

**STUDY OF MRNA PROFILE FOR CYTOKINES
(TNF-A, IFN-Y, IL-2, IL-4, IL-6, IL-10) IN HIV
INFECTED CHILDREN**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

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

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MUMBAI - 400 012**

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*A Thesis submitted
to the
University of Mumbai*

*for the
Ph.D. (Science) Degree
In Microbiology*



*Submitted By
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As required by the University Ordinance 770, I wish to state that the work embodied in this thesis titled "***Study of mRNA profile for cytokines (TNF- α , IFN - γ , IL-2, IL-4, IL-6, IL-10) in HIV infected children***" forms my own contribution to the research work carried out under the guidance of **Dr. (Mrs.) Ranjana A. Deshmukh** at the **Haffkine Institute for Training, Research and Testing, Mumbai -12**. This work has not been submitted for any other degree of this or any other University. Whenever reference have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography



Signature of the candidate
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Writing this gives me an immense pleasure, to thank all those, without whose help, this work would not have seen the light of the day.

I offer my gratitude to Dr. (Mrs.) R. A. Deshmukh, Head, Department of Virology, for being my Research Guide and polishing my work to perfection. I also thank her for channelizing my energies in the right direction and bringing out the best in me.

I take this opportunity to thank Dr. M. A. Phadke, Ex-Director and Dr. S. M. Sapatnekar, the present Director, Haffkine Institute for Training Research and Testing for allowing me to carry out my work in this esteemed Institution.

I thank Dr. S. S. Mahajani, (Training-in-Charge and C.A.O. of the Institute), Mr. J. V. Sawant (Instrumentation Engineer), and Mr. A. Arekar for their timely help during my tenure at Haffkine.

I specially thank Mr. V. D. Mhapsekar and his library staff for availing their wonderful facilities in terms of Books, Internet, Referral papers, Photocopying facilities, etc.

I am deeply indebted to Dr. M. Mangalani and Dr. B. Seth at the Department of Pediatrics, Lokmanya Tilak Memorial Hospital and Medical College, Sion, for allowing me to work with the children enrolled at their HIV OPD and wards. I also thank Dr. P. Tambe and Sis. Mercy, for sharing the samples of healthy children from their clinic.

I feel fortunate to have worked in collaboration with Dr. P. Recordon-Pinson, Associate Professor, Department of Virology, University of

Bordeaux, Paris. I am thankful to Dr. H. Fleury for allowing a part of my work to be carried out at his Department.

It would be unfair on my part to forget the non-technical staff at Haffkine... the attendants at the Department, The Main Office, The Accounts Section, The Stores, without them, my work within the Institute and with the University would have been just impossible.

My friends at Haffkine... I cannot just thank them enough. I thank my Seniors Dr. Rajendra Mahajan and Ms. Meghana Udgire for having oriented me well in the Department.

I thank my Juniors... Kalpita and Shweta for helping a lot with my work, and bearing me, Sandeepan, Suresh and Prathmesh for being at my beck and call, and Swapnaja, Priya and Anuya for keeping me abreast of the Scientific world around me.

Most importantly, I thank my peers, Neena, Rajesh and Renu, for bringing out the competitive spirit in me and giving me a moral high.

I take a moment to thank all children and their parents, who participated in this Study. May God bless their souls and give them the peace of their lives.

Lastly, I thank my parents, my brother Anuj, Trupti maasi (for the generous exploitation of her computer) and Him, for bearing with me throughout this Study and guiding me to the right destination.

---Thank you

Sweta Kothari

Dedicated lovingly to my family...

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Introduction

"The AIDS epidemic has become a moving target and is at risk of spinning out of control".

*Kofi Annan
U.N. Secretary General
On visit to China in October 2002*

The epidemic of Acquired Immunodeficiency Syndrome (AIDS) has claimed more than 4.5 million lives in 2003, and an estimated 5 million people have acquired the Human Immunodeficiency Virus (HIV) in the same year, bringing the total number of people globally living with the virus to 42 million. Of this number, around 70% (3.8-4.6 million) of the infections occur in developing nations like India. It is observed that of the total 69,559 cases reported as on April 2004, Maharashtra harbours 11,726 cases. Mumbai is home to 4342 cases (1).

The HIV prevalence among women attending antenatal clinics in the states like Andhra Pradesh, Karnataka, Maharashtra, Manipur, Nagaland and Tamil Nadu is around 2%, higher than the national average of 1%. Women contribute 25% of the total HIV infections while contributing 21% of AIDS cases. Pediatric AIDS contributes to nearly 10% of total AIDS cases (1).

Around 36% of the HIV infected women belong to the reproductive age, and with the vertical transmission rate of 16-40%, the approximate number of children living in Mumbai alone with HIV would be around 14,000 to 40,000 per year. With the annual infant mortality rate of 3.3 per 1000 live births, there would be more children living morbidly after HIV infection. The statistical data for pediatric HIV infections is however not accurate, since the reporting of the positive cases is very low (1). Unfortunately, none or very few databases are available for the Indian cohort for the pediatric AIDS on global documentation like the WHO / UNAIDS listings.

In most of the pediatric HIV infections, HIV transmission is acquired vertically from their infected mothers. The risk of perinatal transmission is significantly increased if the mother has advanced HIV disease, increased levels of HIV in her bloodstream, or fewer numbers of the CD4+ T cells (2).

According to the Centre For Disease Control (CDC) data, children infected with HIV are classified into a) rapid progressors (20%), who develop serious opportunistic infections in the first year of life progressing very rapidly to AIDS defining conditions, with a rapid loss of CD4+ T cells within the first two years of their lives, death follows by the age of 4 years, b) Intermediate progressors (60-75%) who

develop severe immunosuppression by 7 -8 years of age, with a gradual loss in CD4+ T cells, c) long term survivors (5-10%), who are defined to have a slower rate of disease progression with minimal to no symptoms of HIV disease, normal to minimally decreased CD4 cells count by 8 years of age and no development of serious symptoms of AIDS until adolescence (3).

Normal range CD4+ T cell counts in children varies with age. It is greater than 1500 cells/ μ l for children below 1 year, between 1000-1500 cells/ μ l for the age group 1-5 years and between 500-1000 cells/ μ l for children above 5 years (3).

The wide variations observed in the rate of disease progression in HIV-infected children are due to their viral load and CD4+ T cell counts, their cytokine array as well as the opportunistic infections they suffer from. Nutritional status, personal hygiene and environmental cleanliness of these children also contribute to the disease progression.

Many children with HIV infection do not gain weight, are slow to reach important milestones in motor skills and mental development viz. crawling, walking and speaking. As the disease progresses, many children develop neurologic problems like difficulty in walking, poor school performance, seizures, mental retardation and cerebral palsy (4).

Children with HIV develop life-threatening opportunistic infections (OIs), but the incidence of various OIs differs in adults and children. For example, toxoplasmosis is seen less frequently in HIV-infected children while serious bacterial infections occur more commonly in children as compared to adults. Also, as children with HIV become sicker, they may suffer from chronic diarrhea due to opportunistic enteropathogens (4).

The CDC HIV and AIDS Surveillance data for U.S. reported *Pneumocystis carinii* pneumonia (PCP) as the leading cause of death in HIV-infected children with AIDS. PCP, as well as cytomegalovirus (CMV) disease, usually are new infections in children, whereas in adults these diseases result from the reactivation of latent infections (4).

Children with HIV frequently suffer the usual childhood bacterial infections, which can cause seizures, fever, pneumonia, recurrent colds, diarrhea, dehydration and other problems that often result in extended hospital stays and nutritional problems. HIV-infected children frequently have severe candidiasis, a yeast infection that can cause unrelenting diaper rash and infections in the mouth (oral thrush) and throat that makes eating difficult (4).

In HIV pathogenesis, the qualitative and quantitative abnormality in CD4+T helper cell population in an HIV infected individual has profound effects on immune system. Cytokine induced immune dysregulation of these cells is a central component in progression to AIDS. Based on the cytokine response they generate, the T helper (Th) cells are classified into Th1 and Th2 types (7). A Th1 to Th2 shift in the type of cytokines released after HIV infection has been proposed in this context (5). It is due to the fact that HIV infects Th1 cells easily compared to Th2 cells. This Th1 to Th2 shift results in the inability of host macrophages to get rid of opportunistic intracellular pathogens and this ultimately proves fatal. The loss of T helper cell function in asymptomatic individuals is seen even before the fall in the number of CD4+ T cells. Progression of HIV infection from asymptomatic to symptomatic stage and then AIDS involves shift of Th1 type of responses (cell mediated immune responses) to Th2 responses (humoral immune responses) (6).

It is postulated that the prototypes of T helper cells, viz. Th1 and Th2 play an important part in pathogenesis to AIDS. The Th1 type of CD4+ T cells do not allow the virus to replicate in them and even show the ability to inhibit HIV replication in other HIV infected cells. Thus they are protective in nature.

The Th2 type of cells and the cytokines they produce allow higher virus replication. This in turn leads to preferential killing of Th2 type CD4⁺ T cells and further spreads the infection of HIV (6).

Th1 cells produce Interferon-gamma (IFN- γ), Interleukin-2 (IL-2), Tumor necrosis factor- alpha and beta (TNF- α and TNF- β), responsible for both cell mediated immune responses (education and priming of the plasma cells for antibody production, macrophage activation, antibody dependent cell cytotoxicity (ADCC) and delayed type hypersensitivity (DTH)) and humoral responses. Th2 cells produce IL-4, IL-5, IL-6, and IL-13. These cytokines provide optimal help for humoral immune responses, through production of mast cells, eosinophil growth and differentiation, and facilitation to immunoglobulin A (IgA) synthesis. IL-10 is produced by both Th1 and Th2 cells in humans (7). It is this cytokine that plays a pivotal role during the disease progression to AIDS.

Cytokines produced by Th1 and Th2 cells exert mutual cross-regulatory interactions by autocrine or paracrine functions. For e.g., IFN- γ and IL-4 have antagonistic properties. TNF- α has been postulated to be a key player in AIDS, since evidences indicate that a state of chronic inflammation may be necessary to maintain HIV infection. TNF- α greatly increases the

replication of HIV in T cells and macrophages by inducing a nuclear chemical factor NF- κ B to bind to the proviral DNA and turn on the production of viral proteins (8).

Viral replication allows selective killing of the Th2 cells during the clonal selection procedure. These mechanisms may account for a preferential depletion of T cells that produce IL-4, as is suggested by lack of IL-4 expression in the PBMCs of HIV-infected individuals (8).

T cells from the blood of HIV infected patients are highly susceptible to apoptosis, or programmed cell death, when activated to proliferate. Adding antibodies that block the Th2 cytokines IL-10 and IL-4 could prevent apoptosis of the T cells. Thus, a lack of coordination could result in what could be termed as an "abortive Th1 to Th2 shift"; i.e. Th1 cells dying without the accompanying increase in the number of Th2 cells (247).

The medical literature reviewed above indicates major influence of cytokines on morbidity, mortality and the progression of HIV disease. However, similar data for the pediatric HIV cohort in the Indian context is very scanty. The present study was therefore planned to provide vital data on these parameters.

Review
of
Literature

"It (HIV/AIDS in India) is one of the great areas of concern, clearly".

***Lee Jong Wook
WHO Director General
WHO Report in May 2002***

History and Epidemiology:

Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 as an outbreak of *Pneumocystis carinii* pneumonia (PCP) among homosexual men in the U.S. in 1983, the etiological agent for this syndrome was discovered to be a virus, Human Immunodeficiency Virus (9). Subsequent epidemiological surveys have shown a steep incline in the number of cases of infection and consequent deaths due to infection with HIV. In India, around 3-4 million people are known to be infected with this virus. Of these, children contribute to around 10%. The perinatal transmission rate/risk is reported at 2.82% (1). However, the infection can also be acquired ante-nataly or by breast-feeding from an HIV infected mother.

The HIV prevalence among women attending antenatal clinics in the states like Andhra Pradesh, Karnataka, Maharashtra, Manipur, Nagaland and Tamil Nadu is around 2%. With the advent of newer and more effective life saving media, like

vaccines, food supplements, medicines and drugs, health awareness, etc., there would be more children living morbidly with HIV.

The statistical data for pediatric HIV infections is however not accurate, since the reporting of the positive cases is very low. For example, the National AIDS Control Organisation (NACO) reports 2644 pediatric cases nation-wide to be infected with HIV (1), while around 14,000 HIV seropositive children would be present in Mumbai alone. Also, none or very few databases are available for the Indian cohort for the pediatric AIDS on global documentation like the WHO / UNAIDS listings.

The impact of AIDS has been well documented in Africa. It has shown us the vulnerability of children, especially AIDS orphans (children who have lost both parents to AIDS) to socio-economic restrictions, like schooling, medication, and even basic necessities like food supplementation. The natural history of HIV in pediatric cohorts in our population is rarely documented in Indian medical literature.

Human Immunodeficiency Virus (HIV):

The etiological agent for the development of AIDS is HIV. HIV belongs to the family *Retroviridae* having RNA as the genetic material, and subfamily *Lentivirinae*, a family of "slow-growing" viruses (265). It persists in the host despite a vigorous and an

apparently normal immune response. Few of its unique properties include antigenic diversity, transmission via mucosa and infected cells, resistance to sero-neutralization, ability to integrate into the host genome, rapid emergence of escape mutants and down-regulation of the Major Histocompatibility Complex Class 1(MHC class I) antigens, etc (10). The course of infection with these viruses is characterized by a long interval between the initial infection and the onset of serious symptoms.

There are two types of HIV: HIV 1, the predominant AIDS causing virus in the world, and HIV 2, restricted generally to West Africa. The structure of HIV 2 is similar to that of HIV 1. However, HIV 2 has distinctly different genes and configurations of envelope proteins, particularly gp120 (11).

Structure of HIV:

HIV has a reverse transcriptase (RNA-dependent DNA polymerase) within the virion as the genomic material. It is 100 nm in diameter, with glycoprotein surface projections approximately 8nm in diameter. Within the envelope is an inner shell or capsid, icosahedral in shape, containing helical nucleoprotein. The genome is an inverted dimer of linear, single stranded, positive RNA that dissociates readily into two

or three pieces. Infectivity is sensitive to ether, acid and heat. Replication of the viral RNA involves a DNA *provirus* that is integrated into host cellular DNA.

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

Of the structural genes, *gag* codes for the core proteins p24, p17 and p15 that are cleaved from a p55 precursor protein by viral protease. The RNA-dependent DNA polymerase (reverse transcriptase) is encoded by the *pol* gene, which also codes for the protease (p16) and the integrase/endonuclease (p31). The viral polymerase exists in two forms, p66 and p51. Associated with the polymerase is RNase H, which specifically degrades RNA in RNA-DNA hybrids.

The envelope proteins are responsible for the virus adsorption and seem to be important for the cytopathic effects of HIV. They are encoded by the *env* gene and consist of gp160 and

its two units, gp120 and gp41. gp120 is the outer membrane part of the envelope protein that forms the knobs on the virus surface. gp41 is the transmembrane part by which gp120 is fixed to the viral membrane. gp120 binds to the CD4 receptors of the T helper lymphocytes and other cells, and mediates the adsorption of HIV to the cell surface (265).

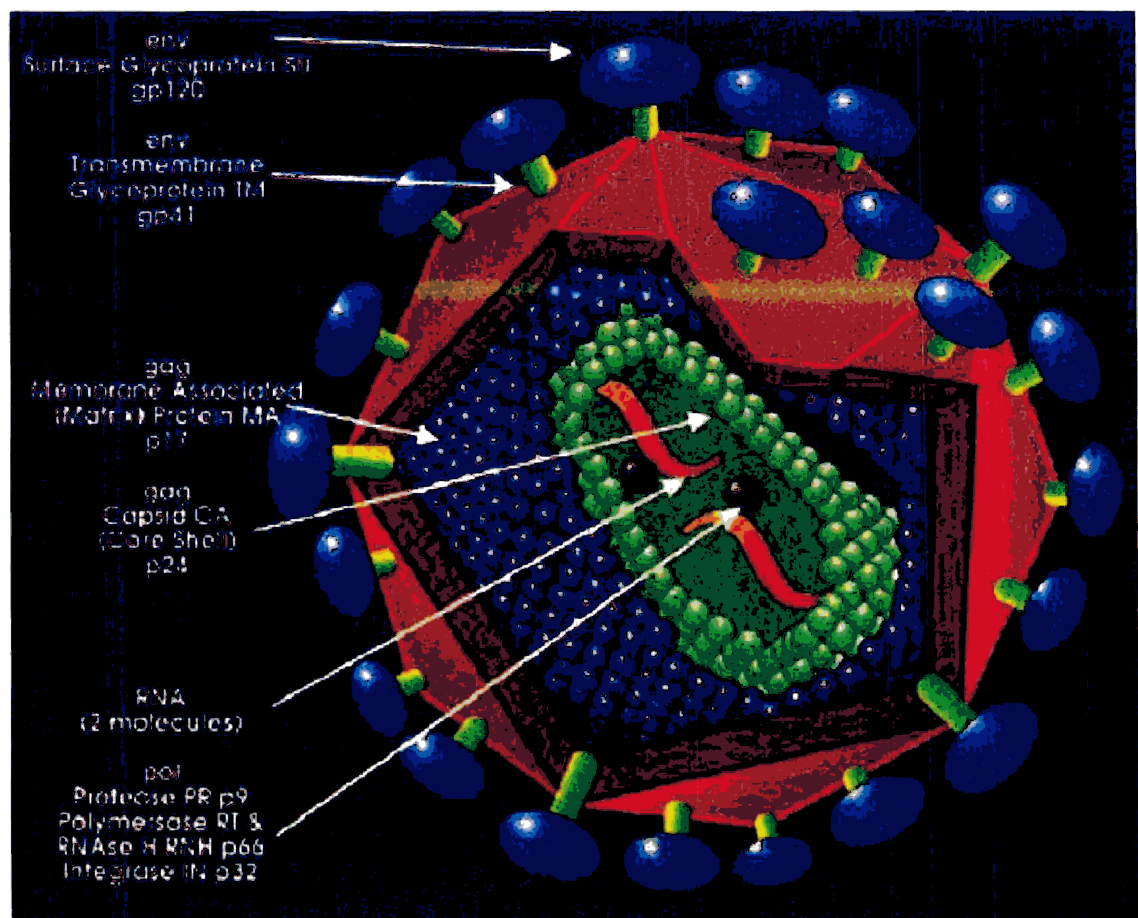


Figure 1: Structure of the Human Immunodeficiency Virus

Prominent among the virally encoded proteins are Tat and Rev. Both are found to be essential for the viral gene expression, replication and cytopathology. Though both proteins mediate their function through viral RNA elements, Tat via the Transactivation Response Element (TAR) and the Rev via the Rev Response Element (RRE), their mechanisms of action are different. Tat modifies the nascent transcription complex allowing for the efficient elongation of transcription, which increases levels of full-length viral transcripts. Rev acts post-transcriptionally to export incompletely spliced viral mRNAs, which code for structural proteins, from nucleus to cytoplasm (12).

Life cycle of HIV:

The mature virion of HIV-1 makes primary contact with CD4 through a constellation of surface gp 120 glycoprotein spikes consisting of variable (V_1 to V_5) and conserved (C_1 to C_5) domains. These glycoproteins are non-covalently linked to a transmembrane gp41 glycoprotein. Critical binding may occur between C_3 and C_4 domains of gp120 and the N-terminal extracellular domains of the CD4 molecule during the primary attachment. Although the V_3 loop of the gp 120 does not bind to the CD4, its role in overall virus binding and entry may be

important. Syncytium induction, neutralization and T cell/macrophage tropism are dependent on the integrity of this region of gp120 (13).

After gp120-CD4 binding, a number of events take place that position the virion for entry. Conformational changes may occur after binding, which uncover domains of the gp41 transmembrane molecule. It is theorized that exposure of the N-terminus of gp41 which contains a stretch of hydrophobic amino acids, allow the host cellular membrane to fuse with the lipid envelope coat of the virion. Cleavage of the gp120-V₃ region by proteases may facilitate exposure of the fusogenic gp41 domain (13).

The entry of HIV-1 takes place by a mechanism of membrane fusion. After insertion, hydrophobic gp41 N-terminal domain fuses with the host cellular membrane. At this point, accessory cellular substances (co-receptors) may facilitate entry of the virus. After fusion of the viral and cellular membranes, the internal virion core is released into the cytoplasm as Ribonucleoprotein (RNP) Complex. Subsequent to the internalization and uncoating of the virion coat, the dimeric genomic RNA is liberated into the cell cytoplasm. The cytoplasm also contains the products of *gag* and *pol* genes. The *pol* gene expresses proteins with Reverse transcriptase

(RT) and Ribonuclease H (RNase H) activity. These proteins generate a linear double stranded cDNA that remains associated with the RNP complex. In addition to the cDNA, the pre-integration complex contains the viral *Gag* matrix, *Pol* integrase and *Pol* RT proteins that were present in the infecting virion. The cDNA either gets integrated into the host cell genome (proviral DNA), or is transcribed to form viral components (13).

Before the virion can be released from the cell, the structural proteins must coordinately assemble. HIV has been observed to assemble both at the plasma membrane and within the intra-cytoplasmic vacuoles. Unspliced viral transcripts are used to synthesize *Gag* and *Gag – Pro – Pol* proteins in the cytosolic compartment. These polyproteins are subsequently transported to the cytoplasmic side of the plasma membrane where they mediate budding, and two copies of the viral genome are incorporated into this particle. Spliced RNA transcripts are used to make *Env* proteins on the membrane of the Rough Endoplasmic Reticulum. These proteins are then transported to the external surface of the cell via the secretory pathway and then move to the sites of budding for packaging. Immediately after budding, the *Gag* and the *Gag – Pro – Pol* proteins within the immature virion are cleaved by viral

protease to produce a mature infectious virion. The *Gag* and the *Gag - Pol* proteins begin to encapsidate the HIV genomic RNA, and the plasma membrane forms a lipid bilayer over the viral core. Precursor gp160 *Env* undergoes glycosylation and oligomerization in the Endoplasmic reticulum. It is then transferred to the Golgi complex where cellular enzymes process the glycoprotein to the surface gp120 and the transmembrane gp41. They then associate non-covalently and are transported to the cell surface. Finally, the inner core shell condenses and is linked with the glycoprotein incorporated lipid membrane (13).

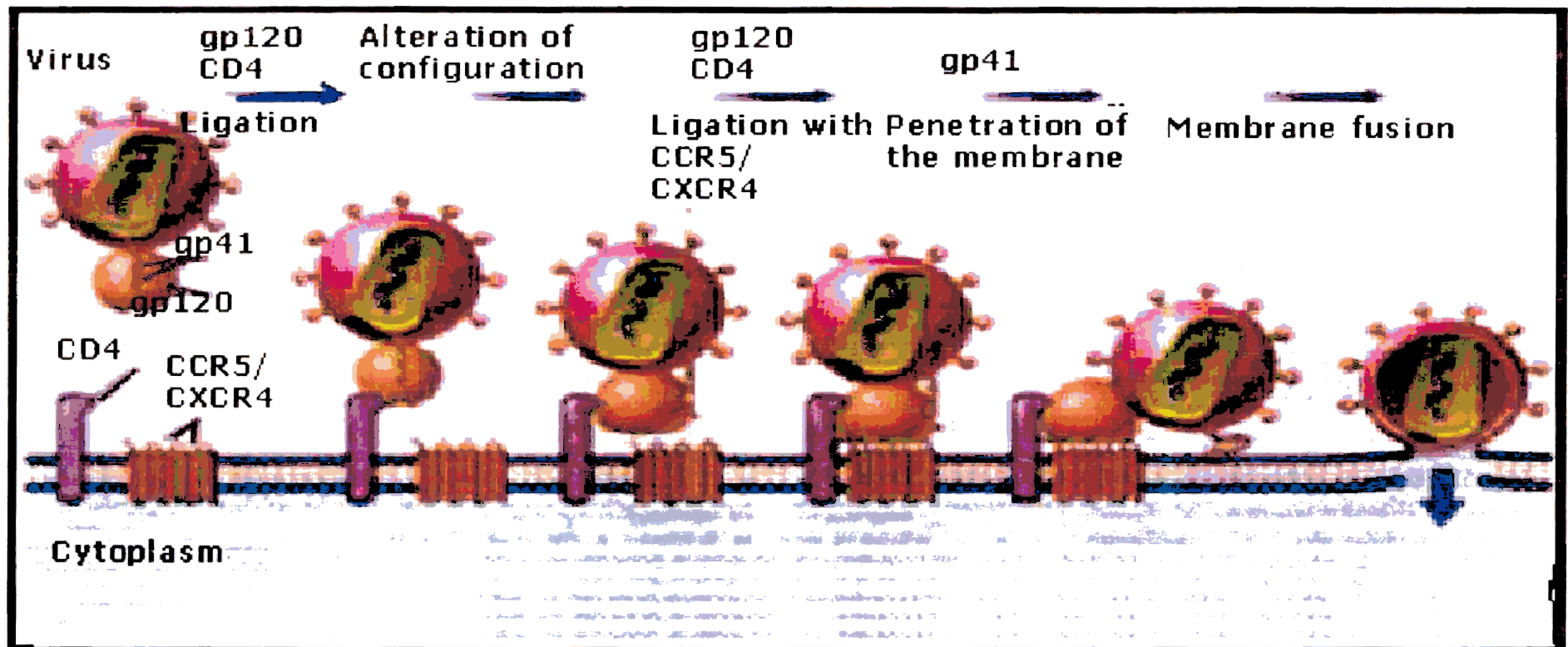


Figure 2: Entry of HIV into the host cells using co-receptors

STAGES OF HIV DISEASE PROGRESSION:

AIDS is a clinical syndrome associated with a progressive deterioration of the immune status of the infected individual. On an average, the development of AIDS from the initial infection may take 10 years. However, the rate of disease progression varies substantially among patients. The disease progression in an HIV infected individual can be predicted by CD4+ T cell counts. The viral load, the cytokine response, development of opportunistic infections, and other such factors may also play an important role in the prognosis of disease progression. HIV disease in adults can be categorized into the following, based on the combination of CD4+T cell counts, and clinical presentations: a) Initial infection b) Early HIV disease c) Intermediate HIV disease d) Late HIV disease e) Advanced HIV disease and f) Terminal HIV disease (14).

A. INITIAL INFECTION (Acute Sero-Conversion Syndrome):

Although the initial infection with HIV may be asymptomatic, as many as 50% of individuals with acute infection report a flu-like or mononucleosis-like illness. Only 20% to 30% of these individuals seek medical attention during this phase.

The symptoms of Acute Sero-Conversion Syndrome (ASCS) usually occur within 2 to 6 weeks with a median of 21 days, after the exposure to the virus. The most common symptoms

reported are fever with lymphadenopathy (LA), pharyngitis, esophagitis, myalgia, diarrhea, nausea, vomiting; while headache is observed less frequently. One of the most distinguishing characteristics of ASCS is the presence of a fleeting, morbiliform skin eruption. Less commonly, neurologic manifestations occur including asymptomatic meningitis, myopathy, peripheral neuropathy, brachial neuritis and rarely self-limiting meningoencephalitis or Guillian-Barre syndrome. Rarely, ASCS is associated with significant immune deficiency, resulting in the development of *Pneumocystis carinii pneumonia* (PCP) and esophageal candidiasis or thrush. Regardless of their type, most symptoms resolve within 14 to 21 days. Individuals who have a longer duration of symptoms have been reported to have a more rapid progression to AIDS (14).

The most striking laboratory feature of ASCS is marked by lymphopenia with depletion of both CD4+ and CD8+ lymphocytes. This initial lymphopenia is often followed by a period of relative lymphocytosis (predominantly CD8+ cells), with a high degree of atypical lymphocytes. Around the time of resolution of symptoms, the CD4+ lymphocyte count increases, but it usually does not return to the baseline levels. In some individuals, the number of CD4 cells remains profoundly

suppressed, resulting in a more accelerated course of disease progression. High levels of HIV p24 antigen are also detected during this acute stage of disease, with values ranging from 100 to 5500pg/ml. During this period of high-level viremia, the immune system begins to recognise and respond to HIV antigens and within 2 -3 weeks after the onset of symptoms, HIV antibodies are detectable (14).

B. EARLY HIV DISEASE:

Early stage HIV disease is defined by a CD4+ cell count greater than 500 cells/ μ l in adults and children above 5 years of age, and greater than 1000 cells/ μ l in children below 5 years of age. Most HIV infected individuals with high CD4 counts are asymptomatic. Among those who have any symptoms of infection, LA is the most common and usually persists from the time of acute seroconversion. The anatomic patterns of enlarged lymph nodes are characteristic and usually involve the cervical, axillary and the inguinal chains, sparing the periaortic and mediastinal area. Significant enlargement of thoracic or abdominal lymph nodes usually indicates an underlying secondary opportunistic infection (OI) process, such as pulmonary or disseminated tuberculosis, *Mycobacterium avium complex* (MAC) disease, histoplasmosis or lymphoma. Dermatologic abnormalities are the most

common infections observed among patients with early HIV disease, the seborrheic dermatitis being the most common manifestation. Oral lesions and Herpes simplex labialis, although more common in the later stages, may also occur in early HIV disease. Aphthous ulcerations may be found in 2% to 5% of patients in the early stages of disease. Oral candidiasis rarely occurs in patients with CD4 cell count more than 500 cells/ μ l (14).

Several studies have demonstrated that 100 million to 1 billion new virions are produced in 24 hours in an infected individual. Less than 5% of the patients with early HIV disease may develop an AIDS defining illness or may die within 18 to 24 months. Ordinarily, an insidious, progressive decline in CD4+T cells (40 - 80 cells/ μ l per year) is expected in the absence of Antiretroviral therapy (ART) (14).

C. INTERMEDIATE STAGE OF HIV DISEASE:

The intermediate stage of HIV disease is defined by a CD4 count between 200 and 500 cells/ μ l. Although the relative risk of developing new OIs is higher among these patients, most of them remain asymptomatic or demonstrate only mild disease manifestations. The frequency of skin and oral lesions becomes more common throughout this stage. Among symptomatic patients, recurrent diarrhea, intermittent fever,

unexplained weight loss, and mild oropharyngeal or vaginal candidiasis represent the usual manifestations of illness at this stage. This symptom complex, previously referred to as AIDS Related Complex (ARC), usually indicates a more rapid disease progression than in the patients who do not develop these symptoms (14).

Other constitutional symptoms such as myalgia, arthralgia, headache and fatigue are often present intermittently. HIV associated nephropathy, a rare disorder characterized by progressive renal insufficiency and high-grade proteinuria, may manifest in some patients during this stage. Previous studies indicate that patients who do not receive anti-retroviral therapy during this stage of HIV disease have a 20% to 30% risk of progression to an AIDS defining condition or death over 18 to 24 months (14).

D. LATE STAGE DISEASE:

Late stage HIV disease is defined by a CD4 cell count between 50 to 200 cells/ μ l. Based on the CDC classification system, all patients with CD4 counts less than 200 cells/ μ l are categorized as having AIDS. The risk of developing a new AIDS defining condition rises dramatically when the CD4 counts drop below 200 cells/ μ l. the most common OIs to affect this group of patients is PCP. Risk of developing OIs,

including *Toxoplasma gondii* encephalitis, cryptosporidiosis, isosporiasis, tuberculosis, B-cell lymphoma, Kaposi's sarcoma, and esophageal candidiasis increases. Constitutional symptoms such as myalgia, arthralgia, low grade fever, and weight loss also become more common, along with other symptoms of ARC. Patients who receive no treatment at this stage have a 50% to 70% chance of developing a new AIDS defining condition or death within the next 18 to 24 months (14).

E. ADVANCED HIV DISEASE:

Advanced HIV disease is defined as a CD4 count of less than 50 cells/ μ l. The risk of developing certain OIs that are associated with more profound immunosuppression becomes significantly higher when the CD4 counts drop below 50 cells/ μ l. MAC disease, cryptococcal meningitis, cytomegalovirus (CMV) retinitis, invasive aspergillosis, progressive multifocal leucoencephalopathy (PML), disseminated histoplasmosis, disseminated coccidiomycosis, disseminated *Penicillium marneffei* infection, become more common during this stage of HIV infection. Patients with advanced HIV disease may develop coexisting OIs, with each OI contributing to the symptom complex (14).

A number of patients with advanced HIV infection develop significant, involuntary weight loss often referred to as the HIV Wasting Syndrome (HWS). Disorders of the brain become especially common in advanced HIV disease. Because of the substantially increased risk of OIs when the CD4 counts drop below 50 cells/ μ l, several strategies for prophylaxis of these OIs are used during this stage. These play a major role in prolonging the survival of the patient (14).

F. TERMINAL HIV DISEASE:

Although the rate of disease progression varied among patients, it is difficult to define a precise moment when an individual makes a transition from the advanced HIV infection to the terminal stage of HIV disease. The diagnosis of the terminal stage HIV disease is usually based on the inability to control the symptoms of the disease, because no treatments are available for the particular disorder, and available treatments become ineffective (14).

HIV PATHOGENESIS:

Once HIV enters the blood stream, it tends to infect activated CD4⁺ (helper) T cells, using the CD4 molecule as a receptor. These cells express the CD4/CD45RO⁺ phenotype or are primed or are memory T cells. HIV also infects monocytes, macrophages and related cells that express CD4-like

molecules (which are lesser in numbers than that in helper T cells). Monocytes and macrophages also get infected by taking up virus-antibody complexes through Fc receptors. This infection is productive but not cytotoxic, and the infected cells become the source of persistent viral infection (15). The lymphoid tissues, i.e. the lymph nodes and the intra-intestinal lymphoid tissue are the main reservoirs for the virus in infected individuals. The virus replicates continuously, even in the early stages of asymptomatic disease. In the central nervous system (CNS), the predominant infected cells are microglial cells of the monocyte-macrophage lineage. The significance of CNS infection in the pathogenesis of AIDS-associated dementia is associated with the loss of neuronal cells. Intestinal mucosal cells are infected possibly through membrane glycolipids. Intestinal infection may cause some forms of chronic AIDS-associated diarrhea (15).

A. EARLY VIRAEMIC STAGE

After entering the early viraemic stage, the virus appears to replicate at a very low level for a variable period of time. A transient decrease of total CD4⁺ T cells and an increase in the circulating HIV-infected T cells may be detected. Soluble p24 may be detected as early as after 5-10 days of infection. Circulating infectious viral particles are detectable for variable

times, usually starting about the same time as appearance of p24, peaking 10-20 days after infection, and persisting until free anti-HIV antibody becomes detectable, i.e. after seroconversion. After this phenomenon, the integrated and soluble viral genomes are detectable by Polymerase Chain Reaction (PCR) (15).

B. ASYMPTOMATIC STAGE

HIV positive individuals remain asymptomatic for variable periods of time, often 10-15 years. During that period, the virus replicates but it is restrained by humoral and cellular immune response.

Various types of antibodies are developed against HIV. The neutralizing antibodies developed against the gp120 and gp41 inhibit the infectivity of free HIV *in-vitro*. These antibodies help in reduction of the rate of progression to AIDS as well as the mortality rate. However, they fail to protect the infected individuals from eventually developing AIDS (15).

Cell mediated immunity may either block infection or prevent the establishment of a productive infection. MHC – 1 restricted CD8+ T lymphocytes recognize a variety of epitopes in *gag*, *env*, *nef*, and *pol* proteins. Cell mediated cytotoxic reactions appear to be prominent in HIV positive individuals who remain asymptomatic for prolonged periods (15).

C. IMMUNOSUPPRESSION

The disease progression towards AIDS is associated with an eventual loss of immunologic protective mechanisms, especially the progressive depletion of CD4⁺ T lymphocytes. This decline is concomitant with the increased HIV replication, suggesting that T-cell activation is necessary for the replication of the pro-viral DNA. The T-cell activation may be brought about by superantigens, activation of HIV-carrying dendritic cells, concurrent infections, and cytokines like TNF- α and IL-6 (15).

D. VIRAEemia

Enhanced viral replication leads to second wave of viraemia that is detectable as many as 14 months before the clinical evolution to AIDS. p24 antibody levels decline coinciding with this reappearance. In the terminal stages, both viraemia and antibody levels may decrease again, reflecting the exhaustion of the CD4⁺ cell population (15).

Despite the immune response generated by the host, the HIV manages to overcome the immune mechanisms and thrives in the cell system. The virus has been observed to launch a multipronged attack on the immune system. This includes the single cell killing, syncytia formation, depletion of CD4⁺ T cells, autoimmune reactions, anergy, programmed cell death

(PCD), generation of superantigens, activation of Cytotoxic T Lymphocytes (CTLs), and most importantly, generation of an array of cytokines and their dysregulation.

A. SINGLE CELL KILLING AND SYNCYTIA FORMATION:

One important virologic sign of progression of HIV infection is the emergence of HIV variants that induce the generation of syncytia in-vitro. Syncytium inducing (SI) HIV variants have been isolated in 50% - 60% HIV seropositive individuals with AIDS, but in less than 10% of HIV seropositive asymptomatic individuals (16). Approximately 70% of HIV seropositive asymptomatic individuals, in whom SI variants could be isolated, progressed to AIDS in a 30 month period (17).

It has been demonstrated that the fast replicating SI HIV variants are preferentially isolated in HIV infected individuals exhibiting a significant reduction in type 1 cytokines production, a significant rise in the type 2 cytokines production and the lowest CD4 counts (18).

In-vitro single-cell killing and syncytia formation occur through direct HIV-mediated cytopathic effect (19). Single-cell killing may result from the accumulation of unintegrated viral DNA or from the inhibition of synthesis of cellular proteins after HIV infection (20). The formation of syncytia involves fusion of the cell membrane of an infected cell with the cell membranes of

uninfected CD4 cells, which result in giant multinucleated cells. A direct relation between the presence of syncytia and the degree of the cytopathic effect of the virus in individual cells has been demonstrated *in-vitro* (21); and HIV isolated during the accelerated phase of infection *in-vivo* has a great capacity to induce syncytia formation *in-vitro* (23). Syncytia have rarely been seen *in-vivo*, however, *in-vitro*, their formation may be regulated by the leucocyte adhesion molecule LFA-1 (lymphocyte-function-associated antigen 1) produced by human CD4+ T lymphocytes inoculated with HIV (22, 24).

B. CD4+ T CELL DEPLETION:

Infection with HIV causes a gradual suppression of immunological function that results ultimately in a diverse array of OIs. The most striking feature of AIDS is a selective depletion of the CD4+ helper/inducer subset of T cells (26). The interaction between HIV and its human host involves an array of complex mechanisms whose end result is destruction of CD4+ T cells (27).

Initial infection with HIV is followed by a long and variable asymptomatic period that is characterized by clinical latency or low levels of circulating virus, stable or slowly declining CD4+ T cell numbers, and functional impairment of the

immune system. The development of AIDS follows activation of HIV expression and rapidly falling CD4⁺ T cell numbers. Since HIV can directly infect and kill CD4⁺ T cells in culture, the elimination of CD4⁺ T cells in-vivo can be assumed to be due to the direct cytopathic effect of the virus.

This effect may arise due to several mechanisms including (a) the destruction of the cell membrane that occurs as a result of massive viral budding (28, 29); (b) the presence of large amounts of non-integrated viral DNA, heterodisperse RNAs and viral core proteins in the cytoplasm of the infected cell that interfere with the proper cell function (30, 31); and (c) the complexing of HIV gp120 with intracellular CD4 molecules (32).

The depletion of CD4⁺ T cells may also be due to the destruction of precursor cells, which give rise to CD4⁺ T cells in-vivo or cells that produce factors essential for CD4⁺ T cell growth (33). An alternative mechanism for CD4 + T cell depletion involves the formation of short lived multinucleated giant cells or syncytia between an HIV-infected CD4⁺ T cell and a cluster of uninfected CD4⁺ T cells. In-vitro, syncytia form by the binding of the CD4 molecule on the surface of the uninfected CD4⁺ T cells to the gp120 molecules that are present on the surface of the HIV-infected CD4⁺ T cell (34).

In addition to its role as the receptor for HIV, CD4 molecule is the natural ligand for the MHC Class II molecules on antigen presenting cells (APCs) (35).

Both MHC Class II molecules and HIV gp120 bind to CD4, due to homology in certain antigenic determinants. Hence, the antibodies raised against HIV gp120 may cross-react with MHC class II molecules. It has been shown that HIV-infected patient sera contain antibodies to a region of the HIV transmembrane glycoprotein (gp41) that cross-react with MHC class II molecules (36, 37). It is possible that these antibodies have also been shown to impair the proliferative responses of CD4+ T cells to soluble antigens and alloantigens (37).

The elimination of CD4+ T cells may also result from the binding of soluble gp120 shed by HIV-infected cells to CD4, and subsequent attack by anti-gp120 antibodies. The attachment of free gp120 to the surface of uninfected CD4 + T cells may render these cells targets for ADCC responses with anti-gp120 antibodies (38). In addition, it has been observed that gp120-specific CD4 + cytotoxic T lymphocytes can kill uninfected CD4 + T cells that have internalized free gp120 molecules that are bound to CD4 and have presented a processed peptide in conjunction with MHC class II molecules (39).

C. HUMORAL IMMUNE RESPONSE TO HIV:

The first substantial antibody response is seen as the initial viraemia decreases (40) and in most people; this persists for 1-2 years before AIDS occurs. The antibody first produced is highly specific for the infecting virus, and antibody of this specificity is present for most of the subsequent time. As time progresses, a more broadly neutralizing antibody is produced. Some investigators have found a strong correlation between this activity and the ability to block the interaction between gp120/160 and soluble CD4. Presumably, the antibody could compete with broadly neutralizing monoclonals (41).

The fact that a significant proportion of patients progresses to AIDS in presence of the specific anti-gp 120/160 and soluble CD4 (s-CD4) attachment-inhibiting antibodies indicates that the presence of antibodies to these specificities alone does not prevent progression of the disease. The frequently accepted reason for this phenomenon is the production of antibody-escape mutants. Thus the antibody found in the host at a given time is specific for the virus which was present at least some months earlier. Also, 1-2 years before AIDS occurs, the germinal centre of lymph nodes has been destroyed and the antibody produced may be of a low affinity (41).

Within 2-4 weeks after initial infection, high levels of circulating free virus can be cultured from the plasma (42,43). This period of high titre plasma viraemia is associated with high levels of p24 antigen. Peak levels of plasma viraemia are generally associated with the developments of acute seroconversion. Within 10-21 days after the onset of the symptoms, a detectable antibody response is noted. The initial antibodies detected are typically immunoglobulin M (IgM) antibodies, which peak within 2 -5 weeks after the onset of clinical symptoms and are undetectable generally within 3 months of appearance (44). The IgG response occurs after the appearance of detectable IgM antibodies, with anti-gp 160 and p24 antibodies detected first, followed by antibodies to gp120 and gp41. Antibodies to the envelope portion of the virus (gp120 and gp41) persist for life, whereas anti-p24 antibody response may diminish to non-detectable levels in the later stages of HIV disease (45).

D. AUTOIMMUNE REACTIONS IN HIV:

Impaired humoral responses may result partly from the lack of helper T cells. In HIV infection, the B cell system seems to be in a state of permanent polyclonal activation, probably as a result of increased release of IL-6 by activated antigen presenting cells and T cells. Although many activated B cells

produce antibodies to HIV antigens, polyclonal activation and loss of immunoregulatory mechanisms probably allow production of unrelated antibodies, including anti-nuclear antibodies and auto-antibodies directed against the platelets and lymphocytes. Anti-lymphocyte auto-antibodies may be cross-reacting anti-viral antibodies. gp120 and the MHC molecule have structural similarities that enable them to interact with CD4. Moreover, an immuno-dominant region of gp41 is homologous to the β 1 region of MHC Class II (15).

Nonpolymorphic determinants of MHC class II molecules, particularly HLA-DR and HLA-DQ share some degree of homology with the gp120 and gp41 proteins of the HIV type 1(37, 38), and antibodies to these proteins may cross-react with HLA class II molecules. In fact, antibodies that react with class II molecules have been found in the serum of patients with HIV infection. These antibodies could prevent the interaction between CD4 and class II molecules expressed on the antigen-presenting cells, thus impairing the cellular interaction required for efficient antigen presentation and inhibiting antigen-specific functions mediated by CD4+T cells (46).

E. ANERGY:

gp120 antigen and antibody complexes bind to CD4 molecules, and the CD4 cells become refractory to further *in-vitro* stimulation through the activation of their CD3 molecules (47). This phenomenon is termed as anergy. Similarly, *in-vitro* peripheral blood mononuclear cells inoculated with HIV no longer respond to stimulation with anti-CD3 antibodies (48). These findings led to the hypothesis that a negative signal is delivered to CD4 molecules reacting with gp120 or gp 120-anti gp120 complexes. In this regard, anti-gp120 antibodies have recently been detected on CD4+ T lymphocytes in patients with AIDS (48).

F. CELL DEATH AND APOPTOSIS:

General assessment of an HIV-1-infected PBMC culture elucidates a diversity of problems. These comprise of: (a) impairment of proliferation of a majority of cells (b) a major disruption of cytokine regulation (c) death of some infected cells by non-apoptotic mechanism (d) death of infected cells by TNF- α , TNF- R and FasL-CD95 (Fas) interactions leading to apoptosis, and (e) death by apoptosis that bypasses the TNF- α and FasL Caspase 8 system and is instead mediated by mitochondrial disruption and a separate Caspase pathway (49).

In-vitro experiments have showed that HIV-1 infection of the Peripheral Blood Mononuclear Cells (PBMCs) markedly impairs T cell proliferation compared to controls, yet only a few cells are infected (50). Ultimately, many of these uninfected cells die by apoptosis (51). The evidence that uninfected cells like CD8+ T naïve T cells decline during disease progression supports this finding (52). It is possible to think about the loss of uninfected cells (a) to be mediated by cell-cell contact (infected cells with uninfected cells), (b) by a viral or host humoral factor, or (c) by more upstream generative abnormalities of the bone marrow and/or thymus, which could also be exogenous and due to infected progenitor cells. The diminished regeneration due to direct killing of precursors of T cells also occurs (53).

In brief, the events that lead to cell death include (a) mechanisms (viral and host) for the fate of the infected cell, (b) mechanisms (cellular and humoral) for anergy and/or apoptosis of 'bystander' cells (c) mechanisms for the generation of the hyperactive state, and (d) mechanisms for the impaired hematopoiesis and thymopoiesis.

The earliest attempts to explain HIV-1 killing of infected cells showed that killing was associated with cells that expressed abundant viral gene products associated with the activated

state of the cell (53). Previous studies have correlated the killing with the amount of CD4 and envelope glycoprotein expressed in the infected cell (54), the evidence for which has been provided by Sodroski and colleagues. They demonstrated that *in-vitro* killing by HIV-1 was associated with a fusion competent envelope expressed concomitantly with CD4 and an appropriate chemokine receptor (55, 56). This evidence is supported by the results of Westerndorp et al., showing that when CD4 of T cells is cross-linked with gp120 in the presence of Tat, the T cells undergo apoptosis (57).

Another mechanism for the death of infected cells is by classic CTL attack on cells expressing viral proteins. However, to do so, the CTL has to overcome several effects of Nef and avoid its own death from the specialized "bullets" HIV has in store for it. Nef contributes to diminished immune function of the infected cell by down-regulation of CD4 (49, 58) and thereby contributing to overall immunodeficiency, while concomitantly promoting more HIV production by removing the CD4 impediment to envelope budding (59). It also contributes to the prevention of super infections, which lead to the rapid death of the cells. Nef also fosters escape of the infected cells from CTLs by downregulating MHC Class I expression (60). Geleziunas et al. have observed that Nef associates with and

inhibits apoptosis signal regulating kinase 1 (ASK-1) (61), which is a crucial intermediate in death signaling mediated by both FasL and TNF- α , whereas Nef enhances expression of FasL (62).

Programmed cell death or apoptosis is a normal mechanism of cell death that has been originally described in the context of the response of immature thymocytes on cellular activation (63). It is a mechanism by whereby the body eliminates autoreactive clones of T cells (64, 65). It has recently been suggested that both qualitative and quantitative defects in the CD4⁺ T cells in patients with HIV infection may be the result of activation-induced cell death or apoptosis (66, 67). There has been a speculation that cross-linking of the CD4 molecule by HIV-gp120 or gp120-anti-gp120 immune complexes prepares the cell for the PCD that occurs when an MHC class II molecule in complex with an antigen binds to the T-cell antigen receptor (68, 69). Thus a mere activation of a prepared cell by a specific antigen or superantigen could lead to the death of the cell, without direct infection with HIV. However, PCD occurs spontaneously *in-vitro*, in absence of an antigenic stimulus in CD8 cells (70). This may help explain the depletion of CD4⁺ T cells without requiring that each depleted cell be infected with HIV (49).

G. GENERATION OF SUPERANTIGENS:

Considerable attention has been given to the possibility that a superantigen, either retrovirally encoded, or unrelated to HIV, may play an important role in the immunopathogenesis of HIV infection (25). Superantigens are microbial or viral antigens that are capable of binding to nearly all T cells that have a specific variable region of the β chain of the T-cell antigen receptor. The conventional antigenic peptides bind in the groove of the MHC class II molecules, requiring the interaction with specific variable components (variable α and β , joining α and β , and diversity β) of the α and β chains of the T-cell antigen receptor, and thus result in the stimulation of only a small fraction of T cells. Superantigens, however by binding only to the variable β region of the T-cell antigen receptor, induce massive stimulation and expansion of the T cells bearing the specific variable β region. This is followed by deletion and anergy of these T cells (72).

The superantigen hypothesis has evolved from the observations that endogenous or exogenous retroviral-encoded superantigens stimulate murine CD4 cells in-vivo, leading to the anergy and deletion of a substantial percentage of CD4+ T cells that have the specific variable β regions (72).

H. HIV-SPECIFIC IMMUNE RESPONSES:

Both humoral and cellular immune responses attribute to the anti-viral immunity (73a). The major anti-viral effects of antibodies are attributed to their neutralizing properties (73b). However, antibodies directed against some regions of the envelope of HIV may have an additional protective function related to their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), after binding to Natural Killer (NK) cells (73b), leading to the killing of HIV-infected cells that express the envelope proteins on their surfaces. HIV-specific cytotoxic T lymphocytes may play an important part in the immune response against HIV. They have been found in substantial percentage of patients with HIV infection (73a). These mechanisms of immunity and the effector cells may have a dual role. The first is a protective role in the initial immune response to HIV infection, at which time these mechanisms may help to control or clear the infection. The second is a pathogenic one during the chronic phase of infection, when the same mechanisms may be involved in the elimination of HIV-infected cells (CD4⁺ T cells, follicular dendritic cells, and macrophages) and may thus contribute to the progressive deterioration of the immune system (73a, 73b).

I. CO-RECEPTORS IN HIV:

Host genetic factors may modify susceptibility to HIV-1 infection and affect the clinical outcome to AIDS and/or death in infected individuals. CD4 is the primary receptor for HIV. CD4 is found in T lymphocytes, monocytes / macrophages, etc. It is a transmembrane glycoprotein of 58 kD. Specific activation of T cells by antigen presentation induces CD4+ T cell receptor formation (11). Evidence for the CD4 molecule as the receptor for HIV infection comes from the fact that HIV infection of the CD4+ cells is blocked by both soluble CD4 and monoclonal anti-CD4 antibodies (74). Expression of human CD4 in human CD4 negative cells by DNA transfection was necessary and sufficient for HIV infection, while mouse cells expressing human CD4 were not infected by HIV (74).

Along with CD4 molecule, HIV-1 requires cellular co-receptors for infecting the target cells. HIV-1 T-cell-tropic (T-tropic) isolates use CXCR4-chemokine receptors, especially CXCR4. On the other hand, the macrophage-tropic (M-tropic) strains utilize CC-chemokine receptors, mainly CCR5. Minor receptors like CCR2b and CCR3 are also engaged. The dual tropic variants can use both types of receptors.

It has been shown that HIV also uses these co-receptors for entry into the target cells in addition to CD4 molecules.

Polymorphisms of some chemokines (Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), Macrophage Inflammatory Protein 1 (MIP-1), etc.) and chemokine receptors may modify transmission and disease progression in HIV (74). It has been observed that a homozygous 32bp deletion in the coding region of the CCR5 gene arrests the expression of the HIV-1 co-receptor. This provides a strong, though not absolute, resistance to M-tropic viral infection (75). The homozygous CCR5- Δ 32 deletion does not exert any protective effect once the HIV-1 switches to other co-receptors like CCR1 to CCR4 and/or CXCR4 (76). The single copy of CCR5- Δ 32 does not seem to protect against HIV-1 transmission, but the protective effect for heterozygotes with CCR5- Δ 32 as compared to wild-type infected adults with CCR5 has been observed during the HIV disease progression (77). This mutation in both alleles has been found to be associated with resistance to HIV infection and individuals heterozygous for Δ 32 mutation in CCR5 gene were found to be long term non-progressors (78).

A point mutation in the CCR2b gene (CCR2-641) has been observed to prolong the AIDS-free survival from 2 to 4 years in HIV-1 seroconvertors (79). Winkler et al. have described the first polymorphism of a chemokine gene, the Stromal Derived

Factor 1 (SDF1) that can ameliorate the clinical outcome of HIV infection. The mutation has been identified as a change from guanine to cytosine in the 3' untranslated region (3'UTR) of the SDF1 β gene transcript (SDF1-3'A) (80).

Mangano et al. have studied the protective effect of CCR2-641 allele in HIV infection by vertical transmission, with a clear delay in progression to AIDS and death in perinatally HIV-1 infected children. The CCR5- Δ 32 and SDF1-3'A polymorphisms do not modify either mother-to-child HIV-1 transmission or clinical outcome to AIDS. However, CCR2-641 polymorphism did not seem to prevent HIV-1 sexual or parenteral transmission (76).

VERTICAL TRANSMISSION OF HIV AND ITS

MANIFESTATION IN CHILDREN:

Most of the studies on HIV have been carried out in adults, and the inconsistent findings derived from these studies can be attributed to multiple host and viral factors. These include the racial background, route of infection, sample size, etc. One of the major causes of variation is the difficulty in determining the exact timing of the viral infection. The perinatally HIV-1 infected cohort is less complex and variable as it has a more precise timing of viral infection. This cohort is

hence a better model to study the host genetic factors on the HIV-1 disease progression (49).

As there has been a global increase in HIV -1 infection in women in childbearing age, there has also been an alarming increase in the number of mother to child transmissions of HIV-1. Use of ART reduces the rate of vertical transmissions, but such therapy is not usually available in developing countries (85).

Co-receptor use corresponds to the biological phenotypes "Non-Syncytium inducing" (NSI) virus and "Syncytium inducing" (SI) virus. The NSI isolates preferentially use CCR5 receptor only, and are called R5 viruses, while the SI isolates use CXCR4 receptor, and are termed X4 viruses. The SI viruses have also been shown to use both the receptors and are designated as R5X4 dualtropic phenotypic viruses (81). Patients who harbor SI or X4 phenotypes progress more rapidly towards AIDS than those who harbor NSI or R5 variants (82). The phenotype of the virus may also influence the transmission to the child. It has been observed that the mothers with the SI strain are more likely to transmit the virus than mothers with the NSI strains (83).

In Asia, subtype C and the circulating recombinant form CRF01_AE are most commonly observed (84).

In a study by Casper et al, all 11 HIV-1 infected children had R5 isolates in the first blood samples collected, including the 2 children born to the mothers who carried HIV-1 with the X4 phenotype. This could have a direct bearing on the hypothesis that certain phenotype of HIV-1 could be preferentially transmitted from the mother to the child in the initial phase of infection (85).

It was also observed that an early R5 → X4 switch in children born to mothers carrying a X4 phenotype is related to transmission of the virus from the mother, whereas a late switch may be related to development of mutations in HIV-1, causing a switch in the child (85).

Vertically acquired HIV infection can cause either extremely rapid symptomatic disease, with death in early childhood, or a chronic condition with varying patterns of disease. The factors that influence the evolution of HIV infection and therefore determine the natural history of the disease in each patient, are likely to include both host and viral factors (86). A substantial number of children survive after early childhood and have different patterns of disease progression. A wide range of clinical features has been observed in children who survived longer than 5 years; 15% were completely free from

any symptoms, 30% had minor clinical manifestations, and 55% had severe disease (86, 172).

Pediatric Human Immunodeficiency Virus infection has a significant impact on the health and welfare of children across the world. It differs from the adult disease in many aspects. Majority of the cases in children occur due to perinatal transmission, i.e. from the mother to the infant. In these infants, the incubation period varies and two groups of children have been identified: those with a short incubation period and the development of disease within the first 2 years of life, and a second group with a more prolonged incubation period (87).

HIV infection in children is considered a chronic, multi-systemic illness with complex health care needs. Pediatric HIV infection is a family unit disease and frequently multiple family members including parents and children are infected. These raise the issues of bereavement, foster care, and adoption. Finally, because the majority of HIV infections in children are perinatally acquired, emphasis is to be placed on the prevention of this perinatal transmission. From a data obtained from the USA, it has been observed that around 86% of the HIV infected children have been infected vertically, i.e. from a seropositive mother (88).

There are some important differences between the children infected perinatally than later in life. Infants tend to progress faster than older children or adults in the development of clinical symptoms, immunodeficiency and related illnesses. In studies carried out with perinatally infected children, the median age of onset of clinical symptoms varied between 5 and 10 months of age (89-92). AIDS defining criteria also appeared earlier in life, generally during the first 2 years. It has been subsequently observed that the overall survival of children with HIV infection has improved, and some children with perinatal infection have survived into the teenage years.

The Pediatric AIDS case definition that includes children from birth through 12 years of age was initially formulated in 1983. It has undergone subsequent revisions (93). The pediatric definition of AIDS is similar to that of adults but also includes clinical diseases that are specific to infants and children, like lymphoid interstitial pneumonitis, and recurrent bacterial infections.

The mechanism of perinatal transmission and the timing are yet not well understood. Perinatal transmission of HIV may occur *in-utero*, intrapartum or postpartum as a result of breast-feeding from an infected mother. It has been suggested that an infant with a positive virus culture or polymerase chain

reaction during the first 48 hours of life is most likely to have been infected *in-utero*. An infant with a positive HIV culture or PCR occurring after 7 days but before 90 days of age acquired the infection intrapartum (94). Preliminary data from prospective studies of infants borne to HIV seropositive mothers suggest that HIV can be cultured in 30-50% of infected infants during the first week of life (95, 96). In addition, a study of twins born to HIV seropositive women showed an increased rate of transmission to the first-born twin, suggesting intrapartum transmission (97).

Postnatal transmission of HIV infection from breast milk has been reported. HIV has been identified from the cell-free extracts of human breast milk from HIV seropositive women (98). There are documented cases of postnatal transmission in children born to women who acquired HIV infection postpartum, usually through transfusion of contaminated blood or through heterosexual contact and breastfed their infants (99-103).

It is postulated that these women are viremic, secrete the virus into their breast milk, and have not yet developed antibodies to the virus, thus potentially exposing the breastfeeding infant to a higher titre of the virus. The risk of

transmission of HIV infection through breastfeeding is estimated to be 29% (104, 105).

Approximately 70% of infants born to HIV seropositive mothers are not infected. The factors associated with perinatal transmission have not been fully elucidated. In most studies, perinatal transmission is not associated with parity, race, maternal age, or maternal mode of acquisition of the virus (106-108). Two prospective studies have shown an association between maternal CD4+ lymphocyte counts (less than 700/mm³ (109) and less than 40/mm³ (110)) and transmission. Some studies have initially found that the absence of maternal antibody to V3 loop peptides in the gp120 envelope protein resulted in a higher likelihood of transmission to their infant (110). Subsequent studies have however yielded conflicting results (111).

Several factors complicate the diagnosis of HIV infection in infants less than 18 months of age. At birth, most infants born to HIV-seropositive mothers have normal clinical and immunological parameters. These infants however, will be HIV seropositive because of passive transfer of the maternal antibody across the placenta. The median time for the disappearance of the maternal antibody is 10 months (89-92),

with the majority of infants converting to seronegative by 18 months of age.

The diagnosis of HIV infection in children less than 18 months of age requires two positive test results using combination of HIV cell culture, HIV PCR HIV p24 antigen detection test. For a child above 18 months of age, a repeatedly reactive ELISA with confirmation by Western blot or immunofluorescence test is necessary (112).

Majority of children recognized to be infected have severe systemic manifestations with growth failure, opportunistic infection, diarrhea, chronic interstitial pulmonary infiltrates, and oral candidiasis. In children infected perinatally with HIV, the time between the acquisition of the virus and development of symptoms is frequently very short. Most infants born to women who are HIV seropositive are asymptomatic at birth, independent of their ultimate infection status. The majority of infected infants develop symptoms of HIV infection within the first year of life (89-92).



Table 1: Non infectious clinical manifestations of HIV-1 infection

System	Clinical Manifestation
General	Failure to thrive, lymphadenopathy, hepatomegaly, splenomegaly, parotitis, dental caries, aphthous stomatitis, oral ulcerations
Pulmonary	Lymphoid interstitial pneumonitis, chronic lung disease
Cardiac	Cardiomyopathy. Pericarditis, arrhythmia
Renal	Proteinuria, nephritic syndrome, acute nephritis, renal tubular acidosis
Nervous system	Developmental delay, learning disabilities, encephalopathy, peripheral neuropathy
Gastro-intestinal	Anorexia, Wasting Syndrome, diarrhea enteropathy, hepatitis
Skin	Atopy, seborrhea, psoriatic skin rash, drug eruptions ,pruritic popular skin rash on lower extremities
Malignancy	Lymphoma, Kaposi's sarcoma, leiomyosarcoma

The above table illustrates the signs and symptoms commonly observed in HIV infected children. Mostly two or more signs and symptoms are present, and are notable by their persistence and severity. During the first year of life, oral candidiasis, hepatosplenomegaly, failure to thrive, generalized

lymphadenopathy, and developmental delay are common findings.

Progression of HIV infection in infants and children occurs earlier and is more rapid than in adults. In infants with perinatal infection, the median age of onset of disease is between 5 and 10 months of age (114). However the expression of disease varies, with some children developing severe disease within the first 2 years of life, whereas others remaining asymptomatic for a longer duration. A few children have been observed not to develop symptoms till early adolescence.

The association between low CD4+ lymphocyte counts and progression of clinical disease is still being defined. Infants with HIV infection as a group have a lower CD4 count than HIV-exposed but uninfected children by 6 months of age (113). The Italian registry has demonstrated that children with HIV infection and low CD4+lymphocyte counts in the first 6 months of life had more rapid progression of clinical disease, than those infected infants with normal lymphocyte counts (114).

CYTOKINE DYSREGULATION IN HIV:

As hypothesized by Mosmann, et al, cytokines can be classified as Th1 (IL-2, IFN - γ) and Th2 (IL-4, IL-6, IL-10). TNF- α is categorized with the Th1 cytokines (126).

1. INTERLEUKIN 2 (IL-2)

Interleukin 2 has been described in 1965 as a T-cell growth factor present in tissue culture supernatants of human peripheral blood lymphocytes stimulated with T cell specific mitogens. IL-2 is primarily produced by activated T helper 1 (Th1) cells. This cytokine promotes the growth of T lymphocytes and is considered to be responsible for activities of other factors like T cell growth factor (TCGF), thymocytes stimulation factor (TSF), thymocytes mitogenesis factor (TMF), T cell replacing factor (TCF) and killer helper factor (KHF) (115).

Studies on the Th1-Th2 models have shown that IL-4 and IL-10 strongly affect IL-2 responses. IL-2 has been reported to participate in the activation, growth and tumoricidal activities of LAK and NK cells. It also augments the growth of B cells and enhances immunoglobulin production. IL-2 has been demonstrated to induce the production of IFN- γ and the production of IL-6 from monocytes. It also modulates the histamine secretion from stimulated basophils (115).

The soluble receptor of IL-2, i.e. sIL-2R, has been studied as a marker for T cell involvement in disease states like autoimmune disorders, malignancies, transplant rejection, and infectious diseases (115).

2. INTERLEUKIN 4 (IL-4)

Interleukin 4 has been first described in 1982 from the supernatants from Phorbol-12-myristate-13-acetate (PMA) stimulated EL-4 thymoma cells capable of supporting the growth of anti-Ig stimulated B cells. The factor in these supernatants is distinctly different from IL-2 and has been termed IL-4 (115).

IL-4, a pleiotropic cytokine produced by T cells, mast cells activated human basophils, regulates the wide range of B cell responses including proliferation, expression of MHC II antigen, and expression of IgG and IgE molecules. IL-4 production is concentrated among the Th2 subset of activated T cells. It has also been described in primed CD8+ T cells. IL-4 along with IL-10 inhibits the cytokine production by Th1 cells, which is of importance during disease progression to AIDS (115).

IL-4 induces the proliferation of thymocytes in presence of PMA and differentiation of CD4+ T lymphocytes into Th2 cells. It acts synergistically with IL-1 as an autocrine growth factor

for antigen specific T cells to enhance antigen presentation and phagocytosis in macrophages. It also enhances the development of CTLs from resting murine cells as well as augmenting LAK cell activity. However, it inhibits the activated macrophages to kill various intracellular and extracellular pathogens (115).

3. INTERLEUKIN 6 (IL-6)

Interleukin 6 is a pleiotropic cytokine produced by fibroblasts, activated macrophages / monocytes, activated T and B cells, endothelial cells, stromal cells and a variety of tumor cells. It regulates immunological responses, hematopoiesis and acute phase reactions. It has been earlier designated as IFN-2 due to its weak anti-viral activity, and later (1988) named IL-6 after the discoveries of IFN-2 and B cell stimulatory factor (BSF) (115).

IL-6 induces proliferation of activated T cells, B cells, mesengial cells, keratinocytes, Kaposi sarcoma cells and synovial fibroblasts. It acts as an inducer for CTL and B cell differentiation and stimulates IgG secretion. This cytokine induces the acute phase proteins like fibrinogen, -1- anti-chymotrypsin, -1- acid glycoprotein, haptoglobulin and C-reactive protein. Thus inhibition of IL-6 may cause immunosuppression.

Blockade of IL-6 induced growth of mesengial cells and Kaposi cells suggests a potential role of IL-6 in alleviating symptoms associated with complications of AIDS' (115).

4. INTERLEUKIN 10 (IL-10)

IL-10 has been originally described as a Cytokine Synthesis Inhibitory Factor (CSIF) produced by Th2 cells. Although detected in supernatants of activated Th2 cells, IL-10 mRNA has been detected in a variety of cell types, including Th1 cells (115).

Studies show IL-10 to be an immunosuppressive agent that inhibits the synthesis of several Th1 type cytokines like IL-2, IFN- γ and TNF- β . IL-10 down regulates expression of MHC Class II, thereby impairing the antigen presenting capacity of monocytes. In contrast, IL-10 stimulates the B cell proliferation and antibody secretion. Also, it immunostimulates the cytokine activated T cells (115).

5. INTERFERON GAMMA (IFN - γ)

Interferons are classified into two groups based on their ability to induce anti-viral response. Type 1 includes leukocyte derived IFN- α and fibroblast derived IFN- β . Additional members of this group include IFN- ω and IFN- λ . IFN- γ is the sole representative of type 2 or immune interferon. It is

produced exclusively by T lymphocytes and NK cells in response to mitogenic or antigenic stimulation (115).

IFN- γ induces MHC Class I and II antigens on the macrophages, T and B cells. It also induces the cytolytic activity of LAK and CTL cells but inhibits the secretion of Th2 cytokines. Tm (T memory) cells are not subject to anti-proliferative effects of IFN- γ (115).

6. TUMOR NECROSIS FACTOR ALPHA (TNF- α)

TNF- α and TNF- β mediate immune and inflammatory responses. Both enhance proliferation of activated T and B cells and induce MHC Class I and II expression. TNF - α is produced by a variety of cells including activated monocytes, macrophages and mast cells. TNF- β , also known as lymphotoxin (LT), is predominantly produced by activated lymphocytes (115).

Signaling through TNF-Receptor1 leads to apoptosis, it also activated NF- κ B to induce proteins that inhibit cell death. A mutant super repressor form of I κ B blocks the activation of NF- κ B and leads to TNF- α induced cell death (115).

TNF- α on one hand acts as a B cell growth factor and induces clonal expansion of B cells during infection, while on the other, it itself increases HIV-1 expression in infected cells. Murine studies have shown that TNF- β deficient mice did not appear

to be immunocompromised. Their CMI responses were normal but the humoral response was impaired. Both TNF- α and TNF- β play an important role in the induction of Graft Versus Host Disease (GVHD) (115).

In HIV, it has been shown that T cell proliferation rate is increased maximally twofold to threefold in the CD4+ population and six-fold to sevenfold in the CD8+ population (116). A technique using deuterated glucose to label DNA in-vivo has shown that the turnover of CD4+ and CD8+ T cells in HIV-infected patients is about three times higher than that of uninfected individuals (117). Two models have been proposed to explain the hyper-activation and increased proliferation of T cells in HIV-1 infection. One model contends that T lymphocyte activation in HIV is driven by antigens from HIV and/or other pathogens (118). Alternatively, increased production of CD4+ T cells may be a homeostatic response to compensate for the loss of CD4 + T cells that are killed by HIV (118).

In HIV infection, two types of cellular antiviral responses associated with CD8+ T cells have been described. One response, mediated by CTLs, involves the cytolysis of infected cells in a HLA - restricted manner (119). Cytolysis results from the targeted release of contents of exocytic granules from the

CD8+ cell. These granules contain perforin and granzymes (serine proteases) that act together to induce apoptosis in the targeted cell (120).

The second type of CD8+ cell-mediated anti-HIV response involves the suppression of HIV replication in cultured CD4+ cells in the absence of cell killing (121). This CD8+ cell non-cytotoxic antiviral response (CNAR) is not HLA-restricted. It blocks the HIV transcription, and is associated with the production of a novel CD8+ cell antiviral factor (CAF). CAF lacks identity with other known cytokines (121).

Study carried out by Mackewicz et al. indicate that the granules of CD8+ T cells from HIV-infected subjects do not contain the anti-viral activity that is associated with CAF. Instead, the antiviral activity resulting from CAF has been found in the cytoplasmic fractions of the antiviral CD8+ cells. These findings suggest that CAF is not stored at substantial levels in the cell but requires new synthesis for its production (122).

Differential cytokine production by CD4 + Th cells during an immune response plays an important role in regulating the nature of the immune response. Human CD4 + T cell clones specific for bacterial antigens and allergen or helminth-specific

T cell clones have been observed to exhibit Th1 or Th2 like cytokine production profiles (123, 130).

T helper cell clones can be divided into two types based on the pattern of cytokines secreted in response to antigen or lectin stimulation. Th1 clones secrete IL-2, IFN γ , and lymphotoxin (LT). Th2 clones express IL-4, IL-5, and IL-6. Both types of clones secrete IL-13, granulocyte-macrophage colony stimulating factor (GM-CSF), TNF- α , pro-enkephalin, etc. The differences between Th1 and Th2 cytokine synthesis patterns lead to markedly different functions (124). Th2 clones are normally better B cell helpers (125), while Th1 clones preferentially induce macrophage activation and delayed type hypersensitivity (DTH). Evidence suggests the existence of other differentiation states of the mouse Th cells, and Th1 and Th2 phenotypes represent activated effector cells (126).

Both murine and human CD4 + T cells with an intermediate (Th0) have also been described. (127) Th1 and Th2 cells may differentiate from a common pool of precursors by the pathways controlled, in part by cytokines produced by lymphocytes and accessory cells. IFN γ has been observed to promote the differentiation of Th1 precursors into Th1 cells (128). On the other hand, IL-4 augments the differentiation of Th2 cells. A reciprocal regulatory role of cytokines in the

differentiation of human Th1 and Th2 cells has been demonstrated (129).

It has been reported that IL-12, in-vitro and possibly, in-vivo is a physiological inducer of the Th1 type of immune responses, with a negative effect on the development of IL-4 producing Th cells. IL-12, in association with IL-2, stimulates IFN γ production by both T and NK cells. IFN γ inhibits both the development of T cells into IL-4 producing clones and the proliferation of Th2 clones (128). Hence, the IL-12 induced T cell differentiation into Th2 like cells could be indirect and mediated by induced IFN γ production (130,131). Cytokines like IFN γ , TNF- α , IL-4 and IL-6 also induce both T cell differentiation and CTL development (132).

Fiorentino et al. have reported the inhibition of IFN γ by Th2 clones stimulated by Concanavalin A (126). The decrease in the IFN γ production may be due to a decrease in the synthesis and/or secretion. It has been observed that secretion was not solely responsible for this decrease as elevated intracellular levels of IFN γ were not observed. Under a similar experiment, the Th1 clones stimulated by Concanavalin A, and depleted of IFN γ and IL-2 did not inhibit IFN- γ production. The IFN γ was observed to be inhibited at mRNA level. The possible mechanisms include reduction of transcription,

accelerated degradation, or altered mRNA processing. The inhibitory factor was labelled as Cytokine Synthesis Inhibitory Factor (CSIF), now known as IL-10 (126).

The action of IL-10 on Th1 cytokine synthesis may be indirect, since it inhibits IFN γ synthesis in response to antigen plus the Antigen Presenting Cell. Also, the inhibition is brought about at later times after stimulation (126).

IL-2 causes increased proliferation of immature thymocyte subsets as well as activated mature T cells (133). IL-4 increases thymocyte maturation and induces differentiation of CD4⁺CD8⁺ and CD4⁻CD8⁺ cells (133). IFN γ affects the expression of the cell surface antigens involved in the immune regulation. This cytokine induces MHC Class II gene expression, and can thus influence antigen presentation to CD4⁺ helper T cells.

IL-10 has been reported to be produced by the CD4⁺ Th2 subset and inhibit the production of cytokines, particularly IFN γ , by Th1 clones (132). This inhibition occurs at the transcriptional level. The inhibition of cytokine(s) production by IL-10 was observed only when macrophages were used as the APCs. IL-10 has been demonstrated to be pleiotropic, and to act on different cell types, including thymocytes, cytotoxic T lymphocytes, mast cells, B cells and macrophages. It inhibits

the antigen specific immune responses by down-regulating the Class II MHC molecules (132).

Maximal IL-10 production occurs 24-48 hours after activation.

This is relatively late as compared to the production of IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α , which are secreted in high amounts between 4-8 hours after activation (134). IL-1 can induce IL-6 production in fibroblasts, thymocytes and monocytes. It is therefore possible that the partial inhibition of IL-6 is due to the impairment in the IL-10 production (135). The inhibitory activity of exogenous IL-10 is more pronounced than that of the endogenous IL-10.

IL-4 has been shown to inhibit the production of IL-10 by at the transcriptional level, but not totally. IL-4 at a very high concentration of 400U/ml has been shown to inhibit 70% of IL-10 activity. IL-4 is also able to impair the production of IL-6 and TNF- α in human monocytes (136).

IL-10 is an autoregulatory cytokine, as seen by the inhibition of IL-10 mRNA synthesis in monocytes activated for 24 hours by exogenous IL-10. Thus IL-10 may have strong down-regulatory effects on the ongoing antigen specific T cell responses. It may be the cytokine that plays a major role in dampening the antigen driven proliferative T cell responses. IL-10 has strong down regulatory effects on the secretion of

the pro-inflammatory cytokines like IL-1, IL-6 and TNF- α . These cytokines modulate the activation and function of the immune system. IL-10 thus plays a major role in the suppression of the immune and inflammatory responses (136). The maturation of B cells into efficient producers of high affinity and high specificity antibodies is regulated by soluble cytokines. *In-vitro* studies have demonstrated the important role of IL-2, IL-4, and IL-10 on B cell maturation (137). IL-10 acts as a growth factor and a differentiation factor for normal B lymphocytes activated through their CD40 antigen or antigen receptor (138, 142), while it inhibits IFN - γ . IL-2 has been observed to be a potent proliferation and differentiation-inducing agent of B-lymphocytes after the cross-linking of their surface immunoglobulin molecules (139). IL-2 has been observed to upregulate its own receptor, whereas IL-4 was described to inhibit the expression of high affinity IL-2 receptors (140).

IL-6 is a T-cell stimulating cytokine like TNF- α , which is released from macrophages (142). This cytokine has been reported to affect the functions of B cells (141), hematopoietic progenitors (142), and plasma cells (143). This suggests that the dysregulation of IL-6 might be involved in the

abnormalities of the humoral immune response during HIV infection.

Enhanced levels of serum IL-6 in advanced HIV disease have been reported in a study carried out by Honda et.al (144). Elevated plasma levels have also been detected in the HIV seropositive and it is suggested that IL-6 overproduction contributes to the polyclonal B cell activation in HIV infection (144). A raised level of IL-6 in serum might be a general phenomenon in diseases with immune pathogenesis, like HIV. For example, raised IL-6 levels have been detected in sera from patients with adult T cell leukaemia; Human T cell lymphotropic virus (HTLV) associated myelopathy and tuberculosis (145).

Monocytes from HIV-infected patients have been observed to be having elevated intracellular levels of IL-1 α , IL-1 β and TNF- α , as compared to uninfected individuals (133). HIV infection of monocyte-macrophages leads to increased levels of IL-6 (133).

IL-6 promotes HIV replication and thus could potentiate circulating viral particles. The increase in IL-6 RNA levels by HIV infection is likely to promote further activation of thymocytes, thereby promoting increased HIV replication. Increased IFN - γ levels in thymus due to HIV infection may be

the result of host defensive mechanisms for the preservation of immature thymocytes. IFN - γ is a known antiviral agent that inhibits RNA and DNA viral synthesis and replication (133). It has been found to inhibit HIV-1 replication in peripheral blood mononuclear cells (133).

Both host and viral factors may influence disease outcome in HIV-infected subjects. Analyses of the host factors have revealed a complex pattern of alterations affecting the cytokine network to be a characteristic of HIV infection (146). Different studies have demonstrated a defective production of IL-2, (147), IL-12 (148) and an increase in the levels of IL-4, IL-6 and IL-10 (149). Clerici et.al have proposed that CMI, mainly stimulated via the production of type 1 cytokines IFN- γ , IL-2 and IL-12 could be more protective than humoral immunity in preventing the progression of HIV infection to AIDS (149). They have observed a decrease in the IL-2 production and a concomitant increase in IL-4 and IL-10 production to be associated with HIV disease progression. The loss of IL-2 production has been demonstrated to be predictive for a decline in CD4 counts, time to AIDS and time to death. Therefore, a strong type 1/ weak type 2 cytokine production would help in slower progression to AIDS (149).

Analyses of viral factors have suggested that different levels of viral activity are associated with different stages of infection and disease progression. Viral indices like circulating p24 antigen, viral isolation from plasma, tissue culture infectious titres, etc., have been utilized to monitor disease outcome in patients at different stages of the disease (149).

It has been reported that the cytokine production of mitogen-stimulated PBMCs from vertically infected pediatric patients with different patterns of disease progression is different in asymptomatic children compared to severely symptomatic children. For example, IL-10 and IL-4 production was significantly higher in the symptomatic group when compared to the asymptomatic group. The Progressive Infection (PI) showed lower levels of IFN- γ and IL-2 as compared to healthy controls or long-term non-progressors (LTNPs) (149).

HIV-infected individuals suffer from severe hematopoietic abnormalities including anemia, granulocytopenia, thrombocytopenia and loss of CD4⁺ cells (150). It has been suggested that abnormal hematopoiesis occurring in HIV-infected patients may be the result of cytokine dysregulation (151). HIV infection is known to alter expression of cellular proteins via modulation of cytokine expression (152), which in turn could alter immune responses to viral infection. A shift in

cytokine profiles from type 1 (IL-2, IL-12 and IFN γ) to type 2 (IL-4, IL-5, IL-6 and IL-10) is observed in HIV-infected individuals (153). A helper T cell type (Th) shift is also observed at single cell level with disease progression (154). These cytokine changes in turn can affect the expression of various other cellular genes. For example, IL-4 upregulates the *in-vitro* expression of the HIV co-receptor CXCR4 (155).

Cytokine production from T cell lineage cells is also altered by HIV infection. Anti-CD3/anti-CD28-activated T lymphocytes from HIV-1 infected individuals have shown decreased levels of IFN γ and IL-13 as compared to uninfected individuals. HIV infection of the human T cell line MT-4 has been shown to cause an elevation of the chemokine macrophage inflammatory protein 1 β (MIP-1 β) and its receptor CCR5 (155).

CD8⁺ T cells are a major defence against viral infections and intracellular parasites, due to their production of IFN γ and TNF- β as well as their cytolytic activity. Th2 cells have been designated as type 2 CD8⁺ cells. It has been reported that the murine CD8⁺ cells can be primed *in-vitro* to produce IL-4 (156). Upon this activation in presence of IL-4, they can switch the development to CD8⁻ CD4⁺ Th2 phenotype that is not cytolytic, and does not produce IFN γ (156). With very low numbers of CD4⁺ circulating T lymphocytes (< 50 cells/ μ l), it

was observed that all CD8⁺ circulating lymphocytes had switched *in-vivo* from the production of Th1 type cytokine IFN- γ to the production of Th2 type cytokines like IL-4, IL-5 and IL-10.

It would be very serious for the host if the CD8⁺ T cells started to produce Th2 type cytokines. It has been observed that in a *Schistosoma mansoni* infection, the Th2 type cytokines that are induced impair the CD8 T cytolytic activity of the host against *Vaccinia* virus and delayed the clearance (157).

Clinical symptoms of a relative dominance of Th2-mediated responses have been described in HIV-1 infected individuals (157). A hypothesis has been proposed that a shifting of CD4⁺ Th cells from the Th1 to Th2 phenotype occurs in the course of HIV-1 and can favour the progression of HIV infection toward the full-blown disease (156). All CD4⁺ CD8⁺ T cell clones derived from HIV infected patients showed a clear-cut Th2-type profile of cytokine secretion, whereas the great majority of CD4⁺ CD8⁺ T cells obtained from HIV-1 seronegative healthy individuals had a Th1 profile. CD8⁺ T cells exhibited markedly reduced cytolytic activity, and were unable to produce IFN- γ , TNF- α and TNF- β . A noticeable proportion produced IL-10, a

powerful inhibitor of Th1 cytokines and IL-4 inhibition of macrophage inflammatory functions (157).

Analysis of mRNA expression revealed that the majority of these CD8⁺T cell cells had lost the ability to express the CD8⁺ molecule on their surface, while a minority failed to express mRNA for both CD4 and CD8 molecules. This suggests that the CD4⁻ CD8⁻, Th2-like, non-cytolytic T cell clones present in HIV infected patients may result from *in-vivo* switching of CD8⁺, Th1-like, cytolytic cells, probably caused by high IL-4 concentrations present in the microenvironment. It has been demonstrated that HIV-1 infection can induce a marked defect in the macrophage production of IL-12 (158) and IFN - γ (159).

It has been demonstrated that the CD8⁺ mature lymphocytes contain precursors of effector cytotoxic T cells that recognize antigens in the context of MHC Class I molecules (160). A number of cytokines have been reported to induce the differentiation of thymic or splenic CD8⁺ T cells into effector cytotoxic T cells. These include IL-2, IL-4, IL-6, IFN - γ and TNF- α . However, none of them individually, or in combination have been observed to induce large scale CTL-p (CTL-precursor) proliferation and differentiation. Suda et al observed that a different cytokine stimulated a strong proliferative response in most thymocyte subsets in presence

of IL-2 and/or IL-4. This cytokine was later designated as IL-10. Experiments of Suda et al revealed that IL-10 induces an increase in the CTL-p frequency, as well as a significant increase in the cytotoxic effector function (161).

Natural killer (NK) cells are a distinct population of lymphocytes, which kill target cells that are neoplastic or infected with intracellular pathogens. NK cells are a major source of IFN - γ , particularly during the initial phase of infection (162). NK cell exposure to Th1 cytokines like IL-2, IL-12 or IL-18 results in IFN - γ expression (163). Also, cytokine co-stimulation with combinations of IL-2, IL-12 and IL-18 has been observed to synergistically enhance IFN - γ expression (163)

The Th2 cytokine IL-4 is viewed as an antagonist of IFN - γ expression in T cells and an inhibitor of IL-12 induced Th1 development (164). There is evidence that supports a cell-type- and/or antigen- specific role for Th2 cytokines like IL-4, IL-10 and IL-13. For example, in T cells, IL-4 has been observed to induce Th1 development in a dose dependent manner.

Studies in a rat model show that Con A and Phytohemagglutinin (PHA) blasts secrete greater amounts of IFN - γ when co-treated with IL-4, although IL-4 inhibits the

development of IFN - γ expressing cells (165, 166). In human myeloid cells, CD40 ligand induced IL-2 expression is enhanced by IL-4 in dendritic cells (167) and by IL-4 and IL-13 treatment in monocytes (167). IL-4 has been shown to have a synergistic role in IL-12-, CD40- or major histocompatibility complex (MHC) class II - induced IFN - γ expression in murine dendritic cells (167).

Although resting NK cells are responsive to cytokines and are capable of MHC-unrestricted cytotoxicity against virus-infected and tumor target cells, IL-2 administration in-vivo and in-vitro augments these responses and results in NK cells proliferation (168). It has been observed that freshly isolated negatively sorted splenic NK cells (DX5+/CD3-), but not T cells (DX5-/CD3-) were responsive to cytokine treatments. Freshly isolated NK cells respond synergistically to IL-2 plus IL-4 and IL-12 plus IL-4 stimulation. However, the effect of IL-4 is not absolute. The failure of naïve, splenic T cells to synthesize IFN - γ in response to cytokines is likely due to the absence of pre-required T-cell activation signal(s) (168).

Cytokine receptor signaling results in rapid activation of JAK/STAT pathways and is a critical mediator of cytokine-dependent gene transcription. In human T cells, IL-4 has been shown to specifically suppress IL-2 induced Stat5 activation

and is an indicative mechanism for IL-4 antagonism of Th1 development (169). Evidence suggests a role for mitogen activated protein kinase (MAPK) family of threonine/serine kinases in cytokine signaling and effector functions in both T and NK cells (169).

Although paradoxical to the conventional dogma, some evidences support the role of IL-4 in Th1 responses. For example, in a mouse model of colitis, IL-4 was found to up-regulate IFN - γ in the colon and increase the severity of the disease. IL-4 has been also shown to have a crucial role in activation of alloreactive T cells in mixed lymphocyte cultures (169). An important role of IL-4 in the promotion of CD4+Th1 antifungal responses has been described (164).

Thus, the data available suggest a dichotomous role for IL-4 in regulating IFN - γ expression and possibly Th1 immune responses that may be cell specific. Also, NK cells may play an important part in driving the Th1 response through the induction of IFN - γ by diverse extracellular signals (164).

Natural killer cell stimulatory factor (IL-12) is produced by monocytes and macrophages, B cells and other accessory cells. It is active on T and NK cells and acts synergistically with other IFN - γ inducers like IL-2 and phorbol diesters in NK and T cells (170). Neutralizing IL-12 antibodies significantly

and often completely inhibit the production of stimulated human PBMNCs. IL-12 is required for the optimal production of IFN γ by resting human T lymphocytes (171). IL-12 also induces the production of TNF- α in NK and T cells although a synergistic effect with IL-2 is not observed. IL-12 has been demonstrated to induce a Th1 type of response and to inhibit the differentiation of IL-4 producing Th2 cells (171). Thus it is evident that IL-12 is a necessary cytokine for Th1 cell differentiation.

IL-10 is secreted by monocytes, B and T cells and is associated with differentiation of Th2 cells (170). IL-10 inhibits the lymphocyte cytokine production, particularly IFN γ . It also impairs the proliferation of T cells. IL-10 is a potent inhibitor of monocyte/macrophage functions, including oxidative burst, nitric oxide production, cytotoxicity, and production of cytokines like IL-1 and TNF- α (170). TNF- α has been shown to be an important co-factor in the induction of IFN γ in murine T and NK cells. Thus IL-10 may partly impair the IFN γ production by inhibiting the TNF- α production (171).

IL-10 has also been observed to inhibit IFN γ by preventing the production of IL-12, an IFN γ inducing cytokine, by accessory cells and APCs.

Vigano et al report that production of IL-2 but not IFN γ is reduced in HIV infected patients compared to uninfected control subjects (172). However the reduction was insignificant when compared within different stages of HIV. IL-4 production for all groups of HIV infected children was increased, while high levels of IL-10 were also observed.

Thus the healthy control subjects differ from the infected children due to higher production of type 1 cytokines and lower amounts of type 2 cytokines. On the other hand, the HIV infected children produce higher levels of type 2 cytokines, particularly IL-4 and IL-10 (172).

The dominant production of type 2 cytokines in children with symptomatic infection could theoretically account for a more rapid progression to AIDS in least two ways. The PBMNCs of HIV infected patients are particularly susceptible to PCD after antigen recognition through their T cell receptor (172). It has been observed that in-vitro addition of exogenous type 1 cytokines like IFN γ , IL-2, IL-12, blocks the T lymphocyte PCD, while IL-4 and IL-10 addition enhances PCD.

Therefore an increase in the concentration of type 2 cytokines could enhance PCD, resulting in the more rapid destruction of CD4⁺ T cells in children with symptomatic HIV infection. Moreover, secretion of type 2 cytokines could have a

stimulating effect on HIV replication because the combination of IL-4 and IL-10 enhances the in-vitro replication of HIV in a monocytic cell line (173).

It has been demonstrated that TNF- α upregulates HIV expression in both human promonocytes and T cell lines (174). HIV in turn has been shown to be a potent inducer of TNF- α (174). The HIV expression upregulated by TNF- α occurs at a level of transcription by binding NF- κ B site (175).

The mRNAs of most cytokines are short-lived and their synthesis is inducible by a myriad of stimuli (176). Thus to establish an appropriate level of cytokine biological activity, it is important to maintain a certain level of mRNA of these cytokines, either by regulating mRNA transcription or mRNA stability. Although transcription is an important event in regulation of gene expression, control of cytoplasmic mRNA turnover at a post-transcriptional level is also critically important in establishing the final level of proteins. The turnover rate of a given mRNA determines the steady state level of mRNA in the cytoplasm (177).

Endothelial growth factor (EGF) and Tissue growth factor (TGF- β) affect the stability of mRNAs of IL-1 α , IL- β and IL-6. This effect is however immediate and transient.

In both humans and mice, IL-1 together with IL-6 preferentially induces CD4⁺ T cell proliferation (176). IL-6 by itself selectively induces CD8⁺ T cell proliferation, and in combination with IL-2 and IFN γ , it induces differentiation of cytotoxic T cells from immature thymocytes (178).

In the light of the above scientific data, it is imperative to study the mRNA profiles of cytokines in HIV infected children.

OPPORTUNISTIC INFECTIONS IN PEDIATRIC HIV DISEASE

TUBERCULOSIS:

From the first isolation of the mammalian tubercle bacilli in 1882 by Robert Koch (181), tuberculosis has become a global problem of enormous magnitude. According to the global estimates, around 90 million cases have been reported in a decade from 1990 to 2000. Tuberculosis is also a leading cause of mortality in developing countries. These countries harbor 95% of global tuberculosis cases (182, 183). India has more than 25 million cases of tuberculosis, with *M. tuberculosis* being the etiological agent. Nearly 1.5 million cases are reported annually. Tuberculosis accounts for 5 lakh deaths annually (184).

The tuberculosis epidemic has closely paralleled the HIV epidemic. Data suggest the resurgence of tuberculosis due to HIV. Poverty, overcrowding, malnutrition and socioeconomic

factors are common to groups and areas affected worst by tuberculosis (185).

The etiological agent, *Mycobacterium tuberculosis* is a slender, non-sporing, non-motile, slightly curved bacillus. The size of the bacillus ranges from 0.3 to 0.6 μm by 1-4 μm . The bacilli may be seen as single slender rods or in groups. They are acid-fast due to the presence of Mycolic acid in their cell wall. They are weakly Gram positive (185). Mycobacteria are sub-grouped into Slow and Rapid growers. A group of miscellaneous bacteria, distinct from human and bovine tubercle bacilli, and of low pathogenicity, termed as atypical Mycobacteria, is mostly an opportunistic pathogen (185).

Based on animal studies, the four stages of pathogenesis of tuberculosis have been described (186).

FIRST STAGE

Virulent tubercle bacilli have to reach the lung alveolar surface to cause an infection. The most important mode of transmission of these bacilli is inhalation. The tubercle bacilli on reaching the alveoli or small bronchioles where they establish a local focus of disease, termed as the "Ghon Focus". The first defense against infection is the resident alveolar macrophages, which scavenge the alveolar surface by ingesting organisms and other inhaled particulate matter,

becoming non-specifically activated in this process. Depending upon the non-specific activation, alveolar macrophages ingesting the tubercle bacilli have variable microbicidal capacity (186).

SECOND STAGE

This is the stage of logarithmic bacillary growth and early tuberculosis lesions. When an innate macrophage microbicidal activity is inadequate to destroy the initial few tubercle bacilli of the droplet nucleus, they replicate within the macrophages, causing the nucleus to rupture. Circulating monocytes are attracted to the focus by various chemotactic factors initiating granuloma formation. No specific reaction develops at this stage, but evidences have shown that gamma and delta T cells respond non-specifically to certain components of the bacillus and cause macrophage aggregation and activation (187). Although immature monocytes ingest the released bacilli, they do not appear to have the capacity to destroy or inhibit their exponential growth (186).

THIRD STAGE

This stage involves the control of pathogenesis by CMI or DTH. The bacilli released from immature macrophages are be taken up and controlled by T-cell activated macrophages. After the establishment of control, mature macrophages accumulate

around the periphery of the caseous lesion, preventing further extension of the disease (186). However at the centre of the caseated material, the mycobacteria may remain viable for a long time and cause reactivation. Tuberculin-like products derived from intracellular bacilli are thought to be an important early stimulus to caseous necrosis, which involved several poorly defined processes in addition to DTH.

DTH, on other hand, is harmful to the host, causing tissue damage that is characteristic of tuberculosis, especially lung cavitation. Depending upon the immune competence of the host, primary tuberculosis is often a sub-clinical, self-limiting illness, but in immunodeficiency, the infection progresses both locally as well as systemically (186).

FOURTH STAGE

In this stage, one observes liquefaction and formation of the lung cavity. This final stage of pathogenesis arises from the second phase of the disease in a host whose CMI fails to control primary tuberculosis. In a normal host, the uncontrolled growth of tubercle bacilli in macrophages stimulates the DTH, leading to caseation and liquefaction, both of which are detrimental to macrophages. When the liquefied caseous lesion discharges its content into a nearby bronchus, a cavity is formed which is a characteristic radiographic pathology of

tuberculosis. The host defenses are ineffective within the cavities and hence the bacilli multiply to great numbers (186).

The patient with cavitary tuberculosis presents with persistent cough and may have hemoptysis. Systemic symptoms of fever, weight loss and anorexia are also associated with this cavitary disease. Defects in the T cell or macrophage function in AIDS leads to reactivation or increased susceptibility to mycobacterial infections (186).

Effectiveness of alveolar macrophages in the initial non-specific response to *M. tuberculosis* infection was found to be limited in persons infected with HIV. It is claimed that occurrence of tuberculosis in HIV seropositive subjects expedites the progression to AIDS. Thus HIV patients are at markedly increased risk for the development of primary and reactivation tuberculosis (189), and the manifestations of tuberculosis in HIV cases are more serious and life-threatening (190).

As compared to adults who are at risk of reactivation of latent infection with *M. tuberculosis*, children with HIV develop primary tuberculosis. Disseminated disease and lymphadenitis are the most common forms of extra-pulmonary tuberculosis. There is ten times higher chance of a person co-infected with HIV and tuberculosis to have an active disease (191). Contact

with adult' tuberculosis is a major source of infection in children. Fever and cough are the most common presentations although clinical findings range from symptomatic pulmonary infection to fatal tuberculosis meningitis. Mantoux test is frequently negative and radiological findings are variable ranging from hilar adenopathy, pleural effusion, cavitation or military pattern. Low CD4 counts predispose the child to infection with tuberculosis.

Therapy with 4-drug ethambutol, isoniazid, rifampicin, pyrazinamide (ETB, INH, RIF, PZA) should be considered for both pulmonary and extra-pulmonary tuberculosis during the initial two months. The duration of the therapy ranges from 6 months for pulmonary and 12 months for extra-pulmonary tuberculosis. Direct Observation Therapy (DOT) is strongly recommended for children during the initial months.

Children who are tuberculin positive but do not show evidence of active disease should receive INH for 12 months. Children with HIV infection appear to have more frequent adverse reaction to anti-tuberculosis therapy must be closely monitored during treatment with second line drugs like ciprofloxacin, ofloxacin, amikacin, kanamycin (192).

CANDIDIASIS:

Candidiasis is the most frequently encountered opportunistic fungal infection (193). The clinical condition presented is "thrush", the fungal etiology of which has been demonstrated by Berg in 1841 and Bennet in 1844 (194). *Candida albicans* has a worldwide distribution, as a commensal in the oral cavity, digestive tract and on the skin of normal healthy adults (195).

Candidiasis is the second commonest OI observed in HIV infected individuals. The incidence of candidiasis has increased with the advent of AIDS pandemic. Its prevalence in AIDS ranges from 12% to 93% (196). It is one of the earliest pre-monitoring sign of HIV infection. Studies have estimated that more than 90% patients with HIV infection will have at least one oral manifestation of candidiasis (197). Various observations have demonstrated oral candidiasis as a marker of disease severity in AIDS patients (198, 199). It is incorporated in HIV/AIDS staging (200).

Candida is a mycelia producing, non-spore forming, yeast-like fungi. Important species of *Candida* responsible for candidiasis in human beings include *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. guilliermondii* (201).

Members of the genus *Candida* are indigenous human yeasts that colonize mucous membranes of normal individuals but produce clinical symptoms only when the natural resistance is lowered (202). During episodes of severe hyperglycemia and poorly controlled diabetes, broad-spectrum antibiotics (203) steroid therapy (204) and in cancer patients, the risk of candidiasis is significantly increased. The clinical manifestations of candidiasis are extremely varied. They can be acute, sub-acute or chronic. Candidiasis may be localized in the mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs or the gastro-intestinal tract. It becomes systemic in case of septicemia, endocarditis or meningitis (205). Oral candidiasis is the most frequent cause of white lesions known as pseudomembranous candidiasis (thrush) and hyperplastic candidiasis (candidial leukoplakia) (206).

Phagocytic cells are the primary host defence against candidial infection. Neutrophils, Monocytes and macrophages are responsible for the killing of *C. albicans* by phagocytosis. But due to impaired immune function in HIV/AIDS, candidiasis becomes an uncontrolled infection with esophageal extension (207).

HIV infected infants are commonly diagnosed with oral thrush but the infections can be extensive and less responsive to

therapy. Severe oral infection may be the presenting manifestation of HIV infection. Oral candidiasis may be present as hypertrophic candidiasis, erythematous candidiasis, and angular cheilitis. Esophageal candidiasis presents as substernal pain, abdominal pain, burning sensation and difficulty in swallowing.

Oral nystatin, clotrimazole, fluconazole and ketokonazole are the drugs of choice for treatment (192).

PNEUMOCYSTIS CARINII PNEUMONIAE:

It is the hallmark opportunistic infection of pediatric AIDS. It is also known as AIDS pneumonia. It is the most common AIDS defining illness in children. PCP is an infection of early infancy and most infants are affected between 4-6 months of age with the median age being 5 months. Clinical features include a tetrad of tachypnea, dyspnea, cough and fever. Physical examination reveals tachycardia, respiratory distress, accelerated tachypnea, and diffuse retractions. In the early stage of the disease, X-Ray shows hyper-infiltration with peribronchial thickening and later shows bilateral alveolar or interstitial infiltrates, usually spreading peripherally from perihilar region to encompass both lungs (192).

Diagnosis necessitates examination of fluid following fibre-optic bronchoscopy with broncho-alveolar lavage or tracheal

wash in an intubated child. Open lung biopsy is reserved for children with worsening clinical status despite treatment.

In view of rapid progression of the disease, treatment is started if significant delay in diagnosis is anticipated. First-line therapy includes intravenous trimethoprim (TMP) and sulfamethoxazole (SMZ). Other prophylactic drugs are dapsone, inhaled/IV pentamidine, atovaquone, etc. (192)

DIARRHEAL DISEASES:

The occurrence of ulcers in the oral cavity presents a diagnostic dilemma because viral infections, such as HSV, CMV and Coxsackie A infection, as well as bacteria, may be causative agents. Biopsy and culture of lesions that are persistent or increasing in size or number is recommended.

Diarrhea is a common manifestation of HIV infection (208). Although an infectious cause is frequently suspected, other important causes include side effects of medications, malignancies, and HIV enteropathy. Anti-bacterial agents are indicated in the treatment of diarrhea associated with enteric pathogens, such as *Shigella*, *Salmonella*, and *Campylobacter*. HIV-infected patients, especially those with low CD4+ counts are at risk for diarrhea caused by *Clostridium difficile*. (209)

Diarrhea is the most frequent gastro-intestinal tract symptom related to opportunistic infections. Diarrhea and weight loss

are independent predictors of mortality. Important pathogens found frequently are *M. avium complex*, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Clostridium*, *Cryptosporidium*, microsporidia, giardia, entamoeba. Viral etiological agents include rotavirus, astrovirus, calicivirus, HIV, adenovirus and herpesvirus (192).

Cryptosporidial diarrhea is observed in 30% to 50% cases in HIV/AIDS patients in India (210). The diarrhea appears to be more voluminous in immunocompromised persons than in immunocompetent individuals. It sometimes contains mucous, but very rarely blood or leukocytes. It is often associated with weight loss. Other symptoms usually observed are abdominal cramps, nausea, vomiting, malaise, myalgia, weakness and headache (211). The onset of diarrhea is accompanied by mild fever, anorexia, muscular pains and weakness. Diarrhea lasts for one to several days. Sometimes, it may persist beyond one month and result in dehydration and death (212).

The pathogenic mechanism involves the decrease in the absorptive capacity of the intestine, especially for water and electrolytes. This may be due to the extensive colonization of the pathogen in the brush-border of the enterocyte, particularly in the small bowel area. The pathogen

subsequently denudates the microvilli and also offers physical blockage, resulting in lower absorption (213).

Secondly, there is fluid accumulation in the small intestine, which in turn has two etiologies. The loss of the brush border enzymes leads to the presence of undigested sugars and increases the osmolarity of the chyme. This results in fluid accumulation. Moreover, hypersecretion into the intestine may occur due to toxins (214).

Water and solute malabsorption and fluid accumulation in the small bowel increase the flow rate into the colon. Colonization of the parasite in the colon and bacterial fermentation of the solutes in colon leads to increased laminar flow rates. This exceeds the normal absorptive capacity of the colon, resulting in diarrhea (212).

VIRAL LOAD:

In HIV-1 infected adults, CD4+ T cell counts and viral load are two key laboratory markers currently being used as predictive markers of clinical outcome of the infection and to make decisions about changing the antiretroviral therapy.

However, CD4+ T cell counts fall naturally with age in uninfected children and even after adjusting for age, CD4+ T cell counts in HIV-1 infected children appear to be much less for predicting the disease progression than in adults. On the

other hand, several studies have reported on the predictive role of viral load in infants.

Although CD8⁺ lymphocytes are believed to be important in the control of HIV-1 infection, only a few studies have examined the prognostic significance of the number of peripheral CD8⁺ cells in adults, and none of them has reported data in children (261).

Although very little is known about the factors that determine the rate and levels of HIV spread in tissue culture systems, even less is understood about the critical parameters affecting virus replication in the infected person. The virus infections are monitored and characterized by using cell free virus stocks. The approach facilitates and standardizes quantitative comparisons of different virus isolates and mutants because the number of extracellular infectious units can be frequently monitored in plaque assays on cultured cell monolayers after the limiting dilutions have been made.

While cell free HIV-1 can be easily detected in tissue culture infections, significant plasma viremia has been difficult to be demonstrated in infected individuals. This is now possible with the advent of extremely sensitive techniques like NASBA, branched chain DNA amplification, etc. In most instances, no cell free virus is detected in asymptomatic seropositive

individuals and is demonstrable only later in the clinical course, when severe immune dysfunction becomes evident (42). The advent of techniques that reliably quantify levels of HIV-1 RNA in patient plasma has been pivotal in the development of new insights into *in-vivo* viral dynamics and HIV disease pathogenesis.

Aims

&

Objectives

The cytokine interplay has a pivotal role in the pathogenesis and progression of HIV disease. The detection of cytokines in body fluids has been hampered by the very short half-life and immediate capture of secreted cytokines by their receptors. Measuring the expression of mRNA would reflect the levels of synthesis of cytokines and the values may not be parallel to those of circulating cytokine levels. This may be due to the regulation of translation of cytokine mRNA or regulation of cytokine protein secretion.

It was therefore envisaged to study the cytokine mRNA levels in the hitherto ignored pediatric cohort and correlate the same with other parameters like the CD4+T cell counts, viral load, opportunistic infections, and morbidity and mortality.

Ethical Committee Clearance

Due permission was obtained from the Ethical Committee of the Lokmanya Tilak Memorial Municipal Hospital, Sion, before the start of the project.

The study was carried out bearing the following aims and objectives:

1. Confirmation of the HIV serostatus of the patients born to HIV seropositive mothers.
2. Estimation of absolute CD4⁺ T cell counts using flow cytometry.
3. Estimation of the mRNA levels of the following cytokines
 - a. Th1 type: IL-2, TNF- α and IFN- γ
 - b. Th2 type: IL-4, IL-6, and IL-10Using semi-quantitative relative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).
4. Determination of viral load using H9 lymphoma cell line and p24 antigen detection.
5. Correlation of the laboratory findings with the clinical presentations.
6. Study of the morbidity and mortality trends in HIV/AIDS patients.

Materials

&

Methods

1. Study population: The study population comprised 128 children between the age of 1-13 years, divided into two cohorts viz. Subjects and Controls.

Subjects comprised of 100 children of either sex, HIV seropositive and attending the OPD or admitted in the wards of Lokmanya Tilak Memorial Municipal Hospital, Sion, Mumbai. All subjects were naïve for any anti-retrovirals. Controls consisted of 28 normal healthy children of either sex, having no symptoms, opportunistic infections, and seronegative for HIV. Detailed clinical history and informed consent was obtained for both the cohorts from their parents/guardians.

2. Blood Collection: Five to ten ml of blood was drawn by venipuncture from all children in the study population. The blood was aliquoted appropriately in K3-EDTA and plain bulbs for obtaining the plasma + cells and serum respectively. The sera were separated and stored at -20°C till further testing.

3. Confirmation of HIV serostatus: Sera samples were tested for the presence of HIV 1 and 2 antibodies by commercially available ELISA kit ("SANDWICH ELISA": SD HIV1/2 ELISA, Ver.3, Standard Diagnostics Inc., and "INDIRECT ELISA": Microlisa HIV, J. Mitra and Co.)



In the 'sandwich' ELISA, the microplate is pre-coated with recombinant HIV 1/2 antigens (gp41, p24, and gp36) on the well. During the first incubation, anti-HIV in patient's serum is bound to the recombinant HIV 1/2 antigens. Following this, all unbound material is removed by aspiration and washing. The recombinant HIV 1/2 antigens-enzyme conjugate (gp41, p24, and gp36) bound to anti-HIV 1/2, thus forming a "sandwich". After a subsequent incubation, the unbound material is removed by aspiration and washing. The residual activity found in the wells is directly proportional to the anti-HIV 1/2 concentration in the patient's serum. After incubation with the Tetramethyl benzidine (TMB) substrate solution, the plate is read colorimetrically at 450nm.

In the 'indirect' ELISA, the recombinant proteins representing the immunodominant epitopes (gp41, C-terminus of gp120, and gp36) of HIV 1/2, are coated onto the microtitre wells. Samples are added to these wells and incubated. Antibodies to HIV 1/2, if present in the specimen, will bind to the specific antigens adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish Peroxidase (HRP) conjugated anti-human IgG is added to each well. This

conjugate binds to the HIV antigen-antibody complex present. Finally, a substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. After incubation, the colour development is read spectrophotometrically at 450 nm.

4. Estimation of absolute CD4+ T cell counts: 100 µl of blood was drawn from K3-EDTA containing bulb. Fifty microlitres each were added to the reagent pair tubes for the estimation of CD4/CD3 and CD8/CD3. After incubation in the dark for 90 minutes, the fixative solution was added to these tubes. The CD4+ T cells were counted using FACScount (Becton-Dickinson). The sorting and analysis of the cells was fully automated.
5. Separation of Peripheral Blood Mononuclear Cells (PBMNCs) (215): The peripheral blood mononuclear cells (PBMNCs) were separated from the blood sample from K3-EDTA containing bulb using the Ficol Hypaque density gradient centrifugation. Three to four ml of blood was overlaid on 3 ml of the Ficol Hypaque-1077 (Sigma) gradient. This was then centrifuged at 2500 rpm for 20 minutes. The buffy-coat layer of PBMNCs was isolated and washed once with sterile RPMI-1640. The cells were then cryopreserved at a density of 10^4 - 10^6 cells/ml at -80°C in

RPMI 1640 containing 5% Fetal Calf Serum and 10% Dimethyl sulfoxide till further use.

6. Extraction of RNA using the TrizolTM reagent method: 800 μ l of PBMNCs was mixed with equal volume of TrizolTM reagent (Life Technologies). 200 μ l of 2.5gm/ml sterile glycogen was added as a co-precipitant and the sample was homogenised thoroughly. The homogenised sample was incubated for 5 minutes at 15⁰C to permit complete dissociation of the nucleoprotein complexes. To, this mix, 200 μ l of chloroform was added. The tubes were agitated vigorously for 15 seconds. They were then centrifuged at 12,000g / 11,000 rpm for 15 minutes at 4⁰C. The mix separated into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase.

The colourless aqueous phase was transferred into a fresh tube and 0.5 ml of isopropanol was added to precipitate the RNA. The samples were incubated for 10 minutes at 15⁰C and centrifuged at 12,000g/11,000 rpm for 10 minutes at 4⁰C. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. This was then centrifuged at 7500g/6800 rpm for 5 minutes at 4⁰C.

The RNA pellet was briefly dried (till complete evaporation of ethanol) and re-dissolved in 15 μ l of Tris EDTA (TE) buffer, pH 7.6 and incubated for 10 minutes at 55^o C.

7. Quantitation of RNA (216): The dissolved RNA was subjected to Spectrophotometric reading at 260 nm and 280 nm. One microlitre of the RNA solution was added to 999 μ l of RNase free water. This was then read using an UV spectrophotometer. The samples having the O.D.₂₆₀ to O.D.₂₈₀ ratio between 1.7-2.0 were selected for the performance of RT-PCR.

8. Evaluation of the cytokine-mRNA profile:

8.1 Th1 cytokines viz. IL-2, TNF- α and IFN- γ , and Th2 cytokines like IL-4, IL-6, and IL-10 were evaluated for their mRNA levels in the present study.

8.2 The RNA samples were diluted appropriately using TE buffer, pH. 7.6, so that 1 μ l of the solution contained 2 μ g of total RNA.

8.3 One-step, relative, gene-specific RT-PCR for the amplification of mRNA of cytokines was performed using the RT-PCR base kit (Qiagen Inc., USA) (217) and gene-specific primers (Ambion, Inc., USA) for the particular cytokine. The samples were

subjected to the RT-PCR for mRNA using the respective primer cocktail of forward and reverse primers. The product obtained was identified using the control DNA. 18S *Classic* primer-competimer set was also employed to amplify the 18S ribosomal RNA. This was used in a primer to competimer ratio of 2:8. This acted as the internal control. The quantitation was brought about by comparing the band intensities of the product and the 18S RNA product by the Gel Documentation software (1D Advanced AAB, Advanced American Biotechnology, USA)

8.3a: IL-2: Primers generated from GenBank
#K02056 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

ii. Cycling conditions:

Pre-heating temperature	50 ⁰ C	2 min	
Reverse transcription	50 ⁰ C	30 min	
Polymerase activation	95 ⁰ C	15 min	
Denaturation	39 cycles	94 ⁰ C	1 min
Annealing		57 ⁰ C	1 min
Extension		72 ⁰ C	1 min
Final extension		72 ⁰ C	10 min

8.3b: IL-4: Primers generated from GenBank
#M23442 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

ii. Cycling conditions:

Pre-heating temperature		50 ⁰ C	2 min
Reverse transcription		50 ⁰ C	30 min
Polymerase activation		95 ⁰ C	15 min
Denaturation	40 cycles	94 ⁰ C	1 min
Annealing		57 ⁰ C	1 min
Extension		72 ⁰ C	1 min
Final extension		72 ⁰ C	10 min

8.3c: IL-6: Primers generated from GenBank
#Y0081 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

ii. Cycling conditions:

Pre-heating temperature	50 ⁰ C	2 min
Reverse transcription	50 ⁰ C	30 min
Polymerase activation	95 ⁰ C	15 min
Denaturation	38 cycles	94 ⁰ C 1 min
Annealing		57 ⁰ C 1 min
Extension		72 ⁰ C 1 min
Final extension		72 ⁰ C 10 min

8.3d: IL-10: Primers generated from GenBank
 # U16720 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

0

ii. Cycling conditions:

Pre-heating	50 ⁰ C	2 min
temperature		
Reverse transcription	50 ⁰ C	30 min
Polymerase activation	95 ⁰ C	15 min
Denaturation	94 ⁰ C	1 min
Annealing	57 ⁰ C	1 min
Extension	72 ⁰ C	1 min
Final extension	72 ⁰ C	10 min

38
cycles

8.3e: IFN- γ : Primers generated from GenBank

J00219, M37265, V00536 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

ii. Cycling conditions:

Pre-heating temperature	50 ⁰ C	2 min	
Reverse transcription	50 ⁰ C	30 min	
Polymerase activation	95 ⁰ C	15 min	
Denaturation	35 cycles	94 ⁰ C	1 min
Annealing		57 ⁰ C	1 min
Extension		72 ⁰ C	1 min
Final extension		72 ⁰ C	10 min

8.3f: TNF- α Primers generated from GenBank
 # Z15026 (218)

i. Reaction Mix:

5x Qiagen buffer	:	10 μ l
5x Q- solution	:	10 μ l
10mM dNTPs	:	2 μ l
5 μ M IL-2 primers	:	5 μ l
3 μ M 18S (2:8) primers	:	3 μ l
Enzyme mix	:	2 μ l
RNase inhibitor	:	0.2 μ l
Random hexamers	:	2 μ l
RNase/DNase free water	:	14.8 μ l
TOTAL	:	49 μ l
Template (sample RNA)	:	1 μ l
FINAL VOLUME	:	50 μ l

ii. Cycling conditions:

Pre-heating temperature	50 ⁰ C	2 min	
Reverse transcription	50 ⁰ C	30 min	
Polymerase activation	95 ⁰ C	15 min	
Denaturation	40 cycles	94 ⁰ C	1 min
Annealing		57 ⁰ C	1 min
Extension		72 ⁰ C	1 min
Final extension		72 ⁰ C	10 min

8.4 Analysis of PCR products using agarose gel electrophoresis: The products obtained after amplification of the mRNA of the cytokines were loaded onto a 2% Low electro-endo-osmotic agarose gel, prepared in Tris Borate EDTA (TBE) buffer, pH 8.0. The gel contained 5µl of 500µg/ml Ethidium bromide. The samples were mixed with the gel loading buffer, containing Bromophenol blue and glycerol. The gel was run in TBE buffer, pH 8.0, at 120V, till such time that the dye ran more than three-quarters of the gel length (~ 45-60 minutes). The gel was then visualized using UV Transilluminator. The bands were detected and quantitated using Gel Documentation software (1D Advanced AAB, Advanced American Biotechnology, USA). This helped in the determination of the template (cytokine mRNA) to control (18S RNA) ratio for quantitation.

9. Determination of the probable viral load:

9.a By p24 antigen capture assay:

The principle underlying this assay is of "limiting dilution". Considering 10^x viral particles to be

present in the serum sample, the dilution factor of 10^{-x} would generate 1 viral particle in the dilute sample. Any dilutions henceforth would have less than 1 viral particle, rendering the sample non-infective. There would therefore be no p24 secreted in the sample (negative result for p24).

H9 is a derivative of HuT78, a culture of T cell lymphoma. These cells are highly susceptible to HIV and the cytopathic effect observed is the formation of cell aggregates, eventually leading to the formation of syncytia. A syncytium is defined as the cell with the diameter larger than diameters of four un-infected cells. In H9, the formation of aggregates can be observed within 30-60 minutes of infection (219). H9 cells used in this assay were procured from National Centre for Cell Sciences, Pune.

The sera samples of the HIV seropositive patients were employed for this assay. They were passed through 0.22μ Millipore filter. Each sample was then diluted ten-folds, viz. from undiluted sample to 10^{-6} viral particles per ml. Two hundred microlitres of each sample were inoculated into H9 suspension

cell culture with density of 10^6 cells per ml. RPMI 1640 with 2mM L-Glutamine, 4.5g/L glucose, 1.5g/L Sodium bicarbonate, 1mM Sodium pyruvate, 10mM HEPES buffer and 10% Fetal Calf Serum was used as the Growth medium. The virus was allowed to adsorb onto the cells for 60 minutes. The cells were then centrifuged and resuspended into fresh Growth medium. This ensured the removal of soluble, virus non-associated p24 antigen. These infected H9 cells were allowed to grow for 4 days. On the 4th day, the cell free supernatant was collected by centrifugation, and was analyzed for p24 antigen (220). This detection of p24 antigen from different supernatants was performed using a commercially available ELISA kit (Zeptometrix, California) as per the kit insert. The dilution of the serum sample that gave a negative result for p24 was marked as the end-point.

9.b By Real time RT PCR method:

9.b.1 Isolation of viral RNA: The viral load estimation by Real Time RT-PCR was carried in two steps. The RNA extraction was carried out at the Department Of Virology, Haffkine Institute

while the amplification was carried out at the Department Of Virology, University of Bordeaux, France. The reagents and kits required for the extraction and the subsequent amplification of viral RNA were generously gifted by Dr. Hervé Fleury, Head, Department of Virology, University of Bordeaux, France.

The RNA was isolated from the serum/plasma samples using the High Pure Viral RNA kit (Roche Diagnostics). One milliliter of serum/plasma was centrifuged at 25,000g at 4°C for 1 hour. The supernatant was discarded, retaining 20µl of the pellet. To this, 180µl of the wash buffer was added and mixed using centrifugation. Four hundred microliters of the binding buffer, supplemented with poly [A] were added to this solution. This was then overlaid on a High Pure Filter column installed on a collection tube. The sample assembly was then centrifuged at 8000g at 4°C for 15 seconds. The collection tube containing the flowthrough was discarded. The Filter column was inserted onto a fresh collection tube. Five hundred microliters of the inhibitor removal buffer were added to the

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

9.b.2 Real Time RT PCR for viral load estimation (221-223):

TaqMan™ One Step RT-PCR kit (Applied Biosystems) was employed for the viral load estimation in HIV. The Long Terminal Repeat (LTR) region of HIV was selected for the designing of the primers.

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

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

The following probe was used for the detection of amplification:

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

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

Each reaction mix consisted of 30µl, comprising of 25µl of 2X Master mix (without UNG*), 1.25µl of Multiscribe RNA mix in 40X RNase inhibitor, 1µl each of 25µM sense and anti-sense primer, 1µl of 10µM probe, and 0.75µl of DNase/ RNase free water. To this, 20µl of the sample RNA were added. The amplification was carried out using the following programme:

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

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

10. Clinical diagnoses: The subjects were examined for their clinical presentations at the Department of Pediatrics, Lokmanya Tilak Memorial Medical College, while the normal children were examined by general physicians as a part of routine check-up.

The symptoms like weight loss, fever, cough/cold, vomiting, diarrhoea and oral thrush were documented. Opportunistic infections like tuberculosis, mycotic dermatitis, *Pneumocystis carinii* pneumonia, otorrhea (fungal/bacterial); Candidiasis (oral/dermal) and gastrointestinal disorders (fungal / bacterial / viral / protozoal) were looked for. Other viral infections like HBV/HCV and meningitis were also observed.

10.a IDENTIFICATION OF MYCOBACTERIA

1. COLLECTION OF THE SPUTUM SAMPLE:

The patients who were suspected for tuberculosis were instructed to expel a deep productive cough. Early morning sputum samples were collected for 3 consecutive days. Samples were then processed for the detection and isolation of causative mycobacteria.

2. SMEAR EXAMINATION:

The sputum samples were examined for the presence of Acid-Fast bacilli (AFB) after staining the smears with Ziehl-Neelsen Carbol fuchsin technique (224).

Common aniline dyes fail to stain the mycobacteria easily. However, basic dyes like Carbol fuchsin have been able to stain the bacilli. On staining, the bacilli retain the dye in spite of treatment with acid-alcohol.

Smears were made from deposits on grease free slides and were immediately heat-fixed. The smear was then flooded with Zeihl-Neelsen Carbol Fuchsin (ZNCF) stain and steamed gently for 7-10 minutes. Boiling or evaporation of stain was avoided by replenishing it whenever required. Smear was washed under running tap water and was discolored using acid-alcohol for 3 minutes. The smear was then rinsed with tap water. The smear was then counter-stained with 1% methylene blue for 1-2 minutes. The slide was then washed with water to remove excess stain and then air-dried. The smear was examined under the oil immersion lens and slender red bacilli were looked for against the background of methylene blue.

3. CONCENTRATION OF THE SPUTUM SAMPLE:

The sputum samples were homogenized and decontaminated using 4.5% sodium lauryl sulphate and 1.5% NaOH (225). The

decontamination agent was used in the ratio of 2:1 w.r.t. the sample and was mixed with constant shaking. This was incubated at 37°C for 30 minutes. The samples were then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the sediment was neutralized by drop-wise addition of the neutralizer till the appearance of blue color.

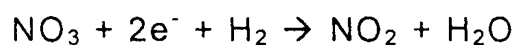
The neutralized sediment was inoculated in duplicate onto sterile slopes of Lowenstein-Jensen (LJ) medium (commercially available) and was incubated at 37°C with 5% CO₂ atmosphere. All slopes were examined for the appearance of growth everyday during the first week of incubation and later every week, for a period of 8 weeks. *M. tuberculosis* grew as white/creamy, rough, tenacious colonies on LJ medium. Smears from such colonies were confirmed for the presence of AFB using ZNCF staining method.

4. BIOCHEMICAL TESTS FOR IDENTIFICATION:

a. Reduction of Nitrate (Virtanen's method) (226):

Mycobacteria produce an enzyme nitroreductase, which reduces nitrate to nitrite that is subsequently broken down to ammonia. The presence of nitrite in the medium is detected by its diazotization, following the addition of sulphanilamide and subsequent coupling with n-naphthyl-ethylene diamine dihydrochloride to form a red colour

complex. If nitrite has been further reduced, the reaction remains colorless. This reduction is detected by the addition of zinc powder. The true negative test is indicated by the development of red colour on addition of the zinc powder, due to the immediate reduction of nitrate to nitrite catalyzed by zinc.



A loopful of growth of actively growing culture was emulsified in 2 ml of substrate solution and incubated at 37°C for 2 hours. One drop of dilute HCl was added followed by the addition of 2 drops each of sulphanilamide solution and n-naphthyl-ethylene diamine dihydrochloride. The tube was then observed for the colour change. The reaction was confirmed by the addition of zinc powder.

The positive result was the development of red colour, which disappeared on addition of zinc. The negative reaction was no colour in the medium, which then turned red on addition of zinc.

The positive control comprised of the medium inoculated with *M. tuberculosis* while un-inoculated medium was used as the negative control.

- b. Niacin production test (Runyon's method) (227): Niacin plays an important role in the oxidation reduction reactions

that occur during metabolic synthesis in all mycobacteria. It is the precursor in the biosynthesis of co-enzyme Nicotinamide Adenosine Dinucleotide Phosphate (NADP). Although all mycobacteria produce nicotinic acid, studies have shown that a blocked metabolic pathway prevents *M. tuberculosis* from utilizing niacin to produce niacin ribonucleotide, and thereby leads to the accumulation of large amounts of niacin as a water soluble by-product. This forms a useful diagnostic method for *M. tuberculosis*. Niacin, a pyridine compound is detected by its reaction with cyanogens bromide in the presence of primary aromatic amine like aniline to produce a yellow colored Schiffs base.

Five hundred microlitres of sterile distilled water is added to the LJ slope, having a growth of more than 4 weeks. The slope is then autoclaved at 15 lbs for 30 minutes. To a 75 x 12 mm test tube, 0.25 ml of 4% aniline solution is added. Equal volume of 10% cyanogen bromide is also added. The solutions are mixed with gentle shaking of the tube and colour development is read within 1 minute. Positive reaction is the development of canary yellow colour, while negative reaction is no development of colour.

Un-inoculated medium forms the reagent control, medium inoculated with *M. avium complex* forms the negative control, and medium inoculated with *M. tuberculosis* forms the positive control.

- c. Tellurite reduction test (Kilburn's method) (228): Among the non-photochromogens, only *M. avium complex* reduces tellurite to metallic tellurium, which is detected by the development of black colour.

One loopful of culture is inoculated into the Middlebrook 7H9 broth containing 0.05% Tween 80 and incubated at 37°C for 7 days. After incubation, 2 drops of 0.2% potassium tellurite solution were added to the broth. The tubes were then re-incubated at 37°C for 72 hours and observed for colour change. The positive reaction was denoted by the formation of black precipitate around sedimented cells.

The positive control comprised of the medium inoculated with *M. avium complex* while un-inoculated medium was used as the negative control.

10.b IDENTIFICATION OF CANDIDA SPECIES

1. COLLECTION OF SAMPLE:

The patients having oral lesions, or dermal lesions suggestive of candidiasis were included in this study. With

the help of sterile cotton swabs, two samples were taken from the lesions on two consecutive days. One sample was used for wet mount in 10% KOH and Grams staining. These preparations were observed for presence of yeasts, hyphae and pseudohyphae.

The other sample was used for inoculation onto two tubes of Sabouraud's Dextrose agar (SDA) containing Chloramphenicol (229). One tube was incubated at 37°C and the other tube at room temperature. The cultures were observed everyday for a week for the appearance of growth. A colony was sub-cultured on SDA for pure culture. This was then used for the identification tests

a. Germ tube test: A colony from pure culture was emulsified into sterile 0.5 ml of horse serum to obtain faint turbidity. This suspension was incubated at 37°C for a maximum period of 4 hours. Few loopfuls of this suspension were microscopically observed at 2 hour, 3 hour and 4 hour duration for the presence of germ tubes (230).

b. Sugar Fermentation Test: A portion of the colony from the pure culture was inoculated into four sugar-fermentation tubes containing glucose, sucrose, maltose and lactose. The tubes were incubated at 37°C for a

maximum of 14 days. They were observed daily for the production of acid (yellow colour) and gas in the Durham's tube (230).

c. Sugar Assimilation Test: One loopful of the pure culture from SDA plate was inoculated on blood agar plate and incubated for 15 days to deprive the organisms from sugars. One loopful of the colony from this blood agar plate was suspended in 0.9 ml of sterile distilled water. Each Assimilation slant was inoculated with this suspension. The slants were incubated at room temperature (25°C) for 15 days. The results were indicated by growth of the organisms and acid production (colour change from purple to yellow) (230).

10.c IDENTIFICATION OF SALMONELLA

The organisms of the genus *Salmonella* are motile and lactose non-fermentors. They are selectively isolated on *Salmonella-Shigella* agar and Brilliant Green Sulphadizine agar. The confirmation of the same is brought about by the differential characterization on the Triple Sugar Iron agar.

COLLECTION OF SAMPLE:

Fresh fecal samples were collected in a suitable container. A loopful of pathological sample was inoculated into 5 ml of

tetrathionate broth for the enrichment of Salmonella. This was incubated at 37°C for 24 hours.

a. Isolation on Salmonella-Shigella Agar: One to two loopfuls of the growth from the tetrathionate broth were streaked onto slants containing Salmonella-Shigella agar. This was incubated at 37°C for 24 hours. Positive results were detected in the form of pale/black colonies on the medium. The black colonies appear due to the production of H₂S by *S. paratyphi B* from the bismuth sulphite in the medium. The colonies on this agar were used further as pure culture.

b. Isolation on Brilliant Green Sulphadizine Agar: One to two loopfuls of the growth from the tetrathionate broth were streaked onto slants containing Brilliant Green Sulphadizine agar. This was incubated at 37°C for 24 hours. Positive results were detected in the form of pale/black colonies on the medium. The black colonies appear due to the production of H₂S by *S. paratyphi B* from the sulphadizine in the medium. The brilliant green present in the medium inhibits the growth of other enteric organisms like *S. typhi*, *E. coli*, *Proteus* spp., *Pseudomonas* spp., etc.

c. Sugar Fermentation Test: A portion of the colony from the pure culture was inoculated into four sugar-fermentation tubes containing glucose, xylose, mannitol and Sodium citrate. The tubes were incubated at 37°C for a maximum of 14 days. They were observed daily for the production of acid (yellow colour) and gas in the Durham's tube

d. Isolation on Triple Sugar Iron Agar: A loopful of the pure culture was isolated on the Triple Sugar Iron agar slant. This was then incubated for 24 hours at 37°C. Positive results were indicated by the production of acidity (yellow colour) in the butt and alkalinity (pink colour) on the slant. Differentiation is brought about by the presence (*S. paratyphi A*) /absence (*S. typhi*) of gas production and the production of H₂S (*S. paratyphi B*) in the tube.

10.d IDENTIFICATION OF SHIGELLA

The organisms from the genus *Shigella* are non-motile, lactose non-fermentors. They can be selectively isolated on Salmonella-Shigella agar. The acid production from complete fermentation of glucose alters the pH of the Glucose Phosphate broth to an extent which is detected by the Methyl red reagent. However, unlike other enteric organisms, it does not bring about incomplete oxidation of

glucose to yield acetyl-methyl carbinol or its reduced product, i.e. 2, 3-butylene glycol. By addition of O'Meara's reagent (Creatinine + KOH + Distilled water), the oxidation of the glycol to the diacetyl compound does not occur and hence a negative reaction (no red/pink colour) is obtained. The differentiation is brought about by the ability of some *Shigella* spp., for example, *Sh. sonnei* to ferment lactose after 48 hours of incubation.

COLLECTION OF SAMPLE:

Fresh fecal samples were collected in a suitable container. A loopful of pathological sample was inoculated into 5 ml of *Shigella* broth base for the enrichment of *Shigella*. This was incubated at 37°C for 24 hours.

a. Isolation on Salmonella-Shigella Agar: One to two loopfuls of the growth from the *Shigella* broth base were streaked onto slants containing Salmonella-Shigella agar. This was incubated at 37°C for 24 hours. Positive results were detected in the form of colourless colonies on the medium. The colonies on this agar were used further as pure culture.

b. Sugar Fermentation Test: A portion of the colony from the pure culture was inoculated into five sugar-

fermentation tubes, containing glucose, lactose, mannitol and two tubes containing Glucose Phosphate Broth (GPB). The two GPB tubes were utilized for the Methyl red – Voges Prausker (MR-VP) test. The tubes were incubated at 37°C for a maximum of 24 hours. Positive results were indicated by acid production with absence of gas in glucose (red colour) and GPB containing methyl red (pink colouration), and no acidity in mannitol and the GPB tube containing O'Meara's reagent / α -naphthol in alcoholic KOH.

Differentiation was brought about depending upon lactose fermentation after 48 hours.

10.e IDENTIFICATION OF CRYPTOSPORIDIA

COLLECTION OF SAMPLE:

Fresh fecal samples were collected in a suitable container on three consecutive days. Samples were processed immediately and wet preparations and smears were made from each sample. The smears were stained with modified cold Kinyoun method as well as Gram staining. Samples not revealing oocysts of parasites were subjected to Sheather's sugar floatation technique (264).

a. Modified Cold Kinyoun Method: It is a modification of ZNCF method. In this method, methanol fixed smears are

flooded with strong Carbol fuchsin and preserved for 15-20 minutes. Smears are then rinsed with tap water. Decolourization is brought about by treatment with 1% hypochloric acid in 95% ethanol for 15-30 seconds. Smears are then rinsed with tap water and counter stained with 0.3% methylene blue for 30-60 seconds. After washing off the excess stain, the smears are air-dried and examined under the light microscope using high power (40X) and oil-immersion (100X) objectives.

Bright red-pink oocysts (4-6 μm) of cryptosporidia are observed against a blue background. The fecal samples not revealing oocysts were subjected to Sheather's sugar floatation technique.

b. Sheather's Sugar Flotation Technique: Approximately 5 gm of sample was emulsified in 100 ml of tap water. The resulting mixture was filtered through gauze in a 10 ml centrifuge. The tubes were centrifuged at 1000g for 5 minutes. The supernatant was decanted and the tube was filled three-fourth with Sheather's sugar solution. The sediment was mixed thoroughly with the sugar solution. Additional sugar solution was added to fill the tube to half-a-centimeter from the brim. The tube was again centrifuged at 310g for 5 minutes. Using a capillary tube,

two drops were siphoned from the surface file and mounted on a glass slide. Cover slips were placed on the drops and examined microscopically at 10X and 40 X magnifications. The cyrptosporidial oocysts (4-5 μ m) appear with a pink hue and with/without a minute residual body.

10.f: IDENTIFICATION OF *Pneumocystis carinii* pneumoniae:

P.carinii has both intracystic and extracystic forms. The cysts are oval to round, approximately 5 - 8 μ m in diameter, and contain 4 - 8 intracystic organisms (sporozoites). These sporozoites are nucleated and measure 1 - 2 μ m in diameter. The extracystic form (trophozoite) measures 2 - 5 μ m in diameter. It is pleomorphic and often has an eccentric nucleus.

The sporozoites and the trophozoites can be clearly stained with Gram, Giemsa or Wright Stains.

Children with PCP present with a tetrad of findings: tachypnea, dyspnea, cough and fever. Physical examination reveals tachycardia and respiratory distress, accelerating tachypnea and diffuse retractions. Pulmonary auscultation is typically non-specific. PCP is characterized by rapid progressive hypoxemia. Early radiographic findings may be normal or can include hyperinflation with

peri-bronchial thickening. As the disease progresses, the chest radiographic findings typically evolve to reveal bilateral alveolar or interstitial infiltrates usually spreading peripherally from the perihilar region to involve both lung fields. Further progression results in the coalescent bilateral airspace disease with air bronchogram. Less typical radiographic findings include focal or patchy infiltrates, cavities or effusions.

Fibre-optic bronchoscopy with bronchoalveolar lavage has been shown to be a safe and sensitive method for the diagnosis of PCP. Other diagnostic methods include sputum, tracheal wash for intubated children and open lung biopsy.

In a laboratory, the presence of PCP can be screened using the Wright/Giemsa stain method.

COLLECTION OF THE SAMPLE

Patients with PCP rarely have a productive cough. Hence special methods are necessary to obtain pulmonary secretions for microscopic analysis. Patients breathe a mist of hypertonic saline (3%) for five to fifteen minutes, and are then asked to produce sputum. The diagnostic sensitivity of induced sputum for diagnosing PCP ranges between 30-90%. Another method for obtaining pulmonary

secretion is the bronchoalveolar lavage (BAL). This procedure identifies PCP in 98-100% of the cases. Typically, 100-200 ml of sterile non-bacteriostatic saline are recovered for laboratory analysis.

DETECTION OF THE ORGANISM

A smear is made from the sputum or the BAL sample and fixed with absolute methanol for 1-5 minutes depending upon the thickness of the smear. The methanol is then washed off by gently rinsing the slide with distilled water.

Around 40 ml of fresh working Giemsa stain (2.5%) is prepared in a staining/ Coplin jar. In another jar, 40 ml of the working Giemsa buffer is added. To this are added 2 drops of Triton X-100. The smear-slides are placed into the working Giemsa stain for 45-60 minutes. The thin smear slides are rinsed by dipping them 3-4 times in the Giemsa buffer, while the thick smears are immersed in the buffer for 5 minutes. The smears are then air-dried and observed under high power (40X) magnification. The trophozoites appear dark blue against a pale blue background (213).

11. Determination of the Mortality in HIV Infected Children:

The HIV infected children were monitored for a period of 18 months. The parents were instructed to visit the hospital

at intervals for the check-up of their children. In case of orphans, the concerned guardian was instructed as above. A Social Worker visited the hospital OPD and wards. Whenever the parents/guardian failed to report to the hospital, the Social Worker visited their residence to check the condition of the child. The parents/ guardians were given post-cards to be posted to the hospital in event of the death of their child.

Clinically, the children were monitored for the change in the CD4+T cell counts, as an index for the progression of HIV disease.

Observations

J

Results



STUDY POPULATION:

Hundred HIV seropositive subjects were enrolled in the study. Of these, 55 were in the age between 1-5 years, while the remaining 45 were above 5 years of age. The Subject cohort comprised of 55 male and 45 female children. All of them were vertically infected with HIV and were not being administered any anti-retroviral therapy.

Twenty-eight normal, healthy children, comprising of 14 children each in the age groups 1-5 years and above 5 years, constituted the Control group. These children were brought to a General Physician as a part of the pre-school clinic for screening for common infections (bacterial, helminthic, etc.) They were being monitored for their hemoglobin levels, Differential White Blood Cell count, etc.

PARENTAL HISTORY OF HIV INFECTED CHILDREN:

Table 2: Parental history of HIV infected children (n=100)

Single parent alive:	
Only mother	28
Only father	12
Both parents alive	29
Both parents expired	13
Parents separated	17
Child abandoned*	1
Total	100

* Brought to the clinic by a social worker

Table 2 illustrates the parental history of the HIV seropositive children. It was observed that only 29 children of the total 100 had both parents alive and living with them. In 40 cases, either of the parents had expired. In 13 cases, both the parents had expired and the children were taken care by the relatives or by orphanages. Parents in 17 cases had separated and the children were living with a single parent. One case of an abandoned child was brought in by the social worker of an orphanage.

CLINICAL MANIFESTATIONS IN HIV INFECTED CHILDREN

Table 3: Clinical Manifestations Observed In The HIV Seropositive Children Enrolled In The Study (n=100)

Clinical manifestations	No. of children
Tuberculosis	19
<i>Pneumocystis carinii</i> pneumonia	25
Diarrhoea	7
Dermatitis	19
Otorrhea	28
Candidiasis	9

It was observed that 44% of the HIV seropositive children in the Study Group suffered from respiratory illness. Tuberculosis was observed in 19% of the cases while PCP was a major

infection, accounting for 25% of the cases. Manifestations like otorrhea (28%) and dermatitis (19%) were also observed. Recurrent diarrhoea was observed in 7% of the cases, while candidiasis was observed in 9%.

CLINICAL STAGING OF THE SUBJECT GROUP:

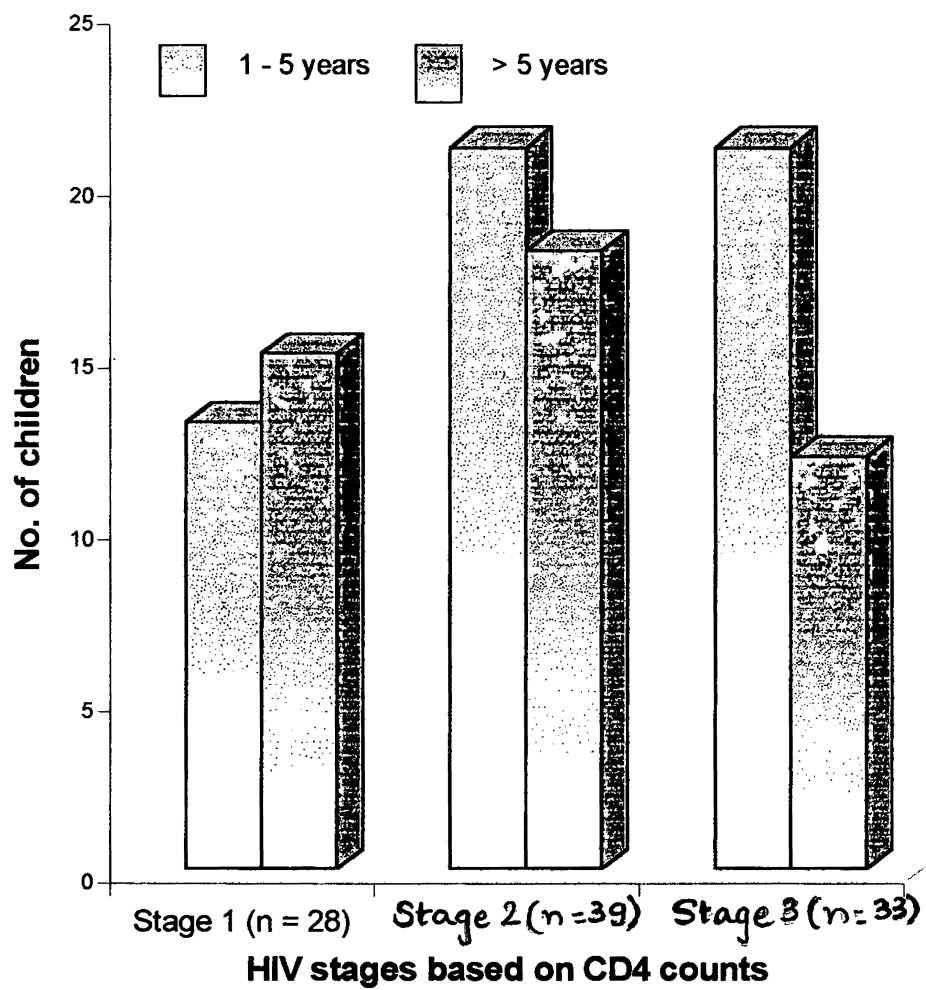
The children were classified into different HIV stages using the CDC Classification System. The categorization is illustrated in Table 4

Table 4: Distribution of the study population based on CD4 counts

Age group	Control group	HIV seropositive Cohort		
		Stage 1	Stage 2	Stage 3
1 - 5 years	14 (>1000)	13 (> 1000)	21 (999-500)	21 (< 500)
> 5 years	14 (> 500)	15 (> 500)	18 (499-200)	12 (< 200)
Total	28	28	39	33

Figures in parentheses indicate the range of CD4 counts for the respective Stage

Of 55 children between 1 - 5 years of age, 13 (32.73%) children were classified to be in Stage 1. Twenty-one (31.18%) children were classified to be in Stage 2 and 21 (31.18%) to be in Stage 3. In 45 children above 5 years of age, 15 (33.33%) children were classified to be in Stage 1. Eighteen (40%) children were classified to be in Stage 2 and 12 (26.67%) to be in Stage 3. The distribution is illustrated in Graph 1.



Graph 1: Distribution of the study population (n=100) into various HIV stages based on CD4 counts

CYTOKINE PROFILE: The cytokines viz. IL-2, IL-4, IL-6, IL-10, IFN- γ and TNF- α were investigated under the present study. Since their circulatory forms have a short half-life, their corresponding nuclear material, i.e. the mRNA was estimated using relative semi-quantitative RT-PCR. The levels were determined as a ratio of the cytokine mRNA to the 18S ribosomal RNA control. The cytokine mRNA levels were correlated with CD4 counts (an index for disease progression), and *Pearson's Correlation Co-Efficient 'r'* was calculated to determine the significance. The mean values of the cytokine mRNA ratio in the control group and different stages of HIV seropositive group were compared using the *Student's 't' Test*. The following mathematical formulae were used to derive the significance value:

a. For correlation:

$$t = r \sqrt{n - 2 / (1 - r^2)}, \quad \text{where } n = \text{Number of samples}$$

$r = \text{Pearson's correlation Co-efficient}$

b. For comparison of means from two different groups:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(\sigma_1^2 / N_1) + (\sigma_2^2 / N_2)}} \quad \text{where } \bar{X} = \text{mean of the group}$$

$N = \text{Number of samples}$

and

$$\sigma^2 = s^2 N / N - 1 \quad \text{where } s = \text{standard deviation}$$

Table 5: Th1 cytokine mRNA profile in HIV seropositive children of age: 1 - 5 years

Cytokine	Control	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-2/18S RNA	0.7121 (0.3-0.89)	0.688 (0.542-0.922) p > 0.05	0.631 (0.489-0.729) p > 0.05	0.474 (0.09-0.627) p < 0.01
IFN- γ /18S RNA	0.0219 (0.00-0.0056)	0.757 (0.55-1.103) p < 0.01	0.501 (0.412-0.616) p < 0.01	0.434 (0.18-0.55) p < 0.01
TNF- α /18S RNA	0.0452 (0.00-0.071)	0.157 (0.08-0.25) p > 0.05	0.520 (0.258-0.69) p < 0.01	0.991 (0.72-1.25) p < 0.01

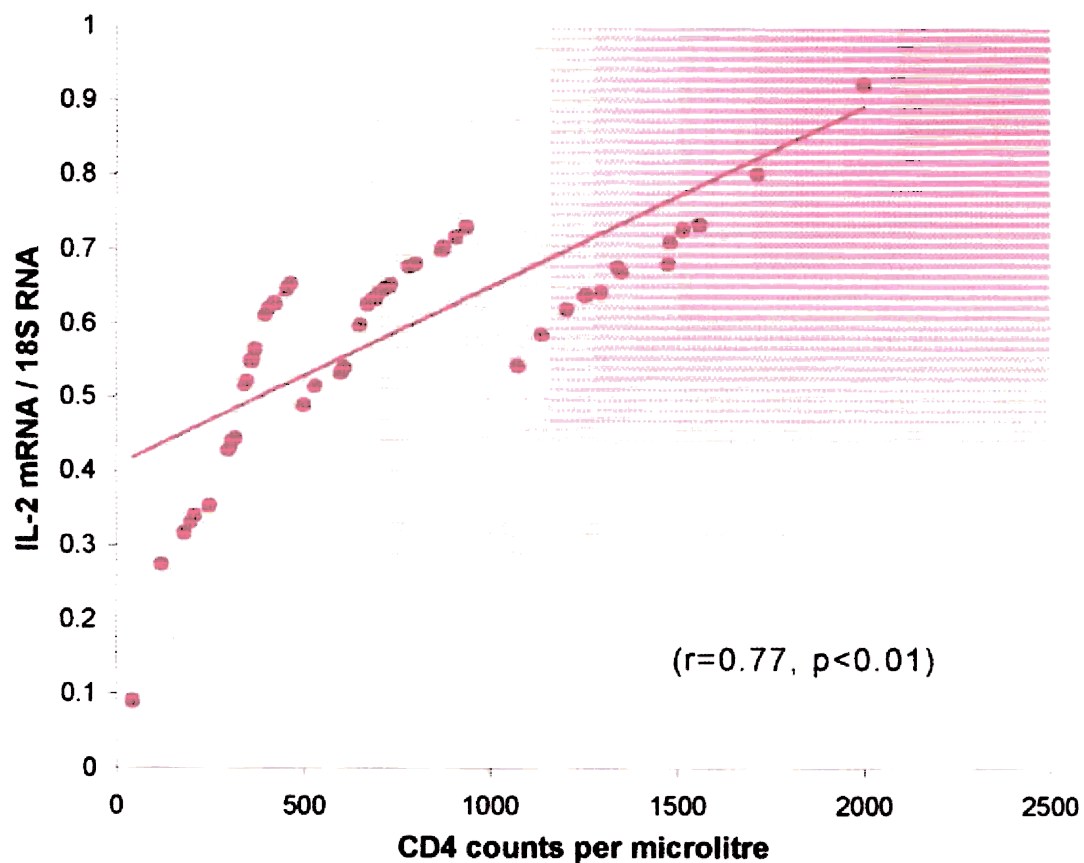
Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

As illustrated in Table 5, in the age group of 1-5 years, the mean ratio of IL-2 mRNA/18S RNA was 0.7121 in the Control group. In Stage1 of the HIV seropositive group, the mean ratio of IL-2 mRNA/ 18S RNA was 0.688 (p>0.05). In Stage 2, the mean ratio was 0.631(p>0.05) and in Stage 3, the mean ratio was 0.474(p<0.01). As shown in Graph 2, the IL-2 levels throughout the Subject Group were in a direct proportion with the concomitant decrease in CD4 counts(r=0.77, p<0.01).

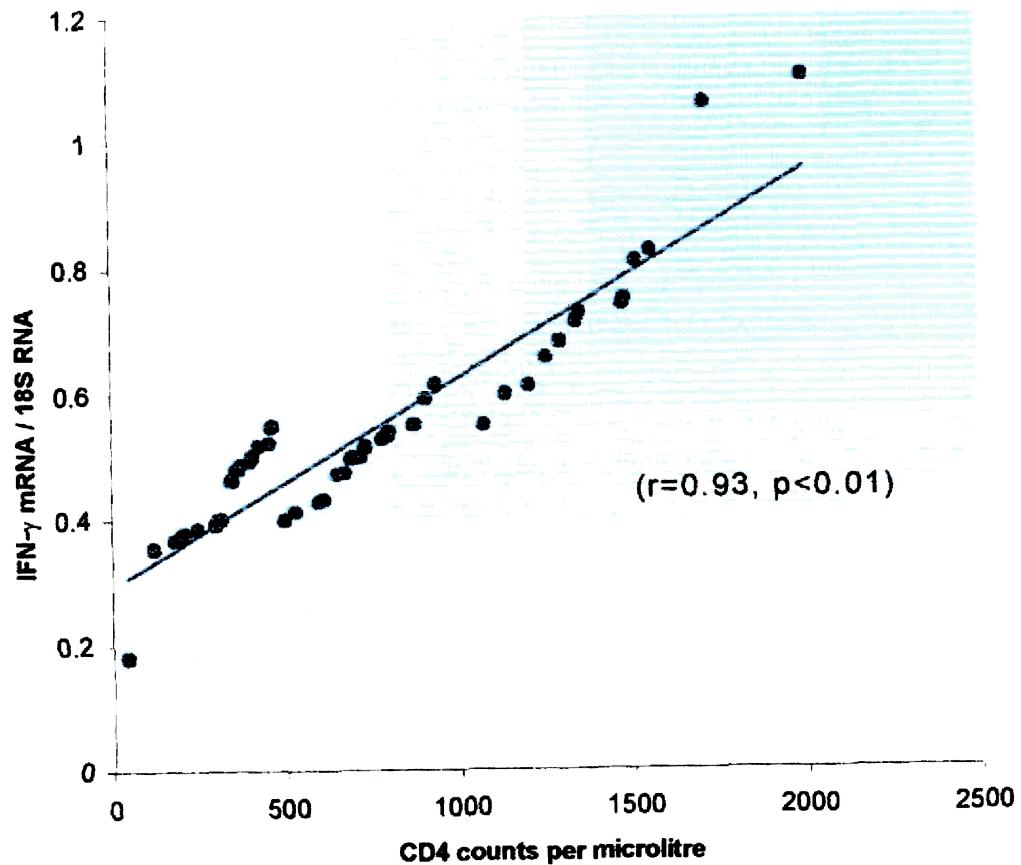
As illustrated in Table 5, the mean ratio of IFN- γ mRNA/18S RNA was 0.0219 in the Control group. In Stage 1 of the HIV seropositive group, the mean ratio of IFN- γ mRNA/ 18S RNA was 0.757 (p<0.01). In Stage 2, the mean ratio was 0.501(p<0.01) and in Stage 3, the mean ratio was 0.434 (p<0.01). As depicted by Graph 3, the IFN- γ levels decreased

with the concomitant decrease in CD4 counts($r=0.93$, $p<0.01$) across the Subject Cohort.

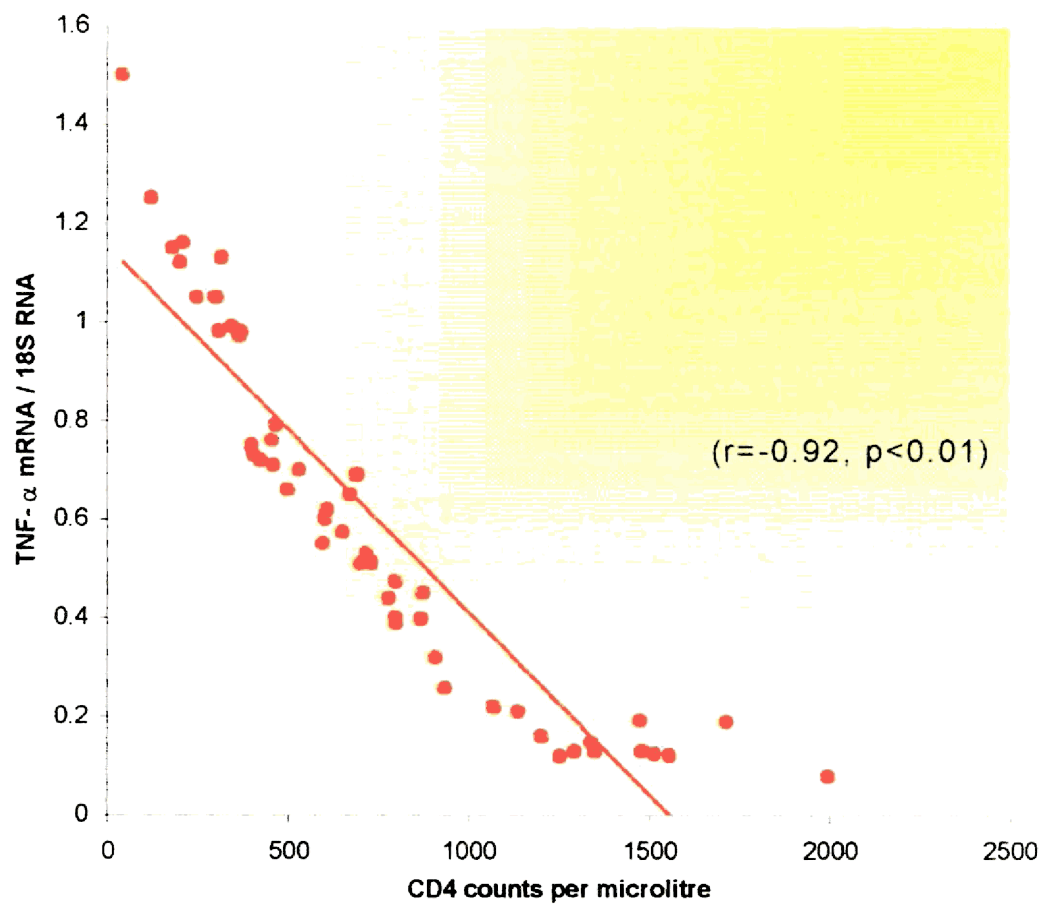
As seen in Table 5, the mean ratio of TNF- α mRNA/18S RNA in the Control group was 0.0452. In Stage 1 of HIV seropositive group, the mean ratio of TNF- α mRNA/ 18S RNA was 0.157 ($p>0.05$). In Stage 2, the ratio was 0.520 ($p<0.01$) and in Stage 3, the ratio was 0.991 ($p <0.01$). TNF- α levels were inversely proportional to the CD4 counts($r= - 0.92$, $p<0.01$), as shown in Graph 4.



Graph 2: IL-2 mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of IL-2 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 3: IFN- γ mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of IFN- γ mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 4: TNF- α mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of TNF- α mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.

Table 6: Th2 cytokine mRNA profile in HIV seropositive children of age: 1- 5 years

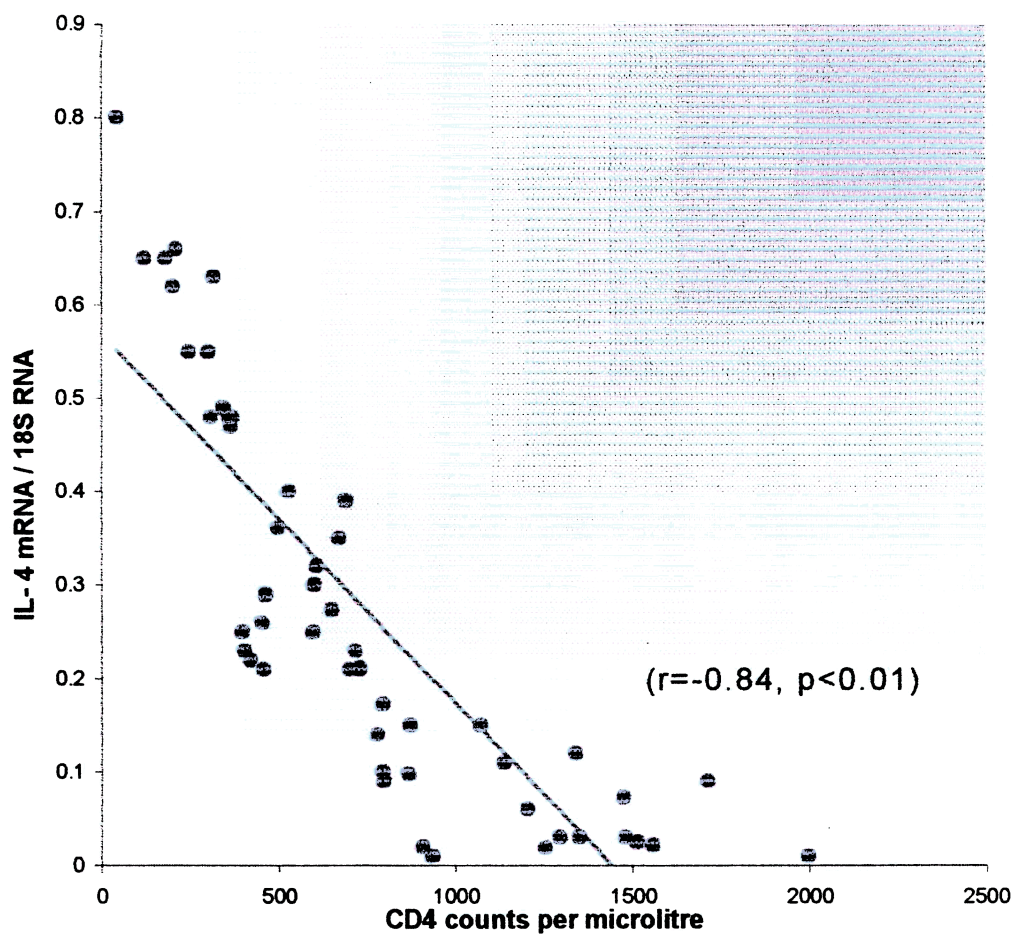
Cytokine	Control	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-4 / 18S RNA	0.023 (0.00-0.056)	0.059 (0.01-0.15) p < 0.01	0.223 (0.09-0.39) p < 0.01	0.477 (0.21-0.80) p < 0.01
IL-6 / 18S RNA	0.320 (0.00-0.54)	0.355 (0.28-0.42) p > 0.05	0.540 (0.278-0.72) p < 0.01	0.790 (0.51-1.30) p < 0.01
IL-10 / 18S RNA	0.0215 (0.00-0.056)	0.116 (0.067-0.207) p < 0.01	0.253 (0.04-0.43) p < 0.01	0.397 (0.13-0.58) p < 0.01

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

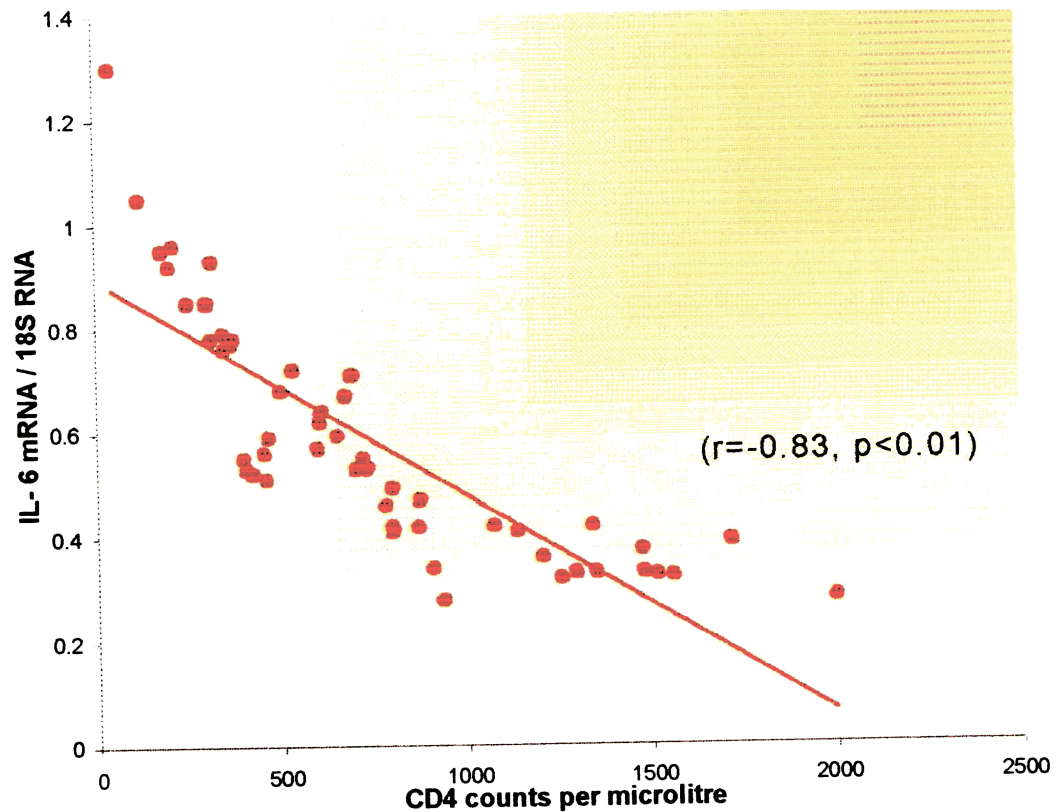
As illustrated in Table 6, in the age group of 1-5 years, the mean ratio of IL-4 mRNA/18S RNA was 0.023 (p<0.01). In Stage 1 of the HIV seropositive group, the mean ratio of IL-4 mRNA/ 18S RNA was 0.059 (p<0.01). In Stage 2, the mean ratio was 0.223 (p<0.01) and in Stage 3, the mean ratio was 0.477 (p <0.01). As shown in Graph 5, IL-4 levels were observed to increase with concurrent decrease in CD4 counts (r= -0.84, p<0.01).

As illustrated in Table 6, mean ratio of IL-6 mRNA/18S RNA in the Control group was 0.320. In Stage 1 of the HIV seropositive group, the mean ratio of IL-6 mRNA/ 18S RNA was 0.355 (p>0.05). In Stage 2, the mean ratio was 0.540 (p<0.01) and in Stage 3, the mean ratio was 0.790 (p <0.01). IL-6 levels were inversely proportional to the CD4 counts (r= - 0.83, p<0.01), as illustrated in Graph 6.

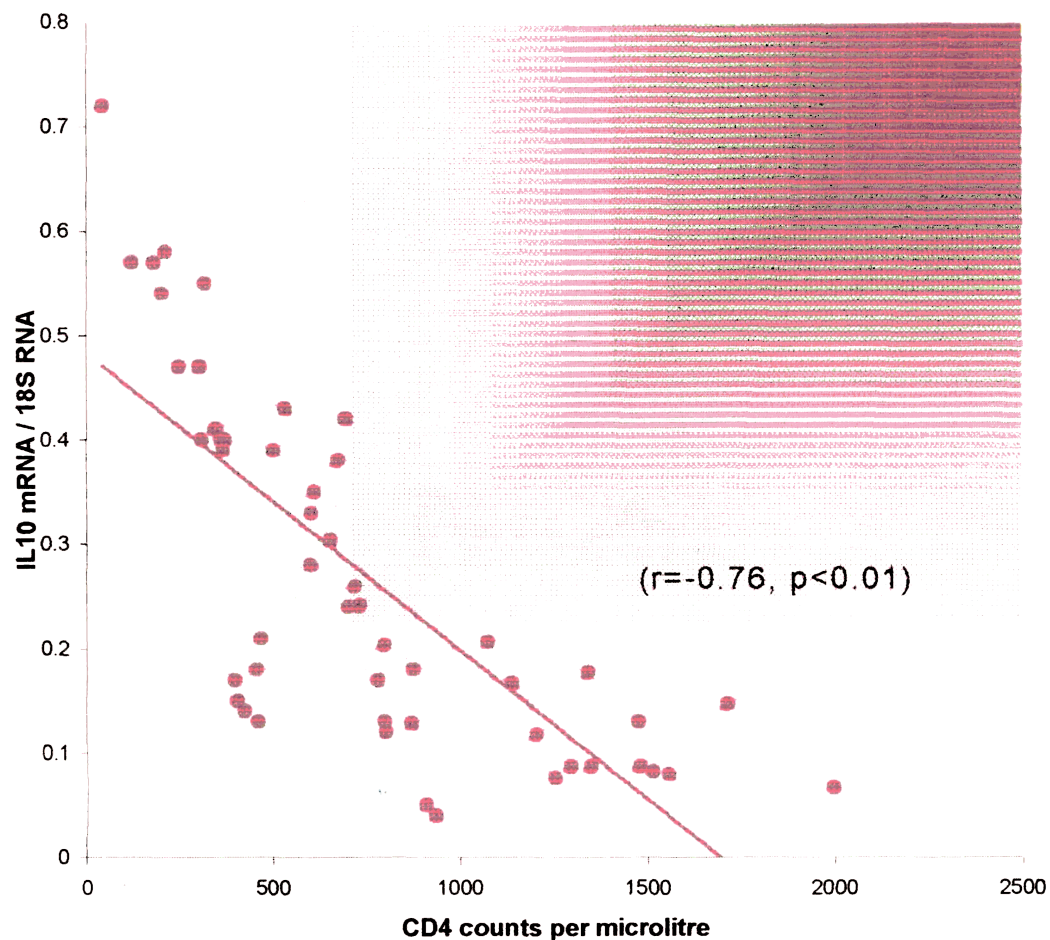
As mentioned in Table 6, the ratio of IL-10 mRNA/18S RNA in the Control group was 0.0452. In Stage 1 of the HIV seropositive group, the mean ratio of IL-10 mRNA/ 18S RNA was 0.116 ($p < 0.01$). In Stage 2, the mean ratio was 0.253 ($p < 0.01$) and in Stage 3, the mean ratio was 0.397 ($p < 0.01$). IL-10 levels were observed to increase with the concomitant decrease in CD4 counts ($r = -0.76$, $p < 0.01$), as shown in Graph 7.



Graph 5: IL-4 mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of IL-4 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 6: IL-6 mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of IL-6 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 7: IL-10 mRNA levels in HIV infected children (n=55) between 1-5 years of age. The levels are expressed as a ratio of IL-10 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.

Table 7: Th1 cytokine mRNA profile in HIV seropositive children of age: >5 years

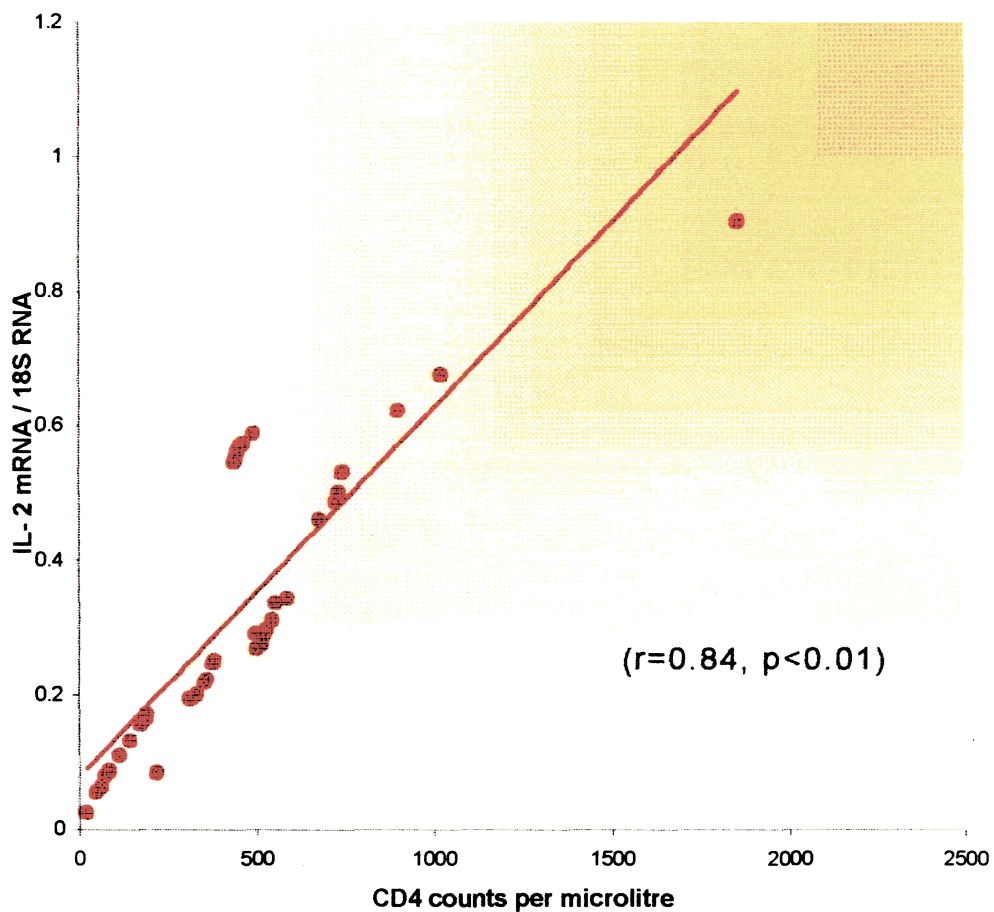
Cytokine	Control group	HIV Seropositive		
		Stage 1	Stage 2	Stage 3
IL-2/18S RNA	0.5529 (0.00-0.86)	0.541 (0.369-1.003) p > 0.05	0.342 (0.083-0.291) p < 0.01	0.107 (0.025-0.172) p < 0.01
IFN - γ /18S RNA	0.0264 (0.00-0.005)	0.758 (0.546-1.55) p < 0.01	0.434 (0.239-0.574) p < 0.01	0.265 (0.102-0.516) p < 0.01
TNF- α /18S RNA	0.0494 (0.00-0.086)	0.354 (0.12-0.433) p < 0.01	0.768 (0.65-0.86) p < 0.01	1.166 (0.902-1.5) p < 0.01

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

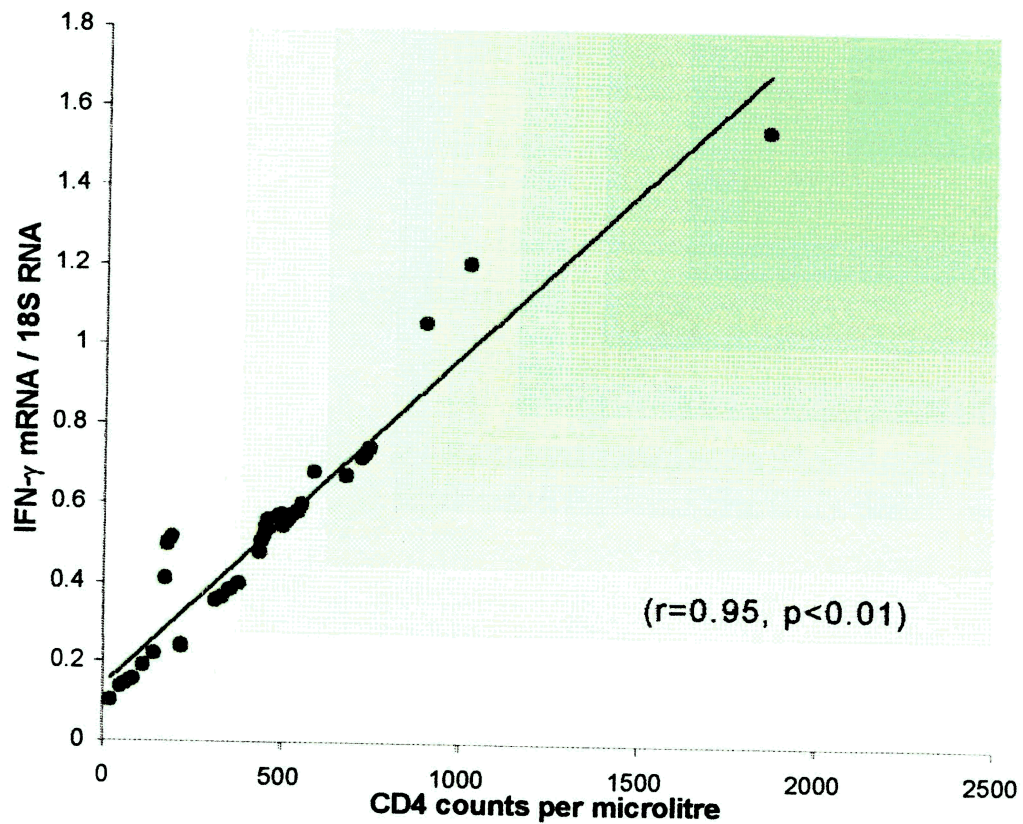
As illustrated in Table 7, in the age group above 5 years, the mean ratio of IL-2 mRNA/18S RNA was 0.5529 in the Control group. In Stage1 of the HIV seropositive group, the mean ratio of IL-2 mRNA/ 18S RNA was 0.541 (p>0.05). In Stage 2, the mean ratio was 0.342 (p<0.01) and in Stage 3, the mean ratio was 0.107(p<0.01). As depicted in Graph 8, IL2 levels were directly proportional to the concomitant decrease in CD4 counts(r=0.84, p<0.01).

As mentioned in Table 7, in the Control group, the mean ratio of IFN- γ mRNA/18S RNA was 0.026. In Stage 1 of the HIV seropositive group, the mean ratio of IFN- γ mRNA/ 18S RNA was 0.758 (p<0.01). In Stage 2, the mean ratio was 0.434(p<0.01) and in Stage 3, the mean ratio was 0.265 (p <0.01). IFN- γ levels were directly proportional to the CD4 counts(r=0.95, p<0.01), as shown in Graph 9.

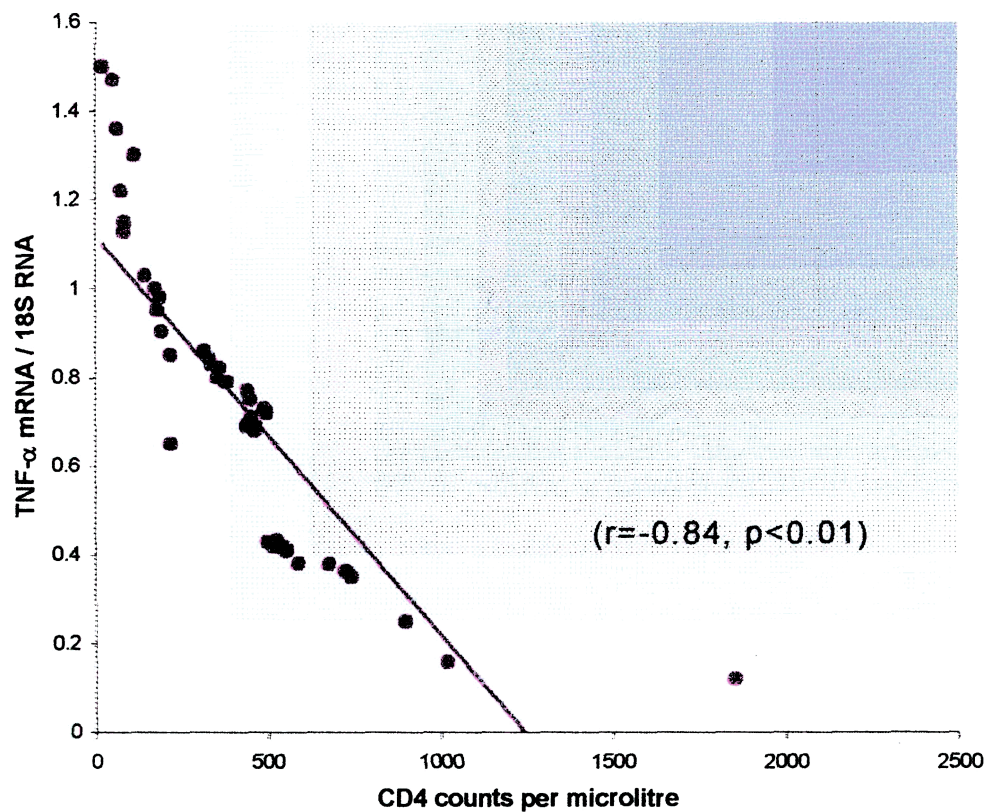
As illustrated in Table 7, the mean ratio of TNF- α mRNA/18S RNA in the Control group was 0.0494. In Stage 1 of the HIV seropositive group, the mean ratio of TNF- α mRNA/ 18S RNA was 0.354 ($p < 0.01$). In Stage 2, the mean ratio was 0.768 ($p < 0.01$) and in Stage 3, the mean ratio was 1.166 ($p < 0.01$). As shown in Graph 10, TNF- α levels increased with the concomitant decrease in CD4 counts ($r = -0.84$, $p < 0.01$).



Graph 8: IL-2 mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of IL-2 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 9: IFN- γ mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of IFN- γ mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 10: TNF- α mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of TNF- α mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.

Table 8: Th2 cytokine mRNA profile in HIV seropositive children of age: >5 years

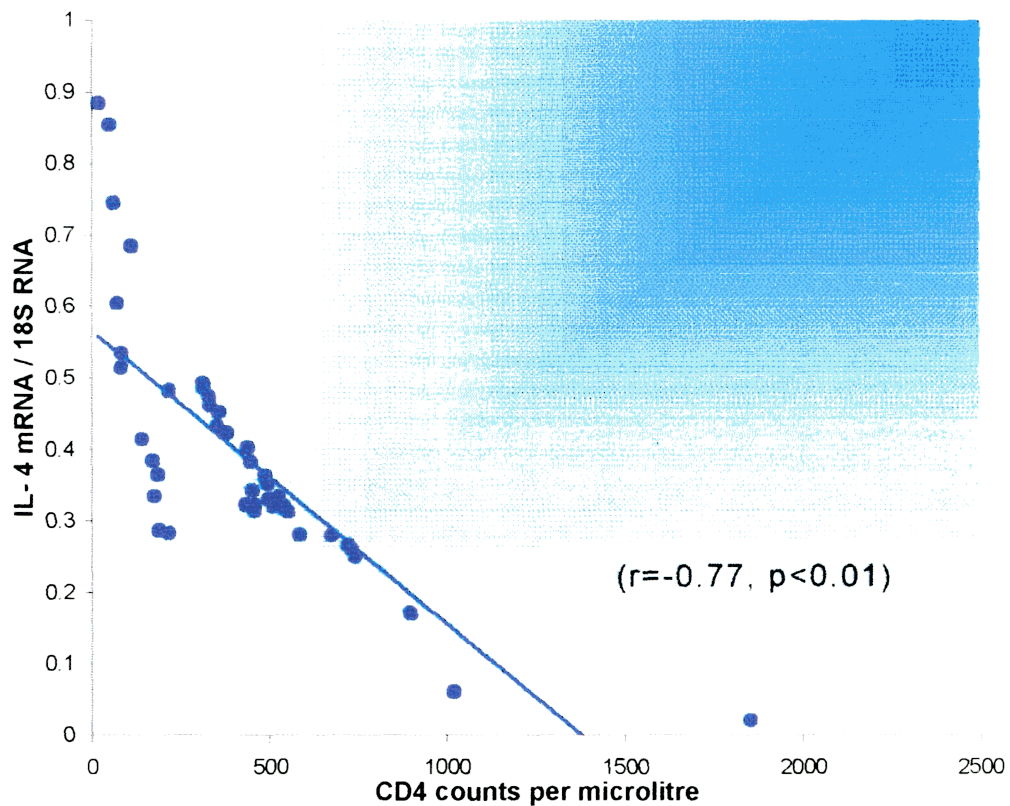
Cytokine	Control group	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-4 / 18S RNA	0.0229 (0.00-0.035)	0.256 (0.02-0.333) p < 0.01	0.400 (0.282-0.492) p < 0.01	0.550 (0.286-0.884) p < 0.01
IL-6 / 18S RNA	0.3621 (0.00-0.69)	0.564 (0.37-0.643) p < 0.01	0.735 (0.62-0.83) p < 0.01	0.976 (0.712-1.31) p < 0.01
IL-10 / 18S RNA	0.0221 (0.00-0.039)	0.276 (0.08-0.35) p < 0.01	0.330 (0.212-0.422) p < 0.01	0.466 (0.202-0.8) p < 0.01

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

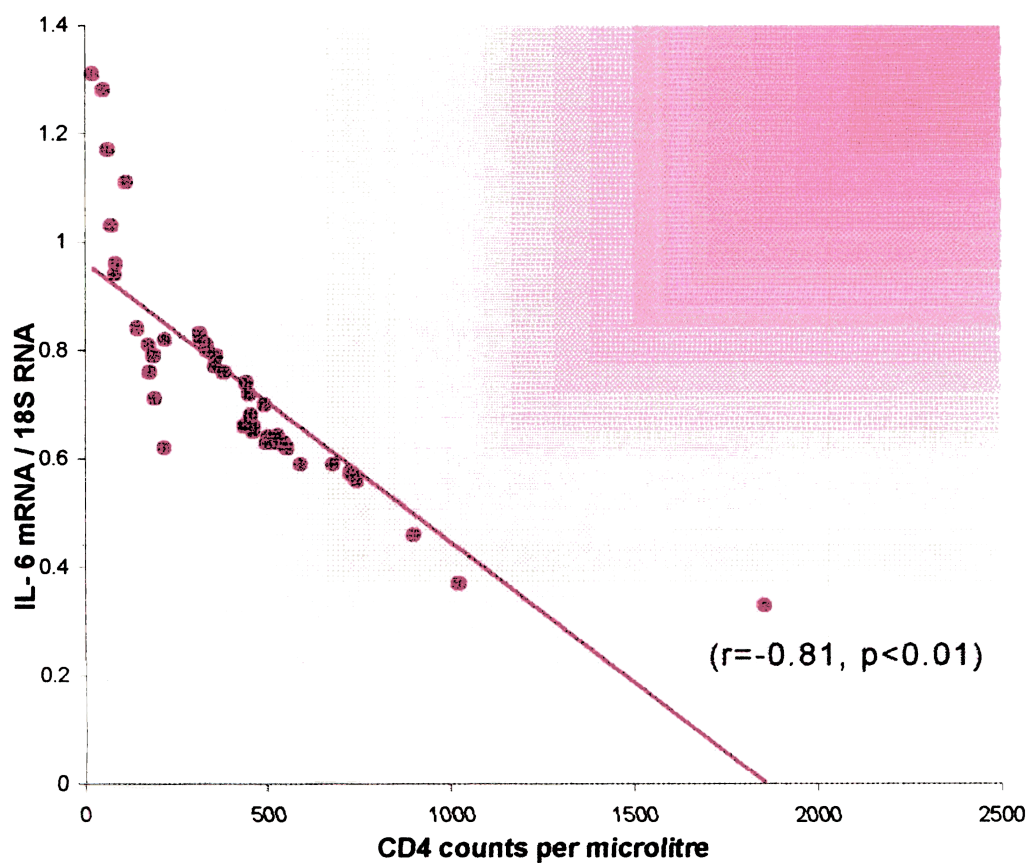
As mentioned in Table 8, in the Control group, the mean normal ratio of IL-4 mRNA/18S RNA was 0.0229. In Stage 1 of HIV seropositive group, the mean ratio of IL-4 mRNA/ 18S RNA was 0.256 ($p < 0.01$). In Stage 2, the mean ratio was 0.400 ($p < 0.01$) and in Stage 3, the mean ratio was 0.550 ($p < 0.01$). As shown in Graph 11, IL-4 levels were observed to increase with concurrent decrease in CD4 counts ($r = -0.77$, $p < 0.01$).

As seen from Table 8, mean ratio of IL-6 mRNA/18S RNA in the Control group was 0.3621. In Stage 1 of HIV seropositive group, the mean ratio of IL-6 mRNA/ 18S RNA was 0.564 ($p < 0.01$). In Stage 2, the mean ratio was 0.735 ($p < 0.01$) and in Stage 3, the mean ratio was 0.976 ($p < 0.01$). As illustrated in Graph 12, IL-6 levels increased with the decrease in CD4 counts ($r = -0.81$, $p < 0.01$).

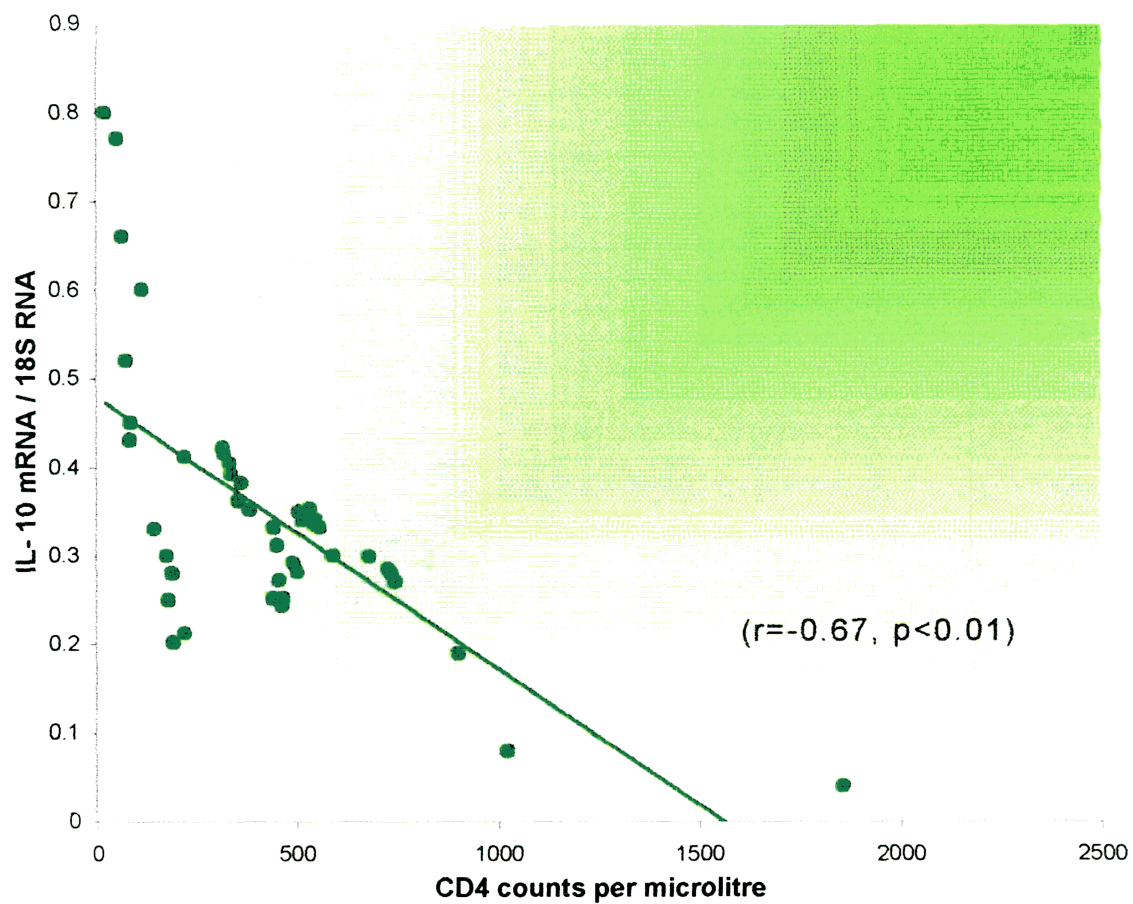
As mentioned in Table 8, the mean ratio of IL-10 mRNA/18S RNA in the Control group was 0.0221. In stage 1 of HIV seropositive group, the mean ratio of IL-10 mRNA/ 18S RNA was 0.276 ($p < 0.01$). In Stage 2, the mean ratio was 0.330 ($p < 0.01$) and in Stage 3, the mean ratio was 0.466 ($p < 0.01$) in Stage 3. As shown in Graph 13, IL-10 levels were thus observed to increase with the concomitant decrease in CD4 counts ($r = -0.67$, $p < 0.01$).



Graph 11: IL-4 mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of IL-4 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 12: IL-6 mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of IL-6 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.



Graph 13: IL-10 mRNA levels in HIV infected children (n=45) above 5 years of age. The levels are expressed as a ratio of IL-10 mRNA/18S RNA and are plotted against the CD4 counts as an index of HIV stage.

**OPPORTUNISTIC INFECTIONS IN HIV DISEASE
PROGRESSION**

Table 9: Opportunistic infections observed in children with respect to CD4 counts

CD4+ T cell counts	Age group											
	1 - 5 years						> 5 years					
	K	D	P	O	C	GI	K	D	P	O	C	GI
>1000 (n ₁ = 13) (n ₂ = 2)	-	-	3	5	-	-	1	1	-	1	-	-
999 - 500 (n ₁ = 21) (n ₂ = 13)	4	4	6	6	-	1	2	2	4	4	1	-
499 - 200 (n ₁ = 18) (n ₂ = 18)	5	4	4	3	4	3	6	5	4	6	3	2
< 200 (n ₁ = 3) (n ₂ = 12)	-	1	1	2	-	-	1	2	3	1	1	1
Total (n = 100)	9	9	14	16	4	4	10	10	11	12	5	1

K = Koch's disease/Tuberculosis

D = Mycotic Dermatitis

P = *Pneumocystis carinii* pneumoniae

O = Otorrhea (fungal/bacterial)

GI = Diarrhoea (fungal/bacterial/viral/protozoal)

C = Candidiasis (oral/dermal)

n₁ = Children in 1-5 years

n₂ = Children >5 years

In the age group, 1-5 years, the most frequently observed infection was the discharge from the ears, i.e. Otorrhea (16/55), followed by *Pneumocystis carinii* pneumoniae (PCP) (14/55), tuberculosis (9/55), dermatitis (9/55), Candidiasis (4/55) and severe diarrhoea (4/55).

In Stage 1 (CD4 counts > 1000/ μ l), opportunistic infections like PCP, tuberculosis, dermatitis and otorrhea were observed. In Stage 2 (CD4 counts 999-500/ μ l), children started presenting multiple infections. In Stage 3 (CD4 T cell counts 499-200/ μ l), the prevalence of multiple infections along with candidiasis and severe diarrhoea was observed. Two patients were also being treated for meningitis and one for hepatitis B.

In the age group of above 5 years, Otorrhea (12/45) and *Pneumocystis carinii* pneumoniae (PCP) (11/45) were the most frequently observed infections. The other important infections were tuberculosis (10/45) and dermatitis (10/45). Candidiasis (5/45) and severe diarrhoea (1/45) were also observed.

In Stage 1 (CD4 counts > 500/ μ l), opportunistic infections like PCP, tuberculosis, dermatitis and otorrhea were observed. In Stage 2 (CD4 counts 499-200/ μ l) and Stage 3 (CD4 T cell counts <200/ μ l), children presented multiple infections including candidiasis and diarrhoea.

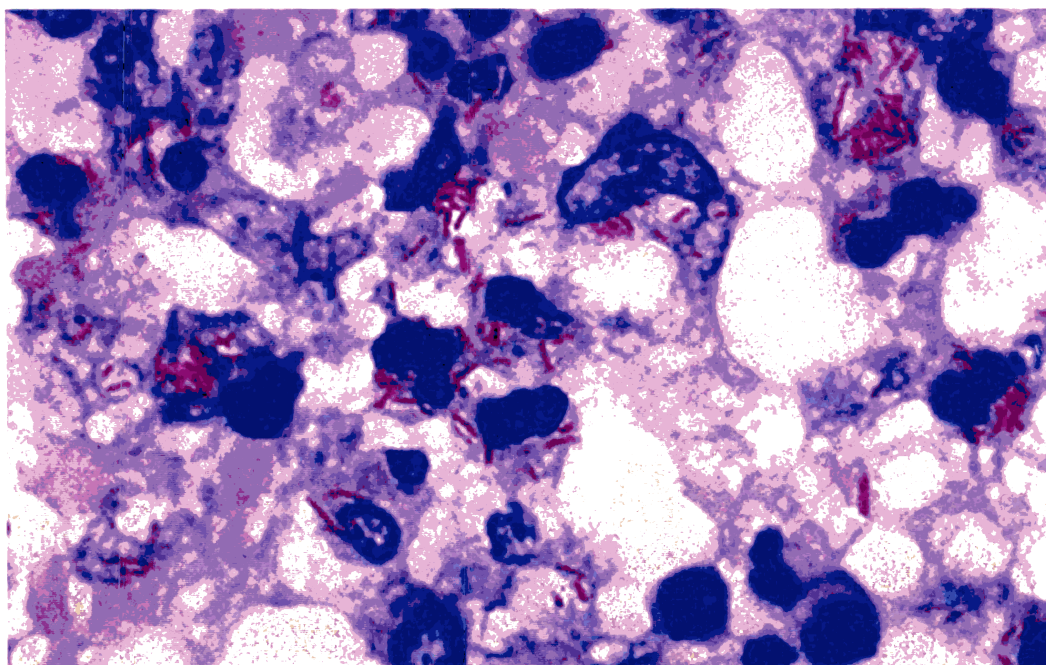


Figure 3: Ziehl - Neelsen Carbol Fuchsin staining of the sputum sample of a HIV infected child suspected for tuberculosis. The smear shows Acid-fast bacilli against blue cells, as observed under 100X magnification



Figure 4: Germ- tube formation by Candida as observed by Gram staining, under 40X magnification

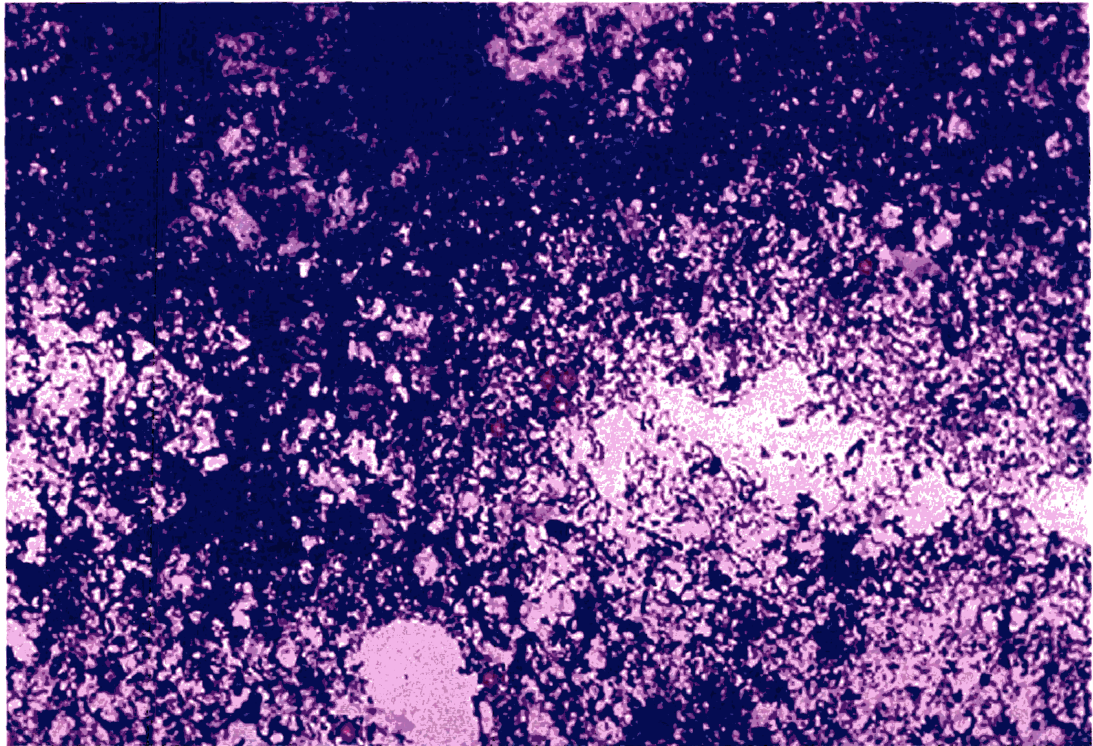


Figure 5: Detection of Cryptosporidial oocysts in the stool sample of an HIV infected child presenting with diarrhoea. The cysts appear spherical in shape and are stained using the Modified Cold Kinyoun Method

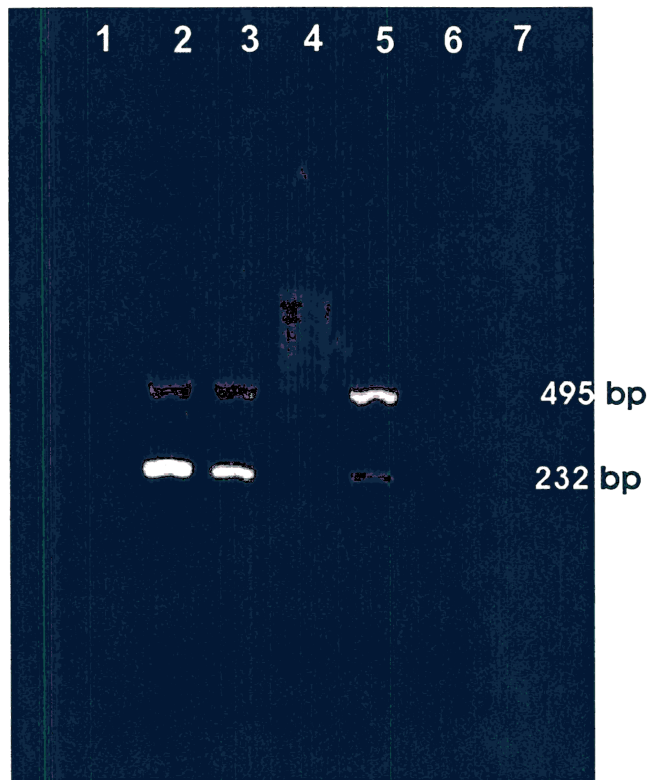


Fig 6: Amplification of the mRNA for IL-2 in HIV infected children

The 2% agarose gel shows two bands in lanes 2, 3, and 5 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (232 bp) is of the IL-2. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.

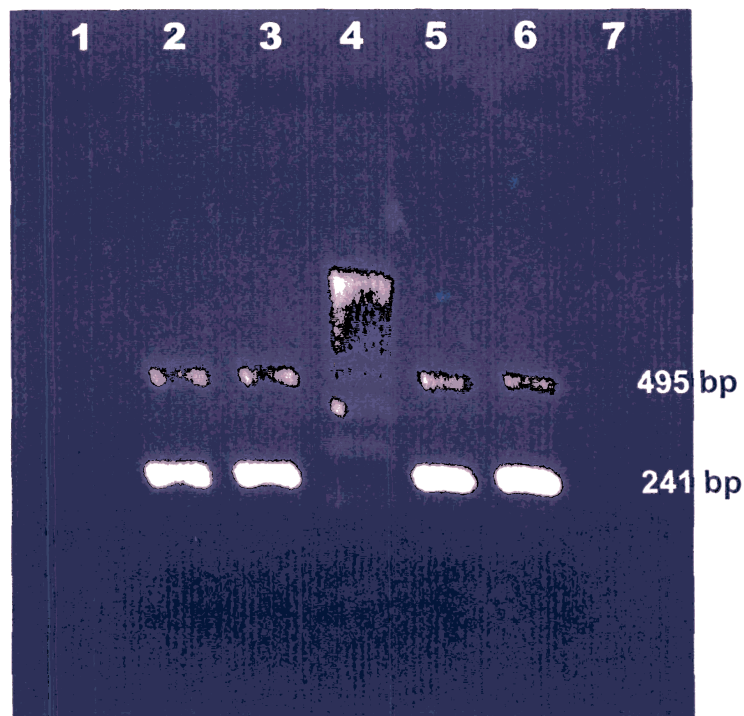


Fig 7 : Amplification of the mRNA for IFN - γ in HIV infected children

The 2% agarose gel shows two bands in lanes 2, 3, 5 and 6 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (241bp) is of the IFN- γ . Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.

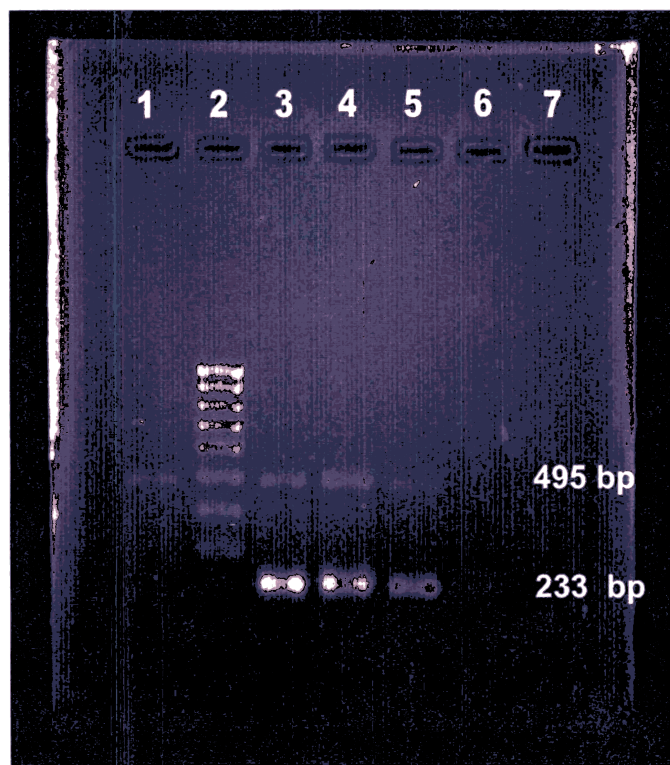


Fig 8 : Amplification of the mRNA for TNF - α in HIV infected children

The 2% agarose gel shows two bands in lanes 3, 4 and 5 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (233 bp) is of the TNF- α . Lane 2 bears the molecular weight marker (DNA ladder) from 100bp-1000bp. Lane 1 shows the sample from a healthy child, where levels of TNF- α are undetectable.

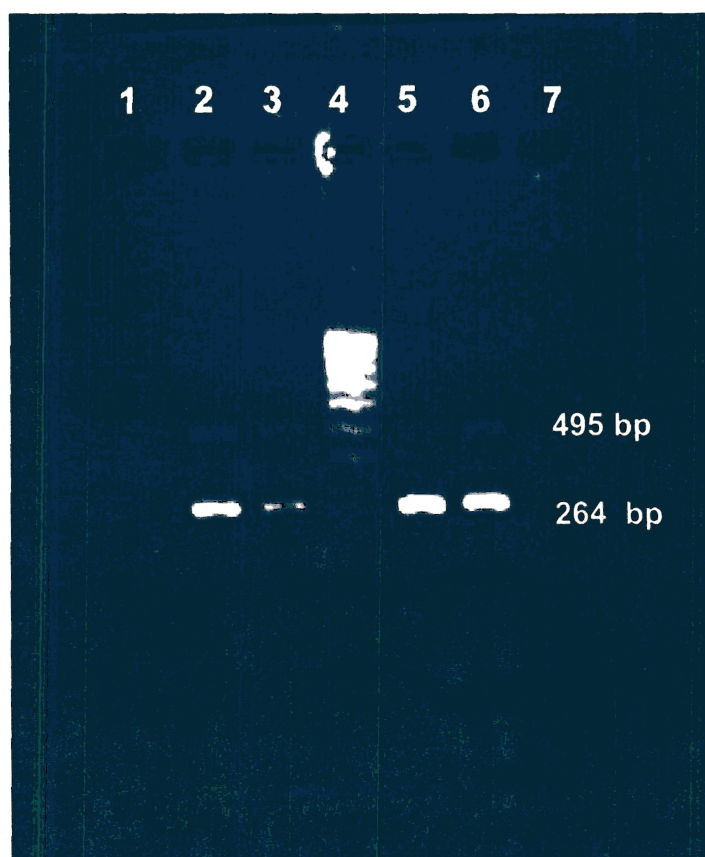


Fig 9 : Amplification of the mRNA for IL-4 in HIV infected children

The 2% agarose gel shows two bands in lanes 2, 3, 5 and 6 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (264 bp) is of the IL-4. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.



Fig 10 : Amplification of the mRNA for IL- 6 in HIV infected children

The 2% agarose gel shows two bands in lanes 2, 3, 5 and 6 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (242 bp) is of the IL- 6. Lane 1 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.



Fig 11 : Amplification of the mRNA for IL- 10 in HIV infected children

The 2% agarose gel shows two bands in lanes 2, 3, 5 and 6 in which the samples have been loaded. One band is for the 18S ribosomal RNA (495bp) while the other (297 bp) is of the IL- 10. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.

MORTALITY IN HIV/AIDS

HIV is a disease with high mortality, especially in the developing countries where the support system (nutrition, medication, anti-retroviral therapy, etc.) is not well developed. Also, the environment bears a plethora of pathogens ready to infect the host at an opportune moment.

Table 10: Mortality trends with respect to CD4 counts

CD4 counts	Mortality	
	1-5 years(n=55)	> 5 years (n=45)
> 1000	1	-nil-
999-500	3	-nil-
499-200	9	-nil-
<200	2	8
Total	15	8

Fifteen children from the age group 1-5 years died during the 18 months of monitoring, while 8 children above 5 years expired during the same period. In the HIV seropositive age group 1-5 years, maximum mortality was observed when the CD4 counts were between 499-200/ μ l. In seropositive children above 5 years, maximum mortality was observed when the CD4 counts were below 200/ μ l. No deaths were reported in the Control group.

VIRAL LOAD ESTIMATION: Viral load estimation was carried out in 10 patients by two methods, viz. the p24 antigen assay and the Real Time RT-PCR. Each Stage of the Subject Group was represented in these samples.

The lowest dilution of the cell supernatant that was detectable for the p24 antigen was taken as the cut-off. The viral load was calculated as follows:

$$\text{Viral load} = (1 / \text{Cut - off} \times 1000) / 200$$

This was then correlated with the results obtained with Real Time RT-PCR.

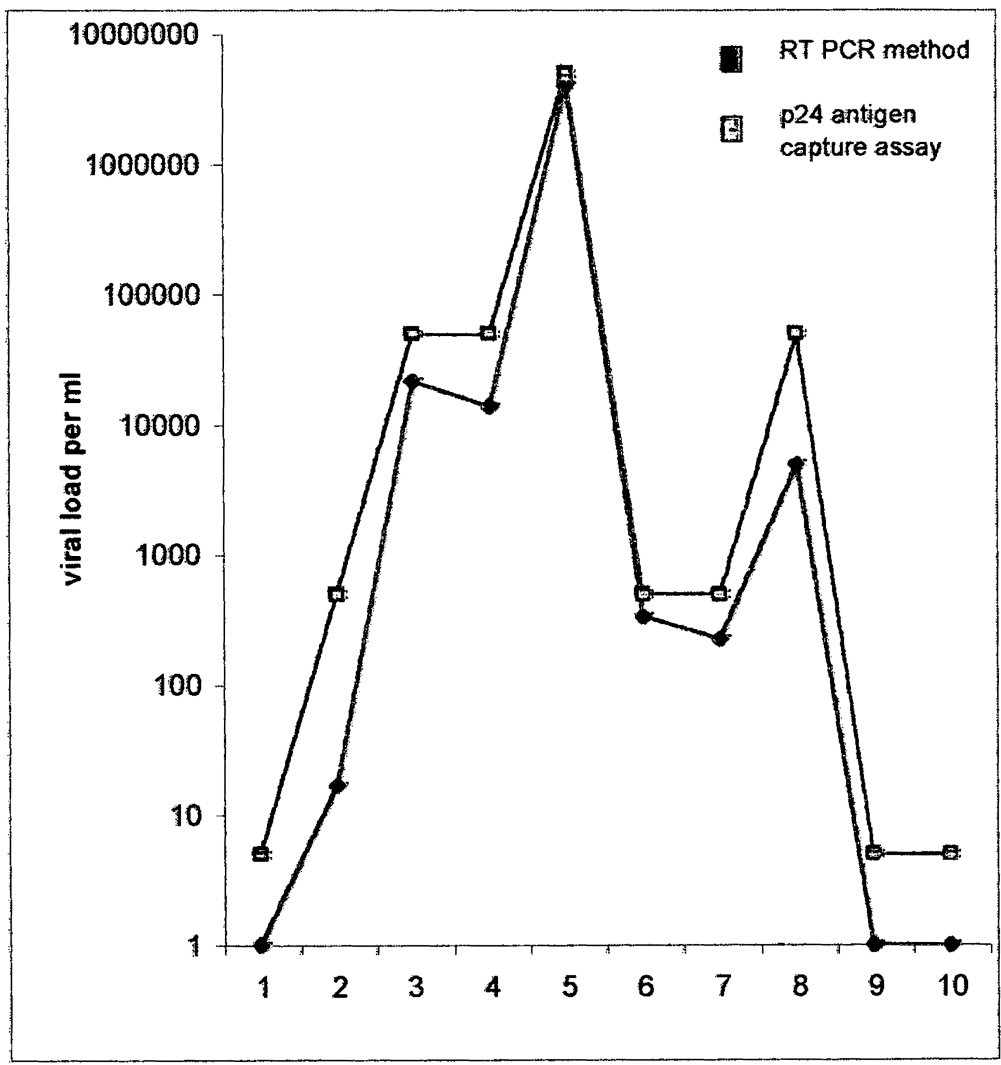
Table 11: Correlation of Two Methods of Viral Load Determination

Sample No.	Viral load / ml	
	p24 antigen assay	RT-PCR
1	5	Undetectable
2	5×10^2	0.169×10^2
3	5×10^4	2.17×10^4
4	5×10^4	1.39×10^4
5	5×10^2	4.08×10^2
6	5×10^2	3.34×10^2
7	5×10^2	2.25×10^2
8	5×10^4	0.5×10^4
9	5	Undetectable
10	5	Undetectable

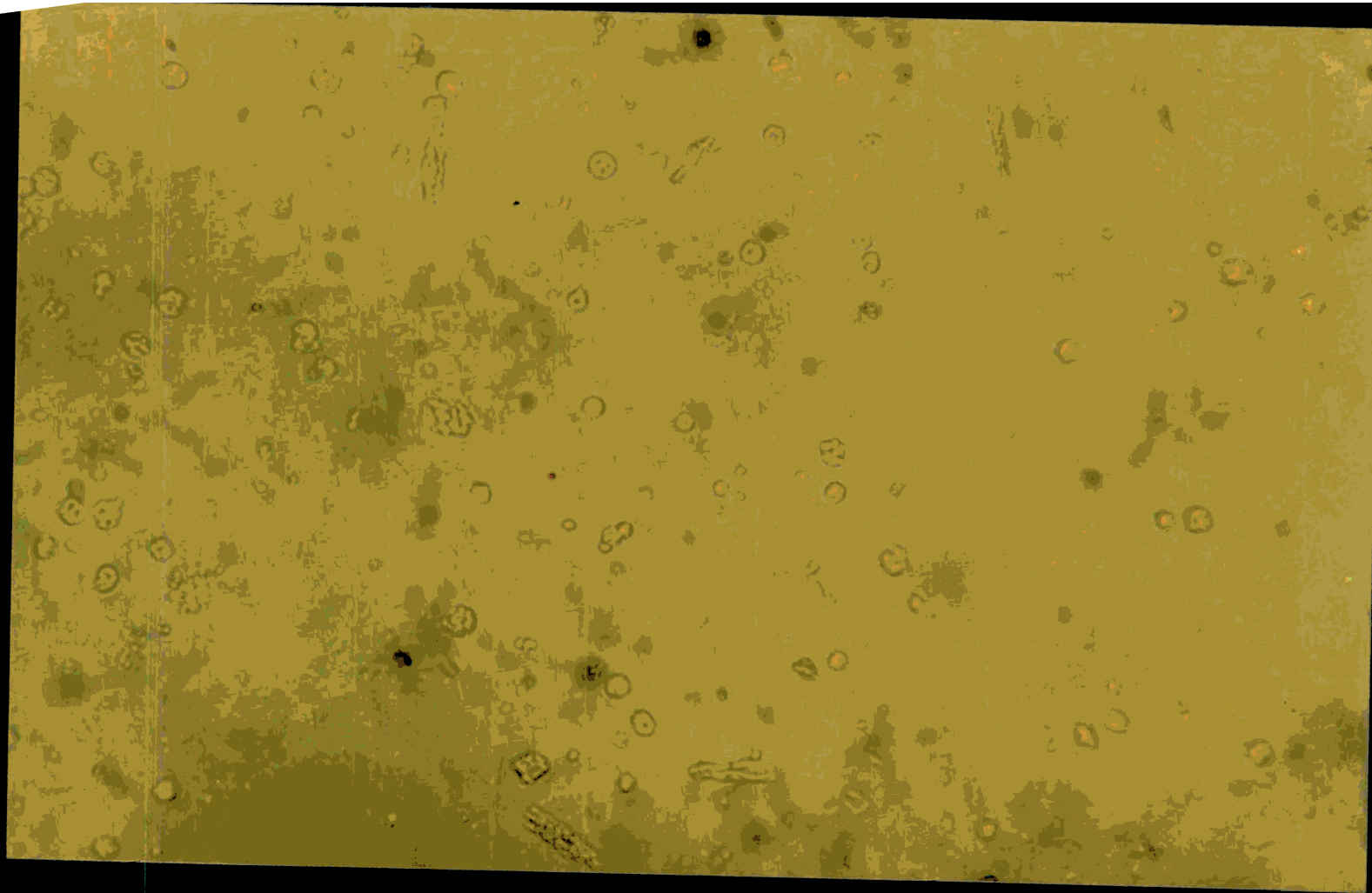
The viral load carried out using p24 antigen detection assay measured the presence of infectious viral particles per milliliter in the serum/plasma samples. On the other hand, the Real Time RT- PCR measured the viral load in terms of the viral RNA copies/ml in the samples. In the estimation of viral

load by RT-PCR method, three samples were found to have undetectable viral copies.

The two assays employed in the study showed a significant correlation ($r = 0.9999$, $p < 0.01$), as illustrated in Graph 14.

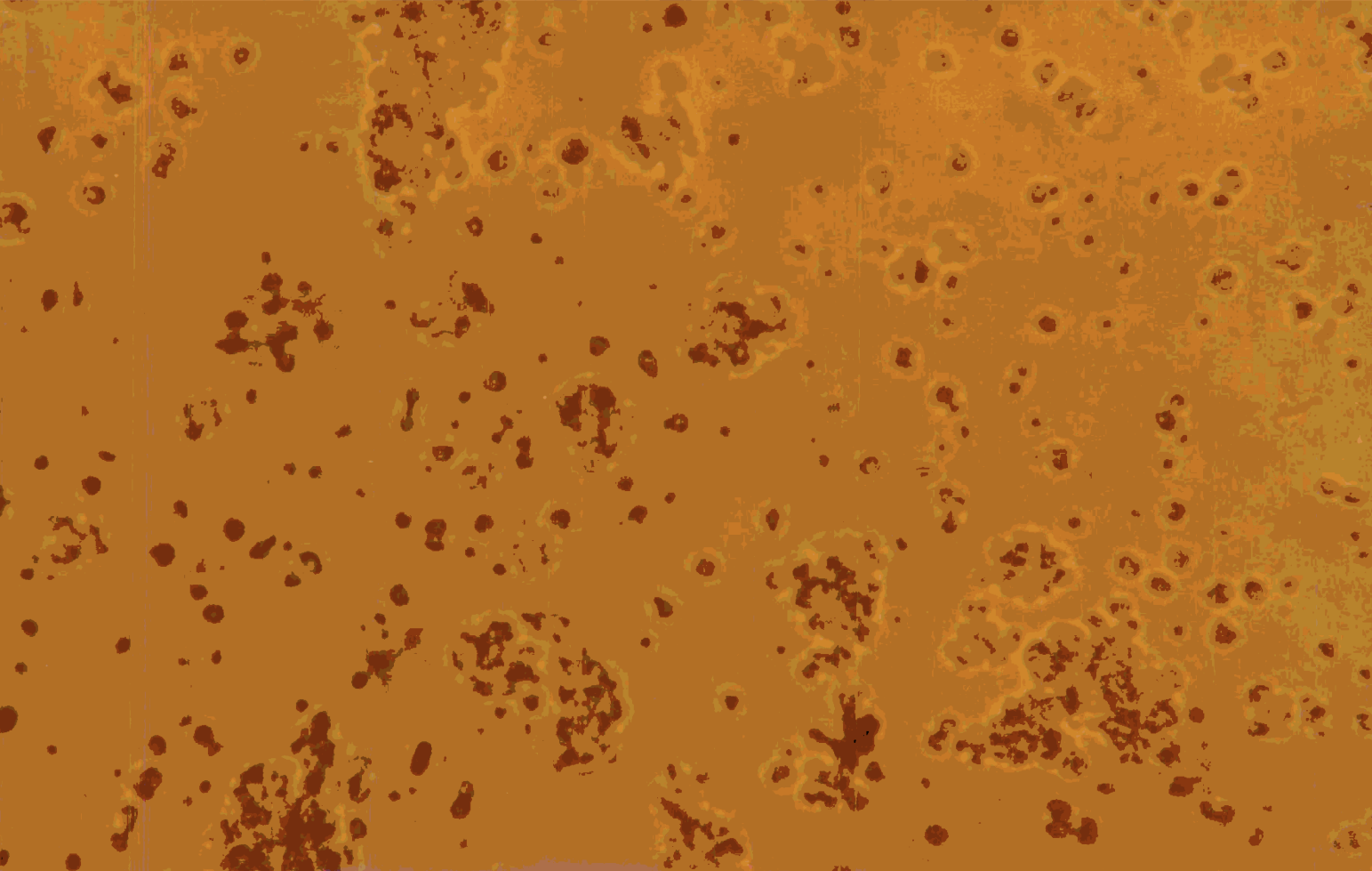


Graph 14: Viral load estimation by a) RT-PCR method and b) p24 antigen capture assay. The above graph shows the correlation between the two methods ($r=0.999$, $p<0.01$). The viral load is estimated as RNA copies per ml in case of RT-PCR method, while in p24 antigen method, it is detected as viral particles per ml.



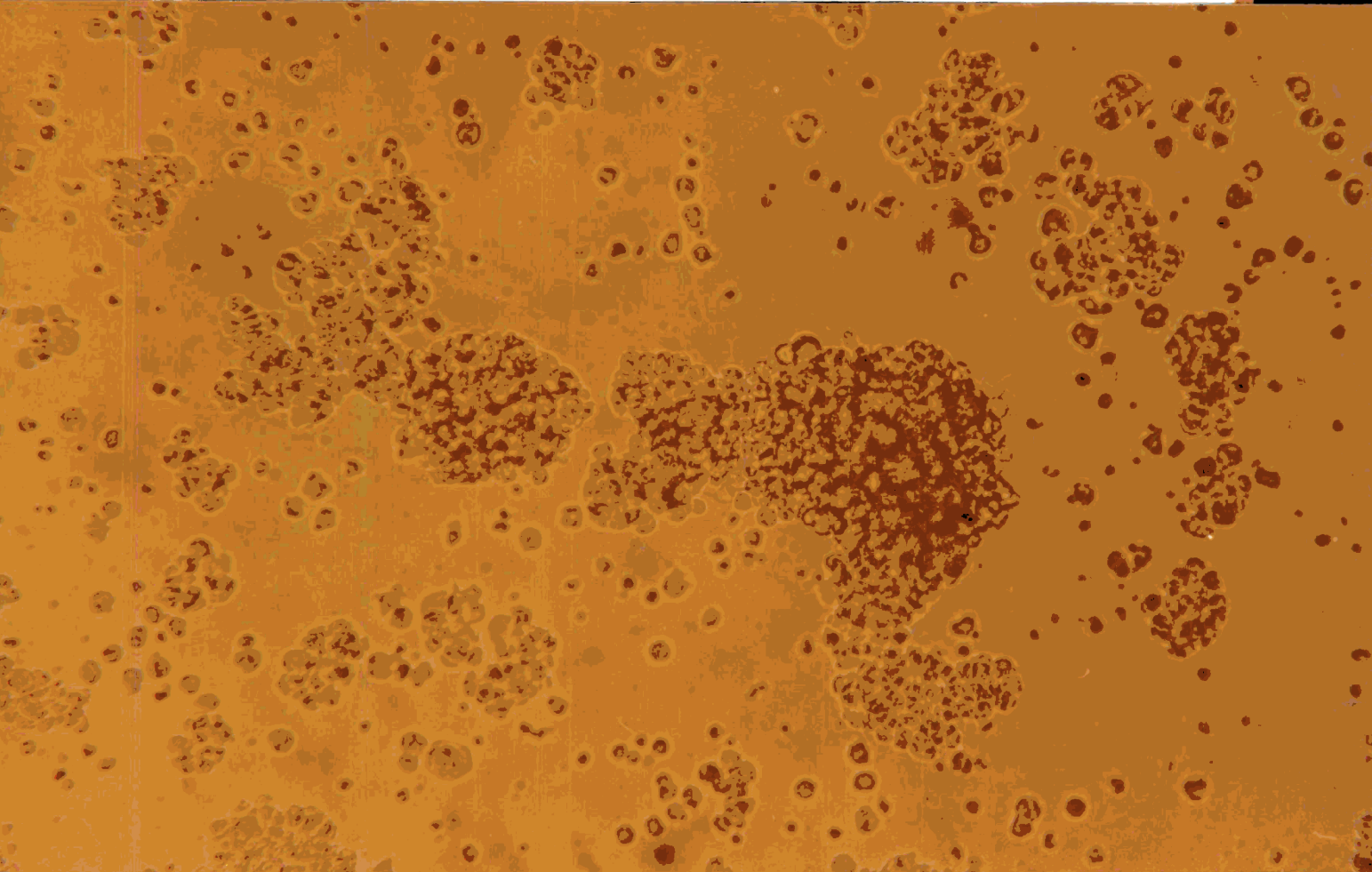
The above photograph shows healthy, circular H9 cells, cultured and cultivated in RPMI 1640 medium with 10% Fetal Calf Serum, 2mM L-Glutamine, 4.5g/L glucose, 1.5g/L Sodium bicarbonate, 1mM Sodium pyruvate, and 10mM HEPES buffer.

Magnification: 20X

A phase-contrast micrograph showing a dense population of cells. Many cells are rounded and some are beginning to cluster together, forming small syncytia. The background is a uniform light brown color.

The above photograph shows H9 cells infected with Human Immunodeficiency Virus. Infection has led to the formation of syncytia or aggregation of the H9 cells. This can be observed from day 2 to day 4 after infection.

Magnification: 20X

A phase-contrast micrograph showing a dense population of cells. Many cells are rounded and some are beginning to cluster together, forming small syncytia. The background is a uniform light brown color.

The above photograph shows H9 cells infected with Human Immunodeficiency Virus. Infection has led to the formation of syncytia or aggregation of the H9 cells. This can be observed day 4 onwards. Complete infection is observed along with a reduction in the number of uninfected cells.

Magnification: 20X

VIRAL LOAD AS A MARKER FOR DISEASE PROGRESSION

Table 12: Correlation of HIV Viral Load with Disease Progression

No.	HIV Stage	CD4 counts / μ l	Viral load per ml	
			p24 assay particles/ml	RT-PCR copies/ml
1	1	734	5×10^2	4.08×10^2
2	2	462	5	Undetectable
3	2	499	5×10^2	3.34×10^2
4	2	464	5×10^4	0.457×10^4
5	3	23	5×10^4	1.39×10^4
6	1	1482	5	Undetectable
7	2	871	5	Undetectable
8	2	501	5×10^2	0.169×10^2
9	2	876	5×10^2	2.25×10^2
10	3	123	5×10^4	2.17×10^4

1 - 5: children below the age of 5 years

6-10: children above the age of 5 years

The viral load as correlated with the corresponding CD4 counts is illustrated in Table 12. It was observed that as the HIV disease progressed from Stage 1 to Stage 3, the viral load increased to $>10^4$ copies/ml. Three samples had undetectable levels of viral copies, when RT-PCR was used.

Discussion

Immunity in humans is the result of the interplay between two immune systems, the innate immune system that initially encounters the antigens, and the adaptive immune system of T and B cells, which responds to the information provided by the innate system. A critical component of the immune response to intracellular pathogens is the innate immunity mediated by the Antigen Presenting Cells and Natural Killer Cells. These cells produce specific cytokines that favour the development of Th1 or Th2 responses.

The helper T cells involved in the immune response are designated Th1 and Th2. A good orchestration of both arms of the T helper cells leads to a desired effect against the pathogen. The Th phenotypes are characterized by the cytokines they produce. The Th1/Th2 phenotype reflects the outcome of naïve T cell activation. The dysregulation of these cytokines by HIV and its accompanying opportunistic infections determine the disease progression and development of AIDS.

An estimated 5 lakh children worldwide are currently infected with HIV (232). It has been observed that children vertically infected with HIV have shorter clinically latent period and more rapid progression to AIDS as compared to other individuals with HIV infection (233). According to the survey in and around

Mumbai, there are around 40,000 HIV infected children (1). The local data for studies carried out on the disease progression in such children is however conspicuous by its absence. Hence, one is compelled to compare the results with either the data from other countries and/ or the data available for HIV infected adults.

In the present study, 100 HIV infected children were investigated as the Study group for their clinical presentations. Of these, 13 children have lost both their (seropositive) parents, i.e. they were AIDS orphans. It is these orphans who have a deep impact on the socio-economic conditions of the society. Forty children were living with a single parent, who was also seropositive. It can be therefore presumed that sooner or later, these children too would become AIDS orphans. These orphan children become an extra burden on the society, as they have to be cared for by their aged grandparents, relatives or local orphanages. This became evident when these children were brought to the HIV clinics with serious complications requiring medical attention or even hospitalization.

In the present study, an array of clinical manifestations was observed in the HIV infected children, which brought them to the hospital. As seen in Table 3, of the 100 children, PCP was

seen as a major opportunistic infection (25%) followed by tuberculosis (19%). Otorrhea (28%) and dermatitis (19%) were the major clinical manifestations. Recurrent diarrhoea (7%) and candidiasis (9%) were also observed.

Pediatric HIV infection has a characteristic epidemiology, mode of transmission and clinical presentations. Majority of the cases are perinatally infected, the incubation is shorted and the clinical manifestations are related to opportunistic infections. Progression of HIV infection is generally faster in children than in adults. Most pediatric patients suffer from HIV-related manifestations at school age (234).

Although the reasons for fast progression are being investigated, it is suggested that age-related immunologic status of the child may contribute to the evolution of the disease. The knowledge about the immunologic events that occur in HIV infected children help to elucidate the mechanisms underlying the disease progression (234).

One of the most important markers for HIV clinical staging and disease progression is the CD4+T cell count. Of the 100 children in the Study group, 55 belonged to the age between 1 and 5 years, while 45 children were above 5 years of age. In both cases, maximum children, i.e. 31.18% in 1-5 years of age, and 40% in the age group above 5 years, were classified

in Stage 2. In a developing country like India, it is but natural that the children from the lower socio-economic strata are brought to the municipal hospitals after the appearance of symptoms requiring medical attention.

The HIV disease progression depends upon various host and viral factors. The most important among them is a complex pattern of alterations affecting the cytokines network, which is very distinctive of HIV.

The cytokine response is studied in terms of the levels of the mRNA, since the life of the circulating cytokines is short. Cytokines like IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ , etc. are important in the HIV disease progression.

IL-2 promotes the growth of T lymphocytes. It also participates in the activation, growth and tumoricidal activities of LAK and NK cells, augments the growth of B cells and enhances immunoglobulin production. Our observations have shown that IL-2 mRNA levels decrease with the HIV disease progression in both the age groups. As illustrated in Table 5, the IL-2 mRNA/18S RNA ratio in children aged 1-5 years dropped from a mean value of 0.688 (0.542-0.922) in HIV Stage 1, to 0.631 (0.489-0.729) in HIV Stage 2, while it dropped significantly ($p < 0.01$) to 0.474 (0.09-0.627) in HIV Stage 3. The Control Group in the same age showed a mean IL-2 value of 0.7121

(0.3-0.89). As illustrated in Table 7, in children above 5 years of age, the IL-2 mRNA/18S RNA ratio decreased from a mean value of 0.541 (0.369-1.003) in HIV Stage 1, to 0.342 (0.083-0.291) in HIV Stage 2, and to 0.107 (0.025-0.172) in HIV Stage 3. The Control Group in the same age showed a mean IL-2 value of 0.5529 (0.0-0.86). Clerici et al, have carried out a study with 31 patients. These are vertically infected pediatric cases. The PBMNCs have been measured for the IL-2 levels. IL-2 levels have been demonstrated to decrease both under stimulated and non-stimulated conditions (235). Another study by Meroni et al. has quantitated the IL-2 levels in 33 HIV seropositive patients and 11 serconverters (who have been reported positive in >3 but < 12 months) (260). They illustrate a drop in the IL-2 levels only after stimulation *in-vitro* with PHA. We have observed that in the age group 1-5 years, in HIV Stage 1 and Stage 2, the drop in IL-2 levels is not significant ($p>0.05$) when compared with levels observed in healthy children. In children above 5 years of age, the decrease in IL-2 levels is not significant ($p>0.05$) only in HIV Stage 1.

The role of IL-2 in the propagation of HIV in IL-2 stimulated PBMNCs probably includes stimulation of cytokine production and lymphocyte proliferation and activation (236). This has

been observed by Tanaka et al. while working with 48 HIV infected patients and 16 HIV negative healthy controls. Earlier studies have demonstrated a decrease in the number of IL-2 producing cells in moderate and advanced HIV infection (237). The deficient IL-2 production in rapid progressors may be explained by the decrease in the proportion of CD4+ T cells within the PBMNC population (238).

IFN- γ , produced by T lymphocytes, induces MHC Class I and II antigens on the macrophages, T and B cells. It also induces the cytolytic activity of LAK and CTL cells but inhibits the secretion of Th2 cytokines. The functional defects of Th, especially Th1 cells, can be observed early after infection. The prototype of Th1 shift to Th2 is the decrease in the IFN - γ production (238). In the present study, the levels of IFN - γ were observed to decrease significantly with disease progression in the Study Group. In children between 1-5 years of age the IFN - γ mRNA/18S RNA ratio increased from a normal value of 0.0219 (0.00-0.0056) to 0.757 (0.55-1.103) in HIV Stage 1. The ratio then dropped to 0.501 (0.412-0.616) in HIV Stage 2 and 0.434 (0.18-0.55) in HIV Stage 3, as depicted in Table 5. In children above 5 years of age, the IFN - γ mRNA/ 18S RNA ratio dropped from 0.758 (0.546-1.55) in HIV Stage 1 to 0.434 (0.239-0.574) in HIV Stage 3 and 0.265

(0.102-0.516) in HIV stage 3. The Control Group in the same age group showed the ratio of 0.0264 (0.00-0.005), as illustrated in Table 7. It can be observed that the Control values for IFN - γ are lower in both the Control Groups when compared to the Study Group. However, within the Study Group, a decline in the IFN - γ levels is seen as HIV disease progresses. It is suggested that diseases caused by bacterial and viral intracellular pathogens are associated with the immunologic unresponsiveness and failure to produce IFN - γ (239).

Infection with HIV is generally observed with one or the other opportunistic infections like tuberculosis. It has been reported in a study that *M. tuberculosis* induces a depression in the production of IFN - γ (239). The referred study has been carried out on 27 patients confirmed of tuberculosis, of which 12 were HIV positive and 15 were HIV negative. Eight healthy individuals served as the controls. It has been observed that INF- γ levels are the highest in the healthy controls, followed by HIV negative tuberculosis patients. The IFN- γ levels are the lowest for patients with a dual tuberculosis-HIV infection.

Depletion of IFN - γ levels has also been observed in the thymocytes of HIV infected individuals, due to the presence of IL-4 (240). This occurs since the absolute numbers of CD4+ T

cells decrease in the thymocytes. The present study shows the decline in the CD4 + T cell counts as the HIV disease progresses and hence the decline in the IFN - γ levels. Also, the capacity of CD4+ and CD8+ cells to secrete IFN - γ decreases. This dual loss accounts for the lowered IFN - γ levels. With the decrease in the levels of IFN - γ , the inhibition of viral entry is compromised and the productive infection of monocyte-derived macrophages occurs (241). This conclusion has been derived from the study involving 74 HIV infected and 30 healthy individuals who had been enrolled for the IFN- γ levels and their disease progression. Thus, a loss of potent antiviral, anti-proliferative and immunomodulatory molecule like IFN - γ appends the severity of the HIV disease.

Macrophages are an integral component of the immune system, functioning not only to digest pathogens, cell debris and foreign particles, but also to regulate the immune response via antigen presentation and cytokine production. HIV-1 binds to these macrophages and replicates within these phagocytes (242). HIV-infected macrophages are able to infect adjacent cells, and regulate immune responses via cytokines.

TNF- α is a cytokine produced by several cell types, primarily by macrophages and activated germinal centre B cells of HIV infected individuals. This may account for the decline in the

absolute CD4+T cell counts as TNF- α augments this decline by T cell dependent ADCC (243). *In-vitro* and *in-vivo* studies have shown that HIV infection can induce secretion and elevation of pro-inflammatory cytokines like TNF- α (244). It has been observed that TNF- α was constitutively expressed in 62 HIV infected individuals. TNF- α is known to act upstream to many pro-inflammatory molecules and contribute to tissue damage.

In the present study, we observed that the PBMNCs of the HIV seropositive children showed significantly higher levels of TNF- α mRNA ($p<0.01$) as the HIV disease progressed. As illustrated in Table 5, the TNF- α mRNA/ 18S RNA ratio increased from a normal value of 0.0452 (0.00-0.071) to 0.157 (0.08-0.25) in HIV Stage 1. The ratio increased significantly ($p<0.01$) to 0.520 (0.258-0.69) in Stage 2 and 0.991 (0.72-1.25) in Stage 3. In children above 5 years of age, the TNF- α mRNA/ 18S RNA ratio increased from a normal value of 0.0494 (0.00-0.086) to 0.354 (0.12-0.433) in HIV Stage 1. The ratio increased significantly ($p<0.01$) to 0.768 (0.65-0.86) in Stage 2 and 1.166 (0.902-1.5) in Stage 3, as depicted in Table 7. The TNF- α mRNA levels increase due to upregulation by HIV. Also, the presence of opportunistic infections like tuberculosis stimulate the monocytes to upregulate the production of TNF- α

(244). Bajaj et al. have carried out a study in 20 patients with HIV, of which 10 suffered from candidiasis. Ten healthy individuals formed the healthy control. The TNF- α levels increased significantly in HIV infected patients, while the increase was enhanced in HIV patients with candidiasis. They have observed that the Th1/ Th2 cytokine imbalance and the reduction in CD4 cell counts were more pronounced in HIV infected patients with candidiasis. They suggest that Candidiasis may have a synergistic immunosuppressive effect in HIV infected patients (245).

TNF- α , by causing CD4+ T cells depletion, anorexia of chronic disease and apoptosis of T cells, and by induction of acute phase proteins, adds to the increased morbidity in HIV infected children.

In 100 HIV seropositive children enrolled in the present study, maximum mortality was observed in HIV Stage 3, where the TNF- α levels were 22 times the normal value in children between 1-5 years of age, and were 23 times the normal value in children above 5 years of age. This increase in the levels of TNF- α may be due to the presence of opportunistic infections like tuberculosis, which by itself leads to secretion of cachectin.

Cellular immunity is considered to be responsible for the eradication of intracellular pathogens like tuberculosis, HIV, etc., with the help of $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and IL-4. In animal models, the balance between Th1 type cytokines, especially $\text{IFN-}\gamma$ and Th2 type cytokines, primarily IL-4 seems crucial for cellular immunity. HIV seronegative individuals suffering from tuberculosis show increased IL-4 levels in CD4^+ as well as CD8^+ cells. However, this immunity also leads to tissue damage. It has been postulated that Th2 type response with increased production of IL-4 in patients with tuberculosis may antagonize host defence and lead to tissue necrosis. (246).

IL-4, a pleiotropic cytokine produced by T cells, mast cells activated human basophils, regulates the wide range of B cell responses including proliferation, expression of MHC II antigen, and expression of IgG and IgE molecules. However, it inhibits the activated macrophages to kill various intracellular and extracellular pathogens. The HIV seropositive cohort under the present study showed elevated mRNA levels of IL-4 as compared to the Control Group. In children aged 1-5 years, the IL-4 mRNA/18S RNA ratio increased from a Control value of 0.023 (0.00-0.056) to 0.059 (0.01-0.15) in HIV Stage 1, as shown in Table 6. In HIV Stage 2, the ratio was 0.223 (0.09-0.39) while in Stage 3, it was 0.477 (0.21-0.80). As shown in

Table 8, in children above 5 years, the IL-4 mRNA/18S RNA ratio increased from a Control value of 0.0229 (0.00-0.035) to 0.256 (0.02-0.333) in HIV Stage 1. In HIV Stage 2, the ratio was 0.400 (0.282-0.492) while in Stage 3, it was 0.550 (0.286-0.884). PBMNCs from HIV infected patients with reduced CD4 counts are expected to produce lower level of serum IL-4 than PBMNCs of asymptomatic HIV infection, HIV patients with normal or slightly reduced CD4 counts (247, 248).

IL-4 mRNA levels increase with disease progression. The decline in circulating IL-4 levels may be due to the ability of HIV *nef* protein to inhibit the protein synthesis by interfering with the translational process (248).

Clerici, et al, have reported an increase in the IL-4 levels with concomitant decrease in the recall antigen stimulated IL-2 response. They suggest that the Th functions assessed by IL-4 production replace the dominant Th1 function of antigen stimulated IL-2 production in disease progression to AIDS (249). HIV-1 productively infects mononuclear phagocytes derived from blood. IL-4 has been shown to stimulate HIV replication in monocytes and macrophages (251).

IL-4 stimulates HIV replication in early stages of infection and may also facilitate virus transmission, thus making it a vital molecule in HIV disease progression.

IL-6 is released in HIV infected individuals by monocytes and activated B cells. It induces the differentiation of B cells and cytotoxic T cells. It also induces the production and action of acute phase proteins (144).

As depicted in Table 6, the IL-6 mRNA/18S RNA ratio increased from a Control value of 0.320 (0.00-0.54) to 0.355 (0.28-0.42) in HIV Stage 1. There was a significant increase ($p<0.01$) in the ratio to 0.540 (0.278-0.72) in HIV Stage 2 and 0.790 (0.51-1.30) in HIV Stage 3. As shown in Table 8, the IL-6 mRNA/18S RNA ratio increased from a Control value of 0.3621 (0.00-0.69) to 0.564 (0.37-0.643) in HIV Stage 1, 0.735 (0.62-0.83) in HIV Stage 2 and 0.976 (0.712-1.31) in HIV Stage 3. Thus, in our HIV seropositive cohort, we observed a rise in the levels of IL-6 mRNA with decline in CD4 counts, as documented elsewhere (144).

IL-6 has been reported to affect the function of B cells, hematopoietic progenitors and plasma cells. Upregulation of IL-6 may be involved in the immune abnormalities of HIV infection (144). Honda et al., have demonstrated a feedback loop involving IL-6 production by HIV infected cells and resulting in enhanced HIV replication. Their study groups comprised of 55 HIV seropositive blood or serum specimens donated by HIV infected individuals, of which 18 had

asymptomatic infection, 16 has AIDS related complex (ARC) and 21 had AIDS. They suggest that increased IL-6 levels in serum may be a general phenomenon in diseases involving immune pathogenesis (144).

In another study, Koka et al, have demonstrated a fifty-fold increase in IL-6 levels in HIV infected thymocytes using Severe Compromised Immunodeficient (SCID) mice (133).

It has been observed that 50% of HIV infected patients have elevated numbers of IL-6 mRNA expressing blood mononuclear cells. Higher levels of IL-6 mRNA expressing cells have been found in the group of patients with CD4 counts < 400 cells/ μ l. This underlines the relationship between high IL-6 levels and HIV disease progression and development of AIDS. IL-6 thus appends the severity of HIV disease (180).

However, there is no agreement as to whether there is any association between the disease stage and the levels of circulating IL-6 (133, 144).

Interleukin 10, formerly called Cytokine Synthesis Inhibitory Factor is produced by T and B cells as well as monocytes. It inhibits cytokines (particularly Th1) production, T cell proliferation and monocyte functions.

IL-10 mRNA levels of our Study Group showed increment as the HIV disease progressed. As shown in Table 6, in children

between 1-5 years of age, the IL-10 mRNA/18S RNA ratio increased from a Control value of 0.0215 (0.00-0.056) to 0.116 (0.067-0.207) in HIV Stage 1, 0.253 (0.04-0.43) in HIV Stage 2 and 0.397 (0.13-0.58) in HIV Stage 3. As shown in Table 8, in children above 5 years of age, the IL-10 mRNA/18S RNA ratio increased from a Control value of 0.0221 (0.00-0.039) to 0.276 (0.08-0.35) in HIV Stage 1, 0.330 (0.212-0.422) in HIV Stage 2 and 0.466 (0.202-0.8) in HIV Stage 3. Study by Chehimi et al shows significant increase in the IL-10 levels in PBMNCs of patients with advanced HIV disease, although they were on Anti-retroviral therapy. Patients with CD4+ T cell counts < 200 cells/ μ l secreted thrice the IL-10 as compared to patients with CD4 counts > 500 cells/ μ l (250).

IL-10 is a central mediator of the depressed immune response in diseases from intracellular pathogens, as pathogens themselves elicit IL-10 production by mononuclear lymphocytes (250). In our study, we observed a ten-fold increase in the IL-10 levels. It may be due to the presence of various opportunistic infections of intracellular pathogens. This probably contributes to a higher inhibition of Th1 responses and faster progression to AIDS.

Clerici et al have demonstrated three-fold rise in the IL-10 levels in the PBMNCs of patients with progressive HIV disease

when compared with Long Term Non Progressors (LTNPs) and healthy controls. This increase in the IL-10 levels enhances the susceptibility of CD4⁺ T cells to Activation Induced Cell Death (AICD), resulting in low CD4⁺ counts, as HIV disease progresses (252). The increase in IL-10 levels accompanied by decline in CD4 counts has been studied in a murine model (235).

IL-10 has an autoregulatory effect as indicated by its ability to decrease IL-10 mRNA synthesis and reduce MHC Class II expression in Lipopolysaccharide activated monocytes (235). IL-10 is known to downregulate macrophage function. Thus on the other hand, IL-10 functions to downregulate HIV replication since macrophages are primary targets for HIV.

A complex array of Th1 and Th2 cytokine responses has been observed in HIV infection. On one hand, the increase in the IL-4 / IFN- γ ratio is responsible for the lowering of CD4 counts (253). On the other hand, we have IL-10 that down-regulates the Th1 cytokine response (250). The Th2 functions indicated by IL-4 production replace the normally dominant Th1 function of antigen stimulated IL-2 production during HIV progression (5). There is also evidence that cytokine dysregulation occurs with AIDS progression, and the overproduction of Th2 cytokines increases the susceptibility to activation induced cell

death whereas Th1 cytokines are protective. While one group of workers has proposed a Th1-Th2 shift in cytokines (5), another group lays the onus on the decrease in the levels of IL-2 producing cells in the CD4/CD8 population in moderate and advanced stages of HIV disease (236).

Similar findings have been observed in a previous study carried out at the Department of Virology, Haffkine Institute. The study involved 210 clinically and radiologically confirmed adult tuberculosis patients. Of these, 25 patients were dually suffering from pulmonary tuberculosis and HIV infection, while 25 HIV seronegative pulmonary tuberculosis patients were also enrolled. The decrement in the Th1 type of response and a concomitant increase in the Th2 cytokine levels were observed in dually infected individuals. The TNF- α levels were seen to rise 10-fold in tuberculosis patients with HIV. In HIV infected patients without tuberculosis, the rise was 5-7 fold (266).

With the above dysregulation in the cytokine responses, varied opportunistic infections are observed in HIV infected individuals. In our HIV seropositive cohort, opportunistic infections, ranging from dermatitis and diarrhoea to tuberculosis and PCP were seen. These develop primarily due to the HIV associated immunodeficiency, reactivation of latent

infections, anemia, as well as poor hygiene, absence of a good nutritional support and lack of adequate medication (4). This is especially true for the lower socio-economic classes of the developing countries like India.

The availability of anti-retroviral therapy (ART) and improved treatment for opportunistic infections have changed the pattern of disease and survival among HIV infected children in most developed countries. However, such therapies have not yet become widely available in developing countries. (254).

Clinical syndrome resulting from infection by HIV type 1 which preferentially infects and destroys CD4 helper/ inducer T cells leads to a severe immunodeficiency involving both cell mediated and humoral immunity. This makes the infected individual highly susceptible to life-threatening bacterial and opportunistic infections and to certain tumors. (209)

A bimodal progression occurs in Indian children. A significant proportion of infected children have severe disease and early death, while children surviving beyond 5 years of age tend to have moderate signs and symptoms, and have a longer time of survival. (255)

The common clinical manifestations of HIV infection such as hepatomegaly, generalized lymphadenopathy and splenomegaly, skin infection, recurrent diarrhoea and failure to

thrive are observed in studies of HIV infected children in both western and tropical countries (255).

The extensive morbidity and mortality seen in HIV infection is due to the respiratory infections. In a study carried out by Madhivanan et al, 34.5% of the total HIV infected children were observed to suffer from pulmonary tuberculosis (256). In the present study, 19% of our Study Cohort of HIV infected children were observed to suffer from tuberculosis as depicted in Table 3. In another study, 14% of HIV infected children were found to suffer from pulmonary tuberculosis (255). This may be because the children are in close contact with tuberculosis infected adults (seropositive parents), and are also at a high risk of nosocomial transmissions. Tuberculosis also forms a major cause of death in HIV infected children.

Other than tuberculosis, PCP is a major respiratory infection observed in HIV. *Pneumocystis carinii* causes an acute and life-threatening pneumonitis in children. Its clinical course is fatal if left untreated. It was observed in the present study that a total of 25% of HIV infected children were suffering from PCP. In a previous study, 8% children were observed to have PCP infection (255), while in another study, only 2.7% of children presented with PCP (256). PCP cases are however, based on presumptive diagnosis. The variation in the

observations of the clinical manifestations may be due to the absence of clinical staging of HIV using CD4 cell counts. Also, the diagnostic procedure employed by different laboratories play an important role in the reporting of the PCP.

As illustrated in Table 3, 9% percent of our study cohort was diagnosed of candidiasis. In a previous study, 26% of HIV infected children were reported for candidiasis (255). HIV infected infants are commonly diagnosed with oral thrush. The infections are extensive and less responsive to therapy. This has been observed in a study where candidiasis persisted for a period of 4 years (256). Severe oral infection is the presenting manifestation of HIV infection. This leads to esophageal candidiasis as the disease progresses.

Chronic diarrhoea resulting in loss of weight is an important prediction of mortality. This opportunistic infection starts from Stage 2 of HIV infection (255, 256). Similar trend has been observed in our Study group as shown in Table 9. In an earlier study, around 30% of HIV infected children has recurrent diarrhoea (255). Also, in a study, where the children were monitored for a period of 4 years, the cases of chronic diarrhoea increased from 5.4% to 18.9% at the end of the monitoring period (256). In the present study, we observed 5% of diarrhoea cases. Although the frequent cause of diarrhoea

is an infectious agent (cryptosporidia, salmonella, shigella, clostridium, etc.), other causes include side effects of medication, malignancies and HIV enteropathy (209).

In HIV-1 infected adults, CD4+ T cell counts and viral load are two key laboratory markers currently being used as predictive markers of clinical outcome of the infection and to make decisions about changing the antiretroviral therapy.

However, CD4+ T cell counts fall naturally with age in uninfected children and even after adjusting for age, CD4+ T cell counts in HIV-1 infected children appear to be much less for predicting the disease progression than in adults. On the other hand, several studies have reported on the predictive role of viral load in infants.

Although CD8+ lymphocytes are believed to be important in the control of HIV-1 infection, only a few studies have examined the prognostic significance of the number of peripheral CD8+ cells in adults, and none of them has reported data in children.

Resino et al., have carried out such a study in 33 HIV-1 infected infants younger than 12 months of age (261). Their results indicate that biologic phenotype of viral isolates, CD8+ T lymphocyte percentages in the peripheral blood as well as viral load and CD4+ T lymphocyte percentages could predict

rapid progression to advanced HIV-1 disease in HIV-1 infected infants.

In a study carried out by Dyer et al., in 7 samples, where unadjusted HIV RNA concentrated was detectable at less than 1,000 copies/ml with at least one assay, all but one had detectable levels by both methods (262).

A growing number of adult and pediatric studies have demonstrated the Human Immunodeficiency Virus type 1 RNA levels have importance in determining the risk of both disease progression and the transmission from the mother to the infant.

The observation of increasing viral loads with the concomitant disease progression is in agreement with these studies. As shown in Table 12, we observed that the viral load increased with the HIV disease progression. This was accompanied by a decrease in the CD4 counts.

In children between 1-5 years, $\approx 10^2$ viral RNA copies per ml were detected in HIV Stage 1. Stage 2 presented with a range of undetectable to $\approx 10^4$ viral RNA copies per ml. A viral load of $\approx 10^4$ viral RNA copies per ml was observed in HIV Stage 3. Similarly, in children above 5 years of age, viral RNA copies were not detected in HIV Stage 1. In HIV Stage 2, a range of undetectable to $\approx 10^2$ viral RNA copies per ml was observed.

Stage 3 presented with a viral load of $\approx 10^4$ viral RNA copies per ml.

The detection of viral particles appeared to be better in the p24 antigen assay that involves the culturing of the virus onto a susceptible cell culture (H9 cells), than in RT-PCR method.

The levels of viral particles detected in plasma are typically 30% to 80% higher than those detected in serum. Thus, it appears that viral load can reasonably be detected in both plasma and serum, but individual studies should consistently use either serum or plasma for RNA detection, with a preference for plasma (263).

It is observed that by weeks 20 to 24, the difference in the RNA levels of the samples stored at -20°C versus the RNA levels of the samples stored at -80°C became statistically significant, with specimens stored at -80°C having around 30-80% more RNA than those stored at -20°C .

By end of 2003, nearly 5.8 lakh children have succumbed to AIDS, the maximum mortality being in Africa, while in the year 2003, another 5 lakh deaths due to pediatric HIV have been reported (254). A high mortality of 23% was observed during the monitoring period of 18 months among these seropositive children included in the present study. This may be due to the reduction in levels of IL-2 and IFN- γ along with the increase in

IL-4, IL-10, and TNF- α levels, making these children more vulnerable for dysregulation of their immune system, leading to rapid disease progression and mortality.

The projections for the predictive mortality in children due to HIV/AIDS are calculated using an "EPIMODEL" designed by Chin and Luanga, epidemiologists with the World Health Organization, in 1991. (257)

The total population of India is 100 million, of which women comprise at least 45%. Thus the total women population would be 45 million. Of these, around 36% are in the reproductive age group of 15-49 years. With the National HIV seroprevalence rate of 1%, the number of HIV infected women in the reproductive age group would be 1.62 lakhs annually. The fertility rate is 3.3 (i.e. ~3 children), thus the number of children born to seropositive mothers would be 4.86 lakhs. The transmission rate of HIV from an infected mother to her child ranges from 14% to 40% (30% for India). Thus 68 thousand to 1.94 lakh children would be born HIV infected at birth (258, 259).

With the median progression to death of 10 years, by 2015, India would account for 1.46 lakh pediatric deaths by HIV/AIDS. It is to be noted that till 2003, 43 lakh children have died worldwide due to HIV/AIDS, while in 2003, 5 lakh

pediatric deaths have occurred globally, thus making HIV disease and its progression in children, a great impending threat on our society. (258, 259)

Studies such as the present aim to throw light on the cytokine spectra, trends of opportunistic infections, mortality, etc. in HIV/AIDS pediatric cases. These will help in lessening the morbidity and mortality due to HIV. This data can be utilized for setting up therapeutic interventions and thus help the medical machinery to tackle the growing problem of HIV/AIDS in children.

Summary

The present study was envisaged to determine the cytokine profile of HIV infected children in Mumbai. Hundred seropositive children vertically infected with HIV were included in the present study. The following can be inferred from our observations:

1. The children were being brought to the municipal hospital only when non-responding clinical manifestations were observed. This was when the children developed Stage 2 and Stage 3 of HIV infection.
2. Multiple opportunistic infections and clinical manifestations were observed in HIV infected children. While PCP and otorrhea presented even in Stage 1 of HIV infection, tuberculosis, dermatitis, candidiasis and diarrhea presented generally from Stage 2. Stage 3 showed maximum mortality (19%), usually due to respiratory infections.
3. The mRNA profiles of the Th1 and Th2 cytokines were determined in the present study. Decrement was observed in IL-2 and IFN γ as HIV disease progressed, while cytokines like IL-4, IL-6, IL-10 and TNF- α increased with progression. The disease progression was measured using CD4 counts as a marker.

4. Viral load was estimated in 10 representative samples using RT-PCR method and by tissue culture using H9 cells. A higher viral load (undetectable in Stage 1 to $\approx 10^4$ in Stage 3) was observed as the HIV disease progressed. A significant correlation was observed between the two methods of detection of viral load. The infectivity assay was able to detect as low as 5 viral particles per ml that remained undetectable with the RT-PCR assay.
5. A high mortality of 23% was observed in our Study Group of 100. The mortality was attributed to the opportunistic infections, seen with the progression of HIV/AIDS.
6. As HIV disease progressed a fall in the CD4 counts was observed, along with the increase in the viral loads. It was observed that the TNF- α levels increased significantly as the HIV disease progressed from Stage 1 to Stage 3. Stage 3 with the CD4 counts <499 in children between 1-5 years of age had the highest TNF- α mRNA/18S RNA ratio of 0.991 (22 fold increase w.r.t. healthy controls, and approximately 6 times increase as compared to asymptomatic HIV cases). Stage 3 with the CD4 counts <200 in children above 5 years of age had the highest TNF- α mRNA/18S RNA ratio of 1.166 (23 fold increase w.r.t. healthy controls, and approximately 3 times increase

as compared to asymptomatic HIV cases). Stage 3 also had the highest mortality (19%). TNF- α levels can thus be used as a marker for disease progression in HIV and an index of mortality in HIV/AIDS.

Our cohort thus presented with faster HIV disease progression, variety of opportunistic infections resulting in a greater mortality. Cytokines play a major role in the mortality and morbidity in HIV disease progression, and thus play an important role as prognostic markers of morbidity and mortality in HIV.

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Appendices

APPENDIX 1

1. ZIEHL NEELSEN CARBOL FUCHSIN (ZNCF) STAIN (115):

Basic fuchsin	1 gm
Phenol	5 gm
Absolute Alcohol	10 ml
Distilled water	100 ml

Dissolve basic fuchsin in absolute alcohol and then add the solution of phenol in water. Filter the stain before use.

2. ACID ALCOHOL (DECOLOURIZER)

Sodium chloride	20 gm
Conc. hydrochloric acid	20 ml
Distilled water	500 ml
Absolute alcohol	1500 ml

Dissolve sodium chloride in distilled water and add concentrated hydrochloric acid, mix thoroughly and then add absolute alcohol

3. COUNTER STAIN

Methylene Blue	1 gm
Distilled water	100 ml

Dissolve the stain completely (keep for 24 hours) and filter before use.

4. 4% ANILINE SOLUTION (120)

Aniline	4 ml
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Absolute alcohol	96 ml
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Store at 5°C in amber coloured bottle. Discard if the solution turns yellow.

5. 10% CYANOGEN BROMIDE SOLUTION (120)

Cyanogen Bromide	5.0 gm
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Distilled water	50 ml
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Cyanogen bromide is a tear gas and therefore should be prepared in a well-ventilated safety cabinet using moistened mask to cover mouth and nose. Niacin test tubes are discarded into disinfectant made alkaline using NaOH to prevent the formation of hydrocyanic acid.

APPENDIX 2

1. SABOURAUD'S DEXTROSE AGAR WITH CHLORAMPHENICOL (126)

Dextrose	40 gm
Peptone	10 gm
Agar	20 gm
Distilled water	1000 ml

Autoclave at 15 psi at 120°C for 10 minutes. Allow cooling to 50°C and add 40 mg of Chloramphenicol powder.

2. CARBOHYDRATE FERMENTATION MEDIUM

a. Yeast Fermentation Medium

Distilled water	1000 ml
Peptone	10 gm
NaCl	5 gm
Beef extract	3 gm
1 N NaOH	1 ml

b. Indicator

Dissolve 0.04 gm bromocresol purple in 100 ml distilled water. Add small volume of 1 N HCl till neutral pH is reached.

Add 100 ml of the indicator to 1 litre of the Yeast fermentation broth.

c. Stock Carbohydrate solutions

Stock carbohydrate solutions are prepared by adding 20 gm of the respective carbohydrate (glucose, sucrose, maltose and lactose) to 100 ml of distilled water. The sugars are dissolved by placing the tubes in a water bath at 56°C for few minutes, and then autoclaved.

d. Preparation of the Sugar-Tubes

18 x 150 mm tubes containing 9 ml of broth indicator and an inverted Durham's tube are sterilized by autoclaving. Five hundred microlitres of the stock carbohydrate are added to the broth indicator tube.

3. SUGAR ASSIMILATION MEDIUM

a. Basal Medium

Bromocresol purple	0.2 ml
0.1 N NaOH	1.0 ml
Agar	2.0 gm
Distilled water	90 ml

Dissolve the medium by heating.

b. Stock Carbohydrate Solution

Carbohydrate (Glucose, sucrose, maltose, lactose)	1.0 gm
Yeast Nitrogen Base	0.67 gm
Distilled water	19 ml

Mix and gently heat to dissolve. Autoclave at 10 psi for 10 minutes at 121°C.

Add the stock carbohydrate solution to the molten agar base. Mix well and adjust the pH to 7.0. Dispense 5 ml in sterile glass tubes. Allow solidifying in slanted position and store at 4°C.

APPENDIX 3

1. KINYOUN CARBOL FUCHSIN

Basic fuchsin	4 gm
Ethyl alcohol	20 ml
Phenol crystals	8 gm
Distilled water	100 ml

Dissolve basic fuchsin in alcohol and add distilled water slowly while stirring. Melt phenol crystals by gentle heating and add to the fuchsin solution.

2. DECOLOURIZER

Ethanol (95%)	99 ml
Conc. HCl	1 ml

3. METHYLENE BLUE STAIN

Methylene blue	0.3 gm
Distilled water	100 ml

Mix with constant shaking

4. SHEATHER'S SUGAR SOLUTION

Sucrose	500 gm
Water	320 ml
Phenol	65 gm

Mix sucrose in water and boil till the solution becomes clear. Carefully add the phenol, stir and cool to room temperature before use.

APPENDIX 4

1. STOCK 100× GIEMSA BUFFER (0.67 M)

Na ₂ HPO ₄	59.24 g
NaH ₂ PO ₄ H ₂ O	36.38 g
Deionized water	1000.00 ml

Autoclave or filter-sterilize (0.2 µm pore). Sterile buffer is stable at room temperature for one year.

2. WORKING GIEMSA BUFFER (0.0067M) PH 7.2

Stock Giemsa Buffer	10.0 ml
Deionized water	990.0 ml

Adjust the pH to 7.2. The buffer is stable for 1 month.

3. 5 % TRITON X-100

Deionized water (warmed to 56°C)	95.0 ml
Triton X-100	5.0 ml

Prewarm the deionized water and slowly add the Triton X-100, swirling to mix.

4. WORKING GIEMSA STAIN (2.5%):

Working Giemsa buffer	39 ml
Giemsa Stain (commercially available)	1 ml
5% Triton X-100	2 drops

APPENDIX 5

1. TRIS EDTA (TE) BUFFER, pH 7.6

Tris HCl 10mM

EDTA 1 mM

Adjust the pH to 7.6

2. TRIS BORATE EDTA (TBE) BUFFER, pH 8.0

Tris HCl 89mM

Boric Acid 89mM

EDTA 2mM

Adjust the pH to 8.0

3. DYE GLYCEROL

EDTA 50mM

Glycerol 30%

Bromophenol blue 0.25%

LIST OF ABBREVIATIONS USED

ADCC: Antibody Dependent Cell Cytotoxicity
AIDS: Acquired Immunodeficiency Syndrome
APCs: Antigen Presenting Cells
ARC: AIDS Related Complex
ASCS: Acute Seroconversion Syndrome
ASK-1: Apoptosis Signal Regulating Kinase 1
BSF: B Cell Stimulatory Factor
CAF: Cell Antiviral Factor
CDC: Center For Disease Control
cDNA: Copy/ Complementary DNA
CMI: Cell Mediated Immunity
CMV: Cytomegalovirus
CNAR: Cell Non-Cytotoxic Antiviral Response
CNS: Central Nervous System
CSIF: Cytokine Synthesis Inhibitory Factor
CTL-p: Cytotoxic T Lymphocyte Precursor
CTLs: Cytotoxic T Lymphocytes
DTH: Delayed Type Hypersensitivity
EGF: Endothelial Growth Factor
ELISA: Enzyme Linked Immunosorbent Assay
FasL: FAS Ligand
GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor
GVHD: Graft Versus Host Disease
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
HLA: Human Leucocyte Antigen
HTLV: Human T Cell Lymphotropic Virus
HWS: HIV Wasting Syndrome
IFN- γ : Interferon Gamma
IgA: Immunoglobulin A
IgE: Immunoglobulin E
IgM: Immunoglobulin M
IL-10: Interleukin 10
IL-2: Interleukin 2
IL-4: Interleukin 4
IL-6: Interleukin 6
JAK: Janus Kinases
KHF: Killer Cell Helper Factor
LA: Lymphadenopathy
LAK CELLS: Lymphokine Associated Killer Cells
LFA-1: Lymphocyte Function Associated Antigen 1
LT: Lymphotoxin

LTNPs: Long Term Non-Progressors
 MAC: *Mycobacterium Avium* Complex
 MAPK: Mitogen Activated Protein Kinase
 MHC-I: Major Histocompatibility Class I
 MHC-II: Major Histocompatibility Class II
 MIP-1: Macrophage Inflammatory Protein 1
 mRNA: Messenger RNA
 NACO: National AIDS Control Organization
 NASBA: Nucleic Acid Sequence Based Amplification
 NF- κ B: Nuclear Factor Kappa B
 NK CELLS: Natural Killer Cells
 NSI: Non-Syncytium Inducing
 OIs: Opportunistic Infections
 PBMCs: Peripheral Blood Mononuclear Cells
 PCD: Programmed Cell Death
 PCP: *Pneumocystis carinii* Pneumoniae
 PCR: Polymerase Chain Reaction
 PHA: Phytohemagglutinin
 PMA: Phorbol-12-Myristate-13-Acetate
 PML: Progressive Multifocal Leucoencephalopathy
 RANTES: Regulated Upon Activation, Normal T Cell Expressed And Secreted
 RNase H: Ribonuclease H
 RNP: Ribonucleoprotein
 RRE: Rev Response Element
 s-CD4: Soluble CD4
 SDF1-3'A: Stromal Derivative Factor 1 Beta Gene Transcript
 SI: Syncytium Inducing
 STAT: Signal Transduced And Activated Upon Transcription
 TAR: Transactivation Response Element
 TCF: T Cell Replacing Factor
 TCGF: T Cell Growth Factor
 TGF-b: Tissue Growth Factor Beta
 Th: Helper T Cells
 Tm: Memory T Cells
 TMF: Thymocyte Mitogenesis Factor
 TNF- α : TNF alpha
 TNF- β : TNF beta
 TNF-R: TNF receptor
 TSF: Thymocyte Stimulation Factor

Synopsis

SYNOPSIS TO THE THESIS TO BE SUBMITTED
TO THE
UNIVERSITY OF MUMBAI
FOR THE DEGRÉE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

TITLE OF THE THESIS : STUDY OF mRNA PROFILE FOR
CYTOKINES (TNF- α , IFN - γ , IL-2, IL-4,
IL-6, IL-10) IN HIV INFECTED
CHILDREN

NAME OF THE CANDIDATE : SWETA T. KOTHARI

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DATE OF REGISTRATION : 16/06/2001

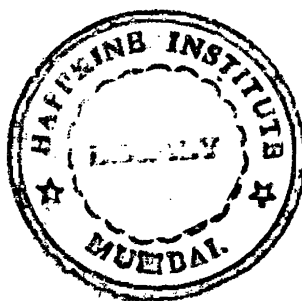
REGISTRATION NO : 50

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STUDY OF mRNA PROFILE FOR CYTOKINES (TNF- α , IFN- γ , IL-2, IL-4, IL-6, IL-10) IN HIV INFECTED CHILDREN

INTRODUCTION:

AIDS epidemic has claimed more than 3 million lives in 2002, and an estimated 5 million people have acquired the Human Immunodeficiency Virus (HIV) in the same year, bringing the total number of people globally living with the virus to 42 million. HIV prevalence among women attending antenatal clinics in the states like Andhra Pradesh, Karnataka, Maharashtra, Manipur, Nagaland and Tamil Nadu is around 2%, higher than the national average. Women contribute 25% of the total HIV infections while contributing 21% of AIDS cases. Pediatric AIDS contributes to nearly 10% of total AIDS cases (1).

Around 36% of the women of the reproductive age group are infected with HIV, and with the vertical transmission rate of 16-40%, the approximate number of children living in Mumbai alone with HIV would be around 14,000 to 40,000. With the infant mortality rate of 3.3 per 1000 live births, there would be more children living morbidly after HIV infection. The statistical data for pediatric HIV infections would however, not be accurate, since the reporting of the positive cases is very low (1). Unfortunately, very few databases are available for the Indian cohort for the pediatric AIDS. The Indian data is conspicuous by its absence on the WHO / UNAIDS listings.

In most of pediatric HIV infections, HIV transmission is acquired vertically from their infected mothers. The risk of perinatal transmission is significantly increased if the mother has advanced HIV disease,

increased levels of HIV in her bloodstream, or fewer numbers of the CD4+ T cells (2).

According to the CDC data, HIV-infected children can be classified into a) Rapid Progressors (20%), who develop serious opportunistic infections in the first year of life progressing very rapidly to AIDS defining conditions. With a rapid loss of CD4+ T cells within the first two years of their lives, death follows by the age of 4 years. b) Intermediate Progressors (60-75%) who develop severe immunosuppression by 7 -8 years of age, with a gradual loss in CD4+ T cells. c) Long term Survivors (5-10%) are defined to have a slower rate of disease progression with minimal to no symptoms of HIV disease, normal to minimally decreased CD4 cells count by 8 years of age and no development of serious symptoms of AIDS until adolescence (3).

Normal range CD4+ T cell counts in children varies with age. It is greater than 1500 cells/ μ l for children below 1 year, between 1000-1500 cells/ μ l for the age group 1-5 years and between 500-1000 cells/ μ l for children above 5 years (3).

The wide variations observed in the rate of disease progression in HIV-infected children are due to their viral load and CD4+ T cell counts, their cytokine array as well as the opportunistic infections they suffer from. Nutritional status, personal hygiene and environmental cleanliness of these children also contribute to the disease progression.

Many children with HIV infection do not gain weight, are slow to reach important milestones in motor skills and mental development viz

crawling, walking and speaking. As the disease progresses, many children develop neurologic problems like difficulty in walking, poor school performance, seizures, mental retardation and cerebral palsy.

Children with HIV develop life-threatening opportunistic infections (OIs), but the incidence of various OIs differs in adults and children. For example, toxoplasmosis is seen less frequently in HIV-infected children while serious bacterial infections occur more commonly in children as compared to adults. Also, as children with HIV become sicker, they may suffer from chronic diarrhoea due to opportunistic entero-pathogens (4).

The CDC HIV and AIDS Surveillance data for U.S. reported *Pneumocystis carinii* pneumonia (PCP) as the leading cause of death in HIV-infected children with AIDS. PCP, as well as cytomegalovirus (CMV) disease, usually are new infections in children, whereas in adults these diseases result from the reactivation of latent infections (4).

Children with HIV frequently suffer the usual childhood bacterial infections, which can cause seizures, fever, pneumonia, recurrent colds, diarrhoea, dehydration and other problems that often result in extended hospital stays and nutritional problems. HIV-infected children frequently have severe candidiasis, a yeast infection that can cause unrelenting diaper rash and infections in the mouth (oral thrush) and throat that makes eating difficult (4).

As has been reported in earlier studies by Clerici, et.al, in HIV pathogenesis, the qualitative and quantitative abnormality in CD4+T

helper cell population in an HIV infected individual has profound effects on immune system. Cytokine induced immune dysregulation of these cells is a central component in progression to AIDS. Based on the cytokine response they generate, the T helper (Th) cells are classified into Th1 and Th2 types (7). A Th1 to Th2 shift in the type of cytokines released after HIV infection has been proposed in this context (5). It is due to the fact that HIV infects Th1 cells easily compared to Th2 cells. This Th1 to Th2 shift results in the inability of host macrophages to get rid of opportunistic intracellular pathogens and this ultimately proves fatal. The loss of T helper cell function in asymptomatic individuals is seen even before the fall in the number of CD4⁺ T cells. Progression of HIV infection from asymptomatic to symptomatic stage and then AIDS involves shift of Th1 type of responses (cell mediated immune responses) to Th2 responses (humoral immune responses) (6).

The prototypes of T helper cells, viz. Th1 and Th2 play an important part in pathogenesis to AIDS. The Th1 type of CD4⁺ T cells do not allow the virus to replicate in them and even show the ability to inhibit HIV replication in other HIV infected cells. Thus they are protective in nature. The Th2 type of cells and the cytokines they produce allow higher virus replication. This in turn leads to preferential killing of Th2 type CD4⁺ T cells and further spreads the infection of HIV (6)

Th1 cells produce Interferon-gamma (IFN- γ), Interleukin-2 (IL-2), Tumor necrosis factor- alpha and beta (TNF- α and TNF- β), responsible for both cell mediated immune responses (antibody production, macrophage activation, antibody dependent cell cytotoxicity and

delayed type hypersensitivity) and humoral responses. Th2 cells produce IL-4, IL-5, IL-6, and IL-13. These cytokines provide optimal help for humoral immune responses, through production of mast cells, eosinophil growth and differentiation, and facilitation to IgA synthesis. IL-10 is produced by both Th1 and Th2 cells in humans (7).

Cytokines produced by Th1 and Th2 cells exert mutual cross-regulatory interactions by autocrine or paracrine functions. For e.g., IFN- γ and IL-4 have antagonistic properties. Folks presumes TNF- α to be a key player in AIDS, since evidences indicate that a state of chronic inflammation may be necessary to maintain HIV infection. TNF- α greatly increases the replication of HIV in T cells and macrophages by inducing a nuclear chemical factor NF- κ B to bind to the proviral DNA and turn on the production of viral proteins (8).

Viral replication allows selective killing of the Th2 cells during the clonal selection procedure. These mechanisms may account for a preferential depletion of T cells that produce IL-4, as is suggested by lack of IL-4 expression in the PBMNCs of HIV-infected individuals (9).

Ameisen reports that T cells from the blood of HIV infected patients are highly susceptible to apoptosis, or programmed cell death, when activated to proliferate. Adding antibodies that block the Th2 cytokines IL-10 and IL-4 could prevent apoptosis of the T cells. Thus, a lack of coordination could result in what could be termed as an "abortive Th1 to Th2 shift"; i.e. Th1 cells dying without the accompanying increase in the number of Th2 cells (8).

The medical literature reviewed above indicates major influence of cytokines on morbidity, mortality and the progression of HIV disease.

However, similar data for the pediatric HIV cohort in the Indian context is very scanty. The present study was therefore planned to provide vital data on these parameters.

AIMS AND OBJECTIVES:

The study was carried out bearing the following aims and objectives:

1. Confirmation of the HIV serostatus of the patients born to HIV seropositive mothers.
2. Estimation of absolute CD4+ T cell counts using flow cytometry.
3. Estimation of the mRNA levels of the following cytokines
 - a. Th1 type: IL-2, TNF- α and IFN- γ
 - b. Th2 type: IL-4, IL-6, and IL-10

Using semi-quantitative relative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

4. Determination of viral load using H9 lymphoma cell line and p24 antigen detection.
5. Correlation of the laboratory findings with the clinical presentations.
6. Study of the morbidity and mortality trends in HIV/AIDS patients.

RATIONALE:

The cytokine interplay has a pivotal role in the pathogenesis and progression of HIV disease. The detection of cytokines in body fluids has been hampered by the very short half-life and immediate capture of secreted cytokines by their receptors. Measuring the expression of mRNA would reflect the levels of synthesis of cytokines and the values may not be parallel to those of circulating cytokine levels. This may be due to the regulation of translation of cytokine mRNA or regulation of cytokine protein secretion (10).

It was therefore envisaged to study the cytokine mRNA levels in the hitherto ignored pediatric cohort and correlate the same with other parameters like the CD4+T cell counts, viral load, opportunistic infections, and morbidity and mortality.

ETHICAL COMMITTEE CLEARANCE: Due permission was obtained from the Ethical Committee of the Lokmanya Tilak Memorial Municipal Hospital, Sion, before the start of the project.

MATERIALS AND METHODS:

1. **Study population:** The study population comprised 128 children between the age of 1-13 years, divided into two cohorts viz. Subjects and Controls.

Subjects comprised of 100 children of either sex, HIV seropositive and attending the OPD or admitted in the wards of Lokmanya Tilak Memorial Municipal Hospital, Sion, Mumbai. All the subjects were born to HIV seropositive mothers. All subjects were naïve for any anti-retrovirals. Controls consisted of 28 normal healthy children of either sex. Detailed clinical history and informed consent was obtained for both the cohorts from their parents/guardians.
2. **Blood collection:** Five to ten ml of blood was drawn by venipuncture from all children in the study population. The blood was aliquoted appropriately in K3-EDTA and plain bulbs for obtaining the plasma + cells and serum respectively. The sera were separated and stored at -20°C till further testing.
3. **Confirmation of HIV serostatus:** Sera samples were tested for the presence of HIV 1 and 2 antibodies by commercially available ELISA kit (SD HIV1/2 ELISA, Ver.3, Standard Diagnostics Inc., and Microlisa HIV, J. Mitra and Co.)
4. **Estimation of absolute CD4+ T cell counts:** 100 µl of blood was drawn from K3-EDTA containing bulb. The CD4+ T cells were counted using FACScout (Becton-Dickinson). The sorting and analysis of the cells was fully automated.

5. Separation of Peripheral Blood Mononuclear Cells (PBMNCs): The peripheral blood mononuclear cells (PBMNCs) were separated from the blood sample from K3-EDTA containing bulb using the Ficol Hypaque density gradient centrifugation. The cells were then cryopreserved at a density of 10^4 - 10^6 cells/ml at -80°C in RPMI 1640 containing 5% Fetal Calf Serum and 10% Dimethyl sulfoxide till further use (11).
6. Extraction of RNA: RNA was extracted from the PBMNCs using the Trizol TM reagent method. The RNA pellet was briefly dried (till complete evaporation of ethanol) and re-dissolved in 15 μl of Tris EDTA buffer, pH 7.2 (12).
7. Quantitation of RNA: The dissolved RNA was subjected to Spectrophotometric reading at 260 nm and 280 nm (12). The samples having the O.D.₂₆₀ to O.D.₂₈₀ ratio between 1.7-2.0 were selected for the performance of RT-PCR for cytokine mRNA.
8. Evaluation of the cytokine-mRNA profile:
 - 7.1 Th1 cytokines viz. IL-2, TNF- α and IFN- γ , and Th2 cytokines like IL-4, IL-6, and IL-10 were evaluated for their mRNA levels in the present study.
 - 7.2 The RNA samples were diluted appropriately using Tris EDTA buffer, pH. 7.2 so that 1 μl of the solution contained 2 μg of total RNA.

7.3 One-step, relative, gene-specific RT-PCR for the amplification of mRNA of cytokines was performed using the RT-PCR base kit (Qiagen Inc., USA) and gene-specific primers (Ambion, Inc., USA) for the particular cytokine. The samples were subjected to the RT-PCR for mRNA using the respective primer cocktail of forward and reverse primers (13). These have been generated from the sequences available with GenBank (14).

Cytokines	GenBank Nos. from which the primers have been generated for use
IL-2	K02056
IL-4	M23442
IL-6	Y0081
IL-10	U16720
TNF- α	J00219, M37265, V00536
IFN- γ	Z15026

The product obtained was identified using the control DNA. 18S *Classic* primer-competimer set was also employed to amplify the 18S ribosomal RNA. This was used in a primer to competimer ratio of 2:8. This acted as the internal control.

The quantitation was brought about by comparing the band intensities of the product and the 18S RNA product by the Gel Documentation software (1D Advanced AAB, Advanced American Biotechnology, USA)

7.4 Analysis of PCR products using agarose gel electrophoresis: The products obtained after amplification of the mRNA of the cytokines were loaded onto a 2% Low

electro-endo-osmotic agarose gel, prepared in Tris Borate EDTA (TBE) buffer, pH 8.0. The gel contained 5 μ l of 500 μ g/ml Ethidium bromide. The samples were mixed with loading buffer containing 0.25% Bromophenol blue and 30% glycerol. The gel was run in TBE buffer, pH 8.0, at 120V, till such time that the dye ran more than three-quarters of the gel length (~ 45-60 minutes). The gel was then visualized using UV Transilluminator. The bands were detected and quantitated using Gel Documentation software (1D Advanced AAB, Advanced American Biotechnology, USA). This helped in the determination of the template (cytokine mRNA) to control (18S RNA) ratio for quantitation.

7.5 Determination of the viral load: The sera samples of the HIV seropositive patients were incubated with H9 cell culture (15) in RPMI 1640 and the p24 antigen secretion in the medium was assayed using a commercially available ELISA kit (Zeptometrix, California). The dilution of the serum sample that gave a negative result for p24 was marked as the end-point (16).

7.6 Clinical diagnoses: The subjects were examined for their clinical presentations at the Pediatric Department of the Lokmanya Tilak Memorial Medical College, while the normal children were examined by general physicians as a part of routine check-up.

The symptoms like weight loss, fever, cough/cold, vomiting, diarrhoea and oral thrush were documented. Opportunistic

infections like tuberculosis, mycotic dermatitis, *Pneumocystis carinii* pneumonia, otorrhea (fungal/bacterial), Candidiasis (oral/dermal) and gastrointestinal disorders (fungal/bacterial/viral/protozoal) were looked for. Other viral infections like HBV/HCV and meningitis were also investigated.

- 7.7 Mortality: The subjects were monitored over a period of 18 months for their CD4 counts for their disease progression and mortality.

The detailed methodology will be discussed in the thesis.

RESULTS:

Study population:

Of the 100 HIV seropositive subjects enrolled in the study, 55 were in the age between 1-5 years, while the remaining 45 were above 5 years of age. The subject cohort comprised of 55 male and 45 female children. Twenty-eight normal, healthy children, comprising of 14 children each in the age groups 1-5 years and above 5 years, constituted the Control group.

Parental history of HIV infected children:

Table 1: Parental history of HIV infected children (n=100)

Single parent alive:	
Only mother	28
Only father	12
Both parents alive	29
Both parents expired	13
Parents separated	17
Child abandoned*	1
Total	100

* Brought to the clinic by a social worker

Table 1 illustrates the parental history of the HIV seropositive children. It was observed that only 29 children of the total 100 had both parents to take care of them. In 40 cases, either of the parents had expired. In 13 cases, both the parents had expired and the children were taken care by the relatives or by orphanages. Parents in 17 cases had separated and the children were living with a single parent. One case of an abandoned child was brought in by the social worker of an orphanage.

The children in the Control Group were being monitored for their hemoglobin levels, Differential White Blood Cell count, etc.

HIV staging:

Table 2: Distribution of the study population based on CD4 counts

Age group	Control group	HIV seropositive Cohort		
		Stage 1	Stage 2	Stage 3
1 - 5 years	14 (>1000)	13 (> 1000)	21 (999-500)	21 (< 500)
> 5 years	14 (> 500)	15 (> 500)	18 (499-200)	12 (< 200)
Total	28	28	39	33

Figures in parentheses indicate the range of CD4 counts for the respective Stage

The children were classified into different HIV stages using the CDC Classification System. Of 55 children between 1 - 5 years of age, 13 (32.73%) children were classified to be in Stage 1. Twenty-one (31.18%) children were classified to be in Stage 2 and 21 (31.18%) to be in Stage 3. In 45 children above 5 years of age, 15 (33.33%) children were classified to be in Stage 1. Eighteen (40%) children were classified to be in Stage 2 and 12 (26.67%) to be in Stage 3.

Cytokine profile:

The cytokines viz. IL-2, IL-4, IL-6, IL-10, IFN- γ and TNF- α were investigated under the present study. Since their circulatory forms have a short half-life, their corresponding nuclear material, i.e. the mRNA was estimated using relative semi-quantitative RT-PCR. The levels were determined as a ratio of the cytokine mRNA to the 18S ribosomal RNA control. The cytokine mRNA levels were correlated with CD4 counts (an index for disease progression), and *Pearson's Correlation Co-Efficient 'r'* was calculated to determine the significance. The mean values of the cytokine mRNA ratio in the control group and different

stages of HIV seropositive group were compared using the *Student's 't' Test*. The following mathematical formulae were used to derive the significance value:

a. For correlation:

$$t = r \sqrt{n - 2 / (1 - r^2)},$$

where n = Number of samples

r = Pearson's correlation

Co-efficient

b. For comparison of means from two different groups:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(\sigma_1^2 / N_1) + (\sigma_2^2 / N_2)}} \quad \text{where } \bar{X} = \text{mean of the group}$$

$$N = \text{Number of samples}$$

and

$$\sigma^2 = s^2 N / N - 1$$

where s = standard deviation

**Table 3: Th1 cytokine mRNA profile in HIV seropositive children
Age: 1 - 5 years**

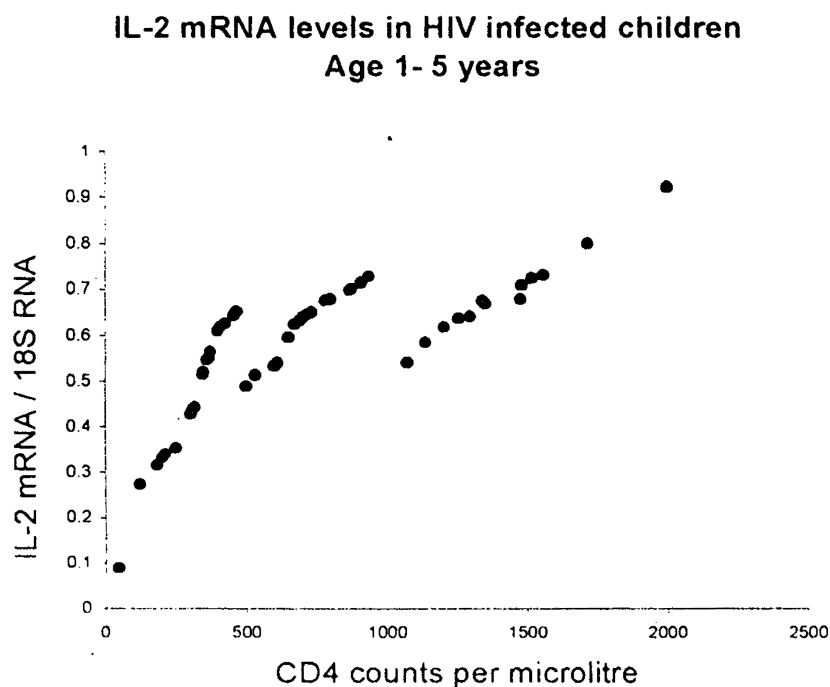
Cytokine	Control	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-2/18S RNA	0.7121 (0.3-0.89)	0.688 (0.542-0.922) p > 0.05	0.631 (0.489-0.729) p > 0.05	0.474 (0.09-0.627) p < 0.01
IFN- γ/18S RNA	0.0219 (0.00-0.0056)	0.757 (0.55-1.103) p < 0.01	0.501 (0.412-0.616) p < 0.01	0.434 (0.18-0.55) p < 0.01
TNF-α/18S RNA	0.0452 (0.00-0.071)	0.157 (0.08-0.25) p > 0.05	0.520 (0.258-0.69) p < 0.01	0.991 (0.72-1.25) p < 0.01

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

As illustrated in Table 3, in the age group of 1-5 years, the mean ratio of IL-2 mRNA/18S RNA was 0.7121 in the control group. In Stage1 of the HIV seropositive group, the mean ratio of IL-2 mRNA/ 18S RNA was

0.688 ($p>0.05$). In Stage 2, the mean ratio was 0.631($p>0.05$) and in Stage 3, the mean ratio was 0.474($p<0.01$).

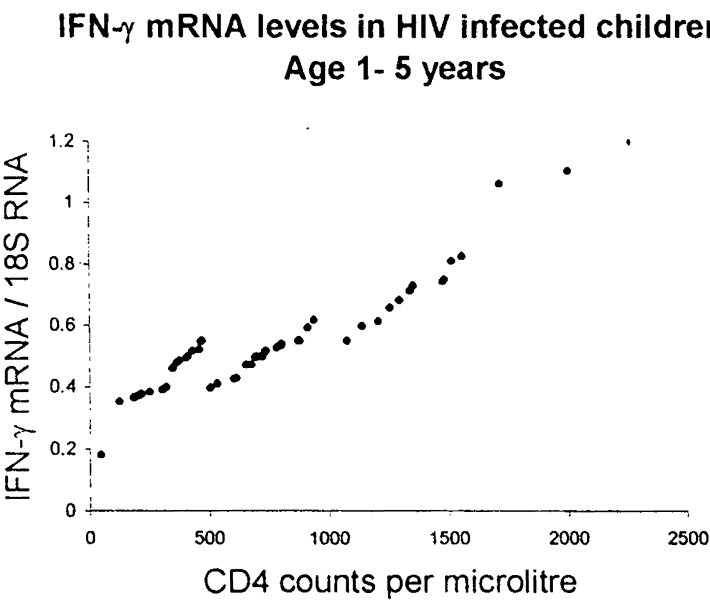
Figure 1:



As shown in figure 1, the IL2 levels showed a direct proportionality with the concomitant decrease in CD4 counts($r=0.77$, $p<0.01$).

As illustrated in Table 3, the mean ratio of IFN- γ mRNA/18S RNA was 0.0219 in the control group. In Stage 1 of the HIV seropositive group, the mean ratio of IFN- γ mRNA/ 18S RNA was 0.757 ($p<0.01$). In Stage 2, the mean ratio was 0.501($p<0.01$) and in Stage 3, the mean ratio was 0.434 ($p <0.01$).

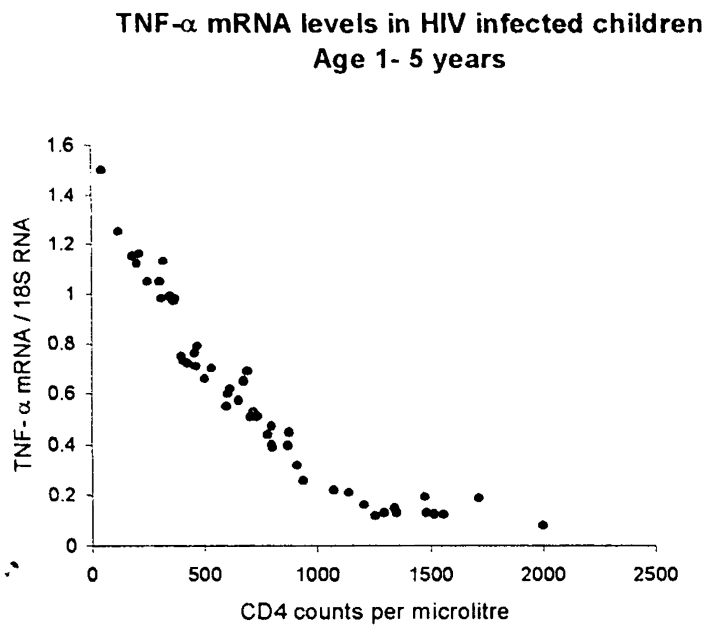
Figure 2:



As depicted by figure 2, the IFN- γ levels decreased with the concomitant decrease in CD4 counts($r=0.93$, $p<0.01$).

As observed in Table 3, the mean ratio of TNF- α mRNA/18S RNA in the control group was 0.0452. In Stage 1 of HIV seropositive group, the mean ratio of TNF- α mRNA/ 18S RNA was 0.157 ($p>0.05$). In Stage 2, the ratio was 0.520 ($p<0.01$) and in Stage 3, the ratio was 0.991 ($p<0.01$).

Figure 3:



TNF- α levels were inversely proportional to the CD4 counts($r= - 0.92$, $p<0.01$), as shown in Figure 3.

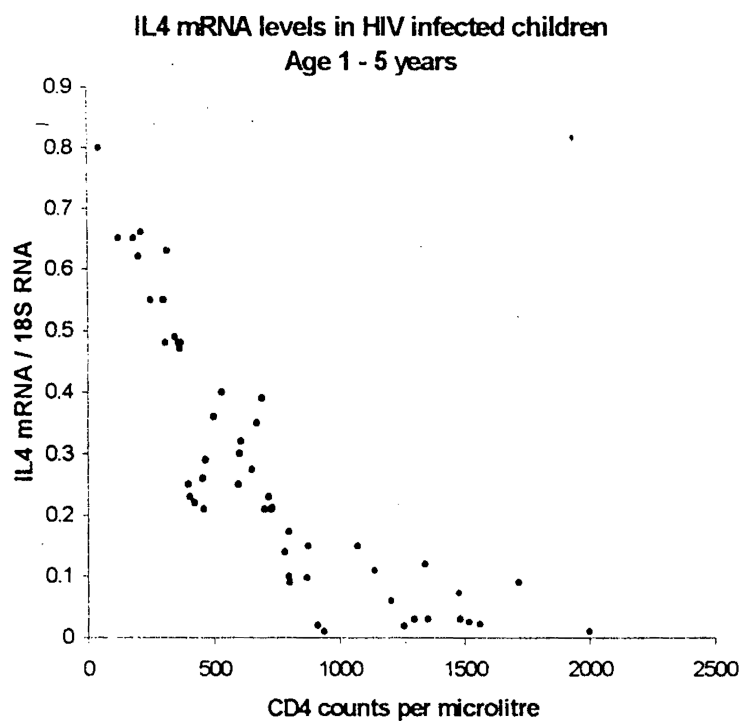
**Table 4: Th2 cytokine mRNA profile in HIV seropositive children
Age: 1-5 years**

Cytokine	Control	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-4 / 18S RNA	0.023 (0.00-0.056)	0.059 (0.01-0.15) $p < 0.01$	0.223 (0.09-0.39) $p < 0.01$	0.477 (0.21-0.80) $p < 0.01$
IL-6 / 18S RNA	0.320 (0.00-0.54)	0.355 (0.28-0.42) $p > 0.05$	0.540 (0.278-0.72) $p < 0.01$	0.790 (0.51-1.30) $p < 0.01$
IL-10 / 18S RNA	0.0215 (0.00-0.056)	0.116 (0.067-0.207) $p < 0.01$	0.253 (0.04-0.43) $p < 0.01$	0.397 (0.13-0.58) $p < 0.01$

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

As illustrated in Table 4, in the age group of 1-5 years, the mean ratio of IL-4 mRNA/18S RNA was 0.023 ($p<0.01$). In Stage 1 of the HIV seropositive group, the mean ratio of IL-4 mRNA/ 18S RNA was 0.059 ($p<0.01$). In Stage 2, the mean ratio was 0.223 ($p<0.01$) and in Stage 3, the mean ratio was 0.477 ($p < 0.01$).

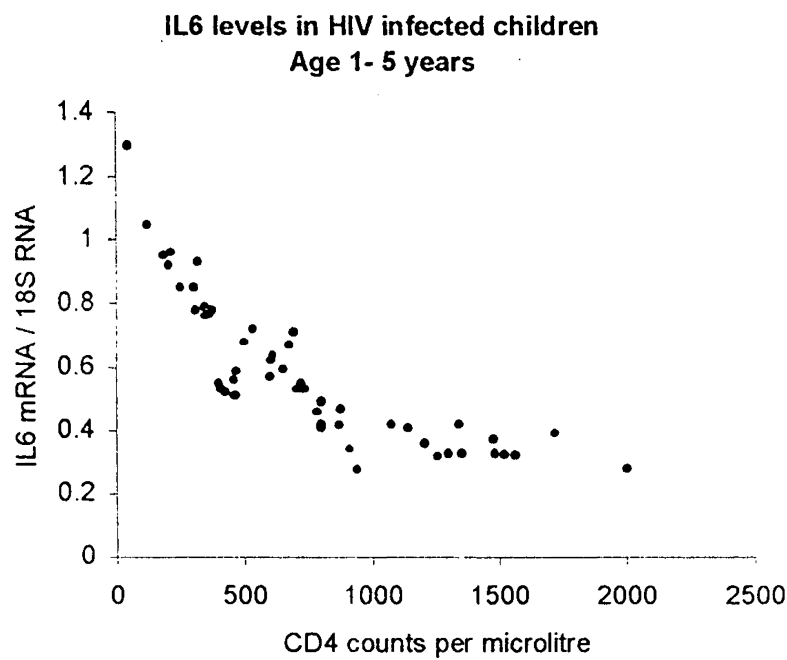
Figure 4:



As shown in figure 4, IL-4 levels were observed to increase with concurrent decrease in CD4 counts ($r = -0.84$, $p < 0.01$).

As illustrated in Table 4, mean ratio of IL-6 mRNA/18S RNA in the control group was 0.320. In Stage 1 of the HIV seropositive group, the mean ratio of IL-6 mRNA/ 18S RNA was 0.355 ($p > 0.05$). In Stage 2, the mean ratio was 0.540 ($p < 0.01$) and in Stage 3, the mean ratio was 0.790 ($p < 0.01$).

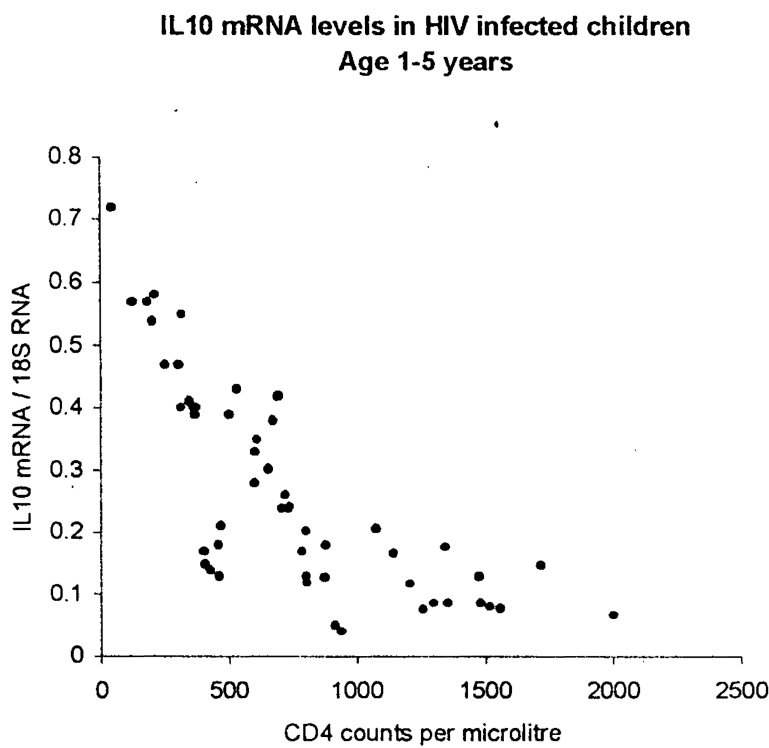
Figure 5:



IL-6 levels were inversely proportional to the CD4 counts($r= - 0.83$, $p<0.01$), as illustrated in figure 5.

As mentioned in Table 4, the ratio of IL-10 mRNA/18S RNA in the control group was 0.0452. In Stage 1 of the HIV seropositive group, the mean ratio of IL-10 mRNA/ 18S RNA was 0.116 ($p<0.01$). In Stage 2, the mean ratio was 0.253 ($p<0.01$) and in Stage 3, the mean ratio was 0.397 ($p <0.01$).

Figure 6:



IL-10 levels were observed to increase with the concomitant decrease in CD4 counts($r= -0.76$, $p<0.01$), as shown in figure 6.

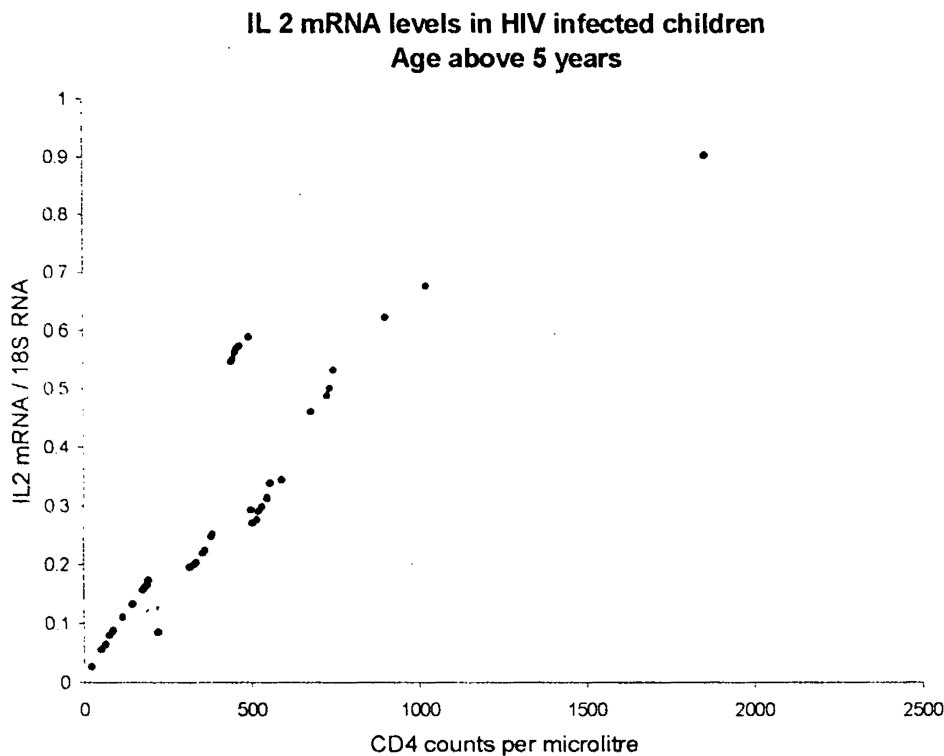
**Table 5: Th1 cytokine mRNA profile in HIV seropositive children
Age: >5 years**

Cytokine	Control group	HIV Seropositive		
		Stage 1	Stage 2	Stage 3
IL-2/18S RNA	0.5529 (0.00-0.86)	0.541 (0.369-1.003) $p > 0.05$	0.342 (0.083-0.291) $p < 0.01$	0.107 (0.025-0.172) $p < 0.01$
IFN- γ /18S RNA	0.0264 (0.00-0.005)	0.758 (0.546-1.55) $p < 0.01$	0.434 (0.239-0.574) $p < 0.01$	0.265 (0.102-0.516) $p < 0.01$
TNF- α /18S RNA	0.0494 (0.00-0.086)	0.354 (0.12-0.433) $p < 0.01$	0.768 (0.65-0.86) $p < 0.01$	1.166 (0.902-1.5) $p < 0.01$

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

As illustrated in Table 5, in the age group above 5 years, the mean ratio of IL-2 mRNA/18S RNA was 0.5529 in the control group. In Stage 1 of the HIV seropositive group, the mean ratio of IL-2 mRNA/ 18S RNA was 0.541 ($p>0.05$). In Stage 2, the mean ratio was 0.342 ($p<0.01$) and in Stage 3, the mean ratio was 0.107($p<0.01$).

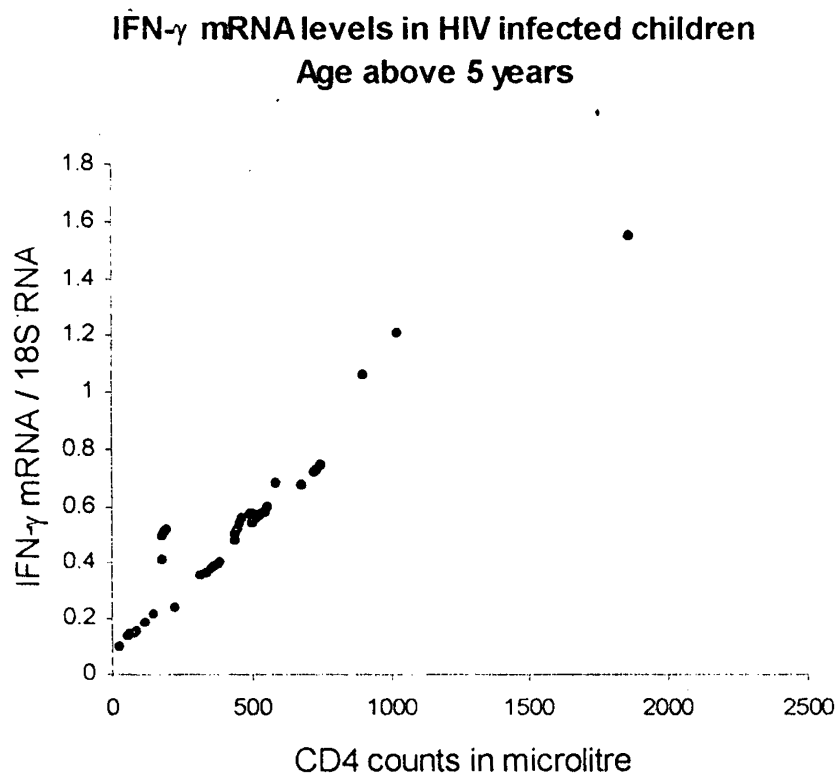
Figure 7:



As depicted in figure 7, IL2 levels were directly proportional to the concomitant decrease in CD4 counts($r=0.84$, $p<0.01$).

As mentioned in Table 5, in the control group, the mean ratio of IFN- γ mRNA/18S RNA was 0.026. In Stage 1 of the HIV seropositive group, the mean ratio of IFN- γ mRNA/ 18S RNA was 0.758 ($p<0.01$). In Stage 2, the mean ratio was 0.434($p<0.01$) and in Stage 3, the mean ratio was 0.265 ($p <0.01$).

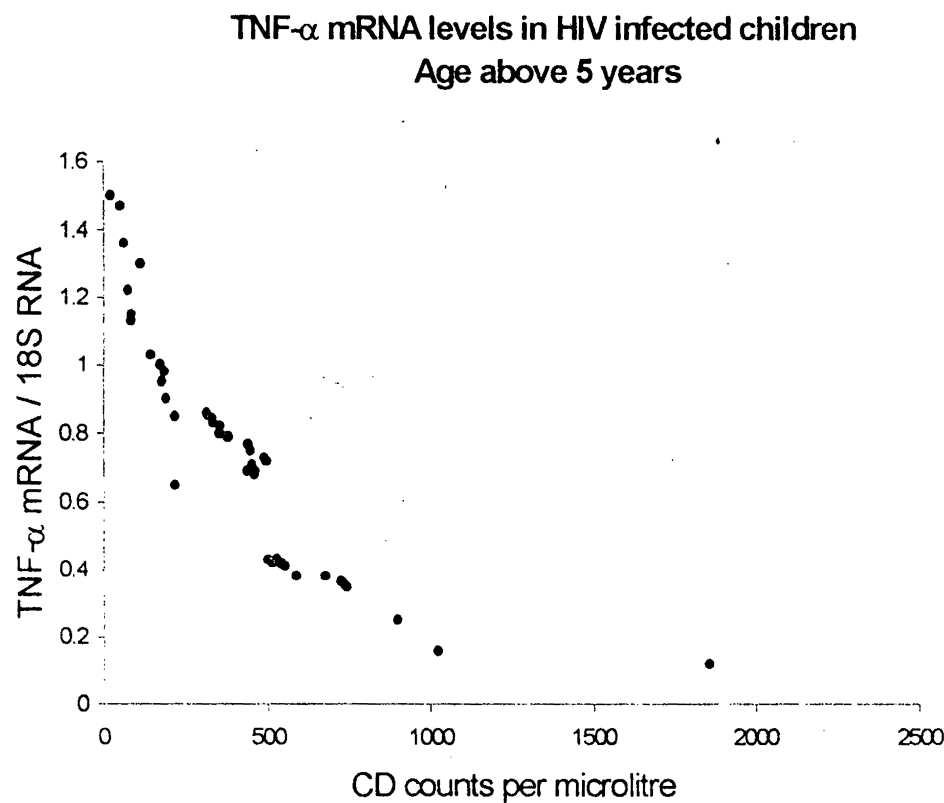
Figure 8:



IFN- γ levels were directly proportional to the CD4 counts($r=0.95$, $p<0.01$), as shown in figure 8.

As illustrated in Table 5, the mean ratio of TNF- α mRNA/18S RNA in the control group was 0.0494. In Stage 1 of the HIV seropositive group, the mean ratio of TNF- α mRNA/ 18S RNA was 0.354 ($p<0.01$). In Stage 2, the mean ratio was 0.768 ($p<0.01$) and in Stage 3, the mean ratio was 1.166 ($p <0.01$).

Figure 9:



As shown in figure 9, TNF- α levels increased with the concomitant decrease in CD4 counts ($r = -0.84$, $p < 0.01$).

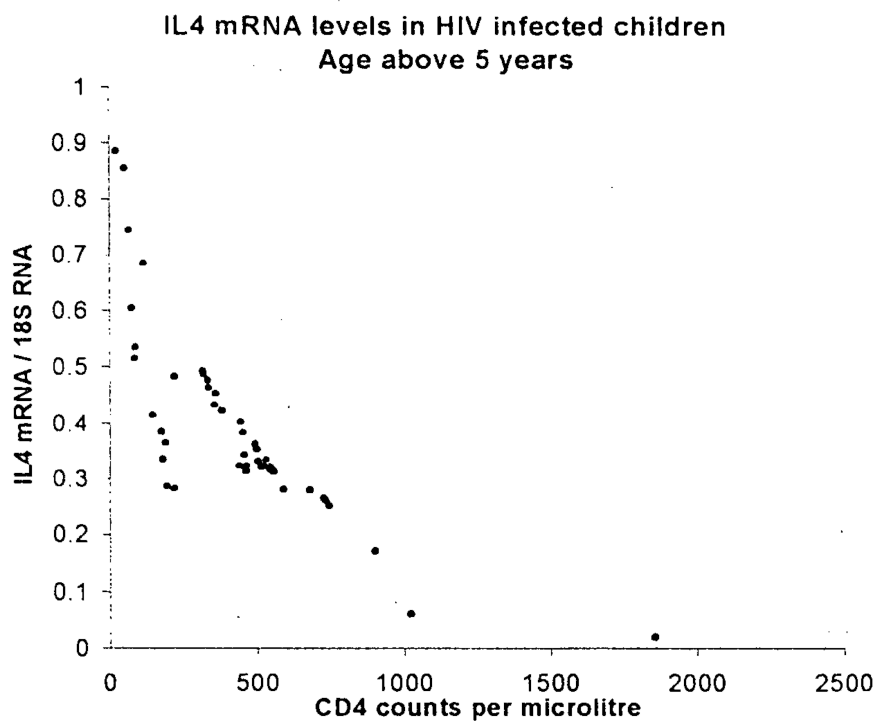
Table 5: Th2 cytokine mRNA profile in HIV seropositive children
Age: >5 years

Cytokine	Control group	HIV seropositive		
		Stage 1	Stage 2	Stage 3
IL-4 / 18S RNA	0.0229 (0.00-0.035)	0.256 (0.02-0.333) $p < 0.01$	0.400 (0.282-0.492) $p < 0.01$	0.550 (0.286-0.884) $p < 0.01$
IL-6 / 18S RNA	0.3621 (0.00-0.69)	0.564 (0.37-0.643) $p < 0.01$	0.735 (0.62-0.83) $p < 0.01$	0.976 (0.712-1.31) $p < 0.01$
IL-10 / 18S RNA	0.0221 (0.00-0.039)	0.276 (0.08-0.35) $p < 0.01$	0.330 (0.212-0.422) $p < 0.01$	0.466 (0.202-0.8) $p < 0.01$

Figures in parentheses indicate the range of cytokine values, with the mean values illustrated in the bold font.

As mentioned in Table 5, in the control group, the mean normal ratio of IL-4 mRNA/18S RNA was 0.0229. In Stage 1 of HIV seropositive group, the mean ratio of IL-4 mRNA/ 18S RNA was 0.256 ($p < 0.01$). In Stage 2, the mean ratio was 0.400 ($p < 0.01$) and in Stage 3, the mean ratio was 0.550 ($p < 0.01$).

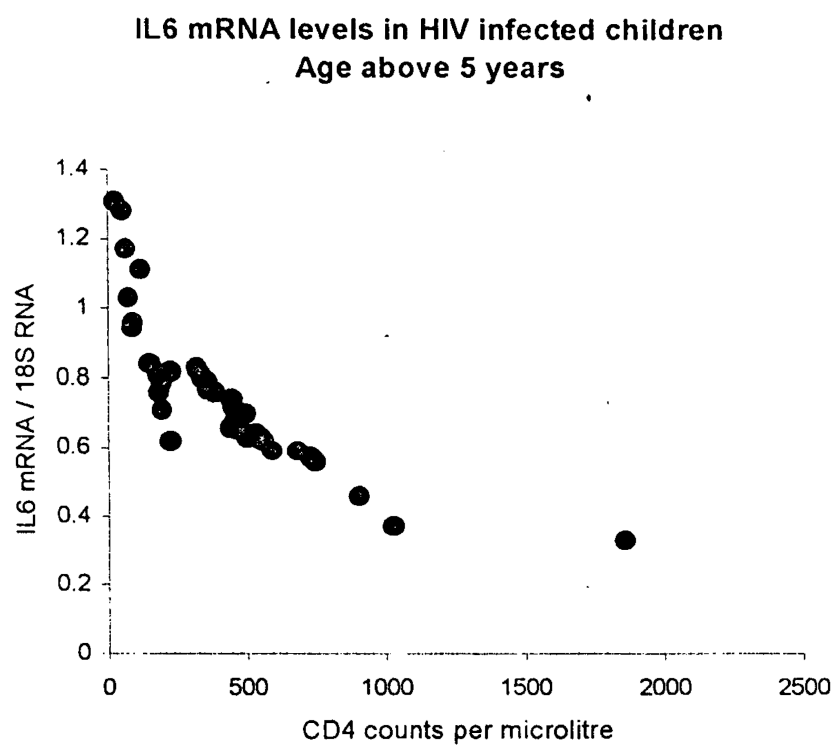
Figure 10:



As shown in figure 10, IL-4 levels were observed to increase with concurrent decrease in CD4 counts ($r = -0.77$, $p < 0.01$).

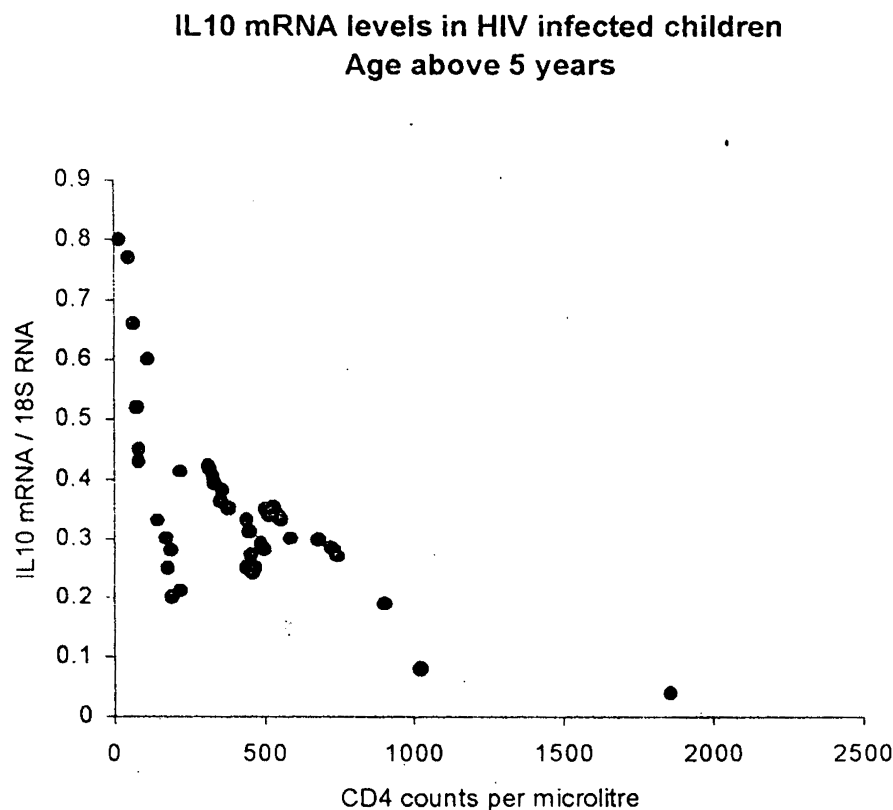
As seen from Table 5, mean ratio of IL-6 mRNA/18S RNA in the control group was 0.3621. In Stage 1 of HIV seropositive group, the mean ratio of IL-6 mRNA/ 18S RNA was 0.564 ($p < 0.01$). In Stage 2, the mean ratio was 0.735 ($p < 0.01$) and in Stage 3, the mean ratio was 0.976 ($p < 0.01$).

Figure 11:



As illustrated in figure 11, IL-6 levels increased with the decrease in CD4 counts($r=-0.81$, $p<0.01$).

As mentioned in Table 5, the mean ratio of IL-10 mRNA/18S RNA in the control group was 0.0221. In stage 1 of HIV seropositive group, the mean ratio of IL-10 mRNA/ 18S RNA was 0.276 ($p<0.01$). In Stage 2, the mean ratio was 0.330 ($p<0.01$) and in Stage 3, the mean ratio was 0.466 ($p <0.01$) in Stage 3.

Figure 12:

As shown in figure 12, IL-10 levels were thus observed to increase with the concomitant decrease in CD4 counts ($r = -0.67$, $p < 0.01$).

Table 6: Opportunistic infections observed in children with respect to CD4 counts

CD4+ T cell counts	Age group											
	1 - 5 years						> 5 years					
	K	D	P	O	C	GI	K	D	P	O	C	GI
>1000 ($n_1 = 13$) ($n_2 = 2$)	-	-	3	5	-	-	1	1	-	1	-	-
999 - 500 ($n_1 = 21$) ($n_2 = 13$)	4	4	6	6	-	1	2	2	4	4	1	-
499 - 200 ($n_1 = 18$) ($n_2 = 18$)	5	4	4	3	4	3	6	5	4	6	3	2
< 200 ($n_1 = 3$) ($n_2 = 12$)	-	1	1	2	-	-	1	2	3	1	1	1
Total ($n = 100$)	9	9	14	16	4	4	10	10	11	12	5	1

K = Koch's disease/Tuberculosis

P = Pneumocystis carinii pneumoniae

GI = Diarrhoea (fungal/bacterial/viral/protozoal)

n_1 = Children in 1-5 years

D = Mycotic Dermatitis

O = Otorrhea (fungal/bacterial)

C = Candidiasis (oral/dermal)

n_2 = Children >5 years

In the age group, 1-5 years, the most frequently observed infection was the discharge from the ears, i.e. Otorrhea (16/55), followed by *Pneumocystis carinii* pneumoniae (PCP) (14/55), tuberculosis (9/55), dermatitis (9/55), Candidiasis (4/55) and severe diarrhoea (4/55).

In Stage 1 (CD4 counts > 1000/ μ l), opportunistic infections like PCP, tuberculosis, dermatitis and otorrhea were observed. In Stage 2 (CD4 counts 999-500/ μ l), children started presenting multiple infections. In Stage 3 (CD4 T cell counts 499-200/ μ l), the prevalence of multiple infections along with candidiasis and severe diarrhoea was observed. Two patients were also being treated for meningitis and one for hepatitis B.

In the age group of above 5 years, Otorrhea (12/45) and *Pneumocystis carinii* pneumoniae (PCP) (11/45) were the most frequently observed infections. The other important infections were tuberculosis (10/45) and dermatitis (10/45). Candidiasis (5/45) and severe diarrhoea (1/45) were also observed.

In Stage 1 (CD4 counts > 500/ μ l), opportunistic infections like PCP, tuberculosis, dermatitis and otorrhea were observed. In Stage 2 (CD4 counts 499-200/ μ l) and Stage 3 (CD4 T cell counts <200/ μ l), children presented multiple infections including candidiasis and diarrhoea.

Table 7: Mortality trends with respect to CD4 counts

CD4 counts	Mortality	
	1-5 years(n=55)	> 5 years (n=45)
> 1000	1	-nil-
999-500	3	-nil-
499-200	9	-nil-
<200	2	8
Total	15	8

Fifteen children from the age group 1-5 years died during the 18 months of monitoring, while 8 children above 5 years expired during the same period. In the HIV seropositive age group 1-5 years, maximum mortality was observed when the CD4 counts were between 499-200/ μ l. In seropositive children above 5 years, maximum mortality was observed when the CD4 counts were below 200/ μ l. No deaths were reported in the Control group.

VIRAL LOAD ESTIMATION: Viral load estimation for the HIV seropositive children is being carried out. The results will be furnished in the thesis.

DISCUSSION AND SUMMARY: According to the recent survey by National AIDS Control Organization, there are approximately 40,000 children infected with AIDS in Mumbai (1). Unfortunately, the morbidity-mortality trends in pediatric HIV, the prevalence of opportunistic infections, etc. are not well documented. The Indian pediatric data is not represented on global AIDS scenario.

In the present study, it was observed that 13 out of 100 children studied had lost both their parents. Forty children were leading their lives with a single parent, who was also seropositive. It can therefore be presumed that sooner or later, these children too would become AIDS orphans. These orphan children became an extra burden on the society as they were taken care by aged grandparents or relatives or by local orphanages and were brought to the HIV clinics with serious complications requiring hospitalization.

Pediatric HIV infection has a peculiar epidemiology, the mode of transmission and clinical features. Most cases are perinatally infected, incubation is usually shorter

than in adults and the majority of clinical manifestations are related to opportunistic infections. Progression of HIV infection is generally faster in children than in adults and most pediatric patients suffer from HIV related manifestations at school age. Although the reasons for fast progression are not well understood, the age-related immunologic status of the young child may contribute to the evolution of the disease. The knowledge of immunologic events that occur in HIV infected children help in elucidating the mechanisms underlying the disease progression (17).

Our observations have shown that the IL-2 mRNA levels decrease with the progression of the disease. The role of IL-2 in the propagation of HIV in IL-2 stimulated PBMNCs probably includes stimulation of both cytokine production and lymphocyte proliferation and activation (18). Earlier studies have demonstrated a decrease in the number of IL-2 producing cells in moderate and advanced HIV infection (19). The deficient IL-2 production in rapid progressors may be explained by the decrease in the proportion of CD4⁺ T cells within the PBMNC population (20).

In the present study, the levels of IFN- γ mRNA were observed to be lower in HIV seropositive cohort as compared to the control group. These levels decreased during the course of the disease. These results are in agreement with an earlier study on IFN- γ in HIV (21). This can be attributed to the decrease in the capacity of CD4 and CD8 cells for secretion of IFN- γ . Also, there is a decline in the total CD4 counts as HIV disease progresses. Thus a dual loss accounts for lowered IFN- γ levels (22). The loss of a potent antiviral, anti-proliferative and immunomodulatory molecule like IFN- γ adds to the progression and severity of the disease.

We observed that the PBMNCs of the HIV seropositive children showed significantly elevated levels of TNF- α mRNA. TNF- α is a cytokine produced by several cell types, but primarily by macrophages and activated germinal centre B cells of HIV

infected individuals. TNF- α may contribute to the CD4+ T cell depletion by augmenting the T cell dependent Antibody Dependent Cell Cytotoxicity (ADCC) (10). Levels of TNF- α mRNA have earlier been reported to increase in HIV disease progression. This may be due to the presence of opportunistic infections like tuberculosis that stimulate the monocytes to upregulate the production of TNF- α (23). TNF- α , by causing CD4 depletion, anorexia of chronic disease and apoptosis of T cells and by induction of acute phase proteins adds to the increased morbidity in HIV seropositive children.

The HIV seropositive cohort under the present study showed elevated mRNA levels of IL-4 as compared to the control group. PBMNCs from HIV infected patients with reduced CD4 counts are expected to produce lower level of serum IL-4 than PBMNCs of asymptomatic HIV patients with normal or slightly reduced CD4 counts (9). Some workers have observed a decrease in the IL-4 levels in constitutive or stimulus induced PBMNCs (24). However, IL-4 mRNA levels happen to increase with the disease progression. The decline in IL-4 levels may be due to the ability of HIV *nef* protein to inhibit the protein synthesis by interfering with the translational process (20). IL-4 stimulates HIV replication in early stages of infection and may also facilitate virus transmission (25), thus making it a vital molecule in HIV disease progression.

IL-6 is released in HIV infected individuals by monocytes and activated B cells. It induces the differentiation of the B cells and cytotoxic T cells. It also induces production and action of acute phase proteins. Patients with low CD4 counts have been observed to have higher levels of IL-6 mRNA expressing cells (10). Our observations, of elevated IL-6 mRNA levels during the course of HIV disease progression are in agreement with the above findings. IL-6 levels are upregulated in the later stages of HIV (10) and this increase appends the severity of HIV disease.

mRNA levels of IL-10 in HIV seropositive cohort in the present study were significantly higher than those in control group. IL-10, previously known as cytokine synthesis inhibitory factor, is thought to play an important role in the cytokine imbalance observed in AIDS patients (5). Significant increase in IL-10 levels has been reported in patients with advanced HIV disease. IL-10 induction has been proposed to be rapid and transient. Low levels of IL-10 fail to check the HIV replication (26). These low levels may act as a feed back mechanism for induction of more IL-10, leading to higher expression and mRNA levels.

A complex array of Th1 and Th2 cytokine responses has been observed in HIV infection. On one hand, the increase in the IL-4 / IFN- γ ratio is responsible for the lowering of CD4 counts (21). On the other hand, we have IL-10 that down-regulates the Th1 cytokine response (27). The Th2 functions indicated by IL-4 production replace the normally dominant Th1 function of antigen stimulated IL-2 production during HIV progression (5). There is also an evidence that cytokine dysregulation occurs with AIDS progression, and the overproduction of Th2 cytokines increases the susceptibility to activation induced cell death whereas Th1 cytokines are protective. While one group of workers has proposed a Th1-Th2 shift in cytokines (5), another group lays the onus on the decrease in the levels of IL-2 producing cells in the CD4/CD8 population in moderate and advanced stages of HIV disease (18).

With the above dysregulation in the cytokine responses, varied opportunistic infections are observed in HIV infected individuals. In our HIV seropositive cohort, opportunistic infections, ranging from dermatitis and diarrhoea to tuberculosis and PCP were seen. These develop primarily due to the HIV associated immunodeficiency, reactivation of latent infections, anemia, as well as poor hygiene, absence of a good nutritional support and lack of adequate medication (4). This is

especially true for the lower socio-economic classes of the developing countries like India.

By end of 2001-2002, nearly 5.8 lakh children have succumbed to AIDS, the maximum mortality being in Africa (28). A high mortality of 23% was observed during the monitoring period of 18 months among these seropositive children included in the present study. This may be due to the reduction in levels of IL-2 and IFN- γ along with the increase in IL-4, IL-10, and TNF- α levels, making these children more vulnerable for dysregulation of their immune system, leading to rapid disease progression and mortality (5).

Studies such as the present aim to throw light on the cytokine spectra, trends of opportunistic infections, mortality, etc. in HIV/AIDS pediatric cases. These will help in lessening the morbidity and mortality due to HIV. This data can be utilized for setting up therapeutic interventions and thus help the medical machinery to tackle the growing problem of HIV/AIDS in children.

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