In the pathogenesis of HIV-1 infection, apoptosis causes a decline in the counts of uninfected bystander CD4+ T cells in HIV infection. The rate of disease progression of HIV infection is considered to be faster in the developing countries. The present study was carried out to investigate certain markers for apoptosis in immunopathogenesis of disease in HIV infected south Indian population.

Methods: Soluble Fas (sFas) antigen and Fas ligand levels in plasma samples from 39 antiretroviral treatment naïve patients was estimated and compared with T cell subsets and HIV-1 viral load.

Results: The mean sFas antigen levels among controls and the CDC A, B and C clinical stages were 2.77, 3.08, 3.26 and 3.28 ng/ml respectively, higher though not significantly among HIV-1 infected individuals compared to controls. The mean sFas ligand levels in CDC A, B and C stages were 0.138, 0.125 and 0.117 ng/ml respectively were higher \((P<0.001)\) than controls (0.073 ng/ml) and positively correlated with total lymphocyte \(\%\) \((r=0.43, P=0.007)\), sFas antigen levels were negatively correlated with total WBC count \((r=-0.34, P=0.04)\), CD4\% \((r=-0.4, P=0.01)\) and CD4:CD8 ratio \((r=-0.37, P=0.02)\). There was an increase in plasma levels of sFas antigen and Fas ligand over time in asymptomatics.

Interpretation & conclusions: The high levels of sFas antigen and Fas ligand seen in HIV infected individuals suggest increased activation and apoptosis of T cells, due to constant stimulation of the immune system by inter-current infections of HIV infected individuals in south India.

Key words: Apoptosis - HIV-1 - pathogenesis - south India

In the pathogenesis of HIV-1 infection, apoptosis causes a decline in the counts of uninfected bystander CD4+ T cells. Uninfected T cells are known to undergo apoptosis via the cross-linking of CD4 molecules by gp120 secreted out of infected cells and activation or by the binding of Fas ligand to the Fas antigen. These events lead to activation of a family of enzymes, nuclear condensation and DNA fragmentation in the cell. The indirect correlation of CD4+ T cell counts and the direct correlation of HIV-1 viral loads with Fas mediated apoptosis has been reported. The CD4+ T cell undergoing apoptosis are known to be memory cells expressing CD28, Fas antigen and Fas ligand. The apoptotic target CD8+ T cells express Fas antigen,
with or without CD28 and Fas ligand expression. HIV-1 triggers Fas ligand mediated apoptosis of uninfected CD4+ T cells, whereas the CD8+ T cell apoptosis is driven by chronic immune activation. Another mechanism involved is the autophagy, wherein bystander CD4+ T lymphocytes come in contact of HIV env complexes with CXCR4, leading to apoptotic cell death contributing to immunodeficiency.

Apoptosis involves other HIV proteins like Tat, Nef, and Vpu. These proteins may upregulate CD95 (Fas) and Fas L levels and thereby enhancing the susceptibility to Fas mediated killing. These proteins can also induce cell death of uninfected T cells. In HIV infected individuals, increased apoptosis was seen in the lymph nodes compared to uninfected individuals. However, the direct cytolytic activity of HIV also contributes the CD4 decline in HIV infection.

The rate of disease progression in HIV infection is thought to be higher and CD4 decline steeper in the developing countries like India. In contrast, a study from Africa has not upheld this observation. It is, however, important to identify the cause for the rapid rate of disease progression and CD4 decline observed among patients from the Indian subcontinent. Immune activation due to inter-current infections could be contributing to the apoptosis of CD4+ T cells that leads to the decline of CD4+ T cells. We have previously shown significantly higher percentage of CD4 cells expressing CCR5 and CXCR4 both in HIV uninfected and infected individuals than in the Western countries. It is now established that activated T cells express more CCR1 through CCR7. In contrast, resting T cells express more CXCR1, 2, and 4. Expression of CCR1, 2 and 5 is upregulated in memory T cells cultured in the presence of IL-2. Furthermore, the increased expression of viral co-receptors could also mean that the T cells of the south Indians are more amenable to infection than the T cells of the western population. We investigated certain markers for apoptosis in the HIV infected south Indian population.

**Material & Methods**

The study was carried out in the department of Clinical Virology, Christian Medical College (CMC) Vellore, a tertiary care centre, between March 1998 and February 2002. Individuals recruited for the study were above 15 yr of age, and belonged to the 4 south Indian States of Tamil Nadu, Kerala, Karnataka and Andhra Pradesh. These individuals were recruited in the course of a multicenter Indian Council of Medical Research funded immunophenotype study and a Department of Biotechnology, Government of India funded study for looking at the subtype profile of HIV-1 in south India which were cleared by the institutional review board. The study subjects were referred to the Clinical Virology Department, either from the Infectious Diseases Clinic of CMC or by general practice physicians, for HIV confirmation or for CD4/CD8 T cell estimation. They were recruited after an informed written and/or verbal consent. Among these individuals, a sub-population of 39 antiretroviral treatment naïve HIV infected individuals were recruited into this study by ‘convenient sampling’. However, there was no separate consent obtained for the estimation of soluble Fas antigen and Fas ligand which was done archived samples. Among the 39 patients 26 were male, and 13 were female with a mean age of 34.7 ± 7.6 and 34.8 ± 12.01 yr respectively. Based on the CDC classification 14 belonged to CDC A and 12 in CDC B and 13 to CDC C clinical criteria. Fifteen normal healthy adults (12 males, 3 females) were recruited as controls, with a mean age of 28.3 ± 3.3 and 30.3 ± 7.5 yr respectively. All these 15 individuals were negative for HIV antibody. Majority of these normal individuals were laboratory staff, who were clinically healthy and they were not screened for any parasites, hepatitis B surface antigen, antibody to hepatitis C virus or Mycobacterium tuberculosis.

Blood samples were collected in two EDTA treated tubes between 0800 and 1000 h. Ten ml of samples were collected in 15 ml collection tubes containing 300 µl of 5 per cent EDTA and two ml of samples in a 5 ml collection tube containing 60 µl of 5 per cent EDTA. From the 10 ml samples the plasma and peripheral blood mononuclear cells (PBMC) were separated by the density gradient technique using Ficoll hypaque and stored in multiple aliquots at -60°C until tested. The 2 ml volume samples were collected for the CD4+/CD8+ T cell estimation. Follow up second samples collected with a mean gap of 25.2 months from 8 treatment naïve HIV infected individuals were also available for the estimation of soluble Fas antigen and Fas ligand.

Fifty-three PBMC samples from 22 HIV-1 infected individuals among the above mentioned 39 individuals were available for detection of apoptosis. More than one sample was available from 13 individuals, while in nine, only one sample was tested.

Plasma soluble Fas antigen and soluble Fas ligand were estimated by commercial ELISA (MBL,
Nagoya, Japan). The tests were carried out as per manufacturer’s instruction. All the samples were tested in duplicate and the mean value was taken for analysis. The standards provided with the kits were used, for soluble Fas antigen it ranged from 0-2 ng/ml while for Fas ligand it ranged from 0-5 ng/ml. An automated microplate washer (Elx 50, Bio-Tek instruments, Vermont, USA) and automated microplate reader (µQuant, Bio-Tek instruments, Vermont, USA) were used. A standard curve was generated with the KC4 software (version 2.7 Bio-Tek instruments, Vermont, USA) and the concentrations of soluble Fas antigen and ligand were estimated.

For HIV-1 infected individuals and controls, T cell subset enumeration was carried out by the standard flowcytometry analysis (FACScan flowcytometer, Becton Dickinson, California, USA). Quantitation of HIV-1 RNA in plasma was carried out with Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, New Jersey, USA).

For the detection of apoptosis in PBMC un-stimulated 1-2 million PBMCs frozen at -60°C was reconstituted and DNA extracted for apoptotic DNA ladder (Roche Diagnostics, GmbH, Germany). Apoptotic U937 cells were used as positive controls. PBMC samples from two HIV uninfected individuals that were stored at -60°C for a long period were used as negative controls to validate the procedure. After electrophoresis in agarose gel the pattern of apoptotic DNA was visualised (Gel Doc 2000, Bio-Rad, California USA).

Epi info 6.04d software was used for most of the statistical analysis. Normality of data was assessed with Describe: Winpepi. Outliers were removed (detected either with Winpepi or Grubbs test at http://www.graphpad.com/quickcalcs/index.cfm) and means calculated. P values for the correlation coefficient were calculated with Pcal.exe (courtesy HPA, Colindale, UK).

Results

Among the 39 patients, two samples with outlier values for both sFas and FasL (one each from CDC A and C) were removed from analysis. The variability of both the assays between the duplicates were very minimal (the mean ± SD of % CV for sFas antigen was 1.9 ± 1.7 while that of sFas ligand was 5.9 ± 6.1). The mean sFas antigen levels among controls and the CDC A, CDC B and CDC C stages of HIV-1 infected individuals were 2.77, 3.08, 3.26 and 3.28 ng/ml respectively. Though the sFas antigen levels were higher among HIV-1 infected individuals compared to controls, the difference was not significance. There was no significant difference between the mean sFas antigen levels among the three CDC groups (Table I). The mean sFas ligand levels among controls and the CDC A, CDC B and CDC C stages of HIV-1 infected individuals were 0.073, 0.138, 0.125 and 0.117 ng/ml respectively. The sFas ligand levels were higher among HIV-1 infected individuals compared to controls and the difference was significant (P<0.001). There was also a significant difference between the controls and CDC A group (P<0.001). There was however no significant difference between the mean sFas ligand levels among the three CDC groups (Table I). sFas antigen levels were negatively correlated with total count (r=-0.34, P=0.04), CD4 per cent (r=-0.4, P=0.01) and CD4:CD8 ratio (r= -0.37, P=0.02). No other significant correlation was established. sFas ligand levels were positively correlated with lymphocyte per cent (r= 0.43, P=0.007). There was a positive correlation of sFas ligand with lymphocyte count (r=0.3, P=0.07) and CD8+ T cell count (r=0.29, P=0.08).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Soluble Fas (ng/ml)</th>
<th>*Soluble Fas ligand (ng/ml)</th>
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<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Controls</td>
<td>15</td>
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</tr>
<tr>
<td>HIV-1</td>
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<tr>
<td>CDC B</td>
<td>12</td>
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<tr>
<td>CDC C</td>
<td>12</td>
<td>3.28</td>
<td>0.753</td>
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</tbody>
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*Controls vs HIV; P<0.001. All the other comparisons between different groups were not significant. †Data on 2 individuals (1 each from CDC A and C) removed from analysis as they are outliers.
In the follow up samples from 8 individuals’ plasma sFas antigen and sFas ligand estimation revealed an increase in both the levels in individuals who remained in CDC A. When individuals progressed to CDC C, there was an increase in sFas and a decrease in sFas ligand. In individuals who remained in CDC C, there was a decline in both sFas and sFas ligand (Table II). Among the patients who were followed up as a group, there was a significant negative correlation between sFas antigen levels and CD4 T cell percentage ($r = -0.56$, $P = 0.002$), CD4 T cell count ($r = -0.41$, $P = 0.03$) and CD4:CD8 ratio ($r = -0.45$, $P = 0.01$). There was also a significant positive correlation between sFas antigen and plasma HIV-1 RNA levels ($r = 0.42$, $P = 0.02$). There was a significant positive correlation of sFas ligand with plasma HIV-1 RNA level ($r=0.41$, $P=0.02$). Two individuals among these 8 (individuals no 3 and 5) progressed from CDC A to CDC C stage during the follow up period. In three individuals there was increase in viral RNA level with two individuals having a significant (>1 log) increase when the first apoptotic sample and the previous sample were compared. There was no significant difference in cell counts, sFas markers when stratified as a sample collected when apoptosis was evident or not.

Among the 53 samples tested from 22 individuals, apoptosis was detectable in 10 PBMC samples from 6 individuals during follow-up. In 8 of the samples, the PBMC was collected when the individual was in CDC A, while in 2 the individual was in CDC C. A representative gel run is shown in the Fig.

### Discussion

In the present study, sFas antigen level was negatively correlated with CD4 T cell percentage and CD4:CD8 ratio in the cross-sectional analysis. When individuals were followed up, there was an increase in sFas antigen over time. Soluble Fas antigen levels were negatively correlated with CD4 per cent, CD4:CD8 ratio, and positively correlated with HIV-1 RNA levels in individuals who were followed up. There was no significant difference between the mean sFas antigen levels among the three CDC groups unlike that found in a Chinese study. This may be a reflection of small sample size and may be significant differences could be shown in a well organized longitudinal study. sFas antigen level was higher in HIV-1 infected individuals compared to controls as reported by the Chinese group. Silvestris et al found that serum titres of sFas antigen and sFas ligand were linearly correlated in 17 severely lymphopenic subjects as compared with other non-HIV patients grouped in relation to their single expression of sFas ligand and sFas antigen, or with double-negative marker expression on T cells by flow cytometry. They also found increased cell death in vitro, in patients with elevations of sFas. Also, the in vitro proliferative rate was inhibited by sFas, in
particular in patients with undetectable levels of the soluble receptor in vivo as well as in normal donors. Functionally exhausted T cells may undergo apoptosis in response to the persistent in vivo stimulation by sFas17. It is also observed that the highly active antiretroviral therapy in HIV infected individuals can decrease the CD95 expression on naive T cells18.

In uncultured PBMC, <3.2 per cent of cells were found apoptotic19. Another group reported a low incidence of apoptosis in un-stimulated PBMCs (2.46%)20. It was also reported that activation induced apoptosis directly correlated with plasma viral load and inversely correlated with CD4 count. The present study also showed a significant positive correlation of sFas antigen and sFas ligand with plasma HIV-1 viral load while there was a significant negative correlation of sFas antigen with CD4+ T cell count. Mortality rate in cultured PBMC was shown to be correlated with disease severity and progression31. Another kind of activation induced cell death of T lymphocytes in HIV that is not Fas dependent and occurs without DNA fragmentation, has been also reported22. Since DNA fragmentation and sFas/sFas ligand levels were investigated separately in the study reported here, it is difficult to comment on the observation of Kottillil et al23.

Though there are several mechanisms described for the depletion of T lymphocytes in HIV infected individuals, it appears that apoptosis is one of the important mechanisms of CD4 decline in HIV infected south Indians as 27 per cent of the studied individuals showed evidence of apoptosis. This finding is probably related to the increased number of activated T cells in the south Indian population, a reflection of constant stimulation of the immune system due to infections. It has been documented that there are two types of population of CD4+ and CD8+ T cells viz., rapidly proliferating cells and the slowly proliferating cells23. It suggests that the rapidly proliferating population represents activated memory or effector cells. Many of these cells having high turnover rate may undergo programmed cell death after activation. The slowly proliferating pool represents naive or long-term memory cells23,25. In HIV infected individuals with low plasma RNA level the rapidly proliferating pool accounts for only <1-10 per cent of the proliferating CD4+ and CD8+ T cells. However, this increases to 10-40 per cent in individuals with the higher viral loads23,25. Both CD4+ and CD8+ T cells decline during the progression of HIV infection and this may result from a relative shift of these cells to the rapidly proliferating pool from the slowly proliferating pool. In response to activation or infection of CD4+ T cells by HIV, the balance of CD4+ T cells may shift to favour gradual depletion of this population of cells. The shorter survival of the individuals with advanced HIV infection may be because of the shorter survival of the T cells associated with T cell activation and elevated Fas expression on CD8+ T cells27. These findings also correlate with the increased presence of chemokine receptors in the south Indian population which are increased with activation of T cells and increase in plasma cytokines10,28.

In conclusion, our findings suggest that increased activation of T cells is an important factor for the increased apoptosis which probably is a reason for the rapid progression of disease in Indian (south) HIV infected individuals. The limitations of this study are the small sample size analysed and certain deficiencies of functional assays.

References


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