Subtype & cytokine profiles of HIV infected individuals from south India

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Background & objectives: The global surveillance of human immunodeficiency virus (HIV) subtypes (clades) helps understand the global distribution and incidence of different HIV subtypes. As knowledge about subtypes circulating in an area is needed for developing a candidate vaccine, prevalence of the subtypes HIV-1 and HIV-2 were studied in south India. The profile of cytokines interleukin 10 (IL10) and interferon gamma (IFNγ) in both types of infection were also analysed as these are considered indicators of disease progression.

Methods: Patients who belonged to the 4 south Indian States i.e. Tamil Nadu, Kerala, Karnataka and Andhra Pradesh were included. HIV-1 subtyping was carried out by the heteroduplex mobility analysis (HMA) while that of HIV-2 was done by direct sequencing. The quantitation of IFNγ and IL-10 was carried out using commercial ELISA kits.

Results: Among the 82 HIV-1 infected individuals subtyped, 78 (95.1%) were subtype C while all 12 HIV-2 strains were subtype A. IL-10 concentration was significantly higher among HIV infected individuals compared to normal healthy controls. IFNγ was significantly higher among symptomatic and AIDS groups compared to asymptomatic HIV-1 infected individuals.

Interpretation & conclusion: HIV-1 subtype C and the HIV-2 subtype A are the major subtypes circulating in south India. The study showed a trend towards a shifting of the cytokine profile from Th1 to Th2/Th0 in HIV-1, HIV-2 infections, and HIV-1 and HIV-2 dual infected individuals as the disease progresses. This trend observed is not unlike that reported from the West, despite the difference in subtype profile.

Currently one of the important aims of the UNAIDS/WHO is to have a global surveillance of human immunodeficiency virus (HIV) subtypes. Based on the phylogenetic analysis of the nucleotide sequences from the env gene, strains belonging to the group M of HIV-1 have been classified into 9 different genetic subtypes A-K. Following studies of full-length genomes of HIV-1, the former subtypes E and I were reclassified as circulating recombinant forms. The epidemiological trends suggest that env based subtype C strains would dominate the HIV pandemic in the coming years. The geographic spread of subtype C strains is also very diverse. It is prevalent in different regions in Africa, Latin America and Asia. It is important to know the subtypes of both HIV-1 and HIV-2 circulating in an area before considering developing a candidate vaccine for trial in that particular region. Moreover, there are reports,
which suggest that the pathogenesis of HIV in infected individuals may differ, depending on the subtype of the infecting virus 2-5.

It is known that cytokines play a crucial role in the pathogenesis of HIV infection 6. Cytokines have a complex effect on the replication of HIV, and conversely in infected individuals HIV directly affects cytokine production. Different studies postulate that the progression of disease in HIV infected individuals may be controlled by the balance between the levels of type 1 (Th1) and type 2 cytokines (Th2) 7-10. Two important cytokines involved in the Th1/Th2 cross-regulation are interferon-γ (IFNγ), a product of Th1 cells and interleukin-10 (IL-10), a product of Th2 cells 11. Majority of these studies are reported from areas where HIV-1 subtype B circulates. In this study, we investigated the plasma IFNγ and IL-10 levels in HIV infected individuals, with reference to the clinical status and virus subtype profile. The HIV-2 subtyping data have been published elsewhere 12 and is referred to here to give a comprehensive picture.

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 included in the study by ‘convenient sampling’ (n=227) were above 15 years of age, and belonged to the 4 south Indian States of Tamil Nadu, Kerala, Karnataka and Andhra Pradesh. The patients 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. Of these, 206 were HIV-1 infected, 14 HIV-2 infected and 7 were dual infected (both HIV-1 and-2) individuals.

Twenty-nine normal healthy adults (HIV seronegative) were included as controls who were volunteers from among the staff of the institution, from the general public or healthy relatives accompanying HIV infected individuals for CD4 counts. All the HIV uninfected volunteers were screened by a questionnaire administered as referred to earlier 13. All the controls were tested by a rapid test capable of detecting both IgG and IgM antibody to HIV (Capillus HIV-1 / HIV-2, Trinity Biosystems, Galway, Ireland).

An informed verbal and/or written consent was obtained from all patients and controls. The approval of the institutional research committee was obtained for the molecular studies and the cytokine testing was done on stored plasma.

About 10ml of blood sample was collected from each individual, in EDTA treated tubes, between 08:00 and 10:00 a.m. to avoid diurnal variation of CD4 counts. An aliquot of whole blood was used for the enumeration of CD4/CD8 T cells. From the remaining blood, plasma was separated and stored immediately. The plasma samples were separated immediately in order to avoid the decay of cytokines and HIV-1 RNA. Peripheral blood mononuclear cells (PBMC) were separated by the density gradient technique using Ficoll hypaque (Amersham Pharmacia Biotech, Sweden). Both plasma and PBMC were stored in multiple aliquots at -60°C until tested.

Samples were collected from 206 HIV-1 infected individuals and CD4/CD8 T cell estimation was carried out. In subpopulations of this group HIV-1 subtyping (n = 82) and plasma cytokine estimation (n = 119) was carried out. The data presented here are on these two groups.

Subtyping of HIV-1: DNA was extracted from uncultured PBMC of 82 individuals using the high pure viral nucleic acid kit (Boehringer Mannheim, Ottweiler, Germany) according to the manufacturer’s instruction. A nested PCR was performed to amplify either a 700 bp or 1.2 Kb portion of the env region as described by Delwart et al 14. The primers used for the first round of amplification were ED3 (5’- TTA GGC ATC TCC TAT GGC AGG AAG AAG CGG-3’) and ED14 (5’- TCT TGC CTG GAG CTG TTT GAT GCC CCA GAC-3’). For the nested round, one of the following pairs of primers were used: ES7 (5’-TGT AAA ACG ACG GCC AGT CTG TTA AAT GGC ATC TCC TAT GGC AGG AAG AAG CGG-3’) and ED14 (5’-TCT TGC CTG GAG CTG TTT GAT GCC CCA GAC-3’). For the nested round, one of the following pairs of primers were used: ES7 (5’-TGT AAA ACG ACG GCC AGT CTG TTA AAT GGC ATC TCC TAT GGC AGG AAG AAG CGG-3’) and ES8 (5’- CAG GAA ACA GCT AGT ACC CAC TTC TCC AAT TGT CCC TCA-3’) for the amplification of a 700 bp product and ED5 (5’- ATG GGA TCA AAG CCT AAA GCC ATG TG-3’) and ED12 (5’- AGT GCT
for the amplification of a 1.2 kb product. After successful amplification of either the 700 bp or 1.2 kb portion of the env region, subtyping was carried out by the heteroduplex mobility assay (HMA) as described by Delwart et al using HMA HIV-1 env subtyping kit (NIH AIDS Research and Reference Reagent program, Bethesda, USA).

**Subtyping of HIV-2:** DNA from PBMC of 12 HIV-2 infected individuals was extracted, PCR amplified and subtyped by direct sequencing, as reported earlier.

**Estimation of cytokines:** For the cross sectional analysis of cytokines (IL-10 and IFN-γ), a total of 140 HIV infected individuals were available. Among these 140 individuals, 119 were HIV-1 infected individuals, 14 were HIV-2 infected individuals and 7 were dual infected individuals with both HIV-1 and HIV-2. The HIV-2 status in both monotypic and dual infected individuals was confirmed by nested PCR. Among the 119 HIV-1 infected individuals categorized by the CDC criteria, 68 were asymptomatic (CDC A), 19 were symptomatic without AIDS (CDC B) and the remaining 32 were in AIDS (CDC C).

As the number of HIV-2 and dual infected individuals were small they were grouped only into asymptomatic and symptomatic groups. There were 11 asymptomatic and 3 symptomatic individuals among HIV-2 infected individuals while 4 of the dual infected individuals were asymptomatic and 3 were symptomatic.

The quantitation of IFN-γ and IL-10 was carried out using ELISA kits (Immunotech-Coulter, Marseille, France). The tests were carried out as per manufacturer’s instruction. All the samples were tested in duplicate and the mean value taken for analysis. The standard curve was constructed for each assay based on the standards provided in the kit and cytokine concentration was calculated by extrapolation from the standard curve.

**T cell subset enumeration and HIV-1 viral load estimation:** For HIV-1 infected individuals and healthy controls, T cell subset enumeration was carried out by the standard flowcytometry analysis (FACScan flowcytometer, Becton Dickinson, CA, USA) with SimulSet software as reported earlier.

During the study at one time, the flow cytometer was not functional in our laboratory, affecting the sample collection for the HIV-1 study. This also coincided with the beginning of sample collection on HIV-2 infected individuals. Hence, an alternative method for estimation of CD4/CD8 T cells was evaluated, and this method was used for HIV-2 infected individuals. The Capcellia CD4/CD8 whole blood kit, an immuno-capture ELISA based assay, from BIO-RAD (BIO-RAD, California, USA) (Formerly Sanofi Diagnostics Pasteur, France) was used to measure CD4 and CD8 T cell counts of HIV-2 and dual infected individuals. The test was carried out as per the manufacturer’s instruction with minor modifications as described earlier. Though the kit manufacturer recommended samples to be tested as singletons, we tested all the samples in duplicate and the average was taken for CD4 and CD8 T cell counts.

**Statistical analysis:** EPI info version 6.04d software was used for statistical analysis. Outliers were removed with the Grubbs’ test only to calculate the means of plasma IL-10 and IFN-γ. Kruskall-Wallis test or ANOVA tests were used to compare means. Pearson’s correlation was calculated with Microsoft Excel, and P values for the correlation calculated with “Pcalc” (Kind courtesy Dr. André Charlette, Health Protection Agency, Colindale).

**Results**

**HIV subtype:** Of the 82 patients subtyped, 66 were male (mean age ±SD 33.6 ± 8.9 yr) and 16 were female (mean age ±SD 33.1 ± 10.7 yr). Forty one, 26, 9 and 6 were from Tamil Nadu, Andhra Pradesh, Karnataka and Kerala, respectively. Seventy eight (95.1%) individuals were infected with subtype C and 3 with subtype A and 1 was untypeable.

Based on the available quantity of the specimens a subset of specimens was tested for genotyping.
Among the three subtype A strains characterized, 1 was A2 and 2 were A3. Of the 67 infected with subtype C whose sample was further characterized, 47 strains were classified as C3, 5 as C2. In 15 samples, the heteroduplexes with two plasmids were close to each other and were classified as C (12 were either C3/C2, 2 were either C3/C1 and 1 was either C1/C2). The HIV-1 subtype A patients were from Tamil Nadu (n = 1) and Andhra Pradesh (n= 2).

Among the 12 HIV-2 infected individuals in whom the virus was subtyped, 8 were male (mean age ± SD 33.2 ± 8.4 yr) and 4 were female (mean age ± SD 35 ± 13.4 yr). Among the 3 symptomatic HIV-2 infected individuals, one had a high IL-10 level of 293 pg/ml contributing to the high SD. The IL-10 level among symptomatic HIV-2 infected individuals was significantly higher than in asymptomatic individuals (P <0.05). IFNγ levels were higher in asymptomatic HIV-2 infected individuals compared to normal healthy controls. IFNγ levels in symptomatics were also higher compared to asymptomatic HIV-2 infected individuals. Both differences were however, not significant, probably a reflection of the sample size. The differences in the IL-10 and IFNγ levels among the asymptomatic and symptomatic dual infected individuals were also not significant (Table II).

The mean CD4 T cell counts and percentages were lower in HIV-1 infected individuals compared to controls while CD8 T cell count and percentages were higher in patients (Table III). There was a significant negative correlation between the HIV-1 plasma RNA level and CD+T cell counts (r = -0.44 P < 0.001), CD4% (r = -0.52 P < 0.001), and CD4:CD8 ratio (r = -0.43, P < 0.001). A significant positive correlation was found between HIV-1 plasma RNA level and CD8% (r = 0.28, P = 0.002). There was a negative correlation between HIV-1 plasma

### Table I. Plasma IL-10 and IFNγ levels in normal controls and HIV-1 infected individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>IL-10 (pg/ml)</th>
<th>IFNγ (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal healthy controls</td>
<td>29</td>
<td>1.25 ± 3.9</td>
<td>80.9 ± 65.5</td>
</tr>
<tr>
<td>HIV-1 infected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>68</td>
<td>11.42 ± 11.45 * †</td>
<td>92.4 ± 115.4 *</td>
</tr>
<tr>
<td>Symptomatic non-AIDS</td>
<td>19</td>
<td>16.84 ± 16.49 * †</td>
<td>505.8 ± 707.5 * § § §</td>
</tr>
<tr>
<td>AIDS</td>
<td>32</td>
<td>18.1 ± 13.45 † §</td>
<td>489.5 ± 704.5 * § §</td>
</tr>
</tbody>
</table>

* Minus one outlier

Values are mean ± SD

† P<0.001 compared to controls

§† P<0.05, §§ P<0.001 compared to asymptomatic

§§§ P<0.01 compared to asymptomatic
RNA level and CD8 count but this was not significant ($r = -0.14$, $P = 0.14$).

There was no significant correlation between IL-10 with CD4 counts or plasma HIV-1 viral load. However, there was a significant negative correlation between plasma IFN$\gamma$ level and CD4 absolute count ($P = 0.023$), CD4% ($P = 0.033$) and CD4:CD8 ratio ($P = 0.045$). CD8% ($P = 0.008$) and plasma RNA levels ($P = 0.017$) correlated positively with IFN$\gamma$ level (Table IV).

For HIV-2 and dual infected individuals, T cell subsets were estimated by the Capcellia test. The counts obtained by this technique have been shown to be higher than when estimated by flowcytometry. In HIV seronegative controls, the mean ± SD of CD4 and CD8 counts are 1048 ± 210 and 595 ± 211 cell/µl respectively. The mean CD4 T cell counts among the 9 asymptomatic HIV-2 infected individuals was 832 ± 228 and for the 3 symptomatic individuals was 326 ± 222 cell/µl. CD4 T cells among the 4 asymptomatic dual infected individuals was 596 ± 177 and the same for symptomatic individuals was 258 ± 176 cell/µl. There was no significant correlation between IL-10 or IFN$\gamma$ levels with CD4 and CD8 T cell count among the HIV-2 and HIV-1 and HIV-2 dual infected individuals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>IL-10 (pg/ml)</th>
<th>IFN$\gamma$ (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal healthy controls</td>
<td>29</td>
<td>1.25 ± 3.9</td>
<td>80.9 ± 65.5</td>
</tr>
<tr>
<td>HIV-2 †</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>11</td>
<td>12.4 ± 28.0</td>
<td>160 ± 216</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>3</td>
<td>105 ± 163 *</td>
<td>232 ± 80</td>
</tr>
<tr>
<td>HIV-1 &amp; HIV-2 dual infected ††</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>4</td>
<td>6.9 ± 10.2</td>
<td>77 ± 135</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>3</td>
<td>24.7 ± 35.2</td>
<td>15 ± 15</td>
</tr>
</tbody>
</table>

Values are mean ± SD †$P<0.05$ ††$<0.01$ compared to controls (IL-10)
* $P<0.05$ compared to asymptomatic

| Table III. CD4 T cell counts, percentage of the normal healthy controls and CD4 T cell counts, percentage and the plasma RNA concentrations observed in the HIV-1 infected individuals |
|---|---|---|---|---|
| Subjects                        | CD4 + T cells Cells/µl | %       | CD8 T cells Cells/µl | %       | CD4:CD8 ratio | Plasma RNA (Copies/ml) |
| Healthy controls (n = 29)       | 790 ± 187.2        | 35.6 ± 6.9 | 696 ± 280.9        | 30.3 ± 8.0 | 1.30 ± 0.31 | - |
| HIV-1 infected (n = 119)        |                    |          |                    |          |               |                  |
| Asymptomatic (n = 68)           | 416 ± 249*†        | 19.1 ± 7.9* | 1161 ± 539.1*↑↑    | 53.1 ± 12.6* | 0.407 ± 0.271* | 135025 ± 186579** |
| Symptomatic non AIDS (n=19)     | 190 ± 119          | 11.2 ± 5.7 | 1129 ± 414.6       | 64.2 ± 11.3 | 0.171 ± 0.08 | 270794 ± 254590 |
| AIDS (n=32)                     | 124 ± 173          | 8.5 ± 7.3  | 709 ± 378.0        | 57.5 ± 14.9 | 0.167 ± 0.176 | 424195 ± 239045** |

*CD4 count/CD4% values were not available in one individual.
**RNA value for one individual each was not available
Values are mean ± SD †$P<0.001$, ††$<0.05$, Compared to healthy controls
The difference in plasma IL-10 levels was not significant between the HIV-1, HIV-2 and dual infected individuals. When asymptomatic and symptomatic infections were compared, IFNγ levels were higher in HIV-1 infected individuals than dual infected individuals. However, this difference was not statistically significant, probably a reflection of the high variation seen in the HIV-1 group. The difference in IFNγ levels was however significant when the symptomatic groups of HIV-2 and dual infection were compared ($P < 0.001$). IFNγ levels of HIV-1 infected individuals with AIDS was higher than symptomatic HIV-2 or symptomatic dual infections, however this difference was also not significant.

**Discussion**

Data from different parts of India show a high prevalence of subtype C. In north India, 78.4% of HIV-1 strains were subtype C, with the majority being subtype C3 (68%) while in western India, 96% of the samples were subtype C, with the majority (66%) of them being C3. Ninety five per cent of HIV-1 strains were subtype C among female sex workers in Kolkata. In this study, 68% showed maximum homology to the C3-Indian reference strain. In the present study, the prevalent HIV-1 subtype was similar to that observed in the other parts of India. Ninety five per cent of our strains had maximum homology with subtype C. Among the subtype C strains that were further characterized, 90.38% had maximum homology with genotype C3. Subtype A was detected in 3.7% of the individuals tested. One individual who had all his exposures in the Middle East had a strain that was untypeable. All the HIV-2 strains subtyped in our laboratory were subtype A, the most predominant HIV-2 subtype demonstrated in areas where HIV-2 is an epidemic. Subtype A has also been reported from western India.

Many reports describe a shift from Th1 to Th2/Th0 cytokine profile during the progression of HIV disease. This is characterized by a decline in the production of IL-2, IL-12 and IFNγ with an increase in the production of IL4, IL6 and IL10. Often a shift from a strong Th1 / weak Th2 to a weak Th1 / strong Th2 profile has been observed. However, there is one report on the lack of evidence of switching of cytokine profile from Th1 to Th2.

To identify a shift from Th1 to Th2, we calculated the IFNγ / IL-10 ratio (data not shown). The ratio was significantly higher in normal controls compared to the HIV-1 infected group. The difference in ratio in the different stages of HIV-1 infected individuals was not significant. These findings demonstrate a shift from Th1 to Th2 / Th0 cytokine profile in HIV-1 infected individuals.

The plasma IL-10 level was significantly higher both in HIV-2 infected and dual infected individuals compared to normal healthy controls. Among the HIV-2 infected individuals, plasma IL10 level was significantly higher in symptomatic than in asymptomatic.
asymptomatic individuals, indicating a shift to the Th2/Th0 cytokine profile. A trend towards the Th2 cytokine profile was also noted in the dual infected individuals as disease progressed. However, the difference in the cytokine levels between symptomatic and asymptomatic individuals were not significant, probably because of the small numbers tested. Unlike HIV-1 or HIV-2 infected individuals dual infected individuals clearly showed a decrease in the IFNγ level with progression, probably the additive effect of both the viruses.

Klein et al.29 reported the production of intracellular IL-10 and IFNγ in stimulation experiments on PBMC. They noticed a progressive increase in the proportion of CD4 T cells expressing IL-10 in HIV-1 infected individuals with AIDS compared to uninfected healthy controls. This may be consistent with the increase in plasma IL-10 seen in our population. CD8 T cells expressing intracellular IL-10 was almost undetectable. On the other hand, they also found an increase in CD8 T cells producing IFNγ as disease progressed, unlike in CD4 T cells where there was a decrease in production. The increase in plasma IFNγ seen here could represent the IFNγ produced by the CD8 T cells, which are a large proportion of T cells as disease progresses. There was a drop in IFNγ level in individuals with AIDS in our population compared to symptomatic individuals, probably because of the drop in the percentage of CD8 T cells. This could explain the significant positive correlation between IFNγ and CD8%.

A previous study from our centre reported a dramatic increase in plasma IL-10 and rapid decrease in CD4 counts as asymptomatic HIV-1 infected individuals progressed to AIDS.10 Similar decline in CD4 T cells was also observed in HIV-1 infected individuals from Western India.2 There are other reports of negative correlation between IL-10 and CD4 T cell counts20 and CD4/CD8 ratios.9

We compared the cytokine levels observed in our population with other populations and found that plasma IFNγ was higher in south Indians than the Sicilian counterparts.31 Similar levels of IL10 were seen in south Indians and Sicilians.31 However, plasma IL-10 levels were lower in south Indians compared to Ethiopians.32 Environmental factors, impact of infections or genetic differences could explain the difference in the baseline cytokine levels. More work needs to be done to assess the influence of the various factors on disease progression in HIV infected individuals.

In conclusion, the major subtype of HIV-1 and HIV-2 circulating in south India are subtype C and subtype A respectively. A shifting trend in the cytokine profile from Th1 to Th2/Th0 was seen in HIV-1, HIV-2 and dual infected individuals with disease progression. Though the subtype profile and certain host factors are different from the West, the cytokine profile appears to be similar to that seen in the West.

Acknowledgment

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References


TH1 and TH2 predominance in HIV-infected individuals. 


