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Estimation of virological and immunological parameters in subjects from South India infected with human immunodeficiency virus type 1 clade C and correlation of findings with occurrence of neurological disease

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Several studies carried out in Western countries have demonstrated that a number of virological and immunological markers such as viral loads, cytokines, β_2-*microglobulin, neopterin, etc., are elevated in the serum and cerebrospinal fluid (CSF) of human immunodeficiency virus (HIV)-infected individuals with neurological disease. The neurological manifestations of HIV infection noted in Indian patients is different from those reported in Western countries. Moreover, few studies have investigated the role of virological and immunological parameters with respect to the progression of HIV-1 clade C infection in India. In this study, we measured virological (HIV-1 RNA levels) and immunological parameters (CD4 cell count and inflammatory markers) in the plasma and CSF of HIV-1–infected neurologically asymptomatic and symptomatic (with opportunistic infections and/or dementia) subjects. By using clade-specific polymerase chain reaction (PCR), we ascertained that all samples used for the study were infected with HIV-1 clade C. Among the various laboratory parameters evaluated, high viral loads in the CSF, low CD4 counts, and higher levels of interleukin (IL)-1α, IL-6, tumor necrosis factor alpha (TNFα), β_2-microglobulin, and neopterin were noted in HIV-infected subjects with neurological disease as compared to asymptomatic subjects. These data suggest that the markers evaluated in plasma and CSF samples correlated with occurrence of neurological disease in symptomatic individuals as compared to asymptomatic HIV infected subjects.* *Journal of NeuroVirology* (2009) **15, 25–35.

Keywords: cytokine; HIV-1 clade C; inflammatory markers; neurological manifestations

Introduction

Viral invasion of the central nervous system following human immunodeficiency virus (HIV) infection occurs very early in the course of infection (Ellovaara, 1995). Productive viral infection within the central nervous system (CNS) in both neurologically symptomatic and asymptomatic individuals is well recognized (Epstein *et al,* 1997). The identification of factors that correlate with, and possibly contribute to, the

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outcome of infection with HIV is important for our understanding of the pathogenesis and natural history of HIV infection in the nervous system as well as in designing effective therapeutic interventions. The role of the relative contribution of such factors is also important. Three categories of prognostic markers are best documented as having significance in relation to prognosis of HIV infection. These include HIV viral load, CD4 T-cell levels, and quantitation of markers of immune activation in the plasma (Fahey *et al*, 1998). The plasma activation markers are products of immune activation and broadly reflect the degree of inflammatory pathology in an infection.

Several reports on the prognostic significance of inflammatory markers of immune activation have focused on neopterin, which is a product of macrophages stimulated by interferon-γ, and on β_2-microglobulin, which represents activation of many immune cells as well as tumor necrosis factor alpha (TNFα), interleukin (IL)-1, and IL-6 (Ashton *et al*, 1998; Fahey *et al*, 1990; Lifson *et al*, 1992; Mellors *et al*, 1996 and Mellors *et al*, 1997). Although these studies have contributed enormously to the understanding of the neuropathogenesis of HIV infection, they may not be directly relevant to the Indian scenario for a number of reasons. Firstly, all these studies have been conducted in developed countries where HIV-1 clades other than clade C predominate. Secondly, there are potentially important differences in the disease pattern noted between the developed countries and those observed in India, which include differences in the prevalence of coinfections, the rate of disease progression, the type and pattern of neurological manifestations, as well as socioeconomic and ethnic differences in the populations under study. Indeed the predominant clade circulating in India is HIV-1 clade C (Siddappa *et al*, 2004; Ramalingam *et al*, 2005), and earlier observations made at our center that opportunistic infections of the CNS predominate over HIV-induced neuropathology (Santosh *et al*, 1995; Satishchandra *et al*, 2000; Shankar *et al*, 2003; Mahadevan *et al*, 2007). Lastly, HIV-associated dementia (HAD) is reported less frequently in India (Satishchandra *et al*, 2000; Wadia *et al*, 2001). We therefore undertook this study to estimate virological and immunological parameters that contribute to occurrence of neurological disease in HIV-1 clade C infection. For this purpose, the conventional approach of estimating laboratory markers, such as HIV viral loads, CD4 counts, cytokines (IL-1α, IL-6, and TNFα), and inflammatory markers (β_2-microglobulin and neopterin) was adopted and a correlation of their levels to the presence or absence of neurological disease was carried out.

Results

Clinical data

The salient clinical details of the two groups of patients included in this study are presented in Table 1 (asymptomatic subjects) and Table 2 (symptomatic subjects). The mean age of the asymptomatic subjects was 31.7 years (range 20 to 45), whereas that of symptomatic subjects was 34.7 years (range 20 to 68). In both of these groups, males predominated, 14/20 in the asymptomatic group and 16/20 in symptomatic group. The mean of the total number of cells in the cerebrospinal fluid (CSF) was 3.85 cells/mm^3 in the asymptomatic group (range 0 to 25 cells/mm^3), whereas it was 148.15 cells/mm^3 (range 1 to 1200 cells/mm^3) in the symptomatic group. None of the patients in the asymptomatic and symptomatic groups had received highly active antiretroviral therapy (HAART), except one patient in the symptomatic group who received HAART for 1 year prior to admission to our hospital (Subject ID S17; Table 2). The predominant neurological manifestation noted amongst symptomatic subjects were opportunistic infections of the central nervous system (16/20; Table 2); 9/20 presented with tuberculous meningitis (TBM), 3/20 with cryptococcal meningitis, 3/20 with progressive multifocal leucoencephaloapthy (PML), and 1/20 patients with cerebral toxoplasmosis. In contrast, 3/20 patients presented with dementia and 1/20 with myelopathy.

Subtyping HIV-1 using clade C–specific PCR

A clade C–specific polymerase chain reaction (PCR) was carried out as described earlier (Siddappa *et al*, 2004) on samples obtained from asymptomatic and symptomatic patient groups. In every run of the clade-specific PCR, plasmids encoding HIV-1 clade B (pNL4-3) and clade C (pIndie) sequences were included as positive controls. Specimens were amplified by PCR, resolved by gel electrophoresis, and clades were identified according to the size of the bands obtained. HIV-1 clade C–positive specimens showed the presence of a 232-bp product universal to all clades as well as a 138-bp clade C–specific product. In contrast, specimens belonging to other clades were positive only for the 232-bp universal product (data not shown). We identified that all the samples in asymptomatic and symptomatic groups were HIV-1 clade C viruses.

Correlation of virological and immunological markers in asymptomatic and symptomatic patient groups

CD4 cell counts

As depicted in Table 3, the mean CD4 and CD8 cell counts and CD4/CD8 ratio of the HIV-infected

Table 1 Clinical and laboratory data of asymptomatic subjects

	Subject ID	Age	Sex	CD4	CD4/CD8 ratio	Plasma Viral load Log copies/ml	C SF Viral load Log copies/ml	CSF cell count cells/mm^3	Neurological manifestations
1	11	31	M	37	0.06	5.19	UD	nil	None
2	13	36	M	67	0.13	5.9	4.53	nil	None
3	25	36	M	111	0.11	5.53	4.20	nil	None
4	36	28	M	166	0.25	5.76	4.49	2	None
5	12	44	F	178	0.09	4.72	4.92	2	None
6	14	26	M	179	0.27	3.46	3.88	2	None
7	32	31	M	187	0.06	0.06	4.85	2	None
8	19	32	M	195	0.41	4.54	5.28	18	None
9	27	27	F	232	0.31	3.86	UD	nil	None
10	23	45	M	246	0.65	3.47	3.89	nil	None
11	33	23	F	254	0.62	5.09	4.76	nil	None
12	7	20	F	284	0.05	4.96	4.48	4	None
13	31	36	M	360	$<$0.18	4.46	4.18	nil	None
14	34	28	M	372	0.3	5.14	4.27	12	None
15	29	37	M	388	0.27	4.94	4.14	nil	None
16	24	30	F	472	0.3	4.8	5.03	nil	None
17	17	30	F	501	0.5	3.38	3.70	nil	None
18	16	30	M	532	0.32	4.07	4.38	25	None
19	26	30	M	600	0.59	4.v04	4.08	10	None
20	28	43	M	840	$<$0.42	4.38	2.73	nil	None

Note. UD = viral load undetected.

asymptomatic group were 310.05 cells/μl, 953.68 cells/μl, and 0.323 respectively. In contrast, subjects in the symptomatic group exhibited a much lower mean CD4 and CD8 cell counts as well as CD4/CD8 ratios (150.94 cells/μl, 817.12 cells/μl, and 0.22, respectively). There were statistically significant differences between CD4 cell count ($P = .003$) and CD4/CD8 ratios ($P = .01$) between the asymptomatic and symptomatic groups. However, there was no statistical significance noted in CD8 cell counts between the asymptomatic and symptomatic groups. The mean CD4 T-lymphocyte counts and CD4/CD8 ratios in symptomatic and asymptomatic group were, however, lower than the normal values (CD4 = 787 cells/μl; CD4/CD8 ratio = 1.35) established for healthy controls in the laboratory.

Table 2 Clinical and laboratory data of symptomatic subjects

	Subject ID	Age	Sex	CD4 Cells/ul	CD4/CD8 ratio	Plasma Viral load Log copies/ml	CSF Viral load Log copies/ml	CSF cell count Cells/mm^3	Neurological manifestations
1	S15	68	F	38	0.05	5.99	5.77	10	Tuberculous meningitis
2	S14	33	M	50	0.06	6.63	5.40	2	Tuberculous meningitis
3	S17	48	M	54	0.37	UD*	UD*	80	Cryptococcal meninigitis
4	S6	48	M	65	0.23	5.40	5.79	90	Dementia
5	S18	30	M	72	0.12	5.88	5.91	nil	Tuberculous meningitis
6	S11	35	M	83	0.52	5.41	5.24	nil	Tuberculous meningitis
7	S8	28	M	90	0.17	4.97	5.76	5	PML
8	S10	30	M	90	0.14	5.60	7.74	60	Tuberculous meningitis
9	S12	35	M	91	0.36	5.95	5.95	80	Tuberculous meningitis
10	S9	35	M	136	0.21	6.33	6.33	60	Myelopathy
11	S1	35	M	162	0.25	5.35	6.66	800	Cryptococcal meninigitis
12	S2	35	M	162	0.08	5.18	6.09	200	Dementia
13	S13	27	F	180	$<$0.09	5.81	6.29	180	Tuberculous meningitis
14	S20	52	M	188	0.16	5.74	5.93	2	Toxoplasmosis
15	S4	33	F	206	0.23	4.97	5.40	1	Cryptococcal meninigitis
16	S7	30	M	258	0.48	5.02	5.71	1200	PML
17	S19	36	M	310	0.15	4.21	4.45	1	PML
18	S3	20	M	316	0.25	4.14	6.78	10	Tuberculous meningitis
19	S16	36	M	317	0.23	4.74	6.63	180	Dementia
20	S5	30	F	ND	ND	3.76	5.97	2	Tuberculous meningitis

Note. UD = viral loads undetected; ND = not done; PML = progresive multifocal leucoencephalopathy.
*Received antiretroviral therapy for 1 year before admission.

Table 3 CD4, CD8 cell counts and CD4/CD8 ratio in HIV-infected asymptomatic and symptomatic patient groups

	Asymptomatics ($n=20$)		Symptomatics ($n=20$)		
	Mean	Range	Mean	Range	*P* value*
CD4 cell count	310.05	37–840	150.94	38–317	.003*
CD8 cell count	953.68	412–2000	817.12	146–2000	.176
CD4/CD8 ratio	0.323	0.06–0.65	0.2233	0.06–0.52	.01*

*Statistical analysis was done using ANOVA. *P* values $<.05$ are considered significant.

HIV-1 RNA levels

Viral loads were estimated using a real-time PCR TaqMan assay (Kamat *et al*, 2007) in the plasma and CSF of all the patients in asymptomatic and symptomatic patient groups. The detection limit of this assay was 180 copies/ml. In 1/20 patients of the symptomatic group (subject S17; Table 1), viral loads were undetectable in the plasma and CSF as he was on antiretroviral therapy for 1 year prior to recruitment into the study. All the remaining patients in the two groups were not on antiretroviral therapy. However, it was observed that viral loads were undetectable in the CSF of 2/20 patients in the asymptomatic group (subject IDs 11 and 27; Table 1).

Viral loads in the CSF of asymptomatic patients ranged from 2.73 to 5.28 log copies/ml (Table 1), whereas in symptomatic patients it ranged from 4.45 to 7.74 log copies/ml (Table 2). The highest CSF viral load (7.74 log copies/ml) was observed in a symptomatic patient with TBM (Table 2). The amplification curve obtained with the CSF of this patient appeared much earlier as compared to the standard reference plasma sample. As depicted in Figure 1 and Table 4, the mean viral loads in the CSF of symptomatic patients (5.99 ± 0.69 log copies/ml) were much higher as compared to the mean viral loads in the CSF of asymptomatic patients (4.32 ± 0.58 log copies/ml). This increase was found to be statistically significant ($P<.001$). A similar observation was made with respect to plasma viral loads between asymptomatic and symptomatic patient groups ($P=.01$). As depicted in Table 5, CSF HIV-1 RNA levels were significantly higher in 52.63% (10/19) of HIV-1–infected individuals with neurological manifestation (>1 log difference in viral RNA levels) as compared to 10.00% (2/20) of HIV-1–infected neurologically asymptomatic subjects. However, the HIV-1 RNA levels in plasma were higher in asymptomatic subjects (45%) as compared to those with neurological manifestations (10.00%). The proportion of individuals who had comparable levels of HIV-1 RNA in the plasma and CSF were more or less equally distributed between the two groups. Additionally, the mean viral loads in the CSF (5.99 log copies/ml) and plasma (5.32 log copies/ml) were 10-fold higher in patients with neurological manifestations (Table 4) as compared to those without

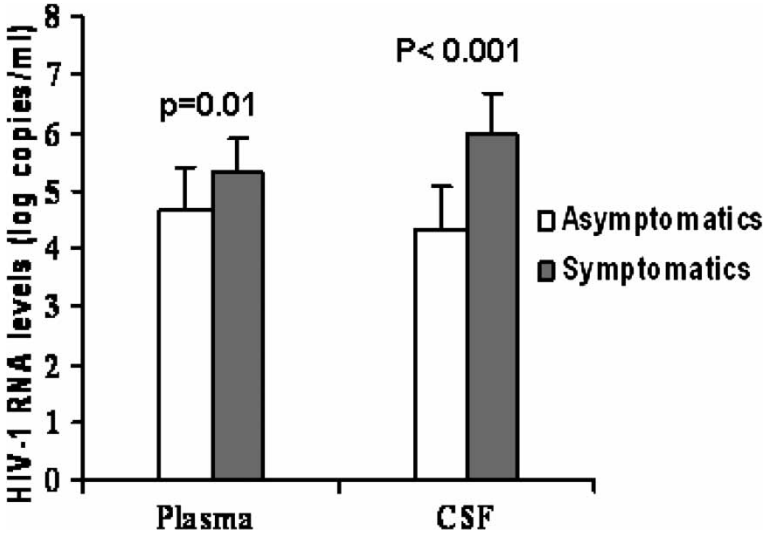

Figure 1 Comparison of mean levels of HIV-1 RNA (log copies/ml; *y*-axis) obtained in plasma and CSF samples (*x*-axis) of asymptomatic (*white bars*) and symptomatic (*gray bars*) group of patients using real-time Taqman assay. Note that mean viral levels obtained in plasma and CSF samples of patients in the symptomatic group were significantly higher compared to the mean levels obtained in patients in the asymptomatic group ($P=.01$ for plasma and $P<.001$ for CSF).

Table 4 Estimation of viral loads, cytokines, neopterin, and β_2-microglobulin levels in plasma and CSF samples of asymptomatic and symptomatic subjects as well as controls

Parameter	Asymptomatic (A)		Symptomatics (B)		Healthy controls (C)		*P* value**					
							A versus B		A versus C		B versus C	
	Plasma*	CSF*	Plasma*	CSF*	Plasma*	CSF*	Plasma	CSF	Plasma	CSF	Plasma	CSF
IL-1α (pg/ml)	68.5 ± 42.8 (30–1050)	7.4 ± 10.49 (2–40)	109 ± 93.07 (20–380)	79.25 ± 56.85 (10–200)	66 ± 96.26 (2–380)	6.75 ± 10.91 (5–30)	> .05	**< .001**	> .05	**< .001**	> .05	**< .001**
IL-6 (pg/ml)	7.35 ± 11.07 (2–22)	10.37 ± 18.47 (2–75)	20.30 ± 20.19 (2–70)	100.5 ± 82.37 (2.5–234.5)	6.50 ± 8.23 (2–22)	3.20 ± 6.06 (2–24)	**< .004**	**< .001**	> .05	> .05	**< .001**	**< .004**
TNFα (pg/ml)	4.9 ± 12.43 (2–50)	2.15 ± 3.63 (2–15)	24.25 ± 31.55 (2–130)	81.25 ± 113.4 (10–500)	2.00 ± 2.23 (2–3.5)	2.00 ± 3.14 (2–4)	**< .03**	**< .01**	> .05	> .05	**.003**	**< .01**
β_2-Microglobulin (g/ml)	3.32 ± 1.49 (1.2–7.7)	3.66 ± 3.47 (0.1–6.2)	4.77 ± 1.88 (2.8–8.8)	8.54 ± 10.50 (1.5–50)	1.66 ± 0.65 (.8–2.9)	0.60 ± 0.48 (.2–1.5)	**< .01**	**< .04**	**< .01**	**< .001**	**< .001**	**< .001**
Neopterin (nmol/L)	18.64 ± 14.79 (7–50)	10.01 ± 9.25 (4.2–66)	44.59 ± 35.29 (7.8–152)	68 ± 45.75 (6.4–142)	9.54 ± 3.24 (5.4–20)	5.41 ± 3.05 (2.8–16)	**< .01**	**< .001**	**.03**	**.04**	**< .001**	**< .001**
Viral load (log copies/ml)	4.64 ± 0.77 (3.47–5.76)	4.32 ± 0.58 (2.73–5.28)	5.32 ± 0.75 (3.76–6.63)	5.99 ± 0.69 (4.45– 7.74)	N/A	N/A	**.01**	**< .001**	N/A	N/A	N/A	N/A

*Mean levels in the group and range in parentheses.
**Statistical significance was obtained using ANOVA followed by Bonferroni multiple comparison test. Statistical values having $P < .05$ are significant (in bold).
N/A = not applicable.

neurological manifestations (plasma 4.64 log copies/ml and CSF 4.32 log copies /ml).

Estimation of inflammatory markers

The levels of inflammatory markers (IL-1 α, IL-6, TNFα, neopterin, and β_2-microglobulin) measured in the plasma and CSF of symptomatic, asymptomatic and healthy subjects is presented in Table 4 and Figure 2A to J. The mean plasma levels of IL-1α, IL-6, and TNFα obtained in healthy controls was 66 ± 96.26, 6.50 ± 8.23, and 2 ± 2.23 pg/ml, respectively. The mean plasma levels of IL-1α, IL-6, and TNFα obtained in asymptomatic patients was 68.5 ± 42.8, 7.35 ± 11.07, and 4.9 ± 12.43 pg/ml, respectively. On the other hand, in symptomatic patients (Table 4) the levels of cytokines were 109 ± 93.07 pg/ml (IL-1α), 20.30 ± 20.19 pg/ml (IL-6), and 24.25 ± 31.55 pg/ml (TNFα). Comparison of the mean cytokine values obtained in the plasma of the three groups revealed that the IL-6 and TNFα levels were significantly higher in the symptomatic group as compared to asymptomatic group and healthy controls (Table 4, A versus B and B versus C; Figure 2C and E).

The mean CSF levels of IL-1α, IL-6, and TNFα obtained in healthy controls was 6.75 ± 10.91, 3.20 ± 6.06, and 2 ± 3.14 pg/ml, respectively. The mean levels of these cytokines in the CSF of asymptomatic subjects was 7.4 ± 10.49 pg/ml (IL-1α), 10.37 ± 18.47 pg/ml (IL-6), and 2.15 ± 3.63 pg/ml (TNFα). In contrast, the mean levels of cytokines in the CSF of symptomatic subjects were found to be higher (79.25 ± 56.85, 100.5 ± 82.37, and 81.25 ± 113.40 pg/ml for IL-1α, IL-6, and TNFα, respectively) as compared to those obtained in healthy controls as well as asymptomatic subjects (Table 4). This increase was found to be statistically significant (Table 4, A versus B and B versus C; Figure 2B, D, and F).

The mean plasma levels of β_2-microglobulin and neopterin in healthy controls were 1.66 ± 0.65 μg/ml and 9.54 ± 3.24 nmol/L, respectively (Table 4). The levels of these two inflammatory markers, on the other hand, were significantly higher ($P < .01$) in the plasma of asymptomatic (3.32 ± 1.49 μg/ml and 18.64 ± 14.79 nmol/L for β_2-microglobulin and neopterin, respectively) and symptomatic (4.77 ± 1.88 μg/ml and 44.59 ± 35.29 nmol/L for β_2-microglobulin and neopterin, respectively) subjects.

The mean CSF levels of β_2-microglobulin and neopterin in healthy controls was 0.60 ± 0.48 μg/ml and 5.41 ± 3.05 nmol/L (Table 4). The levels of these two inflammatory markers, on the other hand, were significantly higher ($P < .05$) in the CSF of asymptomatic (3.66 ± 3.47 μg/ml and 10.01 ± 9.25 nmol/L for β_2-microglobulin and neopterin, respectively) and symptomatic (8.54 ± 10.50 μg/ml and 68.00 ± 45.75 nmol/L for β_2-microglobulin and neopterin respectively) subjects.

Table 5 Comparative analysis of mean levels of viral load, cytokines, and surrogate markers in paired plasma and CSF of HIV-infected asymptomatic ($n = 20$) and symptomatic ($n = 20$) subjects

	Plasma = CSF*		CSF > Plasma*		Plasma > CSF*	
Parameters	Symptomatic subjects	Asymptomatic subjects	Symptomatic subjects	Asymptomatic subjects	Symptomatic subjects	Asymptomatic subjects
Viral load**	7/19 (36.84%)	9/20 (45.00%)	10/19 (52.63%)	2/20 (10.00%)	2/19 (10.52%)	9/20 (45.00%)
IL-1α	2/20 (10.00%)	1/20 (5.00%)	8/20 (40.00%)	0/20	10/20 (50.00%)	19/20 (45.00%)
IL-6	2/20 (10.00%)	4/20 (20.00%)	15/20 (75.00%)	8/20 (40.00%)	3/20 (15.00%)	8/20 (40.00%)
TNFα	6/20 (30.00%)	0/20	13/20 (65.00%)	2/20 (10.00%)	1/20 (5.00%)	2/20 (10.00%)
β_2-Micro globulin	3/20 (15.00%)	6/20 (30.00%)	12/20 (60.00%)	5/20 (25.00%)	5/20 (25.00%)	9/20 (45.00%)
Neopterin	2/20 (10.00%)	2/20 (10.00%)	12/20 (60.00%)	4/20 (20.00%)	6/20 (30.00%)	14/20 (70.00%)

*Assigning a patient into one of the three groups (i.e., Plasma = CSF, CSF > Plasma, and Plasma > CSF) was based on the values obtained in the plasma and CSF for each patient. If the difference in the values between individual plasma and the CSF levels was < 20%, the patient was assigned to the plasma = CSF group. On the contrary, if the difference was > 20%, the patients were assigned to the remaining two groups (i.e., either CSF > Plasma or Plasma > CSF).
**Viral loads were detectable in 19/20 symptomatic subjects. In one subject viral loads were below the detection limit as this patient was on HAART.

Table 5 depicts the comparative analysis of immunological measurements obtained between paired plasma and CSF of patients in the HIV-infected asymptomatic and symptomatic patient groups. Assigning a patient to one of the three groups (plasma = CSF, CSF > plasma, and plasma > CSF) was based on the values obtained in plasma and CSF for each patient. If the difference in the values between plasma and the CSF levels was < 20%, the patient was assigned to the plasma = CSF group. On the contrary, if the difference was > 20%, based on the values the patients were assigned to one of the remaining two groups (CSF > plasma or plasma > CSF). It can be observed from Table 4 that cytokine and inflammatory marker levels were higher in the CSF than in the plasma of the symptomatic subjects as compared to asymptomatic subjects.

Discussion

This study was carried out to investigate if virological and immunological parameters such as HIV viral loads, CD4 counts, and inflammatory markers (IL-1α, IL-6, TNFα, β_2-microglobulin, and neopterin) could be used as laboratory indicators of neurological disease occurrence in HIV-1 clade C–infected individuals. Although several studies have addressed this issue earlier, there have not been any reports that have elucidated the role of all these markers in the same cohort of patients (Gallo *et al*, 1989; Laurenzi *et al*, 1990; Grimaldi *et al*, 1991; Parrella *et al*, 1991, 1992a, 1992b; Tyor *et al*, 1992; Brew *et al*, 1990, 1996, McArthur *et al*, 1992, 1997; Godfried *et al*, 1994; Ellis *et al*, 2000). Moreover, all these investigations have been carried out in countries where viruses other than HIV-1 clade C predominate. The aim of this study was not to construct a precise model of the entire cascade of events leading to neurological damage in HIV infection, rather, to formulate a set of assumptions that would enable the clinician to understand the relative role of laboratory markers in prognosticating the occurrence of neurological disease in HIV-1 clade C–infected individuals.

CD4 lymphocyte count has been viewed as one of the best predictors of the risk of developing acquired immunodeficiency syndrome (AIDS)-related complications. Studies carried out by McArthur *et al* (1997) reported that the mean CD4 cell count for the group with HIV dementia was significantly lower than either the group with HIV minor cognitive/motor disorder or the HIV-infected neurologically asymptomatic group. Similarly in this study, significantly lower CD4 cell counts and CD4/CD8 ratios were observed in HIV-infected group with neurological illness as compared to those who were neurologically asymptomatic (Table 3; $P = .003$ and .01, respectively). In two earlier studies from India, it was noted that the mean CD4 cell counts were lower in AIDS patients as compared to HIV-infected asymptomatic subjects (Sehgal *et al*, 2002; Ghate *et al*, 2000). Clinical conditions correlated well with CD4 cell counts; indeed, CD4 cell counts in HIV-infected individuals with opportunistic infections and those with AIDS were significantly less than those in the asymptomatic patient group (Tables 1 and 2).

Published data regarding correlation between CSF and plasma HIV-1 RNA levels are conflicting. Some investigators reported similar levels in the plasma

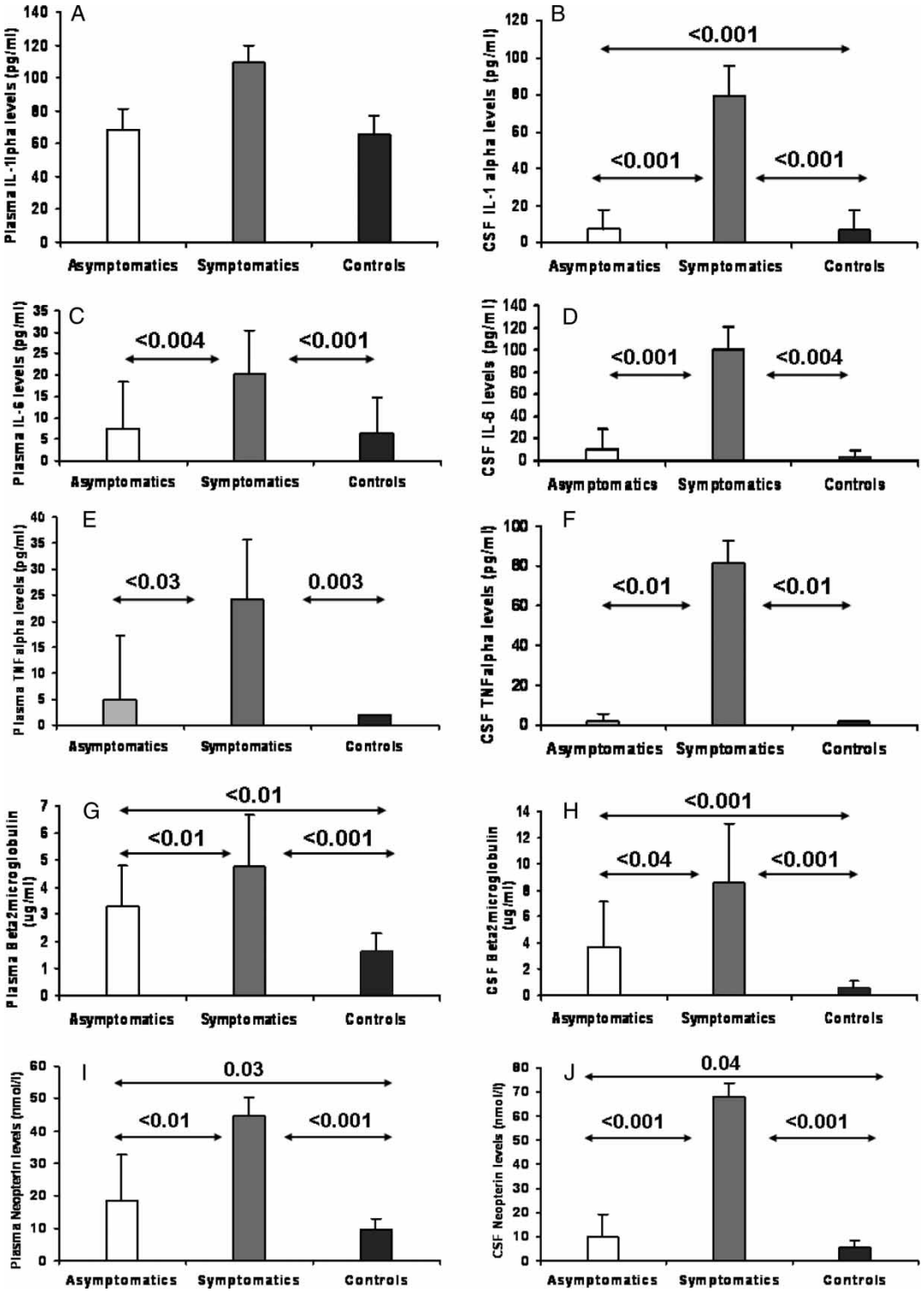

Figure 2 Comparison of mean levels of cytokines and surrogate markers obtained in the serum and CSF of asymptomatic (*white bars*), symptomatic (*gray bars*), and control (*black bars*) subjects. (**A**, **C**, **E**, **G**, and **I**) Mean plasma levels of IL-1 α, IL-6, TNF-α, β_2-microglobulin, and neopterin, respectively. (**B**, **D**, **F**, **H**, and **J**) Mean CSF levels of IL-1 α, IL-6, TNF-α, β_2-microglobulin, and neopterin, respectively. Note that the levels of all the cytokines, with the exception of IL-1α in plasma (**A**), were significantly higher in the symptomatic subjects as compared to asymptomatic as well as control subjects (statistically significant differences are depicted by horizontal bars between groups; **B** to **F**). Similarly, mean plasma and CSF levels of β_2-microglobulin and neopterin were significantly higher in symptomatic subjects as compared to asymptomatic subjects and controls (**G**, **H**, **I**, and **J**, respectively; significant *P* values shown in figure). Further, IL-1α levels in the CSF (**B**) as well as β_2-microglobulin (**G** and **H**) and neopterin (**I** and **J**) levels (both in plasma and CSF) were higher in asymptomatic subjects as compared to controls.

and CSF (Foudraine *et al*, 1998; McArthur *et al*, 1997), whereas others have reported higher levels of HIV-1 RNA in the CSF as compared to plasma (Christo *et al*, 2005; Gisslen *et al*, 1998). Our findings are consistent with those obtained earlier by other investigators who have noted that CSF viral loads were higher in patients with neurological disease as compared to those without active neurological disease (Anderson *et al*, 2001; Christo *et al*, 2005; Ellis *et al*, 2000). Furthermore, the difference in mean CSF and plasma viral loads noted between HIV-infected asymptomatic individuals and symptomatic individuals was statistically significant (Figure 1). Therefore, measurement of HIV-1 RNA levels in the plasma and CSF can be used as a prognostic marker for predicting neurological involvement in HIV infection. Further, it was noted that patients with neurological manifestations showed higher mean levels of HIV-1 RNA in their CSF in this study, as compared to plasma viral levels (Figure 1). High levels of HIV-1 RNA in the CSF reflect increased HIV replication within the brain. In addition, HIV-associated dementia (HAD) has been associated with high levels of HIV CNS viral replication (Ellis *et al*, 2000). The findings of the present study endorse these observations; in three patients, who manifested clinical features of HAD (Table 2), the mean CSF HIV RNA levels were much higher (7.99 log copies per/ml) as compared to those without features of dementia ($n = 17$; mean CSF HIV RNA levels were 5.26 log copies/ml).

The observations summarized in Table 4 indicate that the presence of cytokines and inflammatory markers in the CSF could indeed serve as reliable prognosticators of occurrence of neurological disease. Apart from being consistently detected in the CSF of all symptomatic subjects, the levels of these markers were also significantly higher as compared to HIV-infected asymptomatic subjects as well as controls (Table 4, Figure 2). Additionally, it was interesting to note that the levels of all these markers were higher in the CSF as compared to the plasma of symptomatic subjects, and the reverse, i.e., the levels of all the markers were lower in the CSF as compared to the plasma of asymtomatic subjects, was also true (Table 4). These findings are in concordance with observations made in an earlier study where infiltration of macrophages were noted in the brain of HIV-infected individuals at autopsy (Mahadevan *et al*, 2007).

In this study, increased neopterin levels ($P < .001$) were observed in the CSF of symptomatic individuals as compared to the CSF of asymptomatic individuals and HIV-negative controls (Table 4, Figure 2J). Similar observations have been made earlier by a number of investigators (Anderson *et al*, 2001; Brew *et al*, 1990; Fuchs *et al*, 1989; Griffin *et al*, 1991; Reddy and Grieco, 1989). From these studies, it is apparent that neopterin levels in the CSF increased when neurological disease was present. It is therefore possible that high levels of neopterin in the CSF could provide insights into the pathogenesis of HIV-induced neurological disease.

Apart from neopterin, an increase was also noted with β_2-microglobulin levels in patients with neurological disease as compared to asymptomatic individuals and HIV-negative controls (Table 4, Figure 2G and H). Brew *et al* (1990, 1996) found elevated levels of neopterin and β_2-microglobulin in the CSF of HIV-infected patients with a variety of neurological complications as compared to those without neurological complications. In this study, it was observed that the mean levels of β_2-microglobulin in the CSF (Table 4) was twofold higher than those observed in the plasma of patients with neurological disease as well as in the plasma and CSF of asymptomatic subjects. In an earlier study, CSF and plasma concentrations of β_2-microglobulin were evaluated in 30 patients in various stages of HIV-1 infection (Parrella *et al*, 1991). CSF β_2-microglobulin level and CSF/plasma ratio were significantly higher in patients with neurological complications in comparison to asymptomatic subjects (Parrella *et al*, 1991). These findings indicate that CSF β_2-microglobulin may be a useful marker of neurological involvement in HIV-1 infection.

Very few studies have evaluated the role of IL-1α as a prognosticator of neurological disease in HIV infection. Elevated levels of IL-1α have been reported in the CSF of patients with AIDS dementia complex and IL-1α–positive macrophages have been identified in the brains of these patients (Tyor *et al*, 1992). Circulating levels of IL-1α were much higher than circulating IL-1β levels and therefore IL-1α appears to be the predominant form in circulation (Tyor *et al*, 1992). Significantly higher concentrations of IL-1α ($P < .001$) were noted in the CSF of symptomatic individuals as compared to asymptomatic individuals (Table 4, Figure 2B), suggesting that it could be used as a laboratory marker that indicates neurological involvement in HIV infection.

In the present study, it is noteworthy that mean TNFα levels were more than fourfold higher (24.25 pg/ml) in the plasma of symptomatic subjects (Table 4, Figure 2E) as compared to the mean levels obtained in asymptomatic subjects (4.9 pg/ml). The difference was even more striking with respect to the CSF (Table 4 and Figure 2F) where greater than 40-fold increase was noted in symptomatic subjects (81.25 pg/ml) as compared to asymptomatic subjects (2.15 pg/ml). Consequently, TNFα measurement in the plasma and CSF turned out to be one of the most significant markers of neurological disease in this study. This observation also underscores the fact that higher levels of TNFα in neurologically symptomatic subjects may represent a marker of inflammatory/ infectious activity within the CNS compartment. Indeed, TNFα has been implicated in the neuropathogenesis of HIV infection, probably acting as an important cofactor in the progression

from the asymptomatic stage to clinically manifested AIDS (Godfried *et al*, 1994).

IL-6 is one of the proinflammatory cytokines induced in response to HIV infection. Several studies have reported elevated levels of IL-6 in HIV-infected subjects with neurological disorders (Perrella *et al*, 1992a,b; Gallo *et al*, 1989; Laurenzi *et al*, 1990; Tyor *et al*, 1992). In this study, it was observed (Table 4) that IL-6 levels were markedly elevated in the CSF of HIV-infected patients with neurological disease (mean 100.5 pg/ml, range 2.5 to 234.5 pg/ml) as compared to asymptomatic subjects (mean 10.37 pg/ml, range 2 to 75 pg/ml). In contrast, the difference in IL-6 levels in plasma between these two groups of patients was not very pronounced and there was only a twofold increase in mean IL-6 levels noted in HIV-infected patients with neurological disease as compared to asymptomatic individuals. (Table 4, Figure 2C). It has been reported that IL-6 levels in the CSF are especially high in HIV-infected patients with features of HAD (Perrella *et al*, 1992a,b; Gallo *et al*, 1989; Laurenzi *et al*, 1990). Tyor *et al* (1992), on the other hand, studying the cytokine expression in the brain of AIDS patients did not find high levels of IL-6 in either the plasma or CSF of HIV-infected patients with neurological disease but did find high expression of this cytokine in the cells of brain tissue at autopsy.

In summary, this is the first study to have comprehensively investigated the role of immunological and virological parameters as laboratory markers of neurological manifestation in HIV-1 clade C infection. The findings unambiguously demonstrate that measurement of virological and immunological markers in the plasma and CSF could serve as laboratory markers of the occurrence of neurological disease in HIV-1 clade C infected subjects.

Materials and methods

Patients and samples

This study was carried out at the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, which is a tertiary care hospital and research center in South India. A total of 40 patients were enrolled in the study. Among the 40 patients, 20 were HIV-infected individuals who were neurologically asymptomatic at the time of recruitment (asymptomatic group). All subjects in this group were adults and recruited into a research study investigating the factors responsible for neurological progression and none of them were on antiretroviral therapy (Table 1). Subjects were excluded if they had a previous history suggestive of neurological or psychiatric illness, head injury, substance dependence, hypertension, or diabetes. Subjects with any symptoms of nervous system involvement, including neurocognitive complaints or any recurrent opportunistic systemic infections, were also excluded. The remaining 20/40 patients were HIV-positive adults and presented with neurological opportunistic infections and/or dementia (symptomatic group) and hence were admitted to the neurology wards of NIMHANS hospital for treatment (Table 2). Prior to collection of samples, all the patients underwent a detailed clinical assessment by a neurologist, using a standard neurological evaluation format. All of them underwent computer tomography (CT) scan and/or magnetic resonance imaging (MRI). The salient sociodemographic features and the neurological manifestations noted in these patients are presented in Table 2.

Paired blood and CSF samples were collected from asymptomatic and symptomatic groups at the same time. The CSF samples collected from patients were subjected to routine analysis such as cell counts, glucose levels, tests for cryptococcal culture, antitoxoplasma antibody detection, and culture for *Mycobaterium tuberculosis*. Based on the clinical findings, imaging studies, and laboratory parameters, the symptomatic patients were categorized as those having opportunistic infections, progressive multifocal leukoencephalopathy (PML), dementia, or myelopathy.

To facilitate meaningful interpretation of the data obtained in the study, control CSF samples were collected from HIV-negative individuals (apparently healthy individuals without any neurological symptoms) undergoing spinal anesthesia for minor surgical ailments ($n = 20$), whereas control plasma samples ($n = 20$) were obtained from healthy blood donors (control group). In the control group, plasma and CSF samples were not collected from the same subject. Ethical clearance was obtained from institutional human ethics committee for all participants enrolled in this study and collection of blood and CSF from these groups was carried out after obtaining informed consent. After pretest counseling and obtaining informed consent, 5 ml of peripheral venous blood was collected from these subjects into EDTA-coated vaccutainers. Plasma was separated by centrifuging the blood sample at 1500 rpm for 10 min. HIV infection was confirmed by testing the plasma samples in an initial rapid antibody-screening assay and subsequently by Western blot analysis. HIV-1 subtyping was carried out on all the subjects included in the study using clade C–specific PCR (Siddappa *et al*, 2004).

Estimation of CD4 cell count

CD4 cell count enumeration was carried out by flowcytometry using a fluorescence-activated cell sorting (FACS) count instrument (Becton Dickenson, Singapore). Briefly, 2 ml of patient's blood was collected into potassium EDTA vaccutainers (BD Biosciences, Singapore) and 50 μl of the blood was added to the CD4 and CD8 reagent tube pairs. After

incubation at 37°C for 1 h, 50 µl of fixative solution (5% formalin) was added and the sample was analyzed on the FACS count machine. A paired control reagent tube set containing four calibration beads (zero, low, medium, and high) was included in every run of the assay to verify instrument accuracy and linearity using a blood sample obtained from a healthy individual.

Viral load assay

A TaqMan real-time PCR, described recently by us (Kamat *et al*, 2007), was used to quantify HIV-1 in plasma and CSF samples. Briefly, cDNA was synthesized using the RNA extracted from patient's samples. To control the variability of yield and quality of nucleic acid preparation and reverse transcription, HIV-1 PCR was performed on all samples with a viral reference standard obtained from National AIDS Research Institute, Pune, India. A forward primer (ACC CAT GTT TAC AGC ATT ATC AGA AG) and a reverse primer (GCT TGA TGT CCC CCT ACT GTA TTT) were used to amplify a 80-bp product in the gag region. A highly specific oligonucleotide probe (5′ AGC CAC CCC ACA AGA TTT AAA CAC CAT GT 3′) with a reporter fluorescein dye (FAM) attached to the 5′ end and no quencher (minor groove binding probe) linked to the 3′ end was used for detection.

The PCR was carried out using optical 96-well reaction plates in a 25-µl volume containing each of the 20 × primer probe mix, TaqMan universal master mix, sterile double distilled water, and cDNA (1 to 10 ng) template. For each of the samples PCR was performed in duplicates, whereas for the reference standards PCR was performed in triplicate. Thermal cycling in an ABI Prism 700 machine was initiated with preincubation step at 50°C for 2 min and enzyme activation at 95°C for 10 min. Subsequently, 40 cycles of amplification was carried out at two temperature steps: 15 s at 94°C and 1 min at 60°C.

Estimation of cytokine levels (TNF α, IL-1α, IL-6) in plasma and CSF samples

Cytokine (TNFα, IL-1α, and IL-6) levels in the plasma and CSF were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Diaclone Research, France). The test was carried out according to the manufacturer's instructions. Standard curves were constructed for each of the cytokines by plotting absorbance values obtained with known standards (provided in the kits) against the respective concentrations. The minimum detectable levels of TNFα, IL-6, and IL-1α in the kit was 2, 2, and 5 pg/ml, respectively. The amount of cytokines in plasma and CSF samples of patients and control subjects were subsequently determined by extrapolating absorbance values using the standard curve.

Estimation of inflammatory markers of HIV infection (neopterin and β_2-microglobulin) in plasma and CSF samples

Neopterin and β_2-microglobulin were estimated using commercially available ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Standard curves were constructed for each of the inflammatory markers by plotting absorbance values obtained with known standards (provided in the kit) against the respective concentrations. The minimum detectable level of neopterin and β_2-microglobulin in the kit was 0.7 nmol/L and 0.1 µg/ml, respectively. The amount of the inflammatory markers in plasma and CSF samples of patients and control subjects were subsequently determined by extrapolating absorbance values using the standard curve.

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