ESTIMATION OF mRNA LEVELS OF INTERLEUKIN (IL) -10, TUMOR NECROSIS FACTOR (TNF) - α , IL - 4 AND INTERFERON (IFN) - γ IN HIV INFECTED CHILDREN IN MUMBAI

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ABSTRACT

Cytokines, viral load and opportunistic infections play an important role in HIV-disease progression. Hundred children vertically infected with HIV were enrolled to determine mRNA levels of TNF- α , IL-10, IL-4 and IFN - γ . These levels were estimated by amplifying cytokine mRNA from peripheral blood mononuclear cells. Severity of HIV was staged by the reduction in CD₄⁺ T cells and the onset of opportunistic infections. IL- 10 mRNA levels were observed to increase with the severity. Despite the rising IL-10 mRNA levels, TNF- α mRNA levels increased with severity of HIV and decrease in CD₄⁺ T cell counts. IL-4 mRNA levels increased with the reduction in CD₄⁺ T cell numbers. Depleting mRNA levels of IFN - γ contributed to the worsening of HIV disease. Increase in TNF- α and IL-4 levels appended to the disease severity by upregulation of the viral replication. Increased IL-10 levels and decreased IFN- γ levels predisposed the children to HIV associated opportunistic infections, which in return contributed to cytokine disarray.

KEYWORDS

HIV, disease severity, mRNA levels, cytokines, children

INTRODUCTION

Pediatric HIV infection in children is a chronic, multi-systemic illness with complex health care needs, raising the issues of foster care, and adoption (1). The Acquired Immunodeficiency Syndrome (AIDS) epidemic has claimed 0.51 million children in 2004, and an estimated 0.64 million children have acquired the Human Immunodeficiency Virus (HIV) in the same year. Of 2.2 million infected pediatric cases globally, number, around 70% of the infections occur in developing nations like India. Women account for more than one quarter of new HIV infections in India, according to estimates, and 90% of those who test positive at antenatal clinics say they are in single, long-term relationships (2). Fewer than 10% of pregnant women are offered services of proven effectiveness to prevent HIV transmission during pregnancy and childbirth, while under 3% of orphans and vulnerable

children are receiving public support for their treatment (3). In India, HIV prevalence among women attending antenatal clinics in the Maharashtra is around two percent (4).

The variations observed in the rate of disease progression in HIV-infected children are due to their viral load, CD₄⁺ T cell counts, and cytokine disarray 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. Cytokines are classified as T helper type 1/Th1 (IL-2, IFN - γ) and T helper type 2/Th2 (IL-4, IL-6, IL-10). TNF- α is categorized with the Th1 cytokines (5).

Differential cytokine production by CD4⁺ Th cells during an immune response plays an important role in HIV disease progression (6, 7). A Th1 to Th2 shift in the cytokines released after HIV infection has been proposed (8), due to easy HIV infection of Th1 cells as against Th2 cells. Disease progression in HIV leads to preferential killing of Th2 type CD4⁺ T cells (9).

IFN- γ inhibits the secretion of Th2 cytokines (10) by inhibiting the development of T cells into IL-4 producing clones and proliferation of Th2 clones (11). At the same time, IL-4 inhibits the development of IFN

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- γ expressing cells (12, 13) as studied in a rat model. IL-4 also partially inhibits IL-10 production at the transcriptional level. IL-4 is also able to impair the production of IL-6 and TNF- α in human monocytes (13). IL-4 along with IL-10 inhibits the cytokine production by Th1 cells, which is of importance during disease progression to AIDS (10). IL-10 has strong down regulatory effects on the secretion of the pro-inflammatory cytokines like IL-1, IL-6 and TNF- α that modulate the activation and function of the immune system, thus causing suppression of the immune and inflammatory responses (14). However the impairment of TNF- α is not seen in AIDS, probably since IL-10 is observed to be secreted later than TNF- α after activation (15). TNF- α levels upregulate HIV replication via NF- κ B which in turn increases the TNF- α levels as the disease progresses.

The clinical manifestations in pediatric HIV are related to opportunistic infections (OIs). Most pediatric patients suffer from HIV-related manifestations at school age. OIs like *Pneumocystis carinii*, pneumoniae tuberculosis, diarrhoea, and candidiasis are frequently observed in HIV infected children (16, 17). These result in the increased morbidity and mortality in pediatric HIV. The availability of anti-retroviral therapy (ART) and improved treatment for opportunistic infections have changed the pattern of disease and survival in HIV infected children in most developed countries (19). However, such therapies are beyond the reach of the major population in India.

MATERIALS AND METHODS

1. Study Population

The study population comprised of 128 children between the ages of 1-13 years divided into two cohorts viz. subjects and controls. The Subjects comprised of 100 HIV seropositive children of either sex attending the OPD or admitted in the wards of Lokmanya Tilak Memorial Municipal Hospital, Sion, Mumbai. All subjects were naïve for any anti-retrovirals. The children were categorized into the three Stages of HIV based on their CD4⁺ T cell counts. Controls consisted of 28 normal healthy children of either sex, having no symptoms, and seronegative for HIV. These children visited the General Practitioners for a regular check-up. Detailed clinical history and informed written consent was obtained for both the cohorts from their parents/guardians. Due permission was obtained from the Ethical Committee of the Lokmanya Tilak Memorial Municipal Hospital, Sion, before the start of the project.

2. Blood collection and sera separation

Five milliliters of blood were drawn by venipuncture from all children in the study. The blood was aliquoted appropriately in K3-EDTA and plain bulbs for obtaining

the plasma and serum respectively. The sera were separated and stored at -20°C till further testing.

3. Estimation of CD4⁺ T cell counts

Hundred microlitres 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 estimated by fluorescence activated cell sorter (FACS) analysis using monoclonal antibodies / ligands against CD4⁺ and CD8⁺ cells on a FACScount (Becton-Dickinson Immunocytometry Systems). The sorting and analysis of the cells was fully automated.

4. Estimation of mRNA levels of IL-10, TNF- α , IFN- γ and IL-4 using RT-PCR

a. Extraction of RNA from PBMNCs

The peripheral blood mononuclear cells (PBMNCs) were separated from the blood sample from K3-EDTA containing bulb using the Ficoll Hypaque density gradient centrifugation. They were washed once with sterile RPMI-1640. RNA was extracted from these cells using the Trizol reagent method. The RNA pellet was dried till complete evaporation of ethanol and re-dissolved in 15 μ l of Tris-EDTA (10mM Tris, 1mM EDTA, pH 7.6) and incubated for 10 minutes at 55°C. The RNA was quantified using UV spectrophotometer and the samples were diluted appropriately using TE buffer, so that 1 μ l of the solution contained 2 μ g of total RNA.

b. Amplification of the cytokine mRNA

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 relative RT-PCR primer kit (Ambion, Inc., USA) for the individual cytokine (Refer Table 1).

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

The amplification of the mRNA of the cytokine was carried out on a thermal cycler (Techne, UK.) using a Reverse-Transcriptase polymerase chain reaction (RT-PCR) using a PCR mix comprising of 5X Qiagen buffer, 5X Q-solution, 10 mM dNTPs, 5 mM primer gene specific cocktail, 3 mM 18S primers (already mixed in 2:8 proportion), 2 μ l of PCR enzyme mix, and 0.2 μ l of RNase inhibitor. Two microlitres of random hexamers were added for the Reverse transcription. One microlitre of the template RNA extracted from the PBMNCs was added and the volume was made to

Table 1. Primer sequences for the amplification of cytokines under study

Cytokine	Primer Sequence
IL-10	5' primer: ATCAAGGCGCATGTGAACTCC
	3' primer: CAGAGCCCCAGATCCGATTTT
TNF- α	5' primer: AAGAGTTCCCCAGGGACCTCT
	3' primer: CCTGGGAGTAGATGAGGTACA
IL-4	5' primer: TGCTGCCTCCAAGAACAACACTG
	3' primer: CATGATCGTCTTTAGCCTTTCCA
IFN- γ	5' primer: GAGTGTGGAGACCATCAAGGAA
	3' primer: GCAGGCAGGACAACCATTACTG

50 l using DNase/RNase free water. The mix containing tube was then kept in a thermal cycler pre-heated at 50°C. The reverse transcription was carried out at 50°C for 30 minutes.

This was followed by the activation of Polymerase at 95°C for 15 minutes. Subsequently, amplification cycles comprising of denaturation (94°C for 1 min), annealing (57°C for 1 min) and extension (72°C for 1 min) were carried out. A final extension at 72°C for 10 minutes was carried out.

At the end of amplification, IL-10 (38 cycles) yielded a 297 bp product, TNF- α (40 cycles) yielded a 233 bp product, IL - 4 (40 cycles) gave a 264 bp product while IFN- γ (35 cycles) gave a 241 bp product. The 18S RNA on amplified yielded a 495bp product.

c. Evaluation of the cytokine mRNA levels

The products obtained after amplification of the mRNA of the cytokines were loaded onto a 2% Low electro-osmotic agarose gel, prepared in TBE buffer (89 mM Tris, 89 mM Borate, 2 mM EDTA, pH 8.0). The gel contained 5 l of 500 mg/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.

5. Examination of the children for opportunistic infections

The subjects were examined for their clinical presentations at the Department of Pediatrics, Lokmanya Tilak Memorial Medical College, Sion, Mumbai, while general physicians performed examination of healthy children 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.

6. Statistical analysis

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.

RESULTS

1. Distribution of the Study population based on CD4⁺ T cell counts

The children were brought to the hospital when they presented with persistent HIV associated opportunistic infections. On examination, we observed that their CD4⁺ T cell counts fell to half the normal cut-off. In the age group of 1-5 years, more than 76% of the children brought for observation had deteriorated to HIV Stage 2 and lower. As illustrated in Table 2, 89% of children above 5 years seeking medical attention had reached or crossed HIV Stage 2.

2. Evaluation of the cytokine profile in the Study population

The cytokine levels were determined as the ratio of the cytokine mRNA to the 18S ribosomal RNA, as represented in Figures 1a to 1d.

Mean values of IL-10 mRNA were observed to increase with the HIV disease progression, as described in Table 3. In children between 1-5 years of age, as compared to the mean value in the Control Group, IL-10 mRNA value rose five-fold with the advent of HIV. By the time of the onset of HIV Stage 3, the mean value was 18 times that of the Control Group. As illustrated in Table 4, IL-10 mRNA value in HIV seropositive children above 5 years of age was 10 times the normal value at the onset of HIV, and a 21-fold increment was observed in HIV Stage 3.

Mean TNF- α mRNA values were increased three fold in HIV infected children in age group 1-5 years. However as the HIV disease progressed, a steep

Table 2. Distribution of 100 HIV seropositive children in the age groups 1-5 years and above 5 years based on their CD₄⁺ T cell counts

Age group	Control group	HIV seropositive Cohort		
		Stage 1/ Asymptomatic	Stage 2	Stage 3
1-5 years (n = 69)	14 (>1000 cells/ml)	13 (> 1000 cells/ml)	21 (999-500 cells/ml)	21 (< 500 cells/ml)
> 5 years (n = 59)	14 (> 500 cells/ml)	15 (> 500 cells/ml)	18 (499-200 cells/ml)	12 (< 200 cells/ml)
Total (n = 128)	28	28	39	33

Figures in parentheses indicate the range of normal CD₄⁺ T cell counts for the respective Stage
n refers to the sample size

The children have been categorized on the basis of the CD₄⁺ T cell counts, as per 1994 Revised Classification System for Human Immunodeficiency Virus Infection in Children Less Than 13 Years of Age, September 30, 1994 / 43(RR-12);1-10

Table 3. Cytokine mRNA profile in HIV seropositive children of age 1 - 5 years

Cytokine	Type	Control Group	HIV seropositive Study population		
			Stage 1/ Asymptomatic	Stage 2	Stage 3
IL-10 mRNA / 18S RNA	Th2	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
TNF- α mRNA / 18S RNA	Th1	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
IFN- γ mRNA / 18S RNA	Th1	0.0219 (0.00-0.006)	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
IL-4 mRNA / 18S RNA	Th2	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

Figures in parentheses indicate the range of cytokine values, while the mean values are indicated in bold print.

Figure 1a. Amplification of IL-10 mRNA in HIV infected children using RT-PCR

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 (297bp) is of IL- 10. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.

Figure 1c. Amplification of IFN-γ mRNA in HIV infected children using RT-PCR

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 IFN-T. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp - 1000bp.

Figure 1b. Amplification of TNF-α mRNA in HIV infected children using RT-PCR

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 (233bp) is of TNF-α. Lane 2 bears the molecular weight marker (DNA ladder) from 100 bp-1000 bp. Lane 1 shows the sample from a healthy child, where levels of TNF-α are undetectable.

Figure 1d. Amplification of IL-4 mRNA in HIV infected children using RT-PCR

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 (264bp) is of IL-4. Lane 4 bears the molecular weight marker (DNA ladder) from 200bp-1000bp.

Table 4. Cytokine mRNA profile in HIV seropositive children above 5 years

Cytokine	Type	Control Group	HIV seropositive Study population		
			Stage 1/ Asymptomatic	Stage 2	Stage 3
IL-10 mRNA / 18S RNA	Th2	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
TNF- α mRNA / 18S RNA	Th1	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
IFN- γ mRNA / 18S RNA	Th1	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
IL-4 mRNA / 18S RNA	Th2	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

Figures in parentheses indicate the range of cytokine values, while the mean values are indicated in bold print

incline in the values was observed, with the mean TNF- α mRNA values being 22 times that of the normal value. In HIV infected children above 5 years, the TNF- α levels increased 7 times the Control Group. A 24-fold increase was seen as the children progressed to HIV Stage 3.

In children between 1-5 years, IFN- γ mRNA levels were observed to increase 35 times the normal levels with the initiation of Stage 1 of HIV infection. However, as the disease progressed, a fall was observed, with the IFN- γ mRNA levels in HIV Stage 3 being 20-fold the normal value. Similarly, in children above 5 years, IFN- γ mRNA values were 29 times the values observed in the Control Group, and were reduced in HIV Stage 3 to being 10 times the normal value.

Mean IL-4 mRNA levels in HIV infected children between 1-5 years were 2.5 times the levels in normal children. With the disease progression these values rose to 20 times the values in the Control Group. In children above 5 years of age, the IL-4 mRNA levels increased 11-fold at the onset of HIV infection, which rose to 24 times the normal levels by the time HIV stage 3 was reached.

3. Opportunistic infections observed in the Study population during HIV disease progression

As tabulated in Table 5, of the various clinical manifestations seen in HIV infected children, 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

Table 5. Clinical manifestations in HIV Seropositive children enrolled in the study (n=100)

Clinical manifestations	No. of children infected
Tuberculosis	19
Pneumocystis carinii pneumonia	25
Diarrhoea	7
Dermatitis	19
Otorrhea	28
Candidiasis	9

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%.

As the HIV infection progressed, the children presented with a wide array of opportunistic infections (OIs). The infections observed in different HIV Stages have been illustrated in Table 6. With the onset of HIV Stage 2 (CD4⁺ T cell counts: 999-500 in children between 1-5 years and CD4⁺ T cell counts: 499-200 in children above 5 years), multiple infections were observed, which resulted in death in the next stage of HIV/AIDS. Respiratory infections, i.e. *Pneumocystis carinii* pneumoniae and tuberculosis contributed 44%

Table 6. Opportunistic infections observed in children with respect to CD4 counts (n=100)

CD ₄ ⁺ 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)
n₁ = Children in 1-5 years

D = Mycotic Dermatitis
O = Otorrhea(fungal/bacterial)
C = Candidiasis (oral/dermal)
n₂ = Children >5 years

of the OIs observed.

DISCUSSION

A good orchestration of both Th1 and Th2 types of the T helper cells leads to a desired effect against the pathogen. The Th phenotypes, characterized by the cytokines they produce, and their balance 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. Although the reasons for fast progression are being investigated, it is suggested that age-related immunologic status of the children may contribute to the evolution of the disease, knowing this would help to elucidate the mechanisms underlying the disease progression (16). 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 from the PBMNCs 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 (20).

In the present study, 100 seropositive children, vertically infected with HIV were enrolled and classified in 3 HIV stages. Twenty-eight children were categorized in HIV Stage 1, 39 in HIV Stage 2 and 33

in HIV Stage 3. The stages were determined by the CD4⁺ T cell counts. 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 (21). In the present study, we observed a significant (p<0.01) increase in the IL-10 levels during the HIV disease progression in both age groups (represented in Table 2 and Table 3). Chehimi *et al.* have demonstrated a significant increase in the IL-10 levels in PBMNCs of adult patients with advanced HIV disease, although they were on anti-retroviral therapy. In their study, patients with CD4⁺ T cell counts < 200 cells/ l secreted thrice the IL-10 as compared to patients with CD4⁺ T cell counts > 500 cells/ l (21). In the present study, patients with CD4⁺ T cell counts < 200 cells / l expressed 2.5 times (1.6 - 3.4) times the IL-10 mRNA as compared to patients with CD4⁺ T cell counts > 500 cells / l. A similar finding has been reported by Clerici *et al.*, who 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 (22). The rise in IL-10 levels may be due to the presence of various opportunistic infections of intracellular pathogens. 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 (23). 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

Fig. 2. Cytokine expression in different stages of HIV in children between 1-5 years

The graph illustrates the observation from 55 HIV seropositive children between 1-5 years of age. The cytokine expression is measured as the ratio of the cytokine mRNA levels to the 18S RNA levels. The staging of the HIV disease has been designated based on the CD4⁺ T cell counts. It is observed that TNF-α mRNA levels show a steep incline as we move across the HIV stages. An increase in the values of Th2 cytokine mRNAs for IL-4 and IL-10 also show an increase. The prototype of Th1 response, i.e. the mRNA levels for IFN-γ, demonstrates a decline during the course of HIV infection from Stage 1 to Stage 3.

monocytes (22). IL-10 is known to downregulate macrophage function, thereby down-regulating HIV replication since macrophages are primary targets for HIV. An increase in IL-10 expression contributes to a higher inhibition of Th1 responses and faster progression to AIDS.

TNF-α is known to induce upregulation of many pro-inflammatory molecules and contribute to tissue damage. *In-vitro* and *in-vivo* studies have shown that HIV infection can induce secretion and elevation of pro-inflammatory cytokines like TNF-α. In one such study, TNF-α was observed to be constitutively expressed in 62 HIV infected individuals (24). This may account for the decline in the absolute CD4⁺ T cell counts as TNF-α augments this decline by T cell dependent ADCC (25). In the present study, we have observed the significant rise of TNF-α mRNA

expression once the children between 1-5 years presented with HIV-associated opportunistic infections. However, a significant rise in the expression of TNF-α mRNA was observed even in asymptomatic HIV infected children above 5 years. The presence of opportunistic infections like tuberculosis and candidiasis stimulate the monocytes to upregulate the production of TNF-α (24). Bajaj *et al.* carried out a study with 20 patients with HIV, of which 10 suffered from 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 (26). These increased levels of TNF-α result in increased morbidity in HIV by predisposing the immune system to an active viral attack.

Fig. 3. Cytokine expression in different stages of HIV in children above 5 years

The graph illustrates the observation from 45 HIV seropositive children above 5 years of age. The cytokine expression is measured as the ratio of the cytokine mRNA levels to the 18S RNA levels. The staging of the HIV disease has been designated based on the CD4⁺ T cell counts. A shift from the Th1 (IFN- γ) to a predominant Th2 (IL-4 and IL-10) response is observed. TNF- α levels however show a characteristic increment in the values from HIV Stage 1 to Stage 3.

Cellular immunity is considered to be responsible for the eradication of intracellular pathogens like tuberculosis and HIV with the help of TNF- α , IFN- γ and IL-4, possibly through macrophage activation (37). 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 (27). PBMNCs from HIV infected patients with reduced CD4⁺ T cell counts have been assumed to produce lower level of serum IL-4 than PBMNCs of asymptomatic HIV infected patients with normal or reduced CD4 counts (28, 29). In the present study, we observed an eight fold increment in the IL-4 mRNA levels in HIV Stage 3 as compared to asymptomatic (HIV Stage 1) patients in children between 1-5 years. However, in children above 5 years, a two-fold increase in IL-4 mRNA levels was

observed. Though the IL-4 mRNA levels increase with HIV disease progression, a decline in circulating IL-4 levels has been observed. This may be due to the ability of HIV nef protein to inhibit the protein synthesis by interfering with the translational process, impairing the production of the cytokine (29).

The functional defects of T helper cells, especially Th1 cells, can be observed early after infection with HIV. The prototype of Th1 shift to Th2 is the decrease in the IFN- γ production (30). In the HIV seropositive cohort, in children between 1-5 years, the IFN- γ mRNA levels in HIV Stage 3 were 1.7 times lower as compared to asymptomatic patients. In children above 5 years, these levels were 2.8 times lower. It is suggested that diseases caused by intracellular bacterial and viral pathogens are associated with the immunologic unresponsiveness and failure to produce

IFN - γ . In a study carried out by Gong *et al.*, it has been observed that INF- γ levels were the highest in the healthy controls, followed by HIV negative tuberculosis patients, and the lowest for patients with a dual tuberculosis-HIV infection (31). Depletion of IFN - γ levels in presence of IL-4 has been observed in the thymocytes of HIV infected individuals (32). This depletion occurs since the absolute numbers of CD4⁺ T cells decrease in the thymocytes. Also, the capacity of CD4⁺ and CD8⁺ cells to secrete IFN - γ decreases, as demonstrated by Bailer *et al.* (33). This dual loss accounts for the lowered IFN - γ levels. The present study assumes the decline in the CD4 + T cell counts during HIV disease progression as a factor responsible for the decline in the IFN - γ levels. In a study carried out on 74 HIV infected and 30 healthy individuals enrolled for estimation of IFN- γ levels and their disease progression, it was shown that 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 (33). Thus the loss of INF- γ , a potent anti-viral molecule adds to the severity of HIV infection.

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 can be related to the lowering of CD4⁺ T cell counts (34), while on the other, IL-10 down-regulates the Th1 cytokine response (35). The Th2 functions indicated by IL-4 production replace the normally dominant Th1 function of antigen stimulated IL-2 production during HIV progression (8). 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 (8), 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 (35). In the present study, we observed two characteristic features in the HIV disease progression. First, the TNF- α values increased along with the fall in CD4⁺ T cell counts. Secondly, the IFN- γ /IL-4 ratio fell as the HIV disease traversed from Stage 1 to Stage 3. Both these phenomena were observed in both the age groups. Thus they can serve as prognostic markers for the course of HIV disease.

In another study carried out at the Department of Virology (*unpublished data*), an attempt was made to compare the circulating cytokine levels in HIV infected adults, individuals infected with tuberculosis and patients dually infected with the two diseases. It was seen that the cytokine profile remained similar in all three groups. However, the intensity (increase in TNF- α , IL-10 and a decrease in IFN- γ) was more in patients with dual infection of HIV and tuberculosis. A study

carried out by Bajaj *et al.* (26) has shown the synergistic effect of candidiasis in HIV disease progression. Thus opportunistic infections that arise out of depleted immune defenses contribute to worsening of the cytokine imbalance in HIV disease progression.

With the dysregulation in the Th1 and Th2 responses due to HIV and varied opportunistic infections, cytokine profiles with marked severity in imbalance are observed in HIV infected individuals. On this platform, the present work was embarked upon to study the cytokine profile in HIV infected children in Mumbai. In our HIV seropositive cohort, opportunistic infections, ranging from dermatitis and diarrhoea to tuberculosis and PCP were observed. These develop primarily due to the HIV associated immunodeficiency, reactivation of latent infections, anemia, as well as conditions like poor hygiene, absence of a good nutritional support and lack of adequate medication (36), the latter being true for the lower socio-economic classes of the developing countries like India, where anti-retroviral therapy is yet beyond the reach of common man.

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