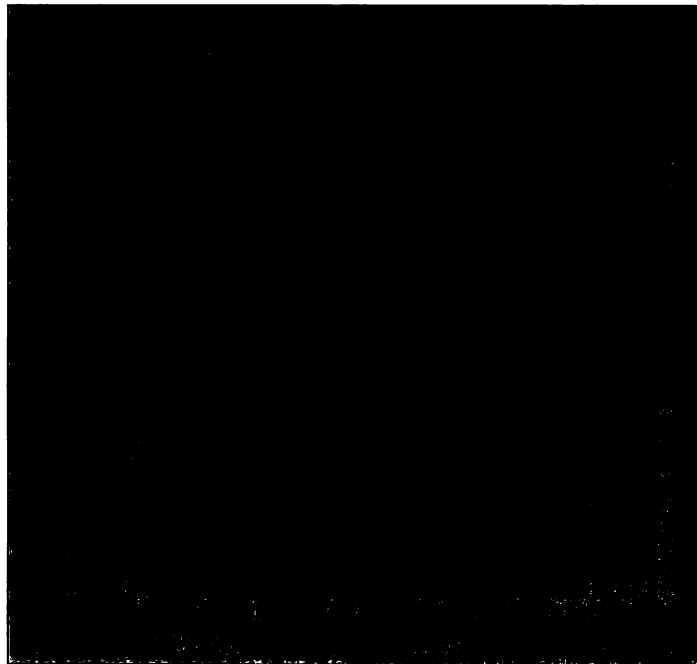


Figure 4: Serologic profile of HIV infection showing three
*Sero-conversion/ primary illness*⁹⁴: The majority of HIV sero-conversions are also clinically silent. In a proportion, a self-limiting nonspecific illness

occurs 6 — 8 weeks after the exposure. Symptoms include fever, arthralgia, myalgia, lethargy, lymphadenopathy, sore throat, mucosal ulcers and occasionally a faint pink maculopapular rash. Neurological symptoms are common, including headache, photophobia, myelopathy, neuropathy and in rare cases encephalopathy. The illness lasts up to 3 weeks and recovery is usually complete. Laboratory abnormalities include lymphopenia with atypical reactive lymphocytes noted on blood film, thrombocytopenia and raised liver enzymes. CD4 lymphocytes may be markedly depleted and the CD4:CD8 ratio reversed. Antibodies to HIV may be absent during this early stage of infection although the level of circulating viral RNA is high and p24 core protein may be detectable.

*Asymptomatic Phase/Clinical Latency*²⁴: The majority of people with HIV infection are asymptomatic for a substantial but variable length of time. However, the virus continues to replicate and the person is infectious. Studies suggest a median time of ten years from infection to development of AIDS, although some patients progress much more rapidly and others have remained symptom-free for up to 15 years. Older age is associated with more rapid progression. A subgroup of patients with asymptomatic infection have Persistent Generalized Lymphadenopathy (PGL), defined as lymphadenopathy (>1 cm) at two or more extralingual sites for more

than 3 months in the absence of causes other than HIV infection. There may be associated splenomegaly.

*Symptomatic HIV infection*⁹⁴: As HIV infection progresses the viral load rises, the CD4 count falls below 500 cells/mm³ and the patient develops an array of symptoms and signs. The clinical picture is the result of direct HIV effects and the associated immunosuppression.

In an individual patient the clinical consequences of HIV-related immune dysfunction will depend on at least three factors:

- *The microbial exposure of the patient throughout life*: Many clinical episodes represent reactivation of previously acquired infection, which has been latent. Geographical factors determine the microbial repertoire of an individual patient. Those organisms requiring intact Cell-mediated immunity for their control are most likely to cause clinical problems.
- *The pathogenicity of organisms encountered*: High-grade pathogens such as *Mycobacterium tuberculosis*, *Candida* and the Herpes viruses I are clinically relevant even when immunosuppression is mild, and will thus occur earlier in the course of the disease. Less virulent organisms occur at later stages of immunodeficiency.

- *The degree of immunosuppression of the host:* When patients are severely immuno-compromised (CD4 counts <200 cells/mm³) disseminated infections with organisms of very low virulence such as *M. avium-intracellulare* (MAI) and cryptosporidium are able to establish themselves.

*AIDS Defining Condition*⁹⁴: The late stage of HIV disease is defined by CD4+ T cell counts of less than 200 cells/mm³. There is a greater risk of manifesting new OI's during this stage that leads to progressive and significant immune deficiency causing AIDS defining conditions and ultimately to death of the patient.

Constitutional symptoms such as low-grade fever, weight loss and myalgia along with other symptoms observed during the advanced stage of HIV disease are more common. This stage of HIV disease is associated with development of protozoal infections such as *Pneumocystis carinii* pneumonia (PCP) and *Toxoplasma gondii* encephalitis. Bacterial infections caused by *Treponema pallidum* and Tubercule multibacilli are predominant OI's. Disseminated histoplasmosis, invasive aspergilosis, and cryptococcal infections are common manifestations in this stage. Viral infections include Cytomegalovirus retinitis, recurrent bouts of oral and genital herpes simplex virus. Studies have indicated the incidence of malignancies

such as Kaposi's sarcoma, anogenital carcinoma and B-cell lymphoma during this stage.

With profound immunosuppression in hand along with advent of a plethora of invasive OI's and their symptom complexes, the patient progresses towards the terminal stage of HIV disease. Patients in the advanced disease stage may develop co-existing OI's with each OI further contributing to the symptom complex. HIV – HBV, HIV – TB, HIV - HSV are common co-infections that aggravate the advent of terminal illness. Involuntary weight loss often referred to, as HIV Wasting Syndrome (HWS) along with brain disorders including AIDS Dementia Complex are common manifestations observed in patients with terminal AIDS.

Since the rate of HIV disease progression is individual specific. Physical health of the individual, genetic constitution, cytokine interplay, viral load, mode of entry, receptor–HIV entry complex, advent of OI's and environmental factors as well are some essential factors which govern the rate of HIV disease progression. Hence, it is difficult to define the precise parameter that causes the transition of an individual through the different stages of HIV disease following infection with the virus.

Partying with the Pirate: OI's in HIV disease

As the CD4 cell count depletes, there arises a plethora of microbial manifestations. These opportunistic infections aid in the progression of HIV disease by interacting with many a host and HIV factors.

Table 2: Major HIV associated pathogens⁹⁴

Protozoa	Bacteria
<i>Toxoplasma gondii</i>	<i>Salmonella spp</i>
<i>Cryptosporidium parvum</i>	<i>Mycobacterium tuberculosis</i>
<i>Leishmania donovani</i>	<i>Mycobacterium avium-intracellulare</i>
<i>Isospora belli</i>	<i>Streptococcus pneumoniae</i>
Viruses	<i>Staphylococcus aureus</i>
Cytomegalovirus (CMV)	<i>Haemophilus influenzae</i>
Herpes Simplex Virus (HSV)	<i>Moraxella catarrhalis</i>
Human Papillomavirus (HPV)	<i>Bartonella Quintana</i>
Hepatitis B Viruses (HBV)	<i>Treponema pallidum</i>
Hepatitis C Viruses (HCV)	
Fungi & Yeasts	
<i>Pneumocystis carinii</i>	
<i>Candida spp</i>	
<i>Cryptococcus neoformans</i>	
<i>Aspergillus fumigatus</i>	

Some of the important OI's and their interplay in the HIV disease progression are discussed below.

*Pneumocystis carinii*⁰⁵: This fungus causes Pneumocystis carinii pneumonia (referred to as PCP). PCP is the main presenting disease seen in AIDS in the industrially developed world. *P. carinii* is ubiquitous in nature and infects a variety of mammals including humans. However the probability of zoonotic infection is rare. *P. carinii* infection in humans is probably transmitted from person to person by the respiratory route. In most people, infection is contained by normal immune mechanisms but remains latent, able to be reactivated should immuno-suppression occur. Until the epidemic of HIV infection, PCP was only seen in patients whose immune system had been depressed by either a known primary or secondary cause. Patients usually develop signs and symptoms insidiously, often giving a 3 — 4 week history of dry, non productive cough, difficult or labored breathing (dyspnoea), chest pain, fever and chills. Some patients have a more fulminate course with a much shorter history and may be in acute respiratory failure and extreme distress.

*Candida spp*⁰⁵: Candidiasis caused by the commensal organism, *Candida albicans*, is the most common oral infection in HIV disease. It presents as reddened patches (erythematous) or white curd-like lesions (pseudomembranous) (thrush). Oropharangeal Candidiasis (OPC) is defined as superficial candidiasis with shallow levels of tissue invasion. As

a result, chewing and swallowing can be difficult. Infections can be acute or recurrent, and are common in immunocompromised patients

*C. albicans*⁹⁶, a ubiquitous dimorphic fungal organism that is part of the normal microflora of the gastrointestinal and reproductive tracts of healthy individuals. As asymptomatic carriers, the approximate rate of oral yeast carriage in HIV-negative persons is 50-60%, with *C. albicans* present in 85% of isolates recovered. In HIV-positive individuals, the asymptomatic colonization rate is higher, approaching 76% Colonization with *C.albicans* is assumed to be responsible for acquired immune responsiveness. Anti-*Candida* antibodies, circulating IgG and mucosal IgA and IgG, can be detected in most healthy individuals. Moreover, greater than 80% of healthy persons have positive cutaneous skin test reactivity to *Candida* antigen, and peripheral blood lymphocytes from over 90% of healthy individuals proliferate *in vitro* to *Candida* antigen. It is presumed that these acquired host responses, in conjunction with innate resistance (*i.e.*, polymorphonuclear leukocytes and macrophages), play a significant role in restricting *C. albicans* to mucosal surfaces in an asymptomatic commensal state. Under immunocompromised conditions, however, *C. albicans* can convert into an opportunistic pathogen with significant morbidity.

A possible association between AIDS and *Candida* infection is the upregulation of CD4 receptors by *Candida* antigens. On the other hand, the shift in the cytokine expression from T_H1 (pro-inflammatory) to T_H2 in HIV/AIDS may have a role in aggravating Candidiasis in these patients⁹⁷.

*Mycobacterium tuberculosis*⁹⁴: Infection with *Mycobacterium tuberculosis* continues to be the most common recognized infectious disease worldwide contributing to significant morbidity and mortality. The interaction of the host with *M. tuberculosis* is likely to be conducive to expression of the virulence of this pathogen. In fact, the very nature of the complex interaction between the host and the organism may underlie the salient features of *M. tuberculosis* infection namely, the persistence of infection within host tissues, and the development of disease. The immune response to *M. tuberculosis* can be characterized to be both salutary to the survival of the host, contributing to control of mycobacterial replication, and damaging to the host, contributing to promotion of tuberculosis. In some infected people, the disease either progresses or, more commonly, reactivates after an asymptomatic period. The most common reactivation from is a chronic pneumonia with fever, cough, bloody sputum and weight loss. A weakened Cell-Mediated Immune system increases the chances of

terminating *M. tuberculosis* latency in the host. TB is one of the most common opportunistic infections associated with the HIV disease. In HIV-TB dual infections there is marked suppression of IFN γ production and increase in TNF α and TGF β . Enhanced TNF α and TGF β activity during such dual infections contribute to augmentation of viral replication and dissemination⁹⁸⁻¹⁰⁰.

*Herpes Simplex Virus (HSV)*⁹⁵: HSV orolabialis is the most common manifestation of HSV1 infection, presenting with a sensory prodrome in the affected area, rapidly followed by the evolution of lesions from papule to vesicle, ulcer, and crust stages on the lips. HSV genitalis is the more common manifestation of HSV2 infection. Perineal lesions on keratinylated skin are similar in appearance and evolution to external orofacial lesions. Local symptoms include a sensory prodrome consisting of pain and pruritis. Ulcerative lesions are usually the only stage observed on vaginal or urethral mucosal surfaces. Mucosal disease is generally accompanied by dysuria, vaginal, or urethral discharge; inguinal lymphadenopathy, particularly in primary infection, is common with perineal disease. In profoundly immunocompromised patients, extensive, deep, non healing ulceration of the perineum/buttocks might occur. These lesions have been most often reported in those with CD4⁺ T lymphocyte

counts of <100 cells/ μL and also might be more commonly associated with acyclovir-resistant virus. It has been demonstrated that HIV is detectable in keratinocytes in non-genital HSV-1 skin lesions in AIDS patients. Human keratinocytes are usually non permissive for HIV replication and appear to be rendered infectable with HIV, by a CD4 independent mechanism. Furthermore, co-infected keratinocytes released large numbers of HIV virion, consistent with activated infection, in the presence of HSV-1. These findings suggest a mechanism by which activation of HIV by HSV could lead to enhanced HIV secretion at epithelial surfaces.

Jack of All, Master of One: Manifold Manifestations of the Microbe

HIV selectively attacks and destroys cells of the immune system, leading to marked immunodeficiency. But apart from having deleterious effects on the immune system, HIV - directly or indirectly, affects major systems of the human host. Some of the manifestations on major systems involved in the HIV disease are discussed below.

*Neurological Disease*⁹⁴: HIV can be carried to the CNS early following initial infection. Entry probably occurs through breaches in the blood-brain barrier. The HIV Tat protein can induce oxidative stress, compromise cellular viability, induce apoptosis, and disrupt tight junctions

in endothelial cells to produce HIV entry as well as ongoing cellular damage. HIV resides within microglial cells or within perivascular macrophages, from which the effects of ongoing HIV replication lead to disease in the brain. The HIV Tat protein induces inflammatory responses that mediate cellular injury. The ongoing cellular damage appears clinically as an encephalopathy or encephalitis.

A clinical syndrome described loosely as "HIV encephalopathy" is often associated with a progressive debilitating dementia, or "AIDS dementia complex." Clinically, AIDS dementia often begins with impaired memory and concentration along with psychomotor slowing. It is progressive and continues to include motor deficits such as ataxia and tremor. Behavioral disturbances range from apathy or withdrawal to frank psychosis.

*Mucocutaneous Manifestations*⁹⁴: The skin is the most common site for HIV-related pathology as the function of dendritic and Langerhan's cells, both target cells for HIV are disrupted. Pruritis is a common complaint in all stages of HIV illness. Generalized dry, itchy, flaky skin is typical and the hair may become thin and dry. Eosinophilic folliculitis presents with urticarial lesions in face, arms and legs. Kaposi sarcoma has been one of the most characteristic skin disease associated with HIV infection. Highly Active Antiretroviral Therapy (HAART) may increase of certain

dermatologic diseases, mostly within the first 3 months after initiation, because of immune restoration that may exacerbate previously dormant conditions such as herpes zoster. Adverse cutaneous drug reactions secondary to HAART may also occur, as does photosensitivity with HAART use.

*Respiratory Manifestations*⁹⁵: Patients with HIV infection frequently present with a wide spectrum of pulmonary complications from opportunistic infections and neoplasms that may be associated with a high mortality rate. Diseases of the respiratory tract account for many deaths from AIDS. The response to therapy in AIDS can be slower and complicated by a greater number of adverse reactions to therapeutic agents than with other immunocompromised states. Cigarette smoking increases the risk for colonization by infectious agents, and smoke decreases alveolar macrophage function, leading to increased numbers of infections or more severe infections. The epiglottis, pharynx, larynx, and trachea can also be affected by AIDS-diagnostic diseases. The commonest are invasive candidiasis and Kaposi's sarcoma. Kaposi's sarcoma has a predilection for the epiglottis. Clinical findings of stridor and hoarseness may suggest KS involvement of the upper airway.

*Cardiac Disease*¹⁰¹: The heart is not a frequent site for opportunistic infectious or neoplastic processes in patients with AIDS. Most AIDS patients are in the third to fifth decades of life, at an age when cardiovascular complications from atherosclerosis are not as frequent as in older patients. Atherosclerotic cardiovascular disease leading to ischemia and infarction can and does occur in some AIDS patients, particularly as the numbers of HIV-infected persons begin to include older persons and those surviving longer on highly active antiretroviral therapy (HAART). However, the chronic debilitated state with cachexia brought on by AIDS may also lead to regression of atherosclerotic lesions. Cardiac lesions are the immediate cause of death in less than 1% of AIDS patients. Clinical cardiac findings may be present in a fourth to three fourths of adult AIDS patients and may be accompanied by findings that include chest pain, tachycardia, electrocardiographic changes including various arrhythmias, effusions, and congestive heart failure. There may be mild cardiomegaly on chest roentgenogram and minimal electro-cardiographic findings.

*Gastrointestinal Disease*¹⁰²: The gastrointestinal (GI) tract is the second most common organ system site for opportunistic infections or neoplasms associated with AIDS. The most frequent clinical symptom resulting from GI involvement is diarrhoea, and the etiologies for this are numerous.

Diarrhoea may appear with acute HIV infection, but typically it is manifested in patients with clinical AIDS. In a few cases this diarrhoea may be life-threatening. In patients receiving highly active antiretroviral therapy (HAART), the frequency of gastrointestinal involvement with opportunistic infections is greatly diminished.

Research has shown important relationship between the HIV virus, the health of the gastrointestinal tract, and AIDS wasting syndrome, involving Tumour Necrosis Factor alpha (TNF α), specific and non-specific immunity in the gut, gut permeability, and oxidative stress.

HIV virus is found in high concentrations in the lamina propria. High concentrations of HIV-RNA are demonstrated in the crypt epithelium, macrophages, lymphocytes, and eosinophils. As the organism invades and replicates, it crosses into the portal circulation and is delivered to the liver, particularly the Kupffer cells. This represents an advance in disease and is associated with progressive global immune suppression secondary to ubiquitous depression of Gut Associated Lymphoid Tissue (GALT) function. With the gut compromised, there is adhesion of previously harmless but potentially enterotoxigenic organisms to the mucosa with ensuing inflammation. Common symptoms include diarrhoea, weight loss, and fever. An immunocompromised individual does not need to ingest a

foreign organism to have inflammatory diarrhoea; an HIV-infected individual will get diarrhoea because the normal balance of intestinal flora and other elements of the nonspecific immune defence system is altered, allowing antigens to cross the leaky gut. In this way, intestinal dysbiosis results in HIV replication by the Protein Kinase C (PKC)/NF κ B HIV replication pathway¹⁰³.

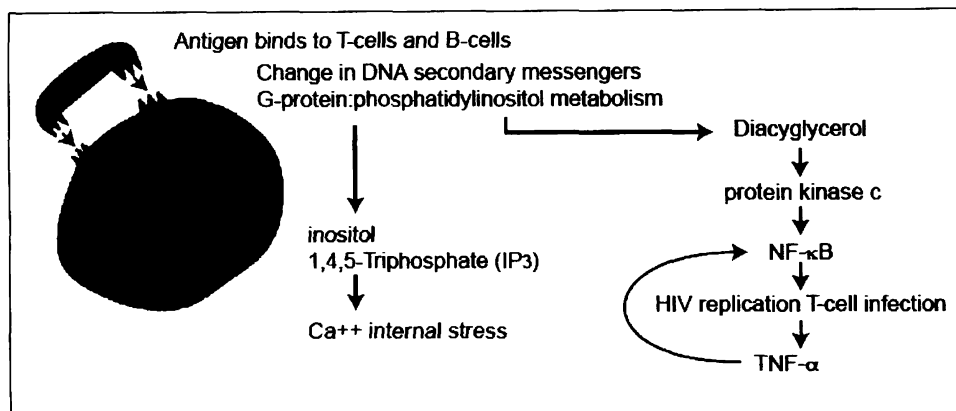


Figure 5: Antigen induction of T-cells and subsequent induction of NF- κ B by PKC

*The Immune System*¹⁰⁴: The most prominent aspect of HIV induced immunodeficiency is AIDS. Some tumours, such as B-cell lymphomas are seen in AIDS patients but these are consequence of the lack of immune surveillance which is responsible for the destruction of tumours of healthy individuals. A great number of different abnormalities of the immune system are seen in AIDS.

Table 3: Immune Abnormalities in HIV infection

Immune Abnormalities in HIV infection
Altered Cytokine Expression
Decreased CTL & NK cell function
Decreased humoral and proliferative response to antigens & mitogens
Decreased MHC-II expression
Decreased monocytes chemotaxis
Depletion of CD4 + Cells
Impaired Delayed Type Hypersensitivity (DTH) Reaction
Lymphopenia
Polyclonal B cell Activation

It is still not clear how much of the pathology of AIDS is caused directly by the virus and how much is caused by the immune system. There are numerous models that have been suggested to explain how HIV causes immunodeficiency. These mechanisms are not mutually exclusive and indeed it is probable that the underlying loss of CD4 + Cells in AIDS is multi-factorial.

Direct cell killing: This was the earliest mechanism suggested based on the behavior of laboratory isolates of HIV. Cell fusion resulting in Syncytium formation is one of the major mechanisms of the cell killing by HIV in-vitro. However, different isolates of HIV may vary considerably in the extent to which they promote the fusion of infected cells.

Subsequent experiments suggested they may not be sufficient like this present in AIDS patients account for all the damage seen, although deal of CD4 + cells may contribute to the overall pathogenesis of AIDS in some circumstances.

Indirect Killing of HIV-infected cells: Indirect effects of infection, e.g. disturbances in cell biochemistry and lymphokine production may also affect the regulation of the immune system. HIV-infected cells can induce autophagy in bystander CD4+ T lymphocytes through contact of the Env protein with CXCR4, leading to apoptotic cell death, a mechanism most likely contributing to immunodeficiency. HIV-1 envelope glycoproteins (Env), expressed at the cell surface, induce apoptosis of uninfected CD4+ T cells. Independently of HIV replication, transfected or HIV-infected cells that express Env induced death uninfected CD4+ T lymphocytes via CXCR4.

Antigenic Diversity: This theory proposes that the continual generation of new antigenic variants eventually swamps and overcomes the immune system, leading to its collapse. There is no doubt that new antigenic variants of HIV constantly arise during the long course of AIDS because of the low fidelity of reverse transcription. It is envisaged that there might be a 'ratchet' effect, with each new variant contributing to the slight but

irreversible decline in immune function. Because of the way virus infections are handled by the immune system, it is probable that variation of T-cell epitopes on target proteins recognized by CTL are more important than B-cell epitopes which generate the antibody response to a foreign antigen. Mathematical models have been constructed which simulate antigenic variation during the course of infection. When primed with known data about the state of immune system during HIV infection, these provide an accurate depiction of the course of AIDS

Recently, it has been shown that there is a simple relationship between virus load and survival time, and that a patient can withstand only $\sim 1,300$ "viral years" of HIV (i.e. copies of the virus genome/ml x survival time in years).

The Superantigen Theory: Superantigens are molecules which short-circuit the immune system, resulting in massive activation of T-cells rather than the usual, carefully controlled response to foreign antigens. It is believed that they do this by binding to both the variable region of the β -chain of the T-cell receptor (V- β) and to MHC II molecules, cross-linking them in a non-specific way. This results in polyclonal T-cell activation rather than the usual situation where only the few clones of T-cells responsive to a

particular antigen presented by the MHC II molecule are activated. The over-response of the immune system produced results in autoimmunity, as rare clones of T-cells which recognize self antigens are activated and immune suppression, as the activated cells subsequently die or are killed by other activated T-cells. It is possible that such superantigens might also induce apoptosis. However, unlike other retroviruses (e.g. Mouse Mammary Tumour virus (MMTV) and the Murine Leukaemia Virus (MLV) responsible for murine acquired immunodeficiency syndrome (MAIDS)) no superantigen has been conclusively identified in HIV, despite intensive investigation. Thus the practical relevance of superantigens in AIDS is thus in doubt. However, it is possible that exposure to superantigens produced by opportunistic infection(s) might play a role in AIDS.

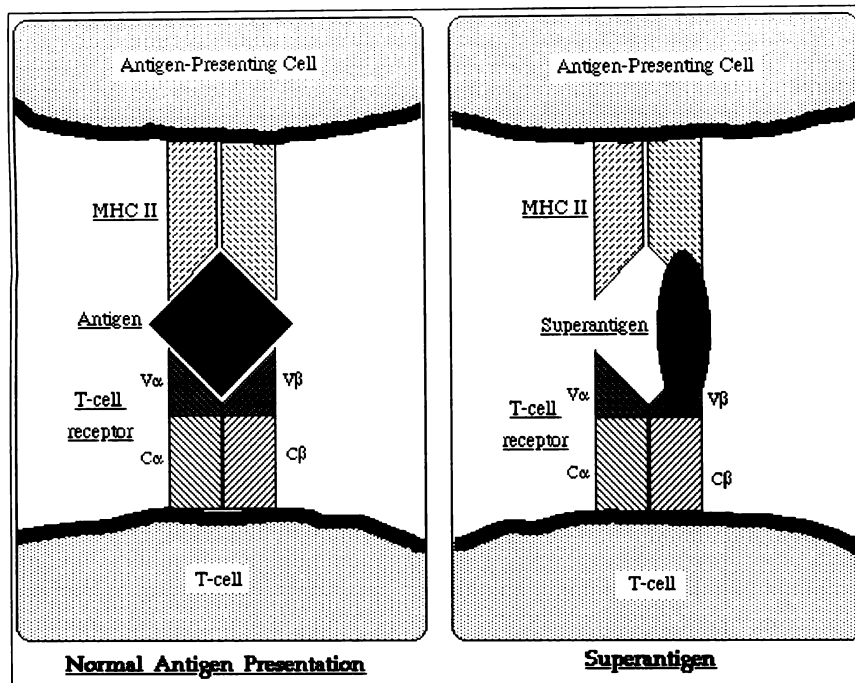


Figure 6: Superantigen Binding

T-cell Anergy: Anergy is an immunologically unresponsive state in which lymphocytes are present but not functionally active. This is usually due to incomplete activation signals and may be an important regulatory mechanism in the immune system, e.g. tolerance of 'self' antigens. In AIDS, anergy could be induced due to HIV infection, e.g. interference with cytokine expression. There is experimental in vitro evidence that gp120-CD4 interactions result in anergy due to interference with signal transduction. Many AIDS patients are anergic, i.e. fail to mount a delayed-type hypersensitivity (DTH) response to skin-test antigens. Impaired DTH responses are directly related to decreasing $CD4^+$ T-

lymphocyte counts. However, there is no strong evidence that this phenomenon is directly related to any aspect of HIV infection in vivo rather than to general depletion of immune functions.

T_H1/T_H2 Imbalance: Early in HIV infection, T_H1-responsive T-cells predominate and are effective in controlling (but not eliminating) the virus. At some point, a (relative) loss of the T_H1 response occurs and T_H2 HIV-responsive cells predominate. Regulation of the immune system depends on a complex network of cells, but central to the process is the role of CD4⁺ T-helper (T_H) cells. Immunological theory suggests that there are two types of these, T_H1 cells which promote the cell mediated response and T_H2 cells which promote the humoral response. This theory suggests that early in HIV infection, T_H1-responsive T-cells predominate and are effective in controlling (but not eliminating) the virus. At some point, a (relative) loss of the T_H1 response occurs and T_H2 HIV-responsive cells predominate. It has been reported that at least some virus variants can inhibit the CTL response to HIV. The hypothesis is therefore that the T_H2-dominated humoral response is not effective at maintaining HIV replication at a low level and the virus load builds up, resulting in AIDS.

Apoptosis (Greek=*Falling Leaves*): Various agents appear to be able to trigger apoptosis (*Apoptosis: A genetically determined destruction of cells from within due to activation of a stimulus or removal of a suppressing agent or stimulus that is postulated to exist to explain the orderly elimination of superfluous cells - also called as programmed cell death*) in CD4⁺ T cells. The viral envelope glycoprotein gp120 and the transactivator protein Tat are involved in priming CD4⁺ T cells for apoptosis¹³. In addition, gp120 alone is able to induce apoptosis in different cell lines bearing CD4 receptors, confirming the ability of this glycoprotein to activate cell suicide programs. Additional candidate mechanisms for apoptosis induction in AIDS are the inappropriate expression of prostaglandins and cytokines i.e. tumor necrosis factor α by other infected cells, such as macrophages^{14,15}. Indeed TNF α participates in the destruction of HIV-infected neuronal cells¹⁶ and favours provirus replication¹⁷. Another example of cytokine-induced apoptosis is the Fas ligand (FasL)-Fas receptor (FasR) system. FasL is a cell surface cytokine belonging to the TNF family, which is able to induce apoptosis in cells bearing FasR. Receptor expression is induced in lymphocytes infected by several viruses, including HIV. Therefore, it has been suggested that FasR-mediated apoptosis could be implicated in AIDS pathology.

Affable Assassination: Apoptosis¹⁰⁵

Apoptosis is a form of programmed cell death in multicellular organisms. It is one of the main types of programmed cell death (PCD) and involves a series of biochemical events leading to a characteristic cell morphology and death, in more specific terms, a series of biochemical events that lead to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. Processes of disposal of cellular debris whose results do not damage the organism differentiate apoptosis from necrosis.

In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis, in general, confers advantages during an organism's life cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose; the result is that the digits are separate. Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult. This process is also responsible for orderly removal of autoreactive lymphocytes in the lymph nodes. In addition to its importance as a biological phenomenon, defective apoptotic processes have been

implicated in an extensive variety of diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer.

The Apoptotic Process: The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly (*extrinsic inducers*) or intracellularly (*intrinsic inducers*). Extracellular signals may include hormones, growth factors, nitric oxide or cytokines, and therefore must either cross the plasma membrane or transduce to effect a response. These signals may positively or negatively induce apoptosis; in this context the binding and subsequent initiation of apoptosis by a molecule is termed positive, whereas the active repression of apoptosis by a molecule is termed negative.

Intracellular apoptotic signalling is a response initiated by a cell in response to stress, and may ultimately result in cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, and hypoxia are all factors that can lead to the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis.

Before the actual process of cell death is carried out by enzymes, apoptotic signals must be connected to the actual death pathway by way of regulatory proteins. This step allows apoptotic signals to either culminate in cell death, or be aborted should the cell no longer need to die. Several proteins are involved, however two main methods of achieving regulation have been identified; targeting mitochondria functionality, or directly transducing the signal via *adapter proteins* to the apoptotic mechanisms. The whole preparation process requires energy and functioning cell machinery.

Mitochondrial Regulation: Apoptotic proteins that target mitochondria affect them in different ways; they may cause mitochondrial swelling through the formation of membrane pores, or they may increase the permeability of the mitochondrial membrane and cause apoptotic effectors to leak out. There is also a growing body of evidence that indicates that nitric oxide (NO) is able to induce apoptosis by helping to dissipate the membrane potential of mitochondria and therefore make it more permeable.

Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cytosol following an increase in permeability. SMAC binds to *inhibitor of apoptosis proteins* (IAPs) and

deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of a group of cysteine proteases called caspases, which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability.

Cytochrome c is also released from mitochondria due to increased permeability of the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis. Once cytochrome c is released it binds with *Apaf-1* and ATP, which then bind to *pro-caspase-9* to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector *caspase-3*.

The mitochondrial permeability is itself subject to regulation by various proteins, such as those encoded by the mammalian *Bcl-2* family of anti-apoptotic genes, the homologs of the *ced-9* gene found in *C. elegans*. *Bcl-2* proteins are able to promote or inhibit apoptosis either by direct action on mitochondrial permeability or indirectly through other proteins. It is important to note that the actions of some *Bcl-2* proteins are able to halt apoptosis even if cytochrome c has been released by the mitochondria.

Direct Signal Transduction: Two important examples of the direct initiation of apoptotic mechanisms in mammals include the *TNF-induced* (tumour necrosis factor) model and the *Fas-Fas ligand-mediated* model, both involving receptors of the *TNF receptor* (TNFR) family coupled to extrinsic signals.

TNF is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF: *TNF-R1* and *TNF-R2*. The binding of TNF to *TNF-R1* has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins *TNF receptor-associated death domain* (TRADD) and *Fas-associated death domain protein* (FADD). Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses. The link between TNF and apoptosis shows why an abnormal production of TNF plays a fundamental role in several human diseases, especially in autoimmune diseases.

The Fas receptor (also known as *Apo-1* or *CD95*) binds the Fas ligand (FasL), a transmembrane protein part of the TNF family. The interaction between Fas and FasL results in the formation of the *death-inducing signaling complex* (DISC), which contains the FADD, caspase-8 and

caspase-10. In some types of cells (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis. In other types of cells (type II), the *Fas*-DISC starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8.

Following *TNF-R1* and *Fas* activation in mammalian cells a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (*Bcl-Xl* and *Bcl-2*) members of the *Bcl-2* family is established. This balance is the proportion of pro-apoptotic homodimers that form in the outer-membrane of the mitochondrion. The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC. Control of pro-apoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood, but it has been found that a mitochondrial outer-membrane protein, VDAC2, interacts with BAK to keep this potentially-lethal apoptotic effector under control. When the death signal is received, products of the activation cascade displace VDAC2 and BAK is able to be activated.

Execution of Apoptosis: Although many pathways and signals lead to apoptosis, there is only one mechanism that actually causes the death of

the cell in this process; after the appropriate stimulus has been received by the cell and the necessary controls exerted, a cell will undergo the organized degradation of cellular organelles by activated proteolytic caspases. A cell undergoing apoptosis shows a characteristic morphology that can be observed with a microscope:

1. Cell shrinkage and rounding due to the breakdown of the proteinaceous cytoskeleton by caspases.
2. The cytoplasm appears dense, and the organelles appear tightly packed.
3. Chromatin undergoes condensation into compact patches against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis.
4. The nuclear envelope becomes discontinuous and the DNA inside it is fragmented in a process referred to as karyorrhexis. The nucleus breaks into several discrete *chromatin bodies* or *nucleosomal units* due to the degradation of DNA.
5. The cell membrane shows irregular buds known as blebs.
6. The cell breaks apart into several vesicles called *apoptotic bodies*, which are then phagocytosed.

Apoptosis progresses quickly and its products are quickly removed, making it difficult to detect or visualize. During karyorrhexis, endonuclease

activation leaves short DNA fragments, regularly spaced in size. These give a characteristic "*laddered*" appearance on agar gel after electrophoresis. Tests for DNA laddering differentiate apoptosis from ischemic or toxic cell death.

*Apoptosis in HIV Disease*¹⁰⁶: The progression of the human immunodeficiency virus (HIV) to AIDS is primarily due to the depletion of CD4+ T-helper lymphocytes, which leads to a compromised immune system. One of the mechanisms by which T-helper cells are depleted is apoptosis, which can be the end-product of multiple biochemical pathways:

1. HIV enzymes inactivate anti-apoptotic *Bcl-2* and simultaneously activate pro-apoptotic *procaspase-8*. This directly does not cause cell death but primes the cell for apoptosis should the appropriate signal be received.
2. HIV products may increase levels of cellular proteins which have a promotive effect on Fas-mediated apoptosis.
3. HIV proteins decrease the amount of CD4 glycoprotein marker present on the cell membrane.
4. Released viral particles and proteins present in extracellular fluid are able to induce apoptosis in nearby "bystander" T-helper cells.

5. HIV decreases the production of molecules involved in marking the cell for apoptosis, giving the virus time to replicate and continue releasing apoptotic agents and virions into the surrounding tissue.
6. The infected CD4⁺ cell may also receive the death signal from a cytotoxic T cell, leading to apoptosis

Some of these key mechanisms are discussed below:

Role of gp120/CD4 interaction¹⁰⁷: The mature HIV-1 envelope glycoproteins are composed of gp120, the exterior envelope glycoprotein, and gp41, the transmembrane glycoprotein assembled as trimer by non covalent interactions. Obviously, the viral envelope can be considered as an extracellular ligand. Consequently, binding of HIV-1 Env gp120/gp41 to its receptors constitutes the primary interface between viruses and T cells and this event is likely able to modulate T cell signaling. Depending on Env presentation and on the complexity of target cell contact, the mechanisms leading to cell death may also be different. Indeed, soluble Env, secreted from infected cells, Env expressed on virions or at the cell surface of infected cells, are able to induce apoptosis of uninfected T cells. Soluble Env resulting from shedding of the surfaces of viral particles or infected cells can be considered as a ligand of CD4 and coreceptor molecules and acts as a signaling molecule through these receptors.

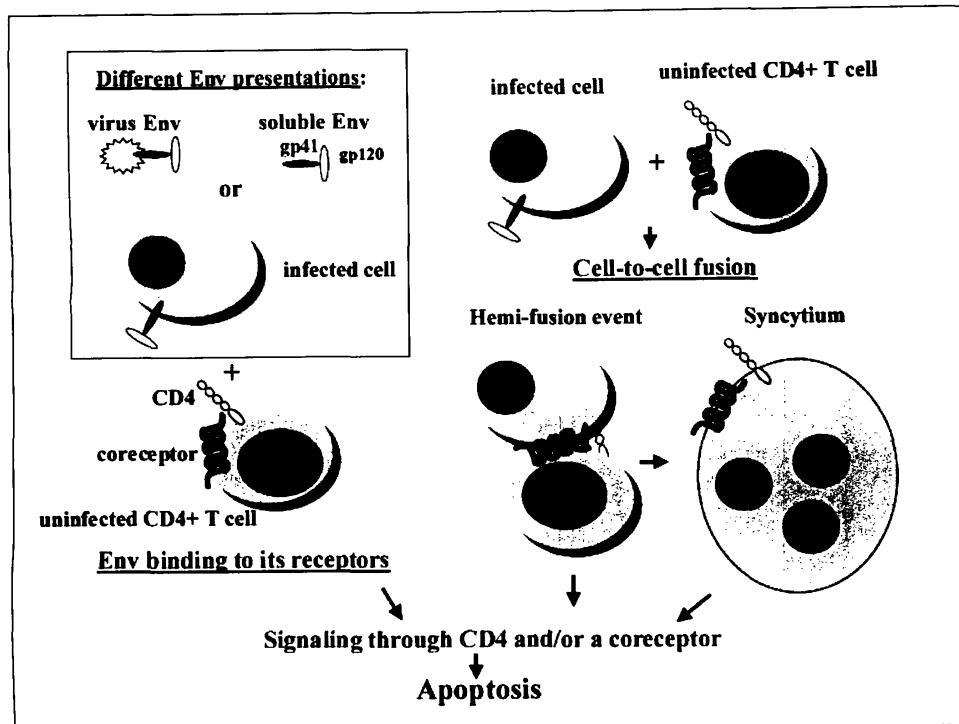


Figure 7: Env Induced T-Cell Apoptosis

Infected cells expressing Env at their surface can interact with uninfected T cells presenting CD4 and coreceptor molecules and can elicit several events, (i) an apoptotic signaling through one of these receptors, (ii) an hemifusion event leading to target cell death or (iii) syncytium formation.

...

T cells from HIV-infected patients are continuously undergoing CD4 cross-linking through interaction with virions or via Env expressed at the surface of infected cells. This phenomenon occurs essentially in lymphoid tissue which is a major reservoir of viral infection in HIV disease and a primary site of antigen presentation and lymphocyte activation. Indeed,

apoptosis is predominantly seen in uninfected bystander cells present in HIV-1 infected individual lymph nodes. When these CD4-cross-linked uninfected T cells encounter antigen-presenting cells in the local environment, they receive stimulatory signals through the TCR, leading to increased apoptosis. This supports the concept that circulating T lymphocytes from HIV-infected patients are in an enhanced state of immune activation, which, in fact, may translate into the observed increased levels of ex vivo spontaneous T cell apoptosis, activation-induced T cell apoptosis and T cell susceptibility to Fas-dependent apoptosis. Another mechanism for depletion of bystander T cells, observed in the lymph nodes of AIDS patients, was suggested when it was discovered that about one-half of the resting CD4⁺ lymphocytes that were pre-exposed to HIV (but not infected) were induced into apoptosis following signaling through receptors necessary for homing to lymph nodes. Several non-immunological complications in HIV disease are attributed to Env induced apoptosis; these include HIV-1 mediated Neurotoxicity and Hepatocyte death and in HIV mediated cardiopathy and nephropathy.

Env mediated apoptosis of CD8⁺ Lymphocytes: HIV infection is characterized by a persistent immune activation and a concomitant decline in both CD4⁺ and CD8⁺ naïve lymphocytes in the early stages

of the disease. In the later stages, both CD4⁺ and CD8⁺ memory T cells decline at similar rates. Notably, apoptosis is seen in peripheral blood CD4⁺ and CD8⁺ T lymphocytes of HIV-infected patients as well as in CD4⁺ and CD8⁺ T cells present in lymph nodes of HIV-infected persons. The degree of apoptosis observed in these cells is significantly higher in infected patients than in uninfected individuals and CD8⁺ as well as CD4⁺ peripheral blood T cells from HIV-infected persons are susceptible to Fas- and activation-induced apoptosis. Furthermore, this cell death correlates with disease progression and severity. In addition to direct CD8⁺ T cell death mediated by the death receptor Fas, CD4 cross-linking by Env interaction in uninfected CD4⁺ lymphocytes prior to TCR stimulation leads to the generation of FasL-expressing CD4⁺ T cells that can trigger CD8⁺ T cell apoptosis. In addition to Fas sensitivity, CD8⁺ T lymphocytes from HIV-infected patients are susceptible to proapoptotic signaling through both tumor necrosis factor receptor TNFR1 and TNFR2, and this is associated with expression of caspase- 8 and -3 and lack of physiological protection by Bcl- 2. IL-15 induces both Bcl-2 and Bcl-xL expression in HIV-specific and total CD8⁺ T cells, and this phenomenon is correlated with apoptosis inhibition and increased cell survival. Thus, reduced Bcl-2 and Bcl-xL

expression found in HIV-specific CD8+ T cells may play an important role in the increased sensitivity to apoptosis.

Fas/FasL Interaction¹⁰⁶: The Fas receptor is a member of the nerve growth factor and the tumour necrosis factor receptor superfamily, whose ability to induce has been well documented. Fas is normally expressed on the surface of many cell types, including T lymphocytes. The effect of HIV on expression of Fas has been evaluated both *in vitro* and *in vivo*. HIV infection of U937 promonocytic cell line results in an upregulation of surface Fas receptor. Similarly, Fas expression in T lymphocytes from HIV infected individuals is increased compared to uninfected controls. Elevated Fas expression correlates with enhanced susceptibility to Fas-mediated induction of apoptosis. The Fas receptor may be triggered both by its naturally occurring ligand (FasL) and by cross-linking antibody. Importantly, Fas stimulation and subsequent death does not induce viral replication, indicating that death is independent of viral replication. Whether the enhanced susceptibility to Fas-mediated apoptosis is a direct effect of HIV-mediated elevation of fas receptor expression or an indirect effect, related to HIV “activating” the cell and rendering it susceptible to apoptosis is not very clear. Evidence supporting the former view is

provided by the observation that Fas expression on CD4 + T cells from HIV-infected patients increases with disease progression.

Tumour Necrosis Factor: Tumor necrosis factor (TNF) and lymphotoxin (LT) are highly pleiotropic cytokines that play a central role in regulating HIV-1 replication. These cytokines express their activities through two membrane receptors, TNFR60 (p55-60) and TNFR80 (p75-80). In latently infected lymphocytic (ACH-2) cells the TNFR60 plays a dominant role in signaling HIV production, although selective activation of TNFR80 by receptor-specific antibodies can also induce HIV production. Unexpectedly, when both TNFRs are activated simultaneously by agonistic antibodies or co-culture with cells expressing a non-cleavable membrane form of TNF, HIV production is downregulated and induction of cell death was enhanced in ACH-2 cells. More relevant, in vitro HIV-infected peripheral blood lymphocytes co-cultured with cells expressing membrane TNF undergoes rapid induction of apoptosis with a subsequent reduced HIV production of these lymphocytes cultures. This is not observed with HIV-infected lymphocytes treated with soluble TNF. There is evidence for the differential trigger potential of membrane versus soluble TNF and show that TNFR80 is an important modulator of TNF responsiveness of HIV-infected T cells via cooperative signaling with

TNFR60. *in vitro* data indicate that (i) HIV infection of either lymphocyte or monocytes, results in TNF α production and (ii) TNF α , acting through NF κ B, activates HIV transcription, thereby creating an autocrine loop, resulting in production of high levels of TNF α and high levels of transcription at the single cell level¹⁰³.

Tuning the tide: TNF α ^{103,108,109,110,111}

When the body is invaded by a foreign organism, an immune response is activated which, in turn, is mediated by cytokines. Common signs of cytokine activity include elevated white count, fever, anorexia, prostration, increased metabolic rate, negative nitrogen balance, hypoalbuminemia, and lymphopenia. TNF- α has a preminent role in initiating the immune response. While it is normally beneficial to the host, in situations of overproduction, TNF- α , itself can kill the host. For example, excess acute levels of TNF- α have been associated with toxic shock syndrome, while chronic over-production is associated with inflammatory bowel disease, rheumatoid arthritis, and cirrhosis of the liver. TNF- α also induces oxidative stress by increasing the metabolic rate. Patients under TNF- α influence exhibit an increase in glycolysis, glycogenolysis, lipolysis, proteolysis, and a negative nitrogen balance. This results in the typical weight loss and wasting seen in AIDS. The reverse may also occur, with

oxidative stress inducing TNF- α , which should be considered when developing a therapeutic plan to prevent progression of HIV to AIDS. As stated above, TNF- α is responsible for an early essential step in the replication of the HIV virus; namely, NF- κ B induction. Further, TNF- α increase the toxic side-effects of the medications used to treat HIV, plays a role in AIDS dementia by its effect on the oligodendrocytes, and suppresses the function of B-cell lymphocytes. In the GI tract, TNF- α has been found in the macrophages in the lamina propria, and is therefore strongly implicated in accelerating replication and dissemination of the HIV virus following inflammation from mucosal adhesion of opportunistic enteropathogens.

At the cellular and subcellular levels, TNF α and other cytokines also increase nitric oxide (NO) production by polymorphonucleocytes (PMNs) and macrophages. NO, in turn, sets into motion cellular events resulting in mitochondrial production of H_2O_2 , inducing NF- κ B, which leads to an increase in the production of cytokines. These events, in turn, result in generation of reactive oxygen species which, if not quenched, increase oxidative stress.

The Irreversible Rust: Oxidative Stress^{112,113,114}

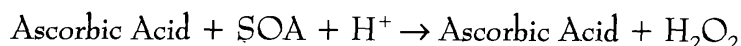
Oxidative stress (*Oxidative Stress: Disturbance of Prooxidant-Antioxidant balance in favour of the former, leading to potential damage*) has been indicated in many a disease process, including Diabetes, Atherosclerosis, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), HIV/AIDS etc.

O₂ is toxic: The problem with oxygen is that it simply oxidizes organic molecules, including foods, plastics, paints, hydrocarbon fuels, human tissues etc. However, the rates of these oxidations are very slow at normal temperatures. They can be increased by enzymes (such as those that catalyze oxidations of foodstuffs in aerobic cells) or by heat, as in combustion of hydrocarbon fuel. Antioxidant defenses may prevent these oxidations by removing catalysts, by being preferentially oxidized (thereby protecting key cell components), or by repairing the damage caused by oxygen.

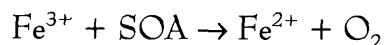
These reactions are carried out by free radicals. A free radical is any species capable of independent existence (hence the term 'free') that contains one or more unpaired electrons. These are highly reactive moieties. When two radicals meet, their unpaired electrons can join to form a pair, and both radicals are lost. However since most molecules

present in living organisms do not have unpaired electrons, any free radicals that are produced will react with non radicals generating new radicals. Hence, free radicals tend to proceed as chain reactions. This is the basic principle of free radical chemistry. Some important free radicals produced in living cells are:

Superoxide Radical (SOA): Superoxide is made by adding one electron on to an oxygen molecule. The added electron pairs with one of the two unpaired electrons already present in O_2 , leaving one unpaired electron. The chemical behavior of SOA differs greatly depending on what it is dissolved in. In water, SOA is not very reactive. It can sometimes act as a weak oxidizing agent, by accepting one more electron. For example, it can oxidize ascorbic acid (Vitamin C).



Superoxide in aqueous solution more often acts as a reducing agent.

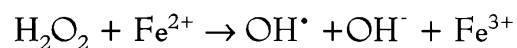


Upon donating its electron, SOA is oxidized and re-forms dioxygen (O_2). However, in organic solvents, SOA is far more reactive and dangerous. Thus any SOA generated in the interior of biological membranes might do considerable damage. In biological systems SOA is generally formed as

a result of oxidative burst reaction, or it may leak out of the electron transport chain during aerobic respiration.

Hydroxyl Radical: the hydroxyl radical is the most reactive oxygen radical known to chemistry. It has tremendous potential to cause biological damage, since it attacks all biological molecules as soon as it comes in contact with them, usually setting up free radical chain reactions.

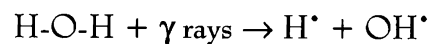
OH[•] can be generated either by the Fenton Reaction:



UV induced hemolytic fission of O-O bond in H₂O₂ can make OH[•], and this could possibly happen to H₂O₂ in sunlight exposed skin.



Ionizing radiation produces OH[•], by the virtue of homolytic fission of water.



These reactive oxygen species (ROS), along with some reactive nitrogen species, interact with many a biomolecule, disturbing their normal structure and hence, function.

SPECIES	COMMON NAME	SYSTEMATIC NAME
Reactive Oxygen Species (ROS)		
$\text{HO}^\bullet$	Hydroxyl Radical	Hydridooxygen
$\text{O}_2^{\bullet-}$	Superoxide Anion	Dioxide($\bullet 1^-$)
$\text{O}_3^{\bullet-}$	Ozonide	Trioxide($\bullet 1^-$)
$\text{O}_2^{2\bullet}$	Oxygen (O_2)	Dioxygen (triplet)
$\text{HO}_3^\bullet$	Hydrogen trioxide	Hydridotrioxxygen ($\bullet$)
Reactive Nitrogen Species (RNS)		
$\text{N}_3^\bullet$	Azidyl Radical	Trinitrogen (2N-N) ($\bullet$)
$\text{NO}^\bullet$	Nitric oxide	Oxidonitrogen
$\text{NO}_2^\bullet$	Nitrogen Dioxide	Dioxidonitrogen

Effects of Oxidation on Biomolecules:

Reactive oxygen species are produced as by products of normal metabolic processes in all aerobic organisms. In physiological conditions the antioxidant defense systems in the body protect the cells and tissues against these species. When the generation of ROS exceeds the ability of the antioxidant defense systems to remove them, such an imbalance can cause oxidative damage to cellular constituents (DNA, proteins, lipids and sugars). Many studies have shown that oxidative stress is responsible for a variety of degenerative processes of some human diseases.

Some of the damage and consequences caused by ROS on various biomolecules are as follows¹¹⁵.

Protein Carbonyls: Protein carbonyl groups are generated by direct oxidation of amino acid residues, particularly lysine, arginine, threonine and proline, or by secondary reaction with the primary oxidation product of Sugars and Lipids. These may alter the proteins structure and hence its function. Protein carbonyls are indicated in oxidative stress associated with neuro-degenerative illness, diabetes and hypercholesterolemia.

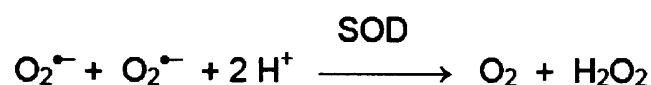
DNA: elevated levels of oxidatively modified DNA are considered responsible for an increased risk of cancer development. The most representative product that may reflect oxidative damage of DNA is 8-hydroxy-2'-deoxyguanosine (8-OHdG). Elevated levels of 8-OHdG have been reported in serum and myocardium of patients with heart failure and in urine of patients with Parkinson's.

Sugars: Advanced glycation end products (AGE's) are products of non enzymatic glycation of proteins by reducing sugars. It has been reported that AGE's accumulate in plasma and tissues with age, diabetes, renal failure, and Alzheimer's disease. Carboxymethyllysine (CML) and pentosidine are products of oxidation accompanied glycation and have been regarded as representative biomarkers of AGE's. in comparison with

healthy subjects, serum levels of CML and pentosidine are about 3 and 10-fold higher in diabetic patients with decreased renal function, respectively.

Lipids: Oxidative damage of lipids can especially have deleterious effects on cell membrane function as lipids form major part of the eukaryotic cell membrane. Various products of lipid peroxidation are indicated in many pathophysiological processes. These include, F_2 Isoprostanes—whose increased levels are indicated in cardiovascular diseases, 4-hydroxy-2-nonenal (4-HNE)— whose increased levels are indicated in atherosclerosis and liver injuries etc. Another important product of lipid peroxidation is Malondialdehyde (MDA). Malondialdehyde is a major oxidation products of peroxidized polyunsaturated fatty acids, has been shown to be of biological significance. MDA is able to form adducts with free amino acids and many more with proteins. MDA introduces cross-links in proteins which may induce profound alteration in their biochemical properties. It has also been proposed that MDA could react physiologically with several nucleosides (deoxy-guanosine, cytidine). Elevated levels of MDA are also indicated in diseases like Tuberculosis and HIV/AIDS.

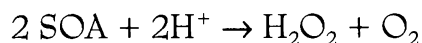
Antioxidant defense mechanisms: The human body has an elaborate antioxidant defense system. Antioxidants are manufactured within the body and can also be extracted from the food humans eat such as fruits, vegetables, seeds, nuts, meats, and oil. There are two lines of antioxidant defense within the cell. The first line, found in the fat-soluble cellular membrane, consists of vitamin E, beta-carotene, and coenzyme Q (10). Of these, vitamin E is considered the most potent chain breaking antioxidant within the membrane of the cell. Inside the cell water soluble antioxidant scavengers are present. These include vitamin C, Superoxide Dismutase (SOD, Glutathione Peroxidase (GPx) and Catalase (4). Superoxide Dismutase (SOD)^{112,113,116,117,118}: Superoxide dismutase (SOD) catalyzes the destruction of the $O_2^{\bullet -}$ free radical.



It protects oxygen-metabolizing cells against harmful effects of superoxide free-radicals. SOD protects hyaluronate against depolymerization by free-radicals and indicated that exogenous SOD might have an anti-inflammatory effect. The $O_2^{\bullet -}$ ion, which has been considered important in aging, lipid peroxidation and the peroxidative hemolysis of red blood cells), is formed by the univalent reduction of O_2 during various enzymatic

reactions or by ionizing radiation. There is also superoxide radical formation during leukocyte phagocytosis. It is indicated that SOD deficiency might lead to Heinz body hemolytic anemia. Depletion of SOD activity has also been reported in HIV/AIDS.

Three superoxide dismutases are characterized by different metal content. A blue-green Cu(II)-Zn(II) enzyme comes from human and bovine erythrocytes, a wine-red Mn(III) protein is found in *E. coli*, and in chicken, and rat liver mitochondria and a yellow Fe(III) enzyme from *E. coli*.



From the above literature we could see that oxidative stress is pronounced in HIV: Oxidative stress occurs following several different viral infections, and may enhance viral replication.

For example, HIV replication is enhanced under oxidative conditions in vitro. Reactive oxygen species activate a nuclear transcription factor, NF- κ B, that is obligatory for HIV replication. NF-KB also acts as a transcription factor for many inflammatory cytokines of the immune system. The effect of oxidants on viral replication has been confirmed by experiments in which both water and lipid soluble antioxidants inhibit replication in vitro. Antioxidants with demonstrated antiviral activity

include N-acetylcysteine (NAC), cysteine, glutathione and glutathione esters, ascorbate, lipoic acid, L-2-Oxothiazolidine-4-carboxylate (OTC), butylated hydroxyl anisole, and oltipraz. However, for unknown reasons, the activity of antioxidants differs across various cell culture systems. In addition, antioxidants appear to be weak antiviral (HIV) agents based on high concentrations employed in vitro. Overall, these studies provide evidence that oxidative stress is involved in viral replication.

We therefore try to evaluate oxidative stress in HIV/AIDS our study population. Also we shall try and investigate the role of apoptosis in oxidative stress induced cell death in our patients' PBMC's.

RATIONALE

Rationale

One mechanism whereby pro-apoptotic factors may potentate the induction of apoptosis in HIV/AIDS patients is by the increase in free radical levels, which in turn could lead to increased oxidative stress and enhanced viral replication in such stressed cells. Hence, understanding the role of oxidants (Superoxide Anion), antioxidants/cytoprotective enzymes (Superoxide Dismutase) and their correlation with pro-apoptotic members such as TNF α could be of great importance in determining the reasons behind significant T cell death especially via Apoptosis in HIV positive patients.

AIMS & OBJECTIVES

Aims & Objectives

- ❑ To confirm the HIV sero-status of the patients.
- ❑ To categorize HIV positive patients on the basis of their CD4 counts.
- ❑ To assess the host pro-inflammatory response by analysing the serum TNF α levels.
- ❑ To gauge the level of oxidative stress in the study cohort (patients + controls) by:
 - Analysing the production of Superoxide Anion in PBMC's by the NBT dye reduction test.
 - Analysing Lipid Peroxidation by measuring serum MDA levels.
- ❑ To study host antioxidant defence mechanism by estimating the activity of Superoxide Dismutase (SOD, EC 1.15.1.1)
- ❑ To correlate the oxidative stress to Cell Apoptosis in the PBMC's.

MATERIALS

8

The Study Population

The study population comprised of 57 HIV seropositive patients (referred hereafter as “patients”) either attending the OPD or admitted to Mathadi Kamgar Hospital, Koperkhairne and Jyothi’s Terminal Care Centre at Kalamboli. The control group comprised of 10 healthy individuals (referred hereafter as “controls”) from Haffkine Institute. Institutional Ethical Committee approval was taken for the project.

Samples were collected after informed written consent. All the individuals were subjected to a structured questionnaire, which included data on demographics and medical history. Presumptive diagnosis of sexually transmitted diseases and other opportunistic infections was based on the reports and medical case history records provided by the hospital.

Patients were grouped as per CDC classification:

Stage 1: Primary Infection and Acute Sero-Conversion Syndrome –

CD4+T cell counts of more than 500 cells/mm³

Stage 2: Symptomatic HIV disease – CD4+T cell count between 200

and 499 cells/mm³

Stage 3: AIDS Defining Condition – CD4 +T cell counts of less than

200 cells/mm³

Collection of Blood

Ten millilitres of blood was drawn by venipuncture from all the individuals. The blood was aliquoted appropriately in K3-EDTA and plain bulbs. The serum was separated from the plain bulb, aliquoted and stored at • 20°C. The whole blood from the EDTA bulb was used for evaluating CD4/CD8 counts and isolation of PBMC's.

Isolation of PBMC's

PBMC's were isolated from whole blood collected in EDTA bulbs by the Ficol Hypaque density gradient centrifugation. Ficol (M/s Sigma-Aldrich Inc.) was overlaid by equi-volume whole blood, which was then centrifuged for 15 min at 1500 rpm. The buffy layer containing the PBMC's was collected and cryo-presereved, using a freezing mixture containing RPMI 1640 (M/s. Gibco), 10% Foetal Calf Serum (FCS; M/s. Himedia, India) and 5% Dimethyl Sulfoxide (DMSO; M/s. SRL, India).

Confirmation of HIV Serostatus

Confirmation of the HIV serostatus for the patients as well as controls were done using HIV1 and HIV2 ELISA, following manufacturers'

instructions. (Sandwich ELISA SD HIV 1/2 ELISA, Ver.3, Standard Diagnostic Inc., and Indirect Elisa, Micro Lisa, M/s. J. Mitra and Co.)

CD4/CD8 Counts

Hundred microlitres of blood was drawn from K3-EDTA bulb and 50 μ l each was added to the reagent pair tubes for the estimation of CD4/CD3 and CD8/CD3. After incubation in the dark for 60–120 min, the fixative solution was added to these tubes. The cells were counted using FACScount (M/s. Becton Dickinson). The sorting and analysis of the cells was fully automated.

Analysis of Cell Apoptosis – DNA Fragmentation

DNA fragmentation is a hallmark of end-stage apoptosis. DNA from cryopreserved PBMC's was extracted using the TRI Reagent (M/s. Sigma-Aldrich Inc.) as per manufacturer's instructions. The cells were counted on a Haemocytometer. 5×10^6 cells were used for the extraction of DNA. The extracted DNA was run on 2% agarose gel prepared in Tris Borate EDTA buffer (TBE), pH 8.0 and stained using Ethidium Bromide. The gel was run in TBE buffer pH 8.0 at 80 V, till the dye ran

more than $\frac{3}{4}$ of the gel length (45–60 minutes). The bands were observed using an AAB gel documentation system.

Estimation of serum TNF α

Estimation of TNF α in serum was performed using commercially available PeliKine human TNF α ELISA kit. TNF α serves as an endogenous mediator of inflammatory, immune and host defence functions. Macrophages and monocytes are thought to be the cells that contribute most to the local and systemic TNF α response to bacterial, viral and parasitic organisms and their products.

Principle: The assay was a "sandwich-type" of enzyme immunoassay in which a monoclonal anti-human TNF α antibody is bound onto polystyrene microtiter wells. Human TNF α , present in a measured volume of sample or standard is captured by the antibody on the microtiter plate. Subsequently, a biotinylated secondary monoclonal antibody to human TNF α is added. The next step is addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biotinylated side of the TNF α sandwich. A substrate solution is then added to the wells. This forms a coloured product in proportion to the amount of TNF α present in the sample or standard.

Determination of Superoxide Anion¹¹⁹

The Superoxide Anion (SOA) is produced by activated monocytes and macrophages. It can work both as a reducing as well as oxidizing agent.

Principle: Superoxide Anion reduces Nitro Blue Tetrazolium (NBT) to an insoluble blue formazan, which can be subsequently re-dissolved and assayed calorimetrically.

Procedure: To 300 μL of patients' PBMC's in RPMI 1640 (Cell Count = 1×10^6 cells), 300 μL of 1 mg/mL NBT solution was added in eppendorf tubes. PBMC's from healthy subjects were used as control. The cells were incubated overnight. Following incubation, the tubes were centrifuged at 1500 rpm for 15 min and the supernatant was discarded. The cell pellet was fixed with 100% Methanol. This was followed by 3 washings with 70% Methanol, to remove excess media. The cells were then treated with 120 μL of 2 N Potassium Hydroxide and 140 μL of DMSO. The tubes were centrifuged at 3000 rpm for 10 min. The supernatant was collected and the absorbance was measured at 650 nm on a Nanodrop ND-1000 UV/Vis Spectrophotometer. Variations in the SOA production in patients and controls were measured as the relative difference in their O/D's.

*Estimation of Serum Malondialdehyde Levels*¹²⁰

Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation.

Principle: MDA reacts with Thiobarbituric Acid (TBA) under acidic conditions, to form an adduct, which can be measured fluorimetrically.

Procedure: Fifty microlitres of serum sample/standard was added in amber glass vials containing 250 μL of 1.22 M Orthophosphoric acid, 450 μL Distilled water and 250 μL of TBA reagent.

Tetramethoxypropane (TMP) was used as the standard as it yields equimolar amounts of MDA under the conditions of the assay. The mixture was incubated at 95°C in a water bath for 60 min. The samples were then cooled on ice followed by addition of 360 μL Methanol and 40 μL of 1 M NaOH to neutralize the sample. Readings were taken on a Nanodrop ND-3300 Fluorospectrometer using white light as excitation source and the emission wavelength set at 553 nm. The Standard curve, of TMP concentration versus Relative Fluorescence Unit (RFU) was plotted, using which, concentration of MDA in serum samples was calculated.

Determination of Superoxide Dismutase Activity¹²¹

Principle: Superoxide Dismutase activity was carried out by the method described by Marklund & Marklund. Pyrogallol (1,2,3-benzene triol) auto-oxidizes very rapidly, especially in alkaline solution. This auto-oxidation primarily involves free radical change by the Superoxide Anion Radical. Superoxide Dismutase univalently reduces Superoxide Anion radical.

Procedure: The assay system consisted of 4 mM Pyrogallol and Tris Cacodylate Buffer (pH = 8.2). The buffer was made up of 62.5 mM Sodium Cacodylic Acid and 1.25 mM Diethylenetriamine penta acetic acid (DTPA). 1 M Tris was used to adjust the pH. 4 mM Pyrogallol prepared in 100 mM HCl was used. The assay was set directly in disposable cuvettes as per the following table.

REAGENTS	BLANK CUVETTE	PYROGALLOL CUVETTE	TEST CUVETTE
TCB*	2 mL	2 mL	2 mL
SAMPLE	—	—	250 µL
D/W	500 µL	250 µL	—
PYROGALLOL	—	250 µL	250 µL

*TCB: Tris Cacodylate Buffer

The reagents were added in successive order. Reaction was started by the addition of pyrogallol at the end. The solutions were mixed well with a

plastic paddle. The rate of pyrogallol auto-oxidation was measured as the incremental difference in A_{420} ($\Delta O/D$) for 1 min on a Jasco V-530 UV/Vis. Spectrophotometer. Enzyme activity was calculated as Units/mL of Serum sample. One Unit of enzyme corresponds to 50% inhibition of the $\Delta O/D$.

RESULTS

The study population consisted of 57 HIV positive individuals (29 Females and 28 Males) within the age group of 22–48 years. Ten HIV negative individuals (8 Males and 2 Females) within the age group of 22–29 years were included as controls. The HIV positive population was grouped into Asymptomatic, Symptomatic and AIDS groups as per the Center for Disease Control (CDC) Classification²⁴.

The statistical analysis was done on SPSS software.

Demography

Table 1 compiles the gender wise distribution of the Study Population.

Immunophenotyping

The CD4+ T cell counts of the entire study cohort are documented in Table 2.¹ The healthy control population had a mean CD+ cell count of 822 cells/mm³ (SD $\pm$ 165.69) [Range: 569–1109], the asymptomatic population had a mean CD+ cell count of 632.70 cells/mm³ (SD $\pm$ 100.05) [Range: 521–837], the symptomatic group had a mean CD+ cell count of 367.96 cells/mm³ (SD $\pm$ 92.18) [Range: 208–494] while the AIDS cohort had a mean CD+ cell count of 103.32 cells/mm³ (SD $\pm$ 56.46) [Range: 7–192].

TABLE 1: DISTRIBUTION OF STUDY GROUP ON THE BASIS OF GENDER

STUDY GROUP	GENDER	
	FEMALE	MALE
STAGE 1 (Asymptomatic) n = 10	8 (80.0%)	2 (20.0%)
STAGE 2 (Symptomatic) n = 25	15 (60.0%)	10 (40.0%)
STAGE 3 (AIDS) n = 22	6 (27.3%)	16 (72.70%)
CONTROL n = 10	2 (20.0%)	8 (80.0%)

Figures in parenthesis indicate percentages in specific groups

TABLE 2: CD4 COUNTS in Cells/mm³

STAGE	N	MEAN	SD (±)	RANGE	
				MIN.	MAX.
ASYMPTOMATIC	10	632.70	100.05	521	837
SYMPTOMATIC	25	367.96	92.18	208	494
AIDS	22	103.32	56.46	7	192
CONTROL	10	822.00	165.69	569	1109

Apoptosis

Apoptosis was demonstrated by fragmentation of Genomic DNA in the PBMC's. Thirty-Two (47.8%) of the samples showed DNA fragmentation. Two samples from the asymptomatic phase (20% of the total asymptomatic population) showed DNA fragmentation, while Twelve samples from the asymptomatic phase (48% of the total symptomatic phase) showed apoptosis. Eighteen of the Twenty-Two AIDS samples showed apoptosis (81.8% of the total AIDS population), while there were no detectable DNA fragmentations in the control population.

Table 3: Distribution of study group on the basis of Apoptosis

STUDY GROUP	APOPTOSIS
STAGE 1 (Asymptomatic) n = 10	2 (20.0%)
STAGE 2 (Symptomatic) n = 25	12 (48.0%)
STAGE 3 (AIDS) n = 22	18 (81.8%)
CONTROL n = 10	0 (0.0%)

Figures in parenthesis indicate percentages in specific groups

Figure 1: DNA Fragmentation Assay

From Left to Right: Well 1 & 2: Standard DNA Ladder
Well 5 & 7: Fragmentation of DNA (DNA extracted from Stage 3 patients)
Well 6: Non-fragmented DNA (DNA extracted from Stage 1 patient)

Biochemical Parameters

Biochemical parameters were studied in Controls and Patient population.

These included the pro-inflammatory molecule, TNF α , Superoxide Anion: an ROS and Malondialdehyde (MDA), product of lipid peroxidation, and the antioxidant enzyme Superoxide Dismutase (SOD).

The Statistical Test used was Multiple Comparison Test with Least Significance Difference.

The following tables summarise the comparative results of the above parameters.

The mean TNF α levels in Stage 1, Stage 2 and Stage 3 were **111.78 pg/mL** [SD $\pm$ 23.30; Range 87.54–163.28], **148.15 pg/mL** [SD $\pm$ 33.83; Range 92.57–223.14] and **229.20 pg/mL** [SD $\pm$ 109.88; Range 186.20–580.89] respectively. These levels were found to be significantly elevated in Stage 1 patients ($p < 0.002$) and in Stage 2 as well as Stage 3 patients ($p < 0.001$) when compared to the normal controls (Mean = **8.88 pg/mL**; [SD $\pm$ 1.16; Range 7.00–11.00]).

TABLE 4: TNF α in pg/mL

STAGE	MEAN	SD ($\pm$)	RANGE	
			MIN.	MAX.
STAGE 1 (Asymptomatic) n = 10	111.78	23.30	87.54	163.28
STAGE 2 (Symptomatic) n = 25	148.15	33.83	92.57	223.14
STAGE 3 (AIDS) n = 22	299.20	109.88	186.20	580.89
CONTROL n = 10	8.88	1.16	7.00	11.00

TABLE 5: MULTIPLE COMPARISONS FOR TNF α

STAGE	MD	SE	SIGNIFICANCE
STAGE 1: (Asymptomatic) n = 10	• 102.90	30.13	<0.002
STAGE 2: (Symptomatic) n = 25	• 139.27	25.21	<0.001
STAGE 3: (AIDS) n = 22	• 290.30	25.69	<0.001

The Mean Difference (MD) is significant at 0.05 level

Mean SOA levels in Stage 1, Stage 2 and Stage 3 patients were **0.227 O/D units** [SD $\pm$ 0.105; Range 0.040–0.317], **0.341 O/D units** [SD $\pm$ 0.046; Range 0.260–0.437] and **0.431 O/D units** [SD $\pm$ 0.047; Range 0.337–0.499] respectively. These levels were found to be significantly elevated ($p < 0.001$) in Stage 1, Stage 2 as well as Stage 3 patients when compared to the normal controls (Mean O/D = **0.114 O/D units**; [SD $\pm$ 0.040; Range 0.063–0.194])

TABLE 6: SOA in O/D units

STAGE	MEAN	SD ($\pm$)	RANGE	
			MIN.	MAX.
STAGE 1 (Asymptomatic) n = 10	0.227	0.105	0.040	0.317
STAGE 2 (Symptomatic) n = 25	0.341	0.046	0.260	0.437
STAGE 3 (AIDS) n = 22	0.431	0.047	0.337	0.499
CONTROL n = 10	0.114	0.040	0.063	0.194

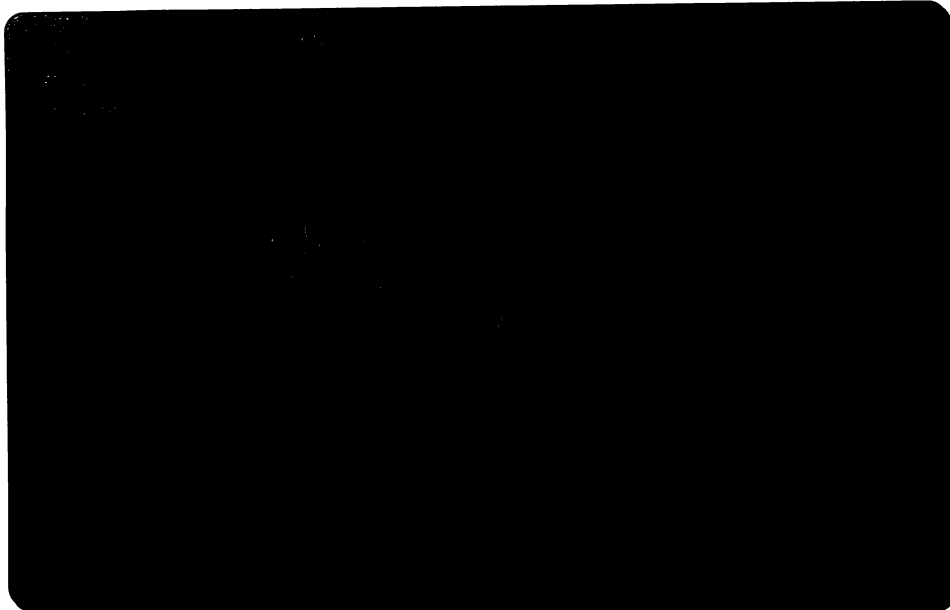
TABLE 7: MULTIPLE COMPARISONS FOR SOA

STAGE	MD	SE	SIGNIFICANCE
STAGE 1: (Asymptomatic) n = 10	• 0.11	0.03	<0.001
STAGE 2: (Symptomatic) n = 25	• 0.23	0.02	<0.001
STAGE 3: (AIDS) n = 22	• 0.32	0.02	<0.001

The Mean Difference (MD) is significant at 0.05 level

There was significant positive correlation between the CD4 counts and the Intracellular SOA levels ($p < 0.001$) with a slope of $\bullet 0.0004$. [Graph 1]

Graph 1: CD4 V/s Intracellular SOA



Mean Serum Malondialdehyde in Stage 1, Stage 2 and Stage 3 patients were **6.26 nM/mL** [SD ± 0.937 , Range 5.28–7.87], **7.39 nM/mL** [SD ± 0.980 ; Range 5.32–8.98] and **8.84 nM/mL** [SD ± 0.409 ; Range 8.13–9.58] respectively. The levels were found to be significantly elevated ($p < 0.001$) in Stage 1, Stage 2 as well as Stage 3 patients when compared to the normal controls (Mean = **3.55 nM/mL** [SD ± 0.166 ; Range 2.43–4.13]).

TABLE 8: MDA in nM/mL

STAGE	MEAN	SD ($\pm$)	RANGE	
			MIN.	MAX.
STAGE 1 (Asymptomatic) N = 10	6.26	0.937	5.28	7.87
STAGE 2 (Symptomatic) N = 25	7.39	0.980	5.32	8.98
STAGE 3 (AIDS) N = 22	8.84	0.409	8.13	9.58
CONTROL N= 10	3.55	0.166	2.43	4.13

TABLE 9: MULTIPLE COMPARISONS FOR MDA

STAGE	MD	SE	SIGNIFICANCE
STAGE 1: (Asymptomatic) n = 10	• 2.71	0.34	<0.001
STAGE 2: (Symptomatic) n = 25	• 3.84	0.29	<0.001
STAGE 3: (AIDS) n = 22	• 5.29	0.29	<0.001

The Mean Difference (MD) is significant at 0.05 level

The Mean Serum Superoxide Dismutase Enzyme activity in Stage 1, Stage 2 and Stage 3 patients were 3.073 U/mL [SD $\pm$ 1.504; Range 0.727–5.090], 2.411 U/mL [SD $\pm$ 0.905; Range 0.533–3.20] and 1.600 U/mL [SD $\pm$ 0.698; Range 0.533–3.20] respectively. These were found to be significantly decreased ($p < 0.001$) in Stage 2 and Stage 3 patients when compared to the normal controls (Mean = 3.709 U/mL

[SD $\pm$ 1.209; Range 2.181–5.818]). However patients the Stage 1 showed non-significantly decreased activity of the enzyme.

TABLE 10: SOD in U/mL

STAGE	MEAN	SD ($\pm$)	RANGE	
			MIN.	MAX.
STAGE 1 (Asymptomatic) N = 10	3.273	1.504	0.727	5.090
STAGE 2 (Symptomatic) N = 25	2.411	0.925	0.533	3.733
STAGE 3 (AIDS) N = 22	1.600	0.698	0.533	3.20
CONTROL N= 10	3.709	1.209	2.181	5.818

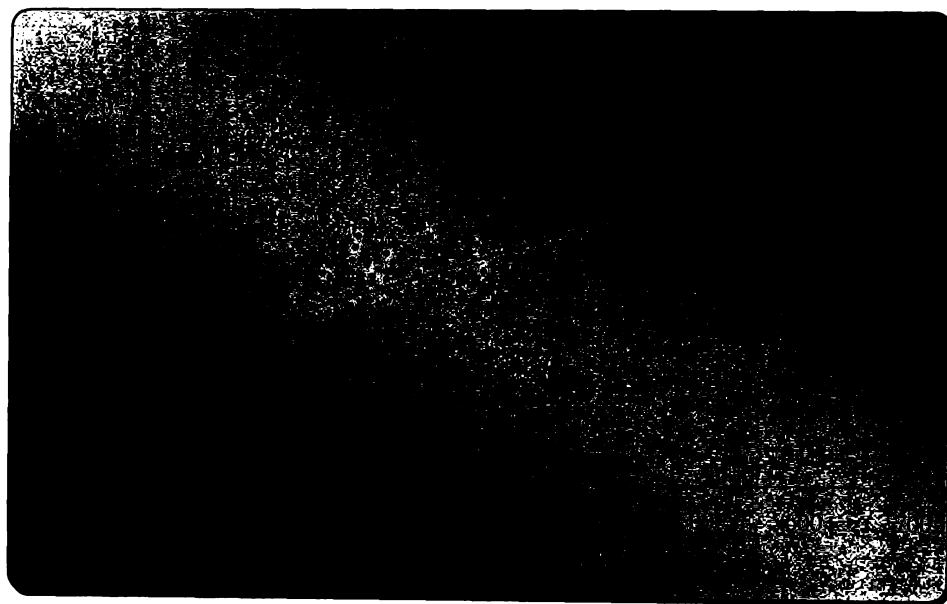
TABLE 11: MULTIPLE COMPARISONS FOR SOD

STAGE	MD	SE	SIGNIFICANCE
STAGE 1: (Asymptomatic) n = 10	-0.436	0.475	0.3830
STAGE 2: (Symptomatic) n = 25	-1.298	0.185	<0.0001
STAGE 3: (AIDS) n = 22	• 2.109	0.149	<0.0001

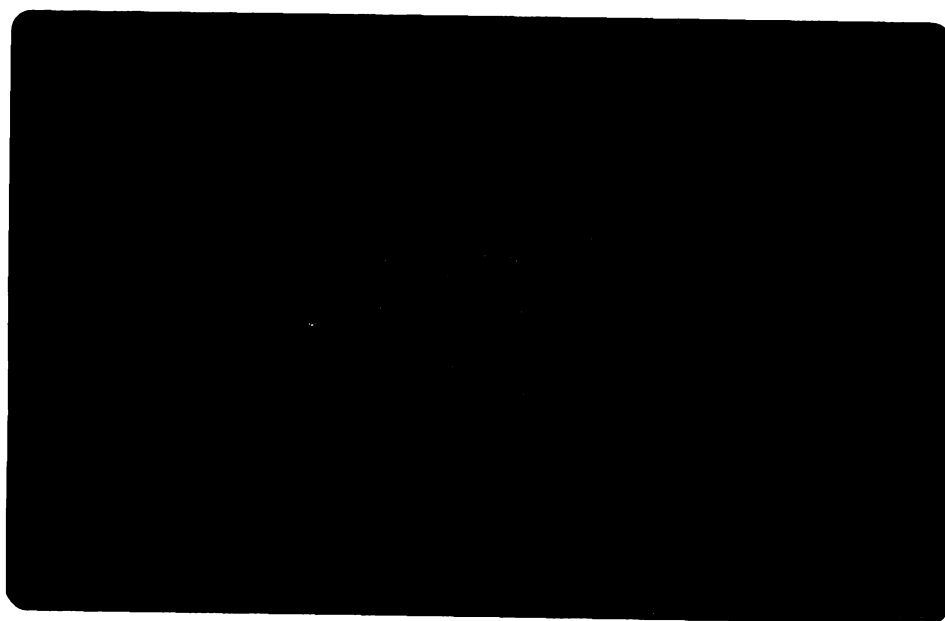
The Mean Difference (MD) is significant at 0.05 level

The pro-inflammatory cytokine, TNF α levels were compared against the intracellular Superoxide Anion production. It showed a significant positive correlation ($p < 0.0001$) with a slope of 0.0006 [Graph 2]

Graph 2: Serum TNF α V/s Intracellular Superoxide Anion



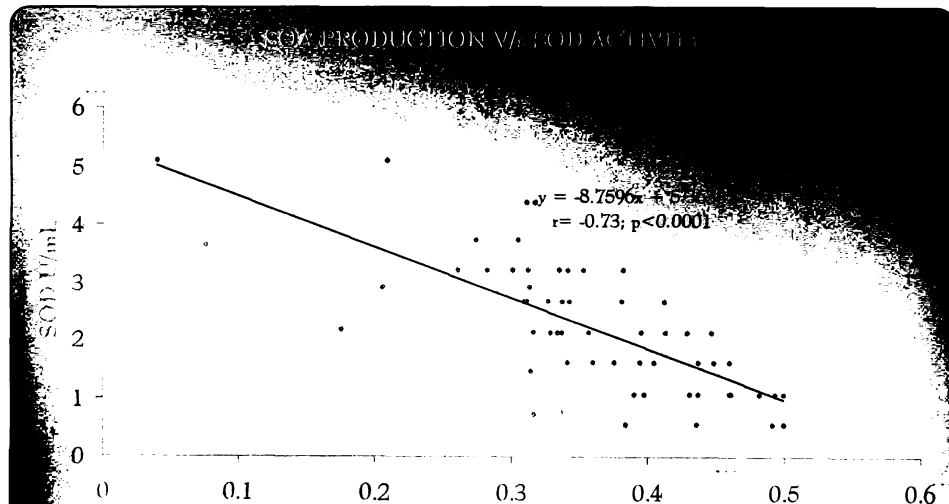
Graph 3: Intracellular Superoxide Anion V/s Serum MDA



When the intracellular Superoxide Anion levels were compared against the serum MDA levels, it showed a significant positive correlation ($p < 0.0001$) with a slope equal to 10.102 [Graph 3]

When Intracellular SOA levels was compared against Serum SOD activity; there was a significant negative correlation observed, with a slope of -8.759 [Graph 4]

Graph 4: Intracellular Superoxide Anion V/s Serum SOD



A compilation of the results for the biochemical parameters is shown in

Table 13

Table 13: Biochemical Parameters

Parameter		Control n=10	Patients		
			Stage 1 n=10	Stage2 n=25	Stage 3 n=22
TNF α	Mean pg/mL	8.88	111.78	148.15	299.20
	Range pg/mL	7.00–11.00	87.54–163.28	92.57–223.14	186.20–580.89
	SD ($\pm$) pg/mL	1.16	23.30	33.83	109.88
	p [wrt Control]	—	<0.002*	<0.001*	<0.001*
SOA	Mean O/D units	0.114	0.227	0.341	0.431
	Range O/D units	0.063–0.194	0.040–0.317	0.260–0.437	0.337–0.499
	SD ($\pm$) O/D units	0.040	0.105	0.046	0.047
	p [wrt Control]	—	<0.001*	<0.001*	<0.001*
MDA	Mean nM/mL	3.55	6.26	7.39	8.84
	Range nM/mL	2.43–4.13	5.28–7.87	5.32–8.98	8.13–9.58
	SD ($\pm$) nM/mL	0.166	0.937	0.980	0.409
	p [wrt Control]	—	<0.001*	<0.001*	<0.001*
SOD	Mean U/mL	3.709	3.273	2.411	1.600
	Range U/mL	2.181–5.818	0.707–5.090	0.533–3.733	0.533–3.20
	SD ($\pm$) U/mL	1.209	1.504	0.925	0.698
	p [wrt Control]	—	0.3830	<0.0001*	<0.0001*

*: statistically significant

Opportunistic Infections

The three major clinical presentations were Tuberculosis (TB), Candidiasis and Diarrhoea. These are represented as CP-1, CP-2 and CP-3 respectively.

Twenty-Five (43.9%) of the 57 HIV positive individuals had TB. Of these, 14 belonged to the symptomatic phase while 11 belonged to the AIDS group. No individual in the asymptomatic phase had TB.

Thirty-Two (56.1%) of the 57 HIV positive individuals had Candidiasis. Of these, 4 belonged to the asymptomatic phase while 10 belonged to the symptomatic phase. Eighteen individuals in the AIDS Group had Candidiasis.

Thirty-One (54.4%) of the 57 HIV positive individuals had Diarrhoea. Of these, 1 belonged to the asymptomatic phase while 11 belonged to the symptomatic phase. Nineteen individuals in the AIDS Group had Diarrhoea.

Table 14: Prevalence of OI's in Patients

STUDY GROUP	CP-1	CP-2	CP-3
STAGE 1 (Asymptomatic) n= 10	0 (0%)	4 (40%)	1 (10%)
STAGE 2 (Symptomatic) n= 25	14 (56%)	10 (40%)	11 (44%)
STAGE 3 (AIDS) n= 22	11 (50.0%)	18 (81.8%)	19 (86.4%)

Figures in parenthesis indicate percentages in specific groups

The above results are examined in the discussion.

DISCUSSION

With over 33.2 million people presently living in the world with HIV, and more adding to the list every passing minute, HIV/AIDS is arguably one of the worst medical disasters of our times. The focal point of the entire episode, the Human Immunodeficiency Virus belongs to the *Retroviridae* family of viruses. These RNA viruses have the unique ability to synthesize DNA from their genomic RNA's, thus reversing the Central Dogma of Molecular Biology—hence the name.

HIV belongs to the family *Lentivirus*, (Slow Viruses) demonstrating typical long periods of latency prior to causing symptomatic disease. HIV targets cells of the immune system and thus, the disease is characterized as immunodeficiency. Two types of HIV have been identified, HIV-1 and HIV-2. These cause clinically indistinguishable disease although the time to disease onset is longer for HIV-2. The worldwide epidemic of HIV and AIDS is caused by HIV-1 while HIV-2 is mostly restricted to West Africa.

The devastating effect that HIV has on the immune system is a result of infecting and killing the immune-regulating Helper T (T_H) lymphocytes. HIV infects and replicates primarily in activated CD4⁺ T lymphocytes and to a lesser extent in macrophages and dendritic cells. Therefore the Centre for Disease Control & Prevention's (CDC) current classification

system, revised in 1993, combines three categories of the CD4 count with three symptom categories to stage HIV positive patients. The definitions of the three CD4+ T lymphocyte categories include Stage 1: > 500 cells/mm³, Stage 2: 200–499 cells/mm³ and Stage 3: <200 cells/mm³. In the present study, patients were classified on the basis of absolute CD4 counts. The pathological indications (Cellular, Biochemical and Molecular) showed a good correlation with this system. However, as has been observed in the past, it must be noted that the CD4 counts are affected by the geographical location, race, ethnic origin, age, gender and changes in total and differential leucocyte counts.

The cell specificity of HIV is due to its envelope (Env) glycoprotein 120 (gp120) binding CD4, along with chemokine receptors CXCR4 or CCR5, for cell entry. Once in the cytoplasm the viral RNA is reverse-transcribed into DNA and randomly integrated into the host cell genome. Activation of the host cell enhances the production of viral proteins, which assemble upon budding out of the cell, utilizing the plasma membrane as the viral envelope.

There are several mechanisms by which HIV can induce cell death directly, in the cell it infects. One of the mechanisms by which T_H cells

are destroyed during the HIV disease is Programmed Cell Death or Apoptosis.

Various agents have been reported to trigger apoptosis in CD4+ T cells of HIV positive individuals. These include viral proteins (i.e. gp120, Tat), inappropriate secretion of inflammatory cytokines by activated macrophages (i.e. Tumor Necrosis Factor α) and products of opportunistic micro-organisms. Kaplan and Sieg¹⁴⁰, Finkel et al¹⁴¹., Debatin et al¹⁴². amongst others have reported apoptosis in PBMC's from HIV infected individuals. Apoptosis has also been indicated as a mode of neuronal cell death in HIV patients, *in vitro* and *in vivo* in the past. AIDS related dementias are ascribed to such events. In the present investigation, DNA fragmentation in PBMC's of the study population (Patients + Controls) was used as an indicator of apoptosis. Wherein 2 out of 10 patient samples (20%) and 12 out of 25 samples (48%) and showed DNA fragmentation, indicative of apoptosis, in Stage 1 (asymptomatic) and Stage 2 (symptomatic) groups respectively, 18 out of 22 samples (81.8%) of Stage 3 (AIDS) patients exhibited DNA fragmentation. None of the subjects from the control group exhibited any DNA fragmentation.

This possibly means that apoptosis in PBMC's may be contributing in a major way, to the loss of CD+ T lymphocytes in the patient group.

TNF α is a pro-inflammatory cytokine, whose roles in various pathophysiological conditions, including apoptosis induction associated with HIV/AIDS are well documented by Badley et.al¹⁴³ and Algecira et.al¹⁴⁴ amongst others. It is documented by Schreck et.al¹¹¹ that several of the AIDS associated pathophysiological changes observed during late stages of the disease, e.g. Wasting, are correlated with and could be due to chronically elevated TNF α levels. Increased levels of serum TNF α in HIV/AIDS have been illustrated earlier by Matsuyama et al. amongst others. We found elevated levels of serum TNF α in the patients from Stage 1 (asymptomatic), Stage 2 (symptomatic) and Stage 3 (AIDS) group. The mean serum TNF α levels in the three groups were 111.78 pg/mL, 148.15 pg/mL and 299.20 pg/mL respectively. These values increase progressively with the disease progression and are significantly higher ($p < 0.002$ for Stage1 (asymptomatic) and $p < 0.001$ for Stage 2 (symptomatic) and Stage 3 (AIDS) groups) than the serum TNF α levels of the control group (Mean = 8.88 pg/mL).

Apart from inducing apoptosis, TNF α has also been indicated to augment replication of the HIV proviral DNA. With respect to TNF-

mediated enhancement of virus production or induction of latent HIV, the critical role of NF- κ B in this process has been shown for T lymphocytes and monocytes/macrophages as well as for neuronal cells by Matsuyama et al. Schreck et al. have reported in the past that high levels of TNF α also lead to Oxidative Stress, which might again encourage HIV production via NF- κ B^{110,111}. Moreover, the role of Oxidative Stress has been pointed out in many pathophysiological conditions associated with HIV/AIDS. Kameoka et al. have demonstrated significant amplification of Superoxide generation in cells of mononuclear phagocytic lineage by HIV-1 infection. Moreover, role of elevated SOA has been pointed out in the cell to cell transmission of HIV in cultured human CD4+ cell lines¹²².

In the present study, a significant increase in the production of the Superoxide Anion has been found in the HIV positive population ($p < 0.001$) when compared to the control group. It is seen that these SOA levels show a significant negative correlation with the CD4 counts—indicators of disease progression ($r = -0.81$, $p < 0.0001$). In addition the SOA values exhibit a strong positive correlation with the serum TNF α values ($r = 0.72$, $p < 0.0001$). Therefore it may be inferred that increased

levels of TNF α and SOA are enhancing disease progression in the patients.

Molecules like SOA directly or indirectly, damage many an important biomolecule. One such significant event is lipid peroxidation. Lipid peroxidation products such as Malondialdehyde (MDA), 4-hydroxy-2-nonenal (4HNE), F₂-Isoprostanes etc. have been associated with cell and tissue damage and are indicated in conditions like atherosclerosis, hepatorenal syndrome, *H. pylori* infections¹²⁴ etc. MDA in particular has been found to be mutagenic and forms DNA adducts. Elevated levels of MDA have been indicated in HIV and Tuberculosis by Madebo et al¹²³. In the present study, serum MDA levels of HIV positive individuals in all three groups were significantly elevated ($p < 0.001$) as compared to HIV negative population. The MDA levels were also shown to have a significant positive correlation with the SOA production ($r = 0.77$, $p < 0.0001$).

The antioxidant defence enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx) etc, protect the cells and tissues from the detrimental effects of the pro-oxidants. SOD scavenges SOA via a dismutation reaction leading to the production of Hydrogen Peroxide. Previous studies have reported depressed levels of SOD in

peripheral blood cells isolated from children infected with HIV^{125,120}. The HIV Tat protein has been shown to down-regulate synthesis and override the induction of the manganese-dependent form of SOD, which is induced by oxidative stress in cultured cells. When we estimated serum SOD activity of our samples, we found that the serum SOD activity was significantly lower in the Stage 2 (symptomatic) and Stage 3 (AIDS) groups ($p < 0.0001$) when compared with the healthy controls. However no significant reduction was observed in the Stage 1 (asymptomatic) cohort.

These results are evidently indicative of Oxidative Stress in the HIV positive study population. However, apart from the HIV-host interactions, Opportunistic Infections (OIs) also have a considerable role to play in the pathogenesis and hence the prognosis of HIV disease.

A number of bacterial, viral, fungal and protozoan agents are seen as OIs during various stages of HIV/AIDS. These contribute considerably to the morbidity and mortalities associated with HIV/AIDS. Many of the OIs are seen to be aiding in the HIV disease progression. In our study we included Tuberculosis (TB), Candidiasis and Diarrhoea. Increasing incidences of TB, Candidiasis and Diarrhoea were seen in patients with disease progression. While there were no incidences of TB in the Stage 1

(asymptomatic) population, 56% of the Stage 2 (symptomatic) and 50% of the patients in the Stage 3 (AIDS) group were suffering from TB. Many protein and non-protein antigens of *M. tuberculosis* have been shown to induce production of TNF α by Valone et al¹²⁷. This enhanced TNF α activity during dual HIV-TB infection may contribute to the augmentation of viral replication and dissemination as has been shown by Janes et al.¹²⁸ have HIV infection leads to a state of anergy in large number of persons with HIV infection, thus confounding tuberculin skin test (TST) interpretation, leading to poor diagnosis of TB in HIV infections.

In this study, occurrence of Candidiasis was seen in all three stages of the infection. Forty percent of the patients in Stage 1 (asymptomatic) and Stage 2 (symptomatic) population were suffering from Candidiasis, whereas 81.8% of Stage 3 (AIDS) patients had oral thrush, indicative of Candidiasis. Candida antigens have been shown to upregulate CD4 receptors on monocytes. This may explain the association of AIDS and Candida infection.

Diarrhoea has been a common problem in patients with HIV. Symptoms arise from a range of pathogens including protozoal, viral or bacterial as well as from the interplay of cytokines (TNF α) in the GI tract as shown

by McGowan et al¹²⁹. Although the exact cause was not determined, in this study, diarrhoea was observed in 10% of the Stage 1 (asymptomatic) population, 44% of the Stage 2 (symptomatic) population and 86.4% of the Stage 3 (AIDS) cohort.

The above results underline the significant contribution of oxidative stress in accentuating the HIV disease, especially causing a decline in the CD4 cell population by inducing apoptosis in the infected cell population. The currently available Highly Active Anti-retroviral Therapy (HAART) has their own side effects; these include subtle symptoms like fatigue, headache and nightmares to more pronounced consequences like anemia, peripheral neuropathy, retinoid toxicity, hypersensitivity, lactic acidosis etc¹³⁰.

In the absence of a therapeutic vaccine or a curative pharmacological agent, and given the side effects of current long term therapies like Highly Active Anti-retroviral Therapy (HAART), an alternative way of managing the epidemic must be considered seriously. Some laboratories have demonstrated the role of antioxidants in slowing the progression HIV disease. *Curcuma longa*,¹³¹⁻¹³³ N-acetylcysteine,^{134,135} α -lipoic acid,¹³⁶ omega-3 fatty acids,¹³⁷ L-carnitine,¹³⁸ and the vitamin E ester, α tocopheryl succinate have been shown to inhibit TNF α and NF κ B. Also

many studies including the one by Allard et al¹³⁰. have underlined the importance of antioxidants in diets, as a useful contributor to reduce oxidative stress and hence a have better prognosis in the HIV disease.

Taking a cue from these studies, development of a defined antioxidant and specific anti-apoptotic regime in HIV becomes imperative. With the growing number of problems associated with the current treatment protocols (drug affordability due to high pricing, drug resistant organisms, side effects etc.) it is only rational that we critically consider the alternatives until the magic molecule is found...

SUMMARY & CONCLUSION

The present study was designed to evaluate the interplay of oxidative stress in apoptosis induced CD4 T cell death, in HIV/AIDS patients. On evaluating 57 HIV +ve patients for various cellular, biochemical and molecular parameters, the following conclusions are drawn:

- Marked oxidative stress was observed in all three stages of HIV infection.
- Intracellular production of Superoxide Anion was significantly higher than the control group in all three groups of patients ($p < 0.001$)
- Serum Malondialdehyde levels (Lipid Peroxidation Product), were also significantly higher than the control group in all three groups of patients ($p < 0.001$)
- The pro-inflammatory cytokine TNF α levels were significantly higher in Asymptomatic Group ($p < 0.002$), and Symptomatic and AIDS group ($p < 0.001$), when compared to the control group.
- In contrast, serum activity of the anti-oxidant enzyme Superoxide Dismutase (SOD) [E.C 1.15.1.1] was significantly lower in Symptomatic and AIDS group ($p < 0.0001$). However, no significant reduction in the enzyme activity was seen in the Asymptomatic group when compared with the control group.

- DNA fragmentation (Hallmark of Apoptosis) of the PBMC's was observed in 20% of Asymptomatic patients. Whereas 48% of Symptomatic and 81.8% of AIDS patients demonstrated DNA fragmentation.
- Incidents of three important clinical manifestations namely – Tuberculosis, Candidiasis and Diarrhoea were pronouncedly increasing with the stages of disease progression.

Hence it can be concluded that Oxidative Stress is an important factor in the progression of HIV disease. Apoptosis, seen prominently in the PBMC's of HIV +ve individuals with decreasing CD4 counts may contribute to CD4 T cell destruction in HIV/AIDS.

APPENDIX

Reagents for Running Agarose Gel

1 M Tris -Cl (pH 7.4)

Final Volume: 10 mL; made up with Distilled Water

Tris -Cl	1.75 gm
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0.5M EDTA

Final Volume: 10 mL; made up with Distilled Water

EDTA	1.86 gm
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Tris Borate EDTA (TBE) Buffer

Final Volume: 1000 mL; made up with Distilled Water

Strength 5X

Tris Base	54 gm
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Boric Acid	27.5 gm
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0.5M EDTA	20 mL
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Gel Loading Buffer

Final Volume: 10 mL; made with Distilled Water

Strength 6X

Bromophenol Blue	25 mg
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Xylene Cyanol	25 mg
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Sucrose	4 gm
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Ethidium Bromide Stock 10 mg/mL

Final Volume: 5 mL; made up with Distilled Water

Ethidium Bromide	50 mg
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Determination of Superoxide Anion

Nitroblue Tetrazolium (NBT) 1 mg/mL

Final Volume: 10 mL; made up with Distilled Water

NBT 10 mg

The solution was freshly prepared, sterile filtered, using 0.22 μ syringe filter and kept in amber glass vials.

2N KOH

Final Volume: 10 mL; made up with Distilled Water

KOH 1.12 gm

Estimation of Malondialdehyde

Thiobarbituric Acid Reagent (TBA)

Final Volume: 10 mL

0.335 gm of TBA was mixed in 5 mL Distilled Water, this was then mixed with 5 mL Glacial Acetic Acid. the solution was freshly prepared and stored in amber bottles.

1N NaOH

Final Volume: 10 mL; made up with Distilled Water

NaOH 400 mg

Estimation of Superoxide Dismutase Activity

10 mM HCl

Final Volume: 100 mL; made up with Distilled Water

HCl 0.084 mL

Pyrogallol Stock 10 mM

Final Volume: 100 mL; made up with 10 mM HCl

Pyrogallol 126.1 mg

Tris Cacodylate Buffer (TCB) (pH 8.2)

Final Volume: 500 mL; made up with Distilled Water

Sodium Cacodylate 6.69 gm

DTPA 245.9 mg

Adjust pH to 8.2 with 1M Tris

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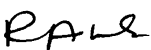
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SYNOPSIS

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What was considered as a gay man's disease in the early 1980's, after two and a half decades, is a household name. With over 40 million people around the globe, living with the disease, HIV/AIDS, for many of us seems to have always been there. Billions of dollars are being spent to exterminate the bug, but the magic pill has nevertheless, remained elusive...

INTRODUCTION

With time the HIV/AIDS pandemic is unfolding and revealing its secrets. Paradoxically, the pandemic is becoming simultaneously more difficult and simpler to comprehend. A modern understanding of the pandemic requires two levels of awareness. First, it is essential to appreciate the enormously complex histories of individual HIV/AIDS epidemics at the community or country level. Yet this level of information is insufficient. It is also necessary to recognize the common features among the HIV/AIDS epidemics, which, at a deeper level, provide insight into the natural history and the shape of the slowly maturing epidemic.

Jonathan Mann and Daniel Tarantola¹

From the beginning of the epidemic, Acquired Immunodeficiency Disease Syndrome (AIDS) exhibited all the classical signs of an infectious disease, and the only convincing explanation for its cause was the emergence of a new infectious agent. An infective aetiology was consistent with the geographical clustering of early cases and epidemiological evidence of case-to case contact. The newness of the disease was the patterns of groups at risk, its occurrence within the same time-scale in the diverse groups affected and, finally, its exponential rise.

By its 25th year (as of January 2006), the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimated that AIDS had killed more than 25 million people since it was first recognized in 1981, making it one

of the most destructive pandemics in recorded history². By the end of 2006 an estimated 39.5 million people were living with Human Immunodeficiency Virus (HIV) of which an estimated 4.3 million were infected in 2006³.

The prevalence however, has not been uniform throughout the globe. While Oceania had 81,000 people living with HIV by the end of 2006, with 0.4 % of its adult population (defined as those aged between 15 – 49 years of age) falling in the category, Sub-Saharan Africa had 24.7 million people testing positive for the Virus, which included an alarming 5.9% of its adult population³. The predicament seems to be more in the developing world than the developed quarter.

India, world's second most populous country is experiencing a highly varied HIV epidemic. Approximately 5.7 million people, of whom 5.2 million are adults, were living with HIV in 2005 in India³. The spread of HIV within India is as diverse as the societal patterns between its different regions, states and metropolitan areas³.

The reasons for this skewed presentation are varied, but apart from being scientific, are also socio-economical⁴.

Anti-Retroviral Therapies (ART's) have come a long way since Azidithymidine (AZT). The Protease Inhibitors, and lately the Fusion Inhibitors have shown to prolong the disease progression considerably. But affordability of these drugs by most people in the developing world still remains unlikely. Albeit, it may be possible for some to meet the expenses for a short term, a failure to do it incessantly over a long-term basis would pose serious problems like evolution of drug-resistant strains⁵.

Social stigmas have been the other major reason for the deadly run of the disease in the third world. Ignorance and egos are further fuelling the inferno.

Prevention has remained the best cure so far. But the magnanimity and heterogeneity of the target population, makes effective implementation of prevention programs difficult. Under such circumstances, understanding the host-pathogen interactions, and providing alternative means of prolonging the disease progression is of key importance in controlling the spread of the disease.

REVIEW OF LITERATURE

THE PATHOGEN

Human Immunodeficiency Virus (HIV) is the aetiological agent of Acquired Immunodeficiency Disease Syndrome (AIDS). Its primary target is the activated CD4+ T helper lymphocyte but it can also infect several other cell types including macrophages. HIV is a lentivirus, a class of retrovirus. The name lentivirus means slow virus, so named because these viruses take a long time to cause overt disease. Most lentiviruses target, cells of the immune system and thus disease is often characterized as immunodeficiency.

Two types of HIV have been identified, HIV-1 and HIV-2. These cause clinically indistinguishable disease although the time to disease onset is longer for HIV-2. The worldwide epidemic of HIV and AIDS is caused by HIV-1 while HIV-2 is mostly restricted to West Africa.

The virions of an HIV-1 consist of an envelope and measure 80-100 nm in diameter. The surface projections are small, at 8 nm in length, but densely dispersed and there are inconspicuous spikes that cover the surface evenly. The nucleoid is concentric while the core is rod-shaped or truncated cone-shaped.

The genome of HIV-1 is dimeric, unsegmented and contains linear, positive-sense, single-stranded RNA. The complete genome is fully sequenced and of one monomer 9200 nucleotides long. The genome has terminally redundant sequences that have long terminal repeats (LTR) of about 600 nucleotides⁶.

THE PATHOGENESIS

The devastating effect HIV has on the immune system is a result of infecting and killing the immune-regulating Th lymphocytes. HIV infects and replicates primarily in activated CD4⁺ T lymphocytes and to a lesser extent in macrophages and dendritic cells. The cell specificity is due to the HIV envelope (Env) glycoprotein 120 (gp120) binding CD4, along with chemokine receptors CXCR4 or CCR5, for cell entry. Once in the cytoplasm the viral RNA is reverse-transcribed into DNA and randomly integrated into the host cell genome. Activation of the host cell enhances the production of viral proteins, which assemble upon budding out of the cell, utilizing the plasma membrane as the viral envelope. HIV plasma levels during all stages of infection range from 50 to 11×10^6 virions/ml⁷. The half-life of most HIV-infected T cells *in-*

vivo is 12–36 hrs^{8,9}, with the mechanisms of cell death being virus- or receptor-mediated apoptosis.

There are several mechanisms by which HIV can induce cell death directly, in the cell it infects. CXCR4-tropic HIV isolates, generally found in the later stages of HIV infection, preferentially infect T cells and induce membrane fusion between cells to form a giant multinucleated cell called a syncytium. Although syncytium formation is not necessary for the progression to AIDS, syncytia have a short lifespan, and the emergence of CXCR4-tropic strains correlates with an increased depletion of T cells⁷. In addition, cell viability can be compromised because the plasma membrane becomes disrupted or more permeable due to the continuous budding of the virion¹⁰, or due to specific HIV proteins such as Vpu that can induce membrane permeability¹¹. Virus replication in the cell also has terminal consequences as cellular toxicity increases due to a build up of un-integrated linear viral DNA¹². Also, through cleavage, the HIV protease can inactivate anti-apoptotic Bcl-2 while simultaneously activating proapoptotic procaspase 8, making the cell more susceptible to mitochondrial dysfunction in response to internal or external death signals⁷.

APOPTOSIS

Various agents appear to be able to trigger apoptosis (*Apoptosis: A genetically determined destruction of cells from within due to activation of a stimulus or removal of a suppressing agent or stimulus that is postulated to exist to explain the orderly elimination of superfluous cells - also called as programmed cell death*) in CD4+ T cells. The viral envelope glycoprotein gp120 and the transactivator protein Tat are involved in priming CD4+ T cells for apoptosis¹³. In addition, gp120 alone is able to induce apoptosis in different cell lines bearing CD4 receptors, confirming the ability of this glycoprotein to activate cell suicide programs. Additional candidate mechanisms for apoptosis induction in AIDS are the inappropriate expression of prostaglandins and cytokines i.e. tumor necrosis factor α by other infected cells, such as macrophages^{14,15}. Indeed TNF α participates in the destruction of HIV-infected neuronal cells¹⁶ and favours provirus replication¹⁷. Another example of cytokine-induced apoptosis is the Fas ligand (FasL)-Fas receptor (FasR) system. FasL is a cell surface cytokine belonging to the TNF family, which is able to induce apoptosis in cells bearing FasR. Receptor expression is induced in lymphocytes infected by several

viruses, including HIV. Therefore, it has been suggested that FasR-mediated apoptosis could be implicated in AIDS pathology. Apoptosis is also induced in CD4⁺ T lymphoblastoid cells under oxidative stress¹³.

Changes in components of the antioxidant defense system and increased levels of oxidation products have been observed in asymptomatic HIV-infected patients and in patients with AIDS. Such evidence points convincingly to accelerated oxidative stress in these patients¹⁸.

OXIDATIVE STRESS

Role of oxidative stress (*Oxidative Stress: Disturbance of Prooxidant-Antioxidant balance in favour of the former, leading to potential damage*) has also been suggested in activation of HIV replication. HIV gene expression can be achieved in-vitro by oxidative stress. H₂O₂ can induce the expression of HIV in human T cell lines by activating NF-κB¹⁹. Also, TNF α, a powerful activator of NF-κB acts via oxidative stress. Furthermore, opportunistic infections can participate in AIDS progression by induction of oxidative stress mechanisms. Oxidative stress has been indicated in a number of

opportunistic infections including Tuberculosis, Candidiasis, Cytomegalovirus, HBV, HSV, etc²⁰. HIV infected individuals have been found to have elevated serum levels of Malondialdehyde (MDA), a product of lipid peroxidation. Moreover the antioxidant defense mechanisms in these patients are diminished. Decreased activities of the antioxidant enzymes Superoxide Dismutase (SOD, EC 1.15.1.1) and Glutathione Peroxidase (GPx, EC 1.11.1.9) of blood plasma and peripheral blood mononuclear cells, as well as the plasma levels of ascorbate, α -tocopherol and β -carotene have been reported²¹.

RATIONALE

One mechanism whereby pro-apoptotic factors may potentiate the induction of apoptosis in AIDS patients is by increase in free radical levels, which in turn could lead to increased oxidative stress and enhanced viral replication in such stressed cells. Hence understanding the role of oxidants (Superoxide Anion), antioxidants/cytoprotective enzymes (Superoxide Dismutase) and their correlation with pro-apoptotic members like TNF- α could be of great importance in determining the reasons behind significant T cell death especially via Apoptosis in HIV positive patients.

AIMS & OBJECTIVES

- To confirm the HIV sero-status of the patients.
- To categorize HIV +ve patients on the basis of their CD4 counts.
- To assess the host pro-inflammatory response by analysing the serum TNF- α levels.
- To gauge the level of oxidative stress in the study cohort (patients + controls) by:
 - Analysing the production of Superoxide Anion in PBMC's by the NBT dye reduction test.
 - Analysing Lipid Peroxidation by measuring serum MDA levels.
- To study host antioxidant defence mechanism by estimating the activity of Superoxide Dismutase (SOD, EC 1.15.1.1)
- To correlate the oxidative stress to Cell Apoptosis in the PBMC's.

MATERIALS & METHODS

STUDY POPULATION

The study population comprised of 57 HIV seropositive patients attending the OPD or admitted to Mathadi Kamgar Hospital, Koperkhairne and Jyothi's Terminal Care Centre at Kalamboli. The control group comprised of 10 healthy individuals from Haffkine Institute. Institutional Ethical Committee approval was taken for the project.

Samples were collected after informed written consent. All the individuals were subjected to a structured questionnaire, which included data on demographics, STD, medical history and sexual behaviour. Presumptive diagnosis of sexually transmitted disease was based on the reports and medical history provided by the hospital.

BLOOD COLLECTION

Ten milliliters of blood was drawn by venipuncture from all the individuals. The blood was aliquoted appropriately in K3-EDTA and plain bulb. The serum was separated from the plain bulb,

aliquoted and stored at -20°C. The whole blood was used for evaluating the CD4-CD8 count.

COLLECTION OF PBMC's

PBMC's were isolated from whole blood collected in EDTA bulbs by the Ficol Hypaque density gradient centrifugation. Ficol was overlaid by equi-volume whole blood, which was then centrifuged for 15 min's at 1500 r.p.m. The buffy coat containing the PBMC's were collected and cryo-preserved, using a freezing mixture containing RPMI 1640, 10% Foetal Calf Serum (FCS) and 5% DMSO.

HIV SEROSTATUS CONFIRMATION

Confirmation of the HIV serostatus was done using HIV1 and HIV2 ELISA. (Sandwich ELISA SD HIV 1/2 ELISA, Ver.3, Standard Diagnostic Inc., and Indirect Elisa, Micro Lisa, J. Mitra and Co.)

CD4-CD8 COUNTS

100µl of blood was drawn from K3-EDTA containing bulb. 50µl each was 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 were counted using FACScount (Becton Dickinson). The sorting and analysis of the cells was fully automated.

ESTIMATION OF SERUM TNF α [PeliKine human TNF α ELISA kit]

TNF α from serum was performed using commercially available PeliKine human TNF α ELISA kit. Tumour necrosis factor α (TNF α) is an extremely potent peptide cytokine which serves as an endogenous mediator of inflammatory, immune and host defence functions. Macrophages and monocytes are thought to be the cells, which contribute most to the local and systemic TNF α response to bacterial, viral and parasitic organisms and products.

PRINCIPLE:

The assay was a "sandwich-type" of enzyme immunoassay in which a monoclonal anti human TNF α antibody is bound onto polystyrene microtiter wells. Human TNF α , present in a measured volume of sample or standard is captured by the antibody on the microtiter plate. Subsequently, a biotinylated second monoclonal antibody to human TNF α is added. Addition of horseradish peroxidase (HRP) conjugated streptavidin, binds onto the

biotinylated side of the TNF α sandwich. a substrate solution is added to the wells. Which forms a coloured product in proportion to the amount of TNF α present in the sample or standard.

DETERMINATION OF SUPEROXIDE²²

The Superoxide Anion (SOA) is produced by activated monocytes and macrophages. It can work both as a reducing as well as oxidizing agent.

PRINCIPLE

Superoxide Anion reduces Nitro Blue Tetrazolium (NBT) to an insoluble blue formazan, which can be subsequently re-dissolved and assayed calorimetrically.

PROCEDURE

To 300 μ L of PBMC's in RPMI 1640 (Cell Count= 1×10^6 cells) 300 μ L of 1 mg/mL NBT solution was added in eppendorf tubes. PBMC's from healthy subjects were used as control. The cells were incubated overnight. Following incubation the tubes were centrifuged at 1500 rpm for 15 minutes and the supernatant was discarded. The cell pellet was fixed with 100% Methanol. This was followed by 3 washings with 70% Methanol, to remove excess

media. The cells were then treated with 120 μ L of 2N Potassium Hydroxide and 140 μ L of DMSO. The tubes were centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and the absorbance was measured at 650 nm on a Nanodrop ND-1000 UV/Vis Spectrophotometer.

ESTIMATION OF SERUM MALONDIALDEHYDE LEVELS²³

Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation.

PRINCIPLE:

MDA reacts with Thiobarbituric Acid (TBA) under acidic condition, to form an adduct which can be measured fluorimetrically.

PROCEDURE:

50 μ L of serum sample/standard were added in amber vials containing 250 μ L of 1.22 M Orthophosphoric acid, 450 μ L Distilled water and 250 μ L of TBA reagent. Tetramethoxypropane (TMP) was used as the standard as it yields equimolar amounts of MDA under the condition of the assay. The mixture was incubated at 95° C in a water bath for 60 mins. The samples were then cooled on ice followed by addition of 360 μ L Methanol and 40 μ L of

NaOH to neutralize the sample. Readings were taken on a Nanodrop ND-3300 Fluorospectrometer with the emission wavelength set at 553 nm.

ANALYSIS OF CELL APOPTOSIS – DNA FRAGMENTATION

DNA fragmentation is a hallmark of end stage apoptosis. DNA from cryopreserved PBMC's was extracted using the TRI Reagent (M/s. Sigma-Aldrich Inc.) as per manufacturers instructions. The cells were counted on a Haemocytometer. 5×10^6 cells were used for the extraction of DNA. The extracted DNA was run on 2 % agarose gel, and stained using Ethidium Bromide. The bands were observed using an AAB gel documentation system.

DETERMINATION OF SUPEROXIDE DISMUTASE ACTIVITY

This assay is yet to be performed; the results will be discussed in the thesis.

RESULTS

The study population consisted of 57 HIV +ve individuals (29 Females and 28 Males) within the age group of 22 – 48 years. Ten HIV –ve (8 Males and 2 Females) within the age group of 22 – 29 years were included as controls. The HIV +ve population was grouped in to Asymptomatic, Symptomatic and AIDS groups as per the Center for Disease Control (CDC) Classification²⁴.

The statistical analysis was done on SPSS software.

Table 1 complies the gender wise distribution of the Study Population.

The CD4 T cell counts of the entire study cohort are documented in Table 2. The healthy control population had a mean cell count of 822 cells/mm³ (SD $\pm$ 165.69) [Range: 569-1109], the asymptomatic population had a mean cell count of 632.70 cells.mm³ (SD $\pm$ 100.05) [Range: 521-837], the symptomatic group had a mean cell count of 367.96 cells.mm³ (SD $\pm$ 92.18) [Range: 208-494] while the AIDS cohort had a mean cell count of 103.32 cells.mm³ (SD $\pm$ 56.46) [Range: 7-192].

TABLE 1: CROSSTABULATION-STUDY GROUP V/s GENDER

STUDY GROUP		GENDER		TOTAL
		FEMALE	MALE	
ASYMPTOMATIC	Count	8	2	10
	% Within Group	80.0%	20.0%	100.0%
	% Within Gender	25.8%	5.6%	14.9%
SYMPTOMATIC	Count	15	10	25
	% Within Group	60.0%	40.0%	100.0%
	% Within Gender	48.4%	27.8%	37.3%
AIDS	Count	6	16	22
	% Within Group	27.3%	72.70%	100.0%
	% Within Gender	19.4%	44.4%	32.8%
CONTROL	Count	2	8	10%
	% Within Group	20.0%	80.0%	100.0%
	% Within Gender	6.5%	22.2%	14.9%
TOTAL	Count	31	36	67
	% Within Group	46.3%	53.7%	100.0%
	% Within Gender	100.0%	100.0%	100.0%

TABLE 2: CD4 COUNTS in Cells/mm³

STAGE	N	MEAN	SD	RANGE	
				MIN.	MAX.
ASYMPTOMATIC	10	632.70	100.05	521	837
SYMPTOMATIC	25	367.96	92.18	208	494
AIDS	22	103.32	56.46	7	192
CONTROL	10	822.00	165.69	569	1109

The three major clinical presentations were Tuberculosis (TB), Candidiasis and Diarrhoea. These are represented as CP-1, CP-2 and CP-3 respectively.

Twenty Five (43.9%) of the 57 HIV +ve individuals had TB. Of these, 14 belonged to the symptomatic phase while 11 belonged to the AIDS group. No individual in the asymptomatic phase had TB.

Table 2 cross-tabulates the Study Population V/s the presence of TB (CP-1).

TABLE 3: CROSSTABULATION-STUDY GROUP V/s CP-1 (TUBERCULOSIS)

STUDY GROUP		CP-1		TOTAL
		- VE	+ VE	
ASYMPTOMATIC	Count	10	0	10
	% Within Group	100.0%	0	100.0%
	% Within CP-1	31.3%	0	17.5%
SYMPTOMATIC	Count	11	14	25
	% Within Group	44.0%	56.0%	100.0%
	% Within CP-1	34.4%	56.0%	43.9%
AIDS	Count	11	11	22
	% Within Group	50.0%	50.0%	100.0%
	% Within CP-1	34.4%	44.0%	38.6%
TOTAL	Count	32	25	57
	% Within Group	56.1%	43.9%	100.0%
	% Within CP-1	100.0%	100.0%	100.0%

Thirty Two (56.1%) of the 57 HIV +ve individuals had Candidiasis. Of these, 4 belonged to the asymptomatic phase while 10 belonged to the asymptomatic phase. 18 individuals in the AIDS Group had Candidiasis. Table 3 cross-tabulates the Study Population V/s the presence of Candidiasis (CP-2).

TABLE 4: CROSSTABULATION-STUDY GROUP V/s CP-2 (CANDIDIASIS)

STUDY GROUP		CP-2		TOTAL
		- VE	+ VE	
ASYMPTOMATIC	Count	6	4	10
	% Within Group	60.0%	40.0%	100.0%
	% Within CP-2	24.0%	12.5%	17.5%
SYMPTOMATIC	Count	15	10	25
	% Within Group	60.0%	40.0%	100.0%
	% Within CP-2	60.0%	31.3%	43.9%
AIDS	Count	4	18	22
	% Within Group	18.2%	81.8%	100.00%
	% Within CP-2	16.0%	56.3%	38.6%
TOTAL	Count	25	32	57
	% Within Group	43.9%	56.1%	100.00%
	% Within CP-2	100.00%	100.00%	100.00%

Thirty One (54.4%) of the 57 HIV +ve individuals had Diarrhoea. Of these, 1 belonged to the asymptomatic phase while 11 belonged to the asymptomatic phase. 19 individuals in the AIDS Group had Diarrhoea. Table 4 cross-tabulates the Study Population V/s the presence of Diarrhoea (CP-3).

TABLE 5: CROSSTABULATION-STUDY GROUP V/s CP-3 (DIARRHOEA)

STUDY GROUP		CP-3		TOTAL
		- VE	+ VE	
ASYMPTOMATIC	Count	9	1	10
	% Within Group	90.0%	10.0%	100.0%
	% Within CP-3	34.6%	3.2%	17.5%
SYMPTOMATIC	Count	14	11	25
	% Within Group	56.0%	44.0%	100.0%
	% Within CP-3	53.8%	35.5%	43.9%
AIDS	Count	3	19	22
	% Within Group	13.6%	86.4%	100.0%
	% Within CP-3	11.5%	61.3%	38.6%
TOTAL	Count	26	31	57
	% Within Group	45.6%	54.4%	100.0%
	% Within CP-3	100.0%	100.0%	100.0%

Biochemical parameters were studied in Normal Controls and HIV +ve patients. These included the pro-inflammatory molecule, TNF- α , Superoxide Anion: an ROS, and Malondialdehyde (MDA), product of lipid peroxidation. The Statistical Test used were Multiple Comparison Test with Least Significance Difference.

The following tables summarise the comparative results of the above parameters.

TNF- α levels were found to be significantly elevated ($p < 0.002$) in asymptomatic patients and ($p < 0.001$) in symptomatic as well as AIDS patients when compared to the normal controls.

TABLE 6: TNF α in pg/mL

STAGE	N	MEAN	SD	RANGE	
				MIN.	MAX.
ASYMPTOMATIC	10	111.78	23.30	87.54	163.28
SYMPTOMATIC	25	148.15	33.83	92.57	223.14
AIDS	22	299.20	109.88	186.20	580.89
CONTROL	10	8.88	1.16	7.00	11.00

TABLE 7: MULTIPLE COMPARISONS FOR TNF α

STAGE	M.D	S.E	SIGNIFICANCE
ASYMPTOMATIC	-102.90	30.13	<0.002
SYMPTOMATIC	-139.27	25.21	<0.001
AIDS	-290.30	25.69	<0.001

Superoxide Anion levels was found to be significantly elevated ($p<0.001$) in asymptomatic, symptomatic as well as AIDS patients when compared to the normal controls.

TABLE 8: SOA in O/D units

STAGE	N	MEAN	SD	RANGE	
				MIN.	MAX.
ASYMPTOMATIC	10	0.227	0.105	0.040	0.317
SYMPTOMATIC	25	0.341	0.046	0.260	0.437
AIDS	22	0.431	0.047	0.337	0.499
CONTROL	10	0.114	0.040	0.063	0.194

TABLE 9: MULTIPLE COMPARISONS FOR SOA

STAGE	M.D	S.E	SIGNIFICANCE
ASYMPTOMATIC	-0.11	0.03	<0.001
SYMPTOMATIC	-0.23	0.02	<0.001
AIDS	-0.32	0.02	<0.001

Malondialdehyde levels was found to be significantly elevated ($p<0.001$) in asymptomatic, symptomatic as well as AIDS patients when compared to the normal controls.

TABLE 10: MDA in nM/mL

STAGE	N	MEAN	SD	RANGE	
				MIN.	MAX.
ASYMPTOMATIC	10	6.26	0.937	5.28	7.87
SYMPTOMATIC	25	7.39	0.980	5.32	8.98
AIDS	22	8.84	0.409	8.13	9.58
CONTROL	10	3.55	0.166	2.43	4.13

TABLE 11: MULTIPLE COMPARISONS FOR MDA

STAGE	M.D	S.E	SIGNIFICANCE
ASYMPTOMATIC	-2.71	0.34	<0.001
SYMPTOMATIC	-3.84	0.29	<0.001
AIDS	-5.29	0.29	<0.001

Apoptosis was demonstrated by fragmentation of Genomic DNA in the PBMC's. Thirty Two (47.8%) of the samples showed DNA fragmentation. Two samples from the asymptomatic phase (20% of the total asymptomatic population) showed DNA fragmentation, while 12 samples from the asymptomatic phase (48% of the total symptomatic phase) showed apoptosis. Eighteen of the 22 AIDS samples showed apoptosis (81.8% of the total IDS population),

while there were no detectable DNA fragmentations in the control population.

TABLE 12: CROSSTABULATION-STUDY GROUP V/s APOPTOSIS

STUDY GROUP		APOPTOSIS		TOTAL
		-VE	+VE	
ASYMPTOMATIC	Count	8	2	10
	% Within Group	80.0%	20.0%	100.0%
	% Within Apoptosis	22.9%	6.3%	14.9%
SYMPTOMATIC	Count	13	12	25
	% Within Group	52.0%	48.0%	100.0%
	% Within Apoptosis	37.1%	37.5%	37.3%
AIDS	Count	4	18	22
	% Within Group	18.2%	81.8%	100.0%
	% Within Apoptosis	11.4%	56.25%	32.8%
CONTROL	Count	10	0	10%
	% Within Group	100.0%	0.0%	100.0%
	% Within Apoptosis	28.6%	0.0%	14.9%
TOTAL	Count	35	32	67
	% Within Group	52.2%	47.8%	100.0%
	% Within Apoptosis	100.0%	100.0%	100.0%

DISCUSSION

By the end of 2005, global expenditure on HIV/AIDS had reached US\$ 8.3 billion²⁵. This was more than the 2005 GDP of 70 countries out of the 180 countries on the International Monetary Fund (IMF) list²⁶. This speaks of the prominence that HIV/AIDS finds on the global agenda. This fund however has not been able to address the core issues and achieve the goals set. In the Declaration of Commitment on HIV/AIDS, adopted at a special session of the UN General Assembly in 2001, leaders from 189 Member States committed to comprehensive, time-bound targets for the delivery of the effective HIV prevention, treatment, care and support needed to halt and begin to reverse the global epidemic by 2015. However by the end of 2005, many of the objectives could not be achieved²⁵. Combinations of antiretroviral agents have readily achieved long-term remission of HIV-1 disease. The suppression of plasma viral loads to less than the limit of quantification of the most sensitive commercially available assays (i.e., less than 50 copies/mL) and the coincident improvement in CD4 T cell counts is associated with resolution of established opportunistic infections and a decrease in the risk of new opportunistic infections. However, prolonged

treatment with combination regimens is difficult to sustain because of problems with adherence and toxic effects. All antiretroviral drugs can have both short-term and long-term adverse events. The risk of specific side effects varies from drug to drug, from drug class to drug class, and from patient to patient²⁷. Hence alternative and support therapies will be of great help in addressing the issue.

Many laboratories have underlined the role of oxidative stress in HIV/AIDS in the past.

Studies of HIV-infected cell lines and observations of HIV-infected patients have provided insights into the possible role of oxidative stress in HIV pathogenesis. The role of oxidative stress in HIV disease appears to be quite broad and involve alterations in viral replication, immune function, apoptosis, weight loss, and critical aspects of redox and glutathione-dependent metabolism¹⁸.

Kameoka, et.al have demonstrated significant amplification of Superoxide generation in cells of mononuclear phagocytic lineage by HIV- 1 infection²⁸.

In the present study a significant increase in the production of the Superoxide Anion has been found in the HIV+ve population ($p < 0.001$). It is also seen that the SOA levels increases with

decrease in the CD4 cell counts i.e. with increase in disease progression.

Elevated MDA levels in serum and other tissues of HIV have been demonstrated by Gil et.al., and Mollace et.al. among others^{29,30}. The present study also found significant elevation of serum MDA levels in the HIV+ve population ($p < 0.001$).

Pro-inflammatory cytokine, TNF α was also significantly elevated in the HIV +ve population ($p < 0.001$), with a negative correlation with the CD4 counts. Also the percentage of samples demonstrating apoptosis correlates inversely to the CD4 counts. And although not investigated in the present study, the TNF α levels may have a bearing on this significant increase in the PBMC apoptosis seen in the HIV +ve population. TNF α has been indicated in having pro-apoptotic activities, especially in HIV disease where elevated TNF α levels makes the CD4 +ve lymphocytes Fas sensitive, thereby inducing apoptosis³¹. Opportunistic Infections, in the past have been demonstrated to contribute in the escalating oxidative stress³². In the present study we see that the percentage of patients demonstrating O.I's increase with a concurrent decrease in the CD4 cell counts. Therefore it

may be said that the O.I's have a role in disease progression via amplification of pathways leading to oxidative stress.

In the absence of a therapeutic vaccine or a curative pharmacological agent, and given the side effects of current long-term therapies (HAART), an alternative way of managing the epidemic must be considered seriously. The above results underline the significant contribution of oxidative stress in accentuating the HIV disease, especially causing a decline in the CD4 cell population by inducing apoptosis in the infected cell population. Some laboratories have demonstrated the role of antioxidants in slowing the progression HIV disease. Taking a cue from these studies, development of a defined antioxidant and specific anti-apoptotic regime in HIV becomes imperative.

With the growing number of problems associated with the current treatment protocols (drug affordability due to high pricing, drug resistant organisms, side effects etc.) its only rational that we critically consider the alternatives until the magic molecule is found...

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