

Chemokine profile among human immunodeficiency virus-1 (HIV-1) infected individuals from southern India

Sandeep Ramalingam, Rajesh Kannangai, O.C. Abraham[†], Swaminathan Subramanian[†]
Priscilla Rupali[†], S.A. Pulimood[‡], Mary V. Jesudason* & Gopalan Sridharan

*Departments of Clinical Virology, [†]Internal Medicine, [‡]Dermatology & *Clinical Microbiology
Christian Medical College, Vellore, India*

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Background & objectives: Individuals infected with HIV-1 have higher levels of chemokine producing cells compared to uninfected individuals. It is important to know the changes in chemokine levels associated with rate of progression of disease. There is a paucity of information on the plasma chemokines in HIV-1 infected individuals from India. We therefore carried out this study to estimate the levels of three chemokines namely macrophage inflammatory protein alpha (MIP1 α), MIP1 β and RANTES, in relation to disease status in HIV-1 infected individuals and compared with uninfected individuals.

Methods: RANTES and MIP1 α were estimated using ELISA in 114 HIV-1 infected and 30 controls, whereas MIP1 β was estimated in 101 HIV infected individuals only and 30 controls. The values were compared to the T cell subsets, HIV-1 viral loads and plasma cytokines (interferon gamma and interleukin-10).

Results: Compared to controls the mean MIP1 α and RANTES level among the HIV-1 infected individuals was higher while MIP1 β level was lower in HIV infected individuals except CDC C groups. There was a significant positive correlation for MIP1 α with HIV-1 viral load and IFN γ , for MIP1 α with viral load and IL10. There was a significant negative correlation between MIP1 α with CD4 count and CD4: CD8 ratio and MIP1 β with CD4 count and CD8 count. There was a negative correlation between RANTES values and CD8 per cent.

Interpretation & conclusions: In conclusion, our study showed a significantly higher level of β chemokines in south Indian HIV-1 infected individuals compared to controls. These β chemokines may have the inhibitory effect on HIV-1 only during the initial period and with the progression of disease this inhibitory effect wanes as shown by the positive correlation of β chemokines with HIV-1 viral load.

Key words Chemokines - HIV-1 - India - MIP1 α - MIP1 β - RANTES

Chemokines are classified into three subfamilies α , β and γ based on the position of four cysteine residues that form disulphide bonds. The monocyte tropic strains of HIV-1 use CCR5 and T cell tropic strains use CXCR4

as co-receptors. Some of the T cell strains are dually tropic. The chemokines that bind to these receptors may play an important part in controlling the extent of the infection in the early pathogenesis of the disease^{1,2}. The

three β chemokines of importance in HIV-1 infection are macrophage inflammatory protein 1 alpha (MIP1 α), macrophage inflammatory protein 1 beta (MIP1 β) and regulated on activation, normally T cell expressed and secreted (RANTES). These are the natural ligands for CCR5. The α -chemokine, stromal derived factor 1 (SDF-1) normally binds to CXCR4³. SDF-1 inhibits infection of peripheral blood mononuclear cells (PBMC) by T-tropic HIV-1, which appear later in the disease (symptomatic)⁴. The β chemokines inhibit M-tropic isolates in lymphocytes from uninfected donors or T cell lines. They act through blocking the entry by binding to the HIV co-receptors⁵.

HIV-1 infected individuals have higher levels of chemokine producing cells compared to uninfected controls^{6,7}. *In vitro* secretion of β chemokines from PBMC can be correlated with a low *in vivo* plasma RNA load and a high CD4 count⁸. It has been found that with progression of disease, and in the presence of a low CD4 count, the strains of HIV-1 isolated were resistant to RANTES probably due to switch in the use of co-receptor to CXCR4⁹. Production of β chemokines by stimulated PBMC was significantly higher in asymptomatic HIV infected individuals compared to those in AIDS¹⁰.

The rate of progression in HIV infection is thought to be higher and CD4+ T cells decline steeper in the developing countries like India^{11,12}. In contrast, a study from Africa has not upheld this observation¹³. It is important to know the changes in cytokine/chemokine levels associated with increased rate of disease progression. Our findings on plasma cytokine levels have been reported earlier¹⁴. No information is available on the plasma chemokines levels in HIV-1 infected Indians. We therefore investigated the levels of three chemokines, MIP1 α , MIP1 β and RANTES to test the hypothesis that the plasma chemokine levels in asymptomatic HIV infected individuals will be higher compared to those with symptomatic HIV infection prior to any antiretroviral therapy.

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 four south Indian States of Tamil Nadu, Kerala, Karnataka and Andhra Pradesh. One hundred and fourteen anti-retroviral treatment naïve HIV infected individuals were recruited

into the study by 'convenient sampling', after an informed consent from a subpopulation of those 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. Among the 114 patients, 75 were male and 39 were female with a mean age of 33.3 yr (range 19.0-72.0 yr). Sixty four of these patients were in CDC A, 19 were in CDC B and 31 were in CDC C clinical stages respectively¹⁵. When the patients became symptomatic with opportunistic infections, co-trimoxazole prophylaxis was started as a matter of hospital infectious disease clinic practice. The numbers of samples from HIV-1 infected individuals tested for MIP1 β was only 101 as the availability of plasma in volume was inadequate for the other 13 samples. There were 30 HIV uninfected controls with mean age of 29.7 yr (range 22.0-44.0 yr) recruited into the study after an informed consent. The controls included 21 male and 9 female, not age and sex matched and were laboratory workers and healthy relatives of HIV infected individuals. Follow up samples were available from 9 individuals for plasma MIP1 α and RANTES estimation over a mean period of 17.5 months. Eight individuals were followed up for MIP1 β over an average of 18.9 months.

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 μ l of 5 per cent EDTA and two ml of samples in a 5 ml collection tube containing 60 μ l of 5 per cent EDTA. The 2 ml volume samples were collected in the 5 ml tubes between 0800 to 1000 h to avoid diurnal variation for the CD4+/CD8+ T cell estimation.

Methods: Plasma MIP1 α , MIP1 β and RANTES were estimated with commercial ELISA kits (R&D systems Inc, MN, USA). 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. All the three assays are based on the principle of quantitative sandwich enzyme immunoassay technique. The standards provided with the kits were reconstituted and serially diluted to achieve different concentrations as recommended by the manufacturer. The standards for MIP1 α , MIP1 β and RANTES ranged from 0-1500, 0-2000 and 0-2000 pg/ml respectively.

Briefly, for MIP1 α , 50 μ l of the diluent was added to the appropriate wells, followed by 200 μ l of the diluted standard or plasma. For MIP1 α , 100 μ l of the diluent was added to the wells, followed by 150 μ l of the diluted

standard or plasma. For RANTES, 100 µl of the diluent was added to the wells, followed by 100 µl of diluted plasma samples and diluted standards.

After incubating at room temperature for 2 h, the wells were washed thrice with a automated microplate washer (Elx 50, Bio-Tek instruments, VT, USA) followed by addition of 200 µl of polyclonal antibody against MIP1 α , MIP1 β and RANTES conjugated to horse radish peroxidase (HRP) for the respective assays. After incubating for a further 2 h (one h for RANTES) and washing thrice more, 200 µl of H₂O₂ - tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by the addition of 50 µl of 2N H₂SO₄ after 20 min of incubation. The reading was taken with an automated microplate reader (μ Quant, Bio-Tek instruments, VT, USA) at a wavelength of 450 nm and a reference wavelength of 540 nm. After subtracting the average zero concentration standard optical density, a standard curve was generated with the KC4 software (version 2.7 Bio-Tek instruments, VT, USA) and the concentrations of the analyte were estimated.

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 earlier¹⁶.

Quantitation of HIV-1 RNA in plasma was carried out with Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, NJ, USA) with a quantifiable range of 400 copies/ml (2.6 log) to 750000 copies/ml (5.9 log). The quantitative measurement of interleukin-10 (IL-10) and interferon gamma (IFN- γ) was carried out by commercial ELISA and it has been already reported¹⁴.

Statistical analysis: Data were entered into Epi info 6.04d¹⁷, and this software was used for most of the statistical analysis. Normality of data was assessed with Describe: Winpepi¹⁸. If normally distributed, the mean was calculated. Where the data were not normally distributed, normality of the logarithmic conversion was assessed with Describe: Winpepi¹⁸. After log conversion if the distribution was normal, the geometric means were used. If the data were still not normally distributed, outliers were removed (detected either with Winpepi or Grubbs test at <http://www.graphpad.com/quickcalcs/index.cfm>) and the means calculated. Means were compared either with t test when the values were normally distributed or the Kruskal-Wallis H where the values were not normally distributed. Pearson's correlation coefficient (r) was calculated with Epi info 6.04d¹⁷. P values for the correlation coefficient were calculated with Pcal.exe (courtesy HPA, Colindale, UK); P < 0.05 was considered significant.

Results

The CD4+ and CD8+ T cell counts with percentages, IL-10, IFN γ and the HIV-1 viral load level obtained for the HIV-1 infected individuals and the controls (except viral load) is shown in Table I. In controls, the mean plasma MIP1 α , MIP1 β and RANTES were 7.94, 49.07 and 4136.7 pg/ml respectively (Table II). Most of the RANTES values both in controls and HIV-1 infected individuals were above the quantifiable limit of the standards provided, and there was insufficient plasma for dilution experiments. Hence, the values given are extrapolated with the help of a polynomial regression curve. There was a significant difference in the levels of MIP-1 α and MIP- β but not for RANTES levels between the three CDC groups

Table I. Clinical characteristics of HIV infected individuals and controls

Analytes	Controls	HIV-1 infected individuals		
		CDC A	CDC B	CDC C
n	30	64	19	31
Mean age (yr)	29.7 \pm 5.5	32.5 \pm 9.2	35.6 \pm 9.0	33.5 \pm 8.6
Sex (M/F)	21/9	40/24	12/7	23/8
CD4+ T cell (counts/ μ l)	791 \pm 184	393 \pm 250	200 \pm 115	124 \pm 175
CD4 (%)	35.6 \pm 6.8	18.7 \pm 8.1	11.5 \pm 5.4	8.5 \pm 7.3
CD8+ T cell (counts/ μ l)	694 \pm 276	1117 \pm 496	1195 \pm 422	704 \pm 392
CD8 (%)	30.2 \pm 7.9	53.6 \pm 13.0	65.6 \pm 11.2	59.2 \pm 11.4
IL-10 (pg/ml)*	1.25 \pm 3.9	16 \pm 22.5	20 \pm 27.1	20 \pm 12.7
IFN γ (IU/l)*	84 \pm 64	102 \pm 141	919 \pm 1829	689 \pm 1337
HIV-1RNA (copies/ml)	N/A	1.5x10 ⁵ \pm 1.9x10 ⁵	2.7x10 ⁵ \pm 2.6x10 ⁵	4.3 x10 ⁵ \pm 2.4x10 ⁵

*One outlier value from controls excluded from analysis
Values are mean \pm SD

Table II. Plasma chemokine levels in controls and HIV-1 infected individuals

Subjects	n [†]	MIP1 α (pg/ml)		MIP1 β (pg/ml)		RANTES (pg/ml)	
		Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
Controls	30	7.94	(4.19 - 11.70)	49.07	(44.86 - 3.28)	4136.7	(3905.4 - 368.0)
CDC A	64	21.02**	(17.05 - 4.99)	40.51 ^{δδ}	(34.95 - 6.96)	4544.4 ^Δ	(4108.2 - 980.2)
HIV-1			(10.54 - 7.58)		(29.72 - 8.20)		(3522.5 - 185.7)
CDC B	19	19.06*	(27.69 - 5.25)	38.96	(60.28 - 9.86)	4354.1	(4166.7 - 442.3)
CDC C	31	36.47*** [†]		73.60 ^δ		4804.5 ^{ΔΔ}	

[†] one outlier excluded from analysis for MIP1 α ,

For MIP1 β only 55, 16 and 30 samples available for testing from CDC A, B and C groups respectively

P * <0.01 ** <0.001 compared to controls; [†]*P* <0.05 compared to CDC A; ^δ*P* <0.05 compared to controls; ^{δδ}*P* <0.001 compared to CDC C; ^Δ*P* <0.05 ^{ΔΔ}*P* <0.01 compared to controls

Table III. Correlation among the HIV-1 infected individuals between plasma MIP1 α , MIP1 β and RANTES with T lymphocyte subsets, HIV-1 viral loads and plasma cytokines

	MIP1 alpha		MIP1 beta		RANTES	
	r	<i>P</i>	r	<i>P</i>	r	<i>P</i>
Total WBC count	-0.19	0.04	-0.08	0.43	0.09	0.3
Lymphocyte %	-0.17	0.07	-0.32	0.001	-0.05	0.6
Lymphocyte count	-0.32	<0.001	-0.37	<0.001	0.02	0.8
CD4%	-0.31	<0.001	-0.26	0.009	0.17	0.07
CD4 count	-0.38	<0.001	-0.27	0.006	0.14	0.1
CD8%	0.18	0.06	-0.03	0.8	-0.22	0.02
CD8 count	-0.17	0.07	-0.34	<0.001	-0.11	0.2
CD4:CD8 ratio	-0.29	0.002	-0.18	0.07	0.18	0.06
HIV-1 viral load	0.19	0.045	0.29	0.004	0.05	0.6
IL-10*	0.17	0.07	0.2	0.045	-0.09	0.3
IFN γ *	0.35	<0.001	0.18	0.07	-0.08	0.4

*Data previously published in Ref 14

(*P* < 0.01). Compared to healthy controls, the levels of MIP-1 α , and RANTES were significantly higher in CDC A group (*P* <0.05). The difference in the levels between CDC A and CDC B was not significant for all the three chemokines. However there was a significant difference between CDC A and CDC C for MIP-1 α and MIP- β (*P* < 0.05) (Table II).

Comparing the correlation of the three chemokines with each other in the HIV-1 infected group, there was a positive correlation between MIP1 α and MIP1 β (*r* = 0.6, *P* <0.001). There was a negative correlation between MIP1 α and RANTES (close to being significant), but no significant correlation between MIP1 β and RANTES. There was a significant positive correlation of MIP1 α with HIV-1 viral load, IFN α . There was also a significant negative correlation of MIP1 α with total count, lymphocyte count, CD4 per cent, CD4 count and

CD4: CD8 ratio. There was a significant positive correlation between MIP1 β and HIV-1 viral load. There was also a significant negative correlation for MIP1 β with lymphocyte per cent, lymphocyte count, CD4 per cent, CD4 count and CD8 count. There was a negative correlation between RANTES values and CD 8 per cent (Table III).

Among the follow up patients MIP1 α (mean) showed a decrease, while MIP1 β and RANTES showed an increase but not significant (Table IV). The mean \pm SD of the base line CD4+ cell count and viral load among the 9 followed up individuals were 257 \pm 158 cells/ μ l (median 340) and 100648 copies/ml (median 67039) respectively. The mean \pm SD of the CD4+ cell count and viral load among the same group at the end of the followed up period was 241 \pm 150 cells/ μ l (median 300) and 100492 copies/ml (median 94796) respectively.

Table IV. Changes in plasma chemokines among followed up patients

Stage at the		Followup		Annual change (pg/ml)		
Start	End	n*	Months*	MIP1 α	RANTES	MIP1 β
A	A	6	17.2	-5.503	43.495	-10.062
A	C	2	19.5	3.103	-0.652	21.909
C	C	1	15.9	-6.719	-1.994	12.244

*In the first group (A-A) only 5 patients followed up for 19.2 months for MIP1 β

Discussion

In this study, the plasma MIP1 α and RANTES were higher in HIV infected individuals than in uninfected individuals. However, MIP1 β levels were lower in CDC A and B of the HIV individuals compared to controls. All the three plasma chemokine levels were highest in CDC C, and were lower in the symptomatic group compared to asymptomatic group.

Ondoa *et al*⁶ reported their findings on chimpanzees, who are relatively resistant to HIV-1 or simian immunodeficiency virus (SIV-1) compared with HIV infected and uninfected humans⁶. They found a higher level of chemokine producing cells in HIV infected individuals compared to uninfected controls. This matches with the findings seen in our study with MIP1 α , MIP1 β and RANTES. They also found a positive correlation between MIP1 α and MIP1 β expressing CD4 and CD8 T cells respectively, similar to the positive correlation seen between plasma MIP1 α and MIP1 β in our study. There was no difference in the serum levels of all three β chemokines obtained from long term non progressors (LTNP) and AIDS patients¹⁹. However, when expressed as a quantity per PBMC, AIDS patients had a significantly higher concentration of all three β chemokines compared to LTNP¹⁹. In our study also all three β chemokine values were highest in the HIV-1 infected group.

Kakkanaiah *et al*²⁰ reported that HIV-infected individuals had significantly lower concentrations of plasma β chemokines than healthy uninfected control group. There are reports which showed no correlation between the concentrations of β chemokines in plasma and HIV-1 viral load in HIV-infected individuals^{21,22} but showed a positive association between monocyte chemo-attractant protein (MCP)-1 and plasma RNA²¹. However, in our HIV-infected individuals there was a significant positive association between the levels of MIP1 α and MIP1 β with HIV-1 viral load but not RANTES.

It is understood that a high level of β chemokines alone is not sufficient to reduce the rate of progression. Both CD4 and CD8 T cells can synthesise β chemokines. It has been shown that clones of CD4 T cells from non progressors retain the ability to secrete β chemokines. It is known β chemokines are present in CD8 cells within cytolytic granules, complexed to proteoglycans, and ready for release²². It was demonstrated that CD4 T cells also secrete MIP1 α , MIP1 β and RANTES that protect it against R5 strain of HIV-1, and SDF1 protects it against the X4 strain of HIV-1²³. While some CD4 T cells are infected and die, those that can secrete the chemokines are protected against infection²³. CD4 T cells do not synthesise and secrete β chemokines in progressors, whereas only CD8 T cells secrete²⁴. Hence, the high levels of β chemokines seen in our population may also reflect the higher CD8+ T cell counts previously documented in our population¹⁶.

It is also shown that MIP1 α and MIP1 β had only a weak inhibitory effect on HIV⁹. Of the two, MIP1 α was more inhibitory to R5 strain than MIP1 β . It was reported that when the early and late isolates were correlated with CD4 counts; the isolates were sensitive to β chemokines when the CD4 counts were relatively high, while R5 HIV-1 variants showed an augmented replicative capacity and reduced sensitivity to β chemokine when the CD4 counts were low^{3,9}. It is also shown that the virus sensitivity to inhibition by β chemokines is also associated with changes in V3 amino acid residues positions 311 and 325¹. These changes in the amino acid residue also correlate with non syncytium inducing (NSI) or syncytium inducing (SI) virus phenotype². Analysis of primary virus isolates obtained from single HIV-1-infected individuals who had been sequentially followed up suggested that loss of sensitivity to β chemokines during the course of infection arise in parallel with a virus phenotype switch from NSI to SI². Hence with progression of the disease virus variants that are less sensitive to β chemokine inhibition might be selected. In our followed up patients there was a decline

in MIP1 α values if the individual remained in the same stage over time, but there was an increase in MIP1 α in those who progressed from CDC A to CDC C. With MIP1 β , there was a decrease in values, if the patient remained in CDC A over the time period. However, if the patient progressed to CDC C or remained in CDC C, there was an increase in MIP1 β level. With RANTES, there was an increase if the patient remained in CDC A, but there was a decline in RANTES levels when the patient progressed to CDC C or remained in CDC C. The group in which there is increased RANTES on follow up correlated with the groups with the lowest decline in CD4 counts and the smallest RNA increases on follow up analysis could be seen. Our study showed a significant positive correlation of plasma levels of MIP1 α and MIP β with HIV-1 viral load, which could mean that the virus is not sensitive to the plasma chemokines. Another reason for this positive correlation of viral load and plasma chemokines could be the increase in number of the β chemokine producing T cells caused by the HIV driven chronic T cell activation^{25,26}.

In conclusion, our study showed a significantly higher level of β chemokines in HIV-1 infected individuals compared to controls. These β chemokines may have the inhibitory effect on HIV-1 only during the initial period and as the disease progresses this inhibitory effect wanes as shown by the positive correlation between β chemokines and HIV-1 viral load.

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Reprint requests: Dr G. Sridharan, Department of Clinical Virology, Christian Medical College & Hospital
Vellore 632 004, India
e-mail: g_sridharan@yahoo.com