[Frontiers in Bioscience 9, 1073-1081, May 1, 2004]

ELEVATED EXPRESSION OF IFN-GAMMA IN THE HIV-1 INFECTED BRAIN

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1. ABSTRACT

We determined the extent of expression of three cytokines (IFN-gamma, IL-4, and TNF-alpha) in brain tissue infected with human immunodeficiency virus-1 (HIV-1). The selections were IFN-gamma as a Th_1 cytokine, IL- 4 as a Th_2 cytokine, and TNF-alpha as a pro-inflammatory cytokine (and because of its prior implication in brain tissue damage due to HIV-1 infection). Based on current models for pathogenesis of HIV-1-associated dementia (HAD), in the periphery, Th1 cytokines are considered to be salutary, whereas Th2 cytokines are regarded as deleterious. However, we hypothesized that in the CNS these roles are reversed. Post-mortem temporal lobe tissue specimens from 16 HIV-1-seropositive patients and 11 HIV-1-seronegative controls were stained for IFN-gamma, IL-4, and TNF-alpha utilizing immunohistochemistry and alkaline phosphatase. HIV-1 infection causes alterations of brain cytokine expression that include increased IFN-gamma expression for HIV-1-seropositive vs. HIV-1-seronegative individuals. There was increased expression of IFN-gamma for HIV-1-seropositive individuals with or without HAD, with or without the broader category of neuropsychiatric impairment (NPI), and with or without opportunistic infections (OIs) compared to HIV-1-seronegatives. A significant inverse correlation between IFN-gamma vs. IL-4 in HIV-1-seropositives with HAD and in seronegative individuals was observed. There was an inverse correlation in seropositives between IFN-gamma vs. TNF-alpha , a positive trend with HAD, significant without HAD, significant with NPI and significant without OIs. Between IL-4 vs. TNF-alpha there was a correlation (trend) in seropositives, a trend with NPI, significant without NPI, and a trend without OI. Due to HIV-1 infection of the brain and neurological disease there is a prominent increased expression of IFN-gamma, an inverse expression of IFN-gamma vs. TNF-alpha , and TNF-alpha vs. IL-4. The inverse correlation between increased IFN-gamma and decreased IL-4 expression is consistent with the stimulation of activated macrophages, and T cells, greater toxicity in the HIV-1-infected brain, and is supportive of the significance of IFN-gamma in HIV-1-infected patients.

2. INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) infects the brain in the early stages of infection (1, 2) and HIV-1 RNA and DNA are detectable in the brains of both symptomatic and asymptomatic patients. HIV-1 associated dementia (HAD) is the most severe manifesting CNS syndrome in HIV-1 infected patients. The spectrum of HIV-1 associated neuropsychological impairments includes sub-clinical cognitive-motor impairment and HIV-1 associated minor cognitive-motor disorder (MCMD) as well as HAD (3, 4, 5, 6). Various pathogenic mechanisms have been proposed to contribute to the development of cognitive impairment including HIV-1 RNA load, HIV-1 proviral load, and its variation across neuroanatomic regions of individuals with evidence for HAD and neurocognitive deficits, HIV-1 strain, and inflammatory processes (7, 8, 9, 10, 11, 12). The pattern of brain imaging techniques related to cognitive-motor dysfunction in HAD suggests that subcortical white matter is the most severely affected (13, 14). Previous histopathologic studies demonstrated that cells infected in the CNS are primarily of monocytes/macrophage lineage (15, 16, 17). Chronic immune activation and increased cytokine expression are associated with neuropathological changes and neuronal damage as well as apoptosis due to HIV-1 infection (18, 19, 20). However, cytokine production is a significant neuropathogenic mechanism that can act at a distance from HIV-1-infected cells (21).

Table 1. Sources of post-mortem brain tissue

No.	A/R/G	HIV Status	DU Status	AT (hrs)	Risk Group/ factor	OI	HAD	NPI	Additional Neurologic Assessment	Cause of Death
1	40/H/M	Positive	DU	27	IDU (c, h)	-	-	+	Disoriented	AIDS, Tuberculosis, PCP
2	35/B/F	Positive	DU	23	IDU	-	+	+	None	AIDS, PCP
3	37/W/M	Positive	No-DU	17.5	HS	-	-	+	Seizures, confusion, hallucinations	AIDS, PCP, Kaposi's Sarcoma
4	44/W/M	Positive	No-DU	8	HS	-	-	+	Severe sensory neuropathy, seizures	AIDS, PCP, Kaposi's Sarcoma
5	43/B/M	Positive	No-DU	8	HS/Corr. Inst	-	+	+	Late HAD, Leukoencephalopathy	AIDS
6	54/B/M	Positive	No-DU	14.5	HTS	-	+	+	HAD, HIV encephalitis	Drowning
7	33/B/M	Positive	No-DU	24	HS/Corr. Inst	-	+	+	Late HAD,Delirium	AIDS, BB, Hypertension, CRF
8	41/W/M	Positive	No-DU	7.5	HS	-	-	-	Numbness of extremities (Peripheral Neuropathy)	AIDS, PCP, Kaposi's Sarcoma
9	51/W/M	Positive	No-DU	9.5	HS	-	-	-	None	AIDS, PCP
10	34/B/M	Positive	No-DU	10	HTS	-	-	-	subacute meningitis with secondary nerve root radiculitis	AIDS
11	14mo/B/M	Positive	No-DU	24	HTS	-	-	-	Calcific Vasculopathy	AIDS, Bronchopneumonia
12	38/B/M	Positive	No-DU	16	HS/Corr. Inst	+	-	-	Toxoplasmosis, CMV	AIDS
13	37/W/M	Positive	No-DU	7	HS/Corr. Inst	+	-	-	Toxoplasmosis, Brain Atrophy	AIDS
14	45/B/F	Positive	DU	14	DU (c)	+	-	-	CMV Ventriculitis, microglial nodule encephalitis	AIDS, Bilateral Pneumonia, Hydrothoraces
15	45 B/M	Positive	No-DU	24	HS/Corr. Inst	+	+	+	NPI, Cryptococcal meningitis, residual com. hydrocephalus	AIDS, Sepsis
16	32/B/M	Positive	No-DU	24	HS/Corr. Inst	+	+	+	NPI, Lymphoma in brain, CMV retinitis	AIDS, PCP
17	24/H/M	Negative	DU	12	DU (c)	-	-	-	trauma	Multiple Blunt Trauma
18	26/B/M	Negative	DU	18	DU (c)	-	-	-	trauma	GSW (head)
19	26/W/M	Negative	DU	13	DU (crk)	-	-	-	none	GSW (heart)
20	19/H/M	Negative	No-DU	16	none	-	-	-	Slight Edema	GSW (several organs)
21	23/W/M	Negative	No-DU	17	none	-	-	-	Depression	Hanging
22	33/W/F	Negative	No-DU	8.5	none	-	-	-	Epilepsy , Right Temporal Lobectomy	Thrombosis
23	60/W/M	Negative	No-DU	10	none	-	-	-	None	Occlusive Coronary Artery Disease
24	24/W/M	Negative	No-DU	12	none	-	-	-	None	Occlusive Coronary Artery Disease
25	21/B/M	Negative	No-DU	14	none	-	-	-	None	Gun Shot Wound to Aorta
26	36/W/F	Negative	No-DU	4.5	none	-	-	-	None	Peritoneal Hemorrhage
27	43/W/M	Negative	No-DU	10	none	-	-	-	None	Arteriosclerotic Coronary Artery Disease

Abbreviations: A: age in years, G: Gender, M: Male, F: Female, R: Race, B: Black, W: White, AT: Autopsy Time, H: Hispanic, DU: Drug user, IDU: Injection drug user, c: cocaine, crk:crack-cocaine, HS: homosexual, HTS: heterosexual, h: heroine, HAD: HIV Associated Dementia, CRF: Chronic Renal Failure, BB: Bilateral, Bronchopneumonia, GSW: Gunshot Wound, PCP: Pneumocystis pneumonia cariniae. Corr. Inst: Correctional Institution. +: presence. -: absence.

Cytokines are detected in normal brains, perform neural functions, and are significant participants in inflammation in both normal and HIV-1 infected brains. In normal brain, cytokines are involved, for example, in synaptic plasticity, neural transmission, and Ca^{+2} signaling (22). Several cytokines, it should be noted, including TNF-alpha and IL-1, are expressed in normal brain in neurons and in glial cells (23, 24, 25). Cytokines are crucial mediators of neuronal injury and destruction in the CNS in HAD. Depending on their effects on the immune system, cytokines may be viewed as "pro-inflammatory" versus "anti-inflammatory." The study of the cytokine profile in brains of patients with HIV-1 infection demonstrates a state of chronic immune activation regardless of the presence or absence of neurological symptomatology (20, 26).

One fundamental question relating to the pathogenesis of HAD is whether there are differences in the consequences of HIV-1 infection of the brain and the peripheral immune system. In the periphery, Th_1 cytokines are considered as salutatory, whereas Th_2 cytokines are considered as deleterious (27, 28, 29). However, in the CNS these roles may be reversed, with their function based on brain macrophage activation centrally (30, 31, 32). Progression of the pathogenic process in HIV-1 infected patients is associated with a switch from Th_1 cell subsets to Th_2 cell subsets in the periphery, when differentiation is present from the Th_0 state. The cytokine production profile of Th_1 cells includes IFN-gamma and IL-2 whereas Th_2 cells profile includes IL-4, IL-5, IL-6, IL-10, and IL-13. In addition, IL-4 stimulates Th_2 cytokine production (27). We selected IFN-gamma as a representative Th_1 cytokine, IL-4 as an example of Th_2 cytokines, and TNF-alpha because of it is a pro-inflammatory cytokine with known destructive effects on myelin (33). We investigated the correlation between expression of IFN-gamma, IL-4, and TNF-alpha in brain tissue specimens from patients with HAD and HIV-1seronegative controls.

3. MATERIALS AND METHODS

3.1. Tissue preparation

Fresh-frozen (cryopreserved) tissue blocks were obtained from autopsied material from the right temporal lobes of 27 individuals, 16 HIV-1-seropositive and 11 HIV-1-seronegative, from the Medical Examiner Offices in Southern Florida, the University of Miami Brain Endowment Bank (Miami, Florida), and the National Human Specimen Research Bank (West Los Angles, CA) Table-1. Samples of cortex,

subcortical, and deep white matter tissue from the temporal lobe were studied in all patients. Tissues were embedded in optimal cutting temperature compound (OCT, Miles, Elkhart, IL), snap frozen in liquid nitrogen, and cryopreserved at -80° C until used. Six-micron (μm) cryostat sections were cut and mounted on "Probe-on plus" silanated slides (Fisher Scientific, Norcross, GA). Sections were fixed in acetone at -20 °C for ten minutes, air dried at room temperature for 15 minutes, and then rehydrated twice in phosphate buffered saline (PBS) for five minutes. Sections were then treated with a protein blocking serum-free solution. A panel of selected monoclonal antibodies was utilized to characterize the expression of IFN-gamma, IL-4, and TNF-alpha utilizing alkaline phosphatase conjugate and fuchsin substrate for these cytokines (Dako Corp.) Detailed methods, sources, as well as dilutions of antibodies used were the same as prior work (8, 12, 34). In addition, Hematoxylin and Eosin stained sections were prepared from each tissue block for morphological assessment. Five repeated determinations (for tissue sections) were performed for each of the three cytokines. All positively stained cells with identifiable Hematoxylin stained nuclei per section were counted using an objective micrometer (Nikon, Tokyo, Japan) at a magnification of 400x. Observers blinded to the study examined sections. The density of positive cells was defined as the number of cells expressing the marker per square millimeter (mm^2). The mean, standard deviation, and standard error were calculated. LPS-stimulated PBMNCs and macrophage/monocyte cell cultures were used for HIV-1-seropositive controls and unstimulated cells as HIV-1-seronegative controls (17). This study focuses on the issue of cytokine expression without addressing the issue of cell identification.

3.2. Statistical Analysis

In order to determine whether positive cell densities were related to total cell densities, and thus should be corrected for different total cell densities among groups, a linear regression analysis was performed for each cytokine-positive and total-cell pair. There was a significant linear trend of positive cells with increasing total cells and the HIV-1 positive group has a larger mean number of positive cells. These findings were similar for IFN-gamma and TNF-alpha . This indicates that an Analysis of Covariance should be used to adjust for differences in positive cells between groups. Tables 2a-5a show this. Correlation analysis (Pearson product-moment) between cytokines was performed as indicated in Tables 2b-5b. Differences between specific subgroups following the ANCOVA utilized the Bonferroni procedure to correct for the number of statistical tests conducted.

4. RESULTS

The HIV-1-seropositive group included fourteen males and two females with a mean age of 38 years (range of fourteen months to 45 years). The control group consisted of nine males and two females with a mean age of 31 years (range of 19 to 60 years). The demographic information is presented in Table 1. None of the HIV-1-seronegative controls had dementia. The HIV-1-seropositive group included six patients with HAD, nine with any level of neuropsychiatric impairment (NPI) (including dementia), and five with opportunistic infections (OI). Three of the HIV-1-seropositive individuals and three of the HIV-1-seronegative individuals were illicit drug users. Ranges in age and autopsy post-mortem interval were similar for HIV-1-seropositive and HIV-1-seronegative individuals.

The number of cells that were immunoreactive for the three cytokines varied among our cases. In general, immunoreactive cells were concentrated more abundantly around blood vessels and fewer in the parenchyma. Generally, cell densities were greater in the white matter than in the cortex. Prior studies demonstrated that multiple brain cell types produce cytokines and our study did not focus on that specific aspect (35).

Cytokine expression in brain tissue is altered because of HIV-1 infection. Our results indicated increased expression of IFN-gamma from 18.5 ± 4.6 cells/mm^2 for HIV-1-seronegative individuals to 233.4 ± 19.6 cells/mm^2 in HIV-1-seropositive individuals ($p < .0001$) (Table 2a). There was no change of expression in TNF-alpha ($p<0.78$) and an increase in expression of IL-4 ($p<0.80$) in HIV-1-seronegative vs. HIV-1-seropositive individuals (Table 2a). There was an increase in the density of cells expressing IFN-gamma for HIV-1-seropositives with (232.5+33.6) or without (234.6+6.5) HAD compared to HIV-1-seronegatives (18.5+4.6) ($p<0.0001$) (Table 3a), with or without the broader category of NPI compared to HIV-1-seronegatives ($p<0.0001$) (Table 4a), and with or without OIs compared to HIV-1-seronegative individuals ($p<0.0001$) (Table 5a). A non-significant increase in densities of cells expressing IL-4 for HIV-1-seropositives without HAD, without NPI, and with OI compared to the other categories in Tables 3a, 4a, and 5a, was observed respectively. There was a non-significant decrease in TNF-alpha for HIV-1-seropositives with OIs (14.4+9.2) (Table 5a) and there were negligible changes for TNF-alpha expression in HIV-1-seropositives with HAD (23.4+7.7) and HIV-1-seropositive individuals with NP (36.9+11.3) (Tables 3a and 4a).

Further splitting the groups among HIV-1-serostatus into HAD and OI or into NPI and OI maintained the significant difference between the HIV-1-seronegative and the HIV-1-seropositive sub-groups. However, this resulted in no significant differences among the stated subgroups for expression of IFN-gamma or the other cytokines.

A comparison of pairs of cytokine expression demonstrated several positive and inverse correlations. There was a significant inverse correlation ($p < .0025$) between IFN-gamma vs. IL-4 in HIV-1-seronegatives (Table 2b). There were no other significant correlations for these individuals. There was a significant inverse correlation ($p < .001$) between IFN-gamma vs. IL-4 in seropositives subjects with HAD (Table 3b) (and for no other seropositive individuals).

There was an inverse correlation between IFN-gamma vs. TNF-alpha , significant for the HIV-1-seropositive group ($p < .001$) (Table 2b), a trend ($p <.0952$) in HIV-1-seropositives with HAD, significant ($p <.0145$) in

Table 2a. Cytokine expression (cells/mm^2): means (± standard error) by HIV-1 Serostatus

Marker	HIV-1 Seronegative		HIV-1 Seropositive		
		n		n	*p
INF-γ	18.5 ± 4.6	6	233.4 ± 19.6	10	0.0001
IL-4	93.0 ± 24.4	5	133.2 ± 21.6	11	.80
TNF-alpha	29.8 ± 9.1	10	29.5 ± 8.1	12	.78

n = number of cases in particular analysis *p = alpha probability from ANCOVA. There is a significant difference for INF-gamma ($F_{1, 13} = 86.5$).

Table 2b. Cytokine expression comparisons by HIV-1 Serostatus

Marker Comparison	HIV-1 Seronegative			HIV-1 Seropositive		
	r	p	n	r	p	n
INF–γ vs IL-4	-0.997	0.0025	4	-0.232	0.616	7
INF-γ vs TNF-alpha	0.073	0.907	5	-0.926	0.0010	8
IL-4 vs TNF-alpha	-0.218	0.725	5	0.643	0.0851	8

n = number of cases in particular analysis. (Pearson correlation coefficient [r] and probability [p]). There are significant inverse correlations between INF-gamma vs IL-4 and INF-gamma vs TNF-alpha . There is a trend relating IL-4 and vs TNF-alpha .

Table 3a. Cytokine expression (cells/mm^2): means (± standard error) by HIV-1 Serostatus and HAD

Marker	HIV-1 Seronegative		HIV-1 Seropositive		HIV-1 Seropositive		
		n	HAD-	n	HAD+	n	*p
INF-γ	18.5 ± 4.6	6	232.5 ± 33.6	6	234.6 ± 6.5	4	< .0001
IL-4	93.0 ± 24.4	5	137.7 ± 28.9	8	121.3 ± 25.0	3	.17
TNF-alpha	29.8 ± 9.1	10	33.9 ± 13.1	7	23.4 ± 7.7	12	.72

Using a 1-way ANCOVA there is no significant difference for IL-4 and TNF-alpha λπηalpha for any of the groups. *p = probability from ANCOVA. There is a significant difference for INF-γ ($F_{2, 12} = 40.5$).

Table 3b. Cytokine expression comparisons by HIV-1 Serostatus and HAD status (Pearson correlation coefficients [r] and probabilities [p])

Marker Comparison	HIV-1 Seronegative			HIV-1 Seropositive HAD$^-$			HIV-1 Seropositive HAD$^+$		
	r	p	n	r	p	n	r	p	n
INF-γ vs IL-4	-0.997	0.0025	4	-0.412	0.491	5	-1.000	0.001[a]	2
INF-γ vs TNF-a	0.073	0.907	5	-0.947	0.0145	5	-0.989	0.0952	3
IL-4 vs TNF-alpha	-0.218	0.725	5	0.665	0.221	5	-0.414	0.728	3

Differences among three or more groups were analyzed using the one-way Analysis of Variance (ANOVA), followed by a pair-wise t-test if the overall F-test was significant. [a] Note that n = only 2 in this comparison. FOR HIV-1 positive HAD-negative subjects INF-gamma vs TNF-alpha varied inversely at a significant level whereas for HIV-1 seropositive HAD-positive individuals there was a trend for their inverse expression.

Table 4a. Cytokine expression (cells/mm^2): means (± standard error) by HIV-1 Serostatus and NPI.

Marker	HIV-1 Seronegative		HIV-1 Seropositive		HIV-1 Seropositive		
		n	NP$^-$	n	NP$^+$	n	p
INF-γ	18.5 ± 4.6	6	263.0 ± 43.6	4	213.6 ± 13.9	6	< .0001
IL-4	93.0 ± 24.4	5	156.6 ± 42.1	5	113.7 ± 18.9	6	.29
TNF-alpha	29.8 ± 9.1	10	19.2 ± 10.9	5	36.9 ± 11.3	7	.57

Using a 1-way ANCOVA there is no significant difference for IL-4 and TNF-alpha for any of the groups. *p = probability from ANCOVA. There is a significant difference for INF-gamms ($F_{2, 12} = 49.2$).

Table 4b. Cytokine expression comparisons by HIV-1 Serostatus and HAD status (Pearson correlation coefficients [r] and probabilities [p])

Marker Comparison	HIV-1 Seronegative			HIV-1 Seropositive NP$^-$			HIV-1 Seropositive NP$^+$		
	r	p	n	r	p	n	r	p	n
INF-γ vs IL-4	-0.997	0.0025	4	-0.319	0.793	3	-0.872	0.128	4
INF-γ vs TNF-a	0.0728	0.907	5	-0.611	0.582	3	-0.943	0.016	5
IL-4 vs TNF-alpha	-0.218	0.725	5	0.999	0.0024	3	0.835	0.079	5

Differences among three or more groups were analyzed using the one-way Analysis of Variance (ANOVA), followed by a pair-wise t-test if the overall F-test was significant. For HIV-1 seropositive and NP-negative subjects INF-gamma co-expressed with TNF-alpha whereas there was a trend for an inverse expression of these variables.

Table 5a. Cytokine expression (cells/mm^2): means (± standard error) by HIV-1 Serostatus and OI.

Marker	HIV-1 Seronegative		HIV-1 Seropositive		HIV-1 Seropositive		
		n	OI$^-$	n	OI$^+$	n	*p
INF-γ	18.5 ± 4.6	6	225.7 ± 13.5	6	244.8 ± 17.9	4	< .0001
IL-4	93.0 ± 24.4	5	111.7 ± 16.4	7	171.0 ± 50.7	4	.44
TNF-alpha	29.8 ± 9.1	10	34.6 ± 10.0	9	14.4 ± 9.2	3	.64

Using a 1-way ANCOVA there is no significant difference for IL-4 and TNF-alpha for any of the groups. p = probability from ANCOVA. There is a significant difference for INF-gamma ($F_{2, 12} = 40.3$).

Table 5b. Cytokine expression comparisons by HIV-1 Serostatus and OI status (Pearson correlation coefficients [r] and probabilities [p])

Marker Comparison	HIV-1 Seronegative			HIV-1 Seropositive OI$^-$			HIV-1 Seropositive OI$^+$		
	r	p	n	r	p	n	r	p	n
INF-γ vs IL-4	-0.997	0.0025	4	-0.270	0.729	4	-0.722	0.486	3
INF-γ vs TNF-a	0.073	0.907	5	-0.919	0.027	5	-0.569	0.615	3
IL-4 vs TNF-alpha	-0.218	0.725	5	0.822	0.088	5	0.980	0.128	3

Differences among three or more groups were analyzed using the one-way Analysis of Variance (ANOVA), followed by a pair-wise t-test if the overall F-test was significant. There was a trend for INF IFN-gamma vs IL-4.

seropositives without HAD (Table 3b), significant (p <.016), in seropositives with NPI (Table 4b), and significant (p <.027) in seropositives without OIs (Table 5b).

There was a correlation between IL-4 vs. TNF-alpha, a trend in HIV-1-seropositives (p < .0851, Table 2b), a trend (p <.079, Table 4b) in HIV-1-seropositives with NPI, significant (p <.0024, Table 4b) in seropositives without NPI, and a trend (p <.088, Table 5b) in HIV-1-seropositives without OIs.

5. DISCUSSION

We found a predominant expression of IFN-gamma compared to IL-4 and TNF-alpha in our HIV-1-seropositive patients with and without HAD, NPI, or OI compared to HIV-1-seronegative controls (36). We have previously demonstrated that HIV-1 penetrates the CNS early in HIV-1 infection since evidence for HIV-1 infection of the brain was found *in vivo* in subjects after development of neuropsychiatric impairment as well as those infected but without impairment (1). As a corollary to this finding and by extrapolation, our current results support the notion of an increased expression of IFN-gamma with HIV-1 infection of the brain. In our study, two other cytokines did not show significant changes because of HIV-1 infection. We found a slight increase in IL-4 but no change in TNF-alpha .

Prior studies have shown different finding of cytokines in the brain. Other investigators (32, 20) did not report a significant expression of IFN-gamma in HIV-1-seropositive patients with HAD. Tyor *et al.* (20) in a study of immunological activation in the brains and CSF of 15 HIV-1 infected patients with AIDS and 11 uninfected controls showed increased expression of MHC-II, IL-1ß, and TNF-alpha in HIV-1-seropositive compared to HIV-1-seronegative controls (20). Their results indicated that generally IFN-gamma staining was more common in HIV-1-seropositive than HIV-1-seronegative specimens although this difference was not statistically significant. However, despite elevated of IFN-gamma in only one CSF sample and no plasma specimens, they detected high levels of neopterin, an indirect measure of IFN-gamma-induced activation of macrophages, in all CSF and plasma specimens. Another study of intracerebral expression of cytokine messenger RNA in brains of 24 HIV-1 infected individuals with and without dementia and 9 HIV-1 uninfected controls demonstrated a strong association between increased TNF-alpha expression and dementia (p=.0035) (32). IL-4 transcripts were undetectable in the brains of demented patients but were detected in brains of many non-demented and control subjects. No significant differences were detected in the amounts of IL-6 or IFN-gamma mRNAs. In both studies, microglia and macrophages showed the highest expression of TNF-alpha and the predominant role of this cytokine in the pathogenesis of HAD was emphasized. Endothelial cells were positive for IL-1 and IFN-gamma and less often for TNF-alpha and IL-6. Additional studies also have demonstrated that macrophage/microglia are the source of TNF-alpha (32, 37, 38, 39).

IFN-gamma is a Th_1 cytokine that has pleotrophic effects that may be part of the mechanism leading to dementia in HIV-1 infection. IFN-gamma activates astrocytes, which in turn enhance their expression of MHC class II HLA-DR antigens (37). These activated astrocytes may present myelin basic protein to encephalitogenic T cell lineages, contributing to the pathogenesis of HAD. In addition, IFN-gamma was shown to augment TNF-alpha and IL-1 production, which leads to further enhancement of HIV-1 replication (40). IFN-gamma also enhances expression of surface MHC class II HLA-DR antigens on astrocytes. IFN-gamma widens the strain susceptibility of macrophage/monocytes from macrophage (M)-tropic (CCR5-dependent) to include T cell (T)-tropic (CXCR4-dependent) HIV-1 infection (41). IFN-gamma associated macrophage stimulation results in the secretion of neopterin that is associated with progression of CNS HIV-1 infection (42) and IFN-gamma-stimulated macrophages increase the production of quinolinic acid, an excitotoxin and convulsant (43).

Our results support a role for IFN-gamma in the pathogenesis of neuropsychiatric disease in HIV-1 infection. Attenuation of macrophage-related toxicity is central in the treatment of HIV-1-associated inflammation in brain, the pathological term for which is HIV encephalitis (HIVE). An example of this type of cell-specific treatment of macrophage/microglia was accomplished using liposome-encapsulated clondrondate in a gerbil model to reduce quinolinic acid in the brain during immune activation (44).

IL-4, a Th_2 cytokine, suppresses macrophage activation; thus, reduction in IL-4 decreases such suppression, increasing the likelihood of macrophage-mediated brain-disease. The neuroprotective effect of IL-4 was found to involve the inhibition of IFN-gamma priming of microglia with a subsequent decrease in the production of TNF-alpha and nitric oxide (45). A decreased expression of IL-4 mRNA in brain tissue of HIV-1 infected individuals with HAD has been reported previously (46). Our results indicated an inverse relationship between IFN-gamma and IL-4 that is associated with compounded activation of macrophages due to the changes in the regulation of both cytokines.

TNF-alpha , the third cytokine in our study is a major pro-inflammatory cytokine and has been frequently studied because of its destructive effects on myelin. A significant elevation of intracerebral expression of TNF-alpha mRNA in HAD has been reported previously and TNF-alpha , has been regarded as the major participant in the development of neurological dysfunction in AIDS patients (31, 32). Sippy *et al* (47) reported increased expression of two specific TNF-alpha receptors in the brains of 13 HIV-1-infected patients, five of whom had clinically documented dementia. In addition, TNF-alpha , as well as IL-1ß and IL-6, is involved in autocrine and paracrine activation of inflammatory process with deleterious effects on neurons and oligodendrocytes (21). However, our results did not reveal such a significant expression of TNF-alpha , in HIV-1 seropositive patients with HAD. This was also observed in HIV-1 seropositive patients with NPI or OI as compared to those without NPI and OI as well as to HIV-1 seronegative controls. This difference between our results and prior reports may be attributed to differences in patient groups, the geography and ethnic composition of the patients in the epidemic, criteria for classification of neuropsychiatric impairment, HIV-1 strains, and differences in laboratory procedures. In addition, many studies were performed *in vitro* and in animal models that may not precisely mimic the human condition (48). It should be noted that one *in vitro* study showed no increased TNF-alpha production, neither due to HIV-1 infection nor by LPS stimulation (49). One explanation may be the stage of the disease at which they died. We know that HIV-1 crosses the BBB early in the course of infection. Perhaps these patients had died at early stages of their disease, while they still had many inflammatory cells that are sources of IFN-gamma. This aspect and the relative roles of Th1 and Th2 cytokines in the brain deserve more investigation. Regarding IL-4 and TNF-alpha , we found in contrast to previous reports (20, 32), only slight differences in these cytokines in HIV-1-seropositives as compared to HIV-1-seronegatives. Their lower level of expression was also observed in HIV-1-seropositive patients with NPI or OI compared to those without NPI or OI and controls.

A recent study on the role of TNF-alpha and IFN-gamma and their relationship in pathogenesis of AIDS also lends further support to our findings. An *et al.* (50) investigated the effects of single nucleotide polymorphisms (SNPs), -179G/T, in the promoter of the interferon-gamma gene (that confers differential TNF-alpha inducibility to the IFN-gamma promoter). This study observed the effects of the polymorphism on the rate of CD4+ T cell depletion in 298 African American HIV-1 seroconvertors. The authors found that the IFN-gamma -179G/T genotype was associated with accelerated decline of CD4+ T cells to less than 200 cells/mm^3 and a diagnosis of AIDS. This indicated that IFN-gamma -179T is a risk factor for AIDS progression. Their results showed that when -179T (that is rarer than -179G) was specifically present, TNF-alpha induced elevated production of IFN-gamma and a more rapid loss of CD^{4+} T cells. Therefore, certain genetic polymorphisms in the IFN-gamma promoter favor greater production of IFN-gamma. This emphasizes its role in the pathogenesis of AIDS rather than the role of TNF-alpha .

Studies of the role of specific SNPs for TNF-alpha in the pathogenesis of HAD have raised additional questions about the role of this cytokine in this disease process. Quasney et al. (51) analyzed HIV-1-infected adults with and without dementia and control populations for a polymorphic site located in the promoter region of the gene coding for TNF-alpha . The A allele at the TNF-alpha -308 site was over-represented among adults with HAD compared to those without dementia (0.28 vs 0.07; OR 5.5; 95% CI 1.8-17.0) and a healthy control population (0.28 vs 0.11). The same of phenomenon of the presence of an adenine at the -308 site was associated with elevated TNF-alpha production in the presence of endotoxin that indicates that genetically, certain individuals secrete higher levels of TNF-alpha, as a general

response to inflammatory reactions rather than a specific response associated with HAD (52). Therefore, the elevation of TNF-alpha level in the presence of HAD only indicates the occurrence of this cytokine as a non-specific marker of inflammation rather than a cytokine unique to HAD. This possibility is the key issue and was not anticipated by earlier studies in this area.

In another study, Brinkman *et al.* (53) addressed whether particular TNF-alpha polymorphisms were associated with clinical course and outcome due to HIV-1 infection. The authors studied the distribution of four TNF-alpha guanine (G) to adenosine (A) transition polymorphisms at positions-376, -308, -238, and -163 in the 5' promoter region of the TNF-alpha gene in a nested case-control study among HIV-1-seropositive individuals of the Amsterdam Cohort. They found that none of the polymorphisms was significantly associated with long-term asymptomatic survival after HIV-1 infection compared with progression to clinical AIDS. Their results did not support a role for known TNF-alpha G to A transition polymorphisms in the clinical course of HIV-1 infection. Another study on the relevance of TNF-alpha promoter polymorphism, a G-to-A polymorphism at position-308 and susceptibility to infection with HIV-1 and progression to AIDS reported that TNF-alpha genotypes do not play a role in HIV-1 disease progression (54). Thus, those TNF-alpha genes lacking the SNPs as well as those with the polymorphisms were not associated with neuropsychological disease in AIDS patients. Based on these observations, there is insufficient evidence to support the role of TNF-alpha in the development of neuropsychiatric manifestations of HAD.

Apart from these data, the presence of TNF-alpha in normal brains has been reported previously (55). This indicates a questionable role of TNF-alpha in the HIV-1 disease process and HAD in spite of the prior findings based on RNA and protein analysis cited above.

We found significant inverse and parallel correlations between pairs of cytokines by HIV-1 serostatus. These comparisons were made because they may be indicative of secondary responses of brain tissue to HIV-1 infection. This type of comparison provides additional information that is not apparent by analysis of each cytokine alone. For example, expression of IFN-gamma varied inversely for IL-4 in HIV-1-seronegatives reflecting their expected mutual Th1- vs. Th2-related inhibition. This relationship exists in the uninfected individuals even at low levels of cytokine expression. IFN-gamma varied inversely for IL-4 in HIV-1-seropositives with HAD. This supports the model in which there is a lessened host-response for IL-4 in HAD and thus no attempt to reverse the predominant increased INF-gamma expression. An increased IL-4 response is mounted in patients without HAD and so they do not exhibit the inverse relationship. The inverse variation of IFN-gamma with TNF-alpha where it occurs is further indicative of the expression of IFN-gamma as the major cytokine in HIV-1-infection. However, the parallel expression of IL-4 with TNF-alpha may reflect a compensatory attempt by the brain to suppress TNF-alpha expression by increasing IL-4 expression that suppresses macrophage activation.

The data in this preliminary study demonstrates that IFN-gamma and not TNF-alpha is significantly expressed in brains of HIV-1-seropositive patients with or without HAD, NPI, or OI. Expression of IL-4 and TNF-alpha both were significantly lower than IFN-gamma. This elevated presence of IFN-gamma supports the hypothesis that in the HIV-1 infection , Th1 (not Th2) cytokines play a major role in the pathogenesis of the brain inflammatory cascade. These effects may be due to the increased activation of macrophages centrally. As pointed out above, there are differences in our cytokine results compared with prior studies as well as differences among the published studies. This may be attributable to several reasons including methods of measurement, differences in clinical diagnoses, and ethnic differences in signs and symptoms. Biological differences may operate among various ethnicities similar to findings in other neuropsychiatric diseases such as Alzheimer's disease (56). In addition, recent evidence from genetic studies supports a different view that there is less involvement of TNFalpha HAD than previously thought.

The findings in this study have potential therapeutic implications and support the use of cytokine modulators and inhibitors for the treatment of brain inflammation (57, 58). Other cytokine therapies such as specific receptor antagonists acting on cytokine networks may provide additional features for the treatment of deleterious brain cytokine expression (59, 60).

6. ACKNOWLEDGEMENTS

We thank Dr. Melvyn Heyes (Curagen, New Haven, CT), Dr. Carol K. Petito, and Dr. Richard Douyon (University of Miami Medical School) for pathologic/clinical analysis, comments, and review and Dr. Wallace W. Tourtellotte (University of California Medical School) and Dr. Deborah C. Mash (University of Miami Medical School) for brain tissue. NIH grants DA 04787, DA 07909, DA 11366, DA 14533, AG 19952, and GM056529 supported this work.

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Key Words: HIV-1, Cytokines, IFN-gamma, IL-4, TNF-alpha, Immunohistochemistry, NeuroAIDS, HIV associated dementia, Neuropsychiatric impairment

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