

**CORRELATION BETWEEN CYTOKINE SERUM LEVELS, NUMBER OF CD4<sup>+</sup> T CELLS/mm<sup>3</sup> AND VIRAL LOAD IN HIV-1 INFECTED INDIVIDUALS WITH OR WITHOUT ANTIRETROVIRAL THERAPY**

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**ABSTRACT.** Seventy-nine HIV-1 infected patients were studied in three groups: Group G<sub>1</sub> – 11 patients with no antiretroviral therapy; G<sub>2</sub> – 40 patients undergoing antiretroviral therapy, 33 with only two nucleoside reverse transcriptase inhibitors (NRTI), and seven with two NRTI and one protease inhibitor (PI), all with viral load (VL) equal or higher than 80 copies of plasma RNA/ml; Group G<sub>3</sub> – 28 patients, 23 on highly active antiretroviral therapy (HAART), 18 with two NRTI and one PI, and five with two NRTI and one non-nucleoside reverse transcriptase inhibitor (NNRTI), the remaining five with combination of two NRTI. All G<sub>3</sub> patients had undetectable viral load for at least the past six months. The control group (G<sub>c</sub>) included 20 normal blood donors without clinical complaints or signs of disease and negative for anti-HIV-1/2 antibodies. Serum cytokine levels pg/ml (TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10) were determined in all patients including controls. CD4<sup>+</sup>T and CD8<sup>+</sup>T lymphocyte counts were made in the 79 patients by flow cytometry; VL determination was by NASBA technology. Analysis of results showed that the number of CD4<sup>+</sup>T and CD8<sup>+</sup>T lymphocytes were higher in G<sub>2</sub> than G<sub>1</sub>, while VL was 0.5 log lower. G<sub>3</sub> patients had similar lymphocyte values to G<sub>2</sub>, however they were chosen for G<sub>3</sub> because their VL was undetectable, different by 4.0 log to G<sub>2</sub>. These results show the effect of antiretroviral treatment in G<sub>2</sub> and G<sub>3</sub> patients with better performance in the latter. Statistical difference was seen between the three groups and controls for serum cytokine behavior: TNF- $\alpha$  [H=48.323; p<0.001;(G<sub>1</sub>=G<sub>2</sub>=G<sub>3</sub>)>G<sub>c</sub>]; INF- $\gamma$ [H=28.992; p<0.001; (G<sub>1</sub>=G<sub>2</sub>=G<sub>3</sub>)>G<sub>c</sub>]; IL-4[H=48.323; p<0.001; (G<sub>1</sub>=G<sub>2</sub>=G<sub>3</sub>)>G<sub>c</sub>]; IL-10[H=47.256; p<0.001; (G<sub>1</sub>=G<sub>2</sub>=G<sub>3</sub>)>G<sub>c</sub>. There was no statistical difference in IL-2 values

between all groups ( $H=6.071$ ;  $p>0.10$ ;  $G_1=G_2=G_3=G_c$ ). In absolute values however,  $G_3$  showed slightly lower TNF- $\alpha$ , IL-4, and IL-10, and higher INF- $\gamma$  and IL-2, to  $G_1$  and  $G_2$ . This suggests a better performance in  $G_3$  patients, especially in IL-2 behavior. For cytokine profile, the three groups showed mature Th0 subset. In  $G_1$  72.73% were mature Th0, and 27.27% Th2;  $G_2$ , 72.50% mature Th0, and 27.50% Th2; and  $G_3$ , 89.29% mature Th0, and 10.71% Th2. There was no statistical difference between groups ( $\chi^2_2=3.014$ ;  $p>0.10$ ;  $G_1=G_2=G_3$ ). Statistical difference was seen between  $G_2$  and  $G_3$  for antiretroviral regimes used ( $\chi^2_1=27.932$ ;  $p<0.001$ ;  $G_3>G_2$ ); HAART with PI predominated in  $G_3$ , suggesting that it was responsible for this better performance. Linear correlation between pairs of variables was made with patient groups only, excluding controls. This was made separately for  $G_1$  and  $G_2$ , 51 patients with detectable VL, and  $G_1$ ,  $G_2$ , and  $G_3$  also including those with undetectable VL. The results showed a strong positive correlation between TNF- $\alpha$  and IL-4; TNF- $\alpha$  and IL-10; INF- $\gamma$  and IL-2; IL-4 and IL-10; IL-2 and CD4<sup>+</sup>. Weak negative correlation was seen between IL-2 and VL. Considering all correlations together, we found that IL-2 had the most correlations – eleven – strong, weak, positive, and negative; it was the only one that correlated with CD4<sup>+</sup> (positively) and LV (negatively). The number of correlations allowed us to evaluate qualitative aspects such as IL-2 correlated positively with INF- $\gamma$  and CD4<sup>+</sup> and negatively with LV; this somehow expresses the compatible profile with subset Th1, which could signify a tendency towards immune response recovery. Determination of cytokine serum values, especially IL-2, could be useful in follow-up of HIV-1 infected patients under HAART together with CD4<sup>+</sup> and VL count.

**KEY WORDS:** serum cytokines, HIV-1/AIDS, HAART.

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## INTRODUCTION

One of the contributing factors to the complexity of HIV-1/AIDS patient follow-up is related to the interference of highly active antiretroviral treatment (HAART). Haase (12) calls our attention to the populations of latently and chronically infected cells where the virus can elude host defenses, perpetuate infection, and escape eradication by HAART. This author reports the dramatic impact of this treatment on suppressing viral replication reducing the pool of stored virus and restoring CD4<sup>+</sup> T cell populations and states that a model of immunity depletion and restoration is based on the limited regenerative capacity of the adult. This model predicts that immune regeneration will be slow, variable, and partial.

Pierson et al. (26) emphasized that more than 60 years of treatment are necessary to eradicate mechanisms of viral persistence to ARV therapy and immune response, based on the half-life of latent HIV-1 reservoirs, represented by latently infected cells with a 43 month half-life.

The progression of HIV-1 infection would appear to depend on the immunogenetics of the host (34) and in the other hand with virologic factors (17).

In view of this, progression markers of HIV-1 infection are important for HAART follow-up. Several studies have indicated the importance of CD4<sup>+</sup> T/mm<sup>3</sup> lymphocyte count (22,28), the determination of viral load (VL) (20,21,22); these being markers of the natural history of HIV-1 infection, biological activity, and therapeutic efficacy. However, they are quantitative indices and do not always show immune function compromise.

Clerici and Shearer, in 1993 (5), reported Th-1/Th-2 polarization to associate the progression of AIDS with loss of IL-2 and INF- $\gamma$  production, together with increased IL-4 and IL-10. They also mentioned that seronegative individuals exposed to HIV-1 showed strong Th-1 response. Other authors have highlighted the Th-1/Th-2 paradigm (30), the transcriptional regulation of Th-1/Th-2 polarization (29), and the relationship of these profiles with infectious diseases (14).

The most important fact in the natural history of HIV-1 infection is the infected organism's incapacity to sustain the vigorous immune response expressed immediately after infection and during the primary infection phase, with or without acute disease; this is characterized by the proliferation of specific cytotoxic T lymphocytes and increased levels of complement (C') binding antibodies. These immunological events precede viremia decline, with the beginning

of the asymptomatic or oligosymptomatic phase that can last years and be followed by AIDS, which results from immunosuppression, establishes itself, and is characterized by the decline of CD4<sup>+</sup> T lymphocytes to below 200/mm<sup>3</sup> and increased viremia (25).

The HAART has produced changes in patient behavior, resulting from the viral population dynamics response to this therapy, many of which change to undetectable viral load (12). However, although there may be control, infection is not eradicated. There is a concomitant clinical improvement in the patient who becomes asymptomatic, the opportunistic infections become more easily controlled, and the CD4<sup>+</sup> T lymphocytes increase, but without reaching normal values. According to Carcelain et al. (4), there are two moments of increased CD4<sup>+</sup> T lymphocytes related to HAART treatment. In the first, the number of CD4<sup>+</sup>/mm<sup>3</sup> cells increases in the first two months of treatment, with a 1.0 to 2.0 log concomitant reduction in VL; this is due to the recirculation of CD4<sup>+</sup> cells, which were retained in the lymphatic system. The second increase is related to VL reduction and stability over time. Thus a 4.0 log reduction in VL can be associated with a 40% increase in CD4<sup>+</sup>/mm<sup>3</sup> cells after 2 years treatment (8). When VL reduction is 1.0 log, this increase is only 5% (8).

The biggest problem for those treating HIV-1/AIDS patients is obtaining information on the functional state of immune response; they need other markers to see if there is immunological recovery or not (13,33).

Cytokine behavior has shown that inhibition of viral replication resulting from ARV can reverse IL-2 and INF- $\gamma$  suppression, at the same time that there is a reduction in IL-4 and IL-10 production (13,31,36). Meira et al. (19) have observed that in AIDS patients with cytokine serum levels of less than 200 CD4<sup>+</sup> T lymphocytes, TNF- $\alpha$  and IL-10 were much higher, while those with more elevated INF- $\gamma$  and IL-2 had more than 350 CD4<sup>+</sup> T/mm<sup>3</sup> lymphocytes. These authors (19) determined cytokine serum levels by ELISA, a simple, low cost procedure. The objective of this study was to evaluate the role of TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10 serum cytokines in HIV-1 infected individuals, whether or not they were sick or under combined antiretroviral therapy, and correlate them with number of CD4<sup>+</sup> T/mm<sup>3</sup> lymphocytes and VL. A cross-sectional study was performed on HIV-1 infected patients with or without ARV treatment at the Tropical Diseases Clinic, Botucatu School of Medicine, UNESP.

## **PATIENTS AND METHODS**

### ***Patients***

Between November 1997 and August 2001, 79 HIV-1 infected individuals, with or without AIDS, were seen at the Special Out-patient Clinic and Tropical Disease Infirmary of Botucatu School of Medicine, UNESP. All had a history compatible with HIV-1 infection, with positive ELISA or Western Blot. Thirty-eight were male, 41 female; ages varied from 18 to 63 years (mean 35.5 years).

Twenty normal individuals were also included, 18 male and 2 female, aged from 24 to 57 years (mean 36.4 years); blood donors from the Botucatu Hemocentre who had given informed consent to participate.

### ***Methods***

#### ***Groups***

Control Group (G<sub>c</sub>): 20 blood donors without clinical complaints and negative for anti-HIV-1/2 antibodies. None of them showed any sign of the disease.

Group 1 (G<sub>1</sub>): 11 HIV-1 infected individuals, with or without AIDS, who had never received ARV. These patients or did not had yet ARV indication, or they had had the HIV-1 infection diagnosis few days before their inclusion in this study.

Group 2 (G<sub>2</sub>): 40 HIV-1 infected individuals, sick or not, on antiretroviral treatment, 33 with just two reverse transcriptase inhibitors analogous to nucleotides (NRTI), and seven on HAART with two NRTI and one protease inhibitor (PI), and viral load (VL) equal to or higher than 80 copies of plasma RNA/ml.

Group 3 (G<sub>3</sub>): 28 HIV-1 infected individuals on antiretroviral treatment, 23 on HAART, 18 with two NRTI and one PI, and 5 with two NRTI and one reverse transcriptase inhibitor non-analogous to nucleotides (NNRTI), the remaining 5 with two NRTI. All G<sub>3</sub> patients had undetectable viral load for at least the past 6 months.

### ***Considered parameters***

#### ***Clinical***

All 79 HIV-1 infected individuals (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) were submitted to full clinical observation including associated opportunistic diseases at time of blood collection for cytokine detection.

In the 68 treated patients (G<sub>2</sub> and G<sub>3</sub>), the specific ARV regimens were also considered: double – two NRTI; or HAART – two NRTI plus one PI, or one NNRTI.

The 20 normal individuals (G<sub>c</sub>) were clinically screened; all were asymptomatic without any signs of the disease.

#### ***Laboratory***

All 79 HIV-1 infected individuals (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) were submitted at the same time to: VL determination; CD4<sup>+</sup> T and CD8<sup>+</sup> T/mm<sup>3</sup> lymphocyte count; and TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10 serum cytokine determinations. Controls (G<sub>c</sub>) were also submitted to TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10 serum cytokine determinations; five controls were chosen at random for *in vitro* cytokine determination.

#### ***HIV-1 plasma viral load determination***

Blood (10ml) from each infected individual was tested for HIV-1 plasma VL by the NucliSens HIV-T (Bio Merieux) test, characterized by amplification of nucleic acids based on NASBA technology (2,16,32).

#### ***CD4<sup>+</sup> T CD8<sup>+</sup> T/mm<sup>3</sup> lymphocyte counts***

Quantitative determination of T lymphocyte subpopulation with CD4<sup>+</sup> and CD8<sup>+</sup> markers was performed using flow cytometry and Coulter Monoclonal Reagents. We collected 3ml of venous blood from each patient (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) using a Vacutainer system, in a tube with EDTA anticoagulant (2mg/ml blood). The sample was kept at room temperature, processed 4 hours after collection, and submitted to Coulter T-890 automatic counter. Next, 100 $\mu$ l of blood was incubated with 10 $\mu$ l CD4<sup>+</sup> (RD1) and CD8<sup>+</sup> (FITC) specific monoclonal antibodies (Coulter Monoclonal Reagents), at room temperature for 10 minutes. Then the Q-prep system (Coulter) was used and analyzed by flow cytometry. Results were shown in relative and absolute numbers of CD4<sup>+</sup> T and CD8<sup>+</sup> T/mm<sup>3</sup> lymphocytes. Controls were mouse IgG, subclasses IgG1, conjugated with RD1, fluorochrome, and FITC.

### ***Serum cytokine determination***

We collected 8ml of blood, in dry tube, from the 79 HIV-1 (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) and the 20 normal (G<sub>c</sub>) individuals; serum was separated, aliquoted, and stored at -70°C. TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10 cytokines were determined by ELISA, using Quantkine IL-2E Kits (R&D Systems, Mineapolis, MN). Initially 96-well microplates were sensitized with determined anti-cytokine monoclonal antibody (TNF- $\alpha$ , INF- $\gamma$ , IL-2, IL-4, and IL-10). Next, 200 $\mu$ l of test, positive control, and negative control sera were added (dilution 1:2); they were incubated at 37°C for periods ranging from 30 to 60 minutes depending on cytokine. Four washes were performed with detergent solution containing 2-chloroacetamide (0.1%). This was repeated until the phase preceding substrate addition. Later, biotin marked plates received streptavidine-peroxidase. After incubation, a substrate of hydrogen peroxide (0.02%) and tetramethylbenzine (2%) was added. Reaction was interrupted at room temperature with 2N sulfuric acid. Results were evaluated by reading optical density (OD) in a Titertek Multiscan automatic ELISA reader at 450nm. Serum cytokine concentrations were calculated from a standard curve (19,23).

### ***In vitro cytokine determination***

We collected 15ml of blood in dry tube with 0.1 heparin from five randomly chosen G<sub>c</sub> individuals, to obtain mononuclear cells by separation in Ficoll-Hypaque gradient (3). The lymphocyte and monocyte rich ring was initially washed in ice-cold EDTA-PBS solution for five minutes at 200g, and then with RPMI culture medium for another five minutes at 200g. After this, the cell suspension was resuspended in 1640 RPMI (Gibco Laboratories, Grand Island, NY) supplemented with 2mM L-glutamine (Sigma Chemical Co. USA), 40 $\mu$ g/ml gentamicine, and 10% deactivated human AB serum (complete cell culture medium: CCCM); mononuclear cell identification and assessment of viability were made by count in 5% Turk and monocyte isolation by neutral red (50 $\mu$ l aliquots of cell suspension were incubated at 37°C for 10 minutes with 0.45ml staining solution at 0.02%). Next, the cell suspension was distributed in 96-well plates at different concentrations according to experiment. For monocyte isolation, after 1 hour incubation at 37°C in 5% CO<sub>2</sub> tension, the non-adherent cells were eliminated by washing the plates with RPMI culture medium. Total mononuclear cells (2x10<sup>6</sup>/ml) were incubated at 37°C in 5% CO<sub>2</sub> tension. The supernatants were aspirated and

centrifuged at 2000 rpm; aliquots were then stored at -70°C until used for cytokine determination. This was performed by ELISA, using commercial kits from R & D Systems as described for serum cytokines.

### ***Statistical analysis***

Comparison between groups G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub>, and G<sub>c</sub> according to serum cytokine determination was made by analysis of variance (ANOVA) for entirely randomized experiments (ERE), and by non-parametric Kruskal-Wallis test (35). Groups G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> were also compared according to CD4<sup>+</sup> T and CD8<sup>+</sup> T/mm<sup>3</sup> lymphocytes, and with VL determination by ANOVA for ERE, and the non-parametric Kruskal-Wallis test (35). Linear comparison between pairs of studied variables was made by Spearman's coefficient (35). Serum cytokine profiles were obtained by mean+2SD of their normal values (35). Thus Th1 subset (31) was defined when IL-2 and INF-γ determinations were higher than mean+2SD of G<sub>c</sub> values while the IL-4 and IL-10 showed values lower than mean+2SD of G<sub>c</sub> values. The mature Th0 subset (31) was considered when the INF-γ and IL-4 values were higher than mean+2SD of G<sub>c</sub> values independent of other elevated cytokines (IL-2 and/or IL-10). Subset Th2 (31) was considered when IL-4 and IL-10 were higher than mean+2SD of G<sub>c</sub> values, while IL-2 and INF-γ were lower than mean+2SD of G<sub>c</sub> values. The proportions of Th1, mature Th0, and Th2 subsets in G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>, patients and proportions of HAART and double antiretroviral treatment regimes in G<sub>2</sub> and G<sub>3</sub> patients were compared by the  $\chi^2$  test (35).

This study was approved by the Ethics Research Committee of Botucatu School of Medicine, UNESP.

## **RESULTS**

### ***Normal values of serum cytokines***

Serum cytokine normal values (pg/ml) were obtained from mean+2SD of values from the 20 normal individuals (G<sub>c</sub>). Thus, normal values in pg/ml were respectively: TNF-α (variation=49-143; mean=97.05; SD=27.68; mean+2SD=152); INF-γ (variation=98-310; mean=204.15; SD=52.87; mean+2SD=309); IL-2 (variation=61-183; mean=109.25; SD=33.76; mean+2SD=176); IL-4 (variation=0-15; mean=7.55; SD=4.03; mean+2SD=15);

IL-10 (variation=0-27; mean=6.05; SD=8.11; mean+2SD=22). Therefore, normal cytokine values (pg/ml) obtained by ELISA, with Quantikine IL-2 E kits (R & D Systems Minneapolis, MN) were: TNF- $\alpha$ =152; INF- $\gamma$ =309; IL-2=176; IL-4=15; and IL-10=22.

#### ***Definition of Th1; mature Th0; and Th2 subsets (31)***

Serum cytokine profiles were defined based on normal values; subset Th1 was considered when cytokine values in pg/ml were: INF- $\gamma$ >309; IL-2>176; IL-4<15; and IL-10<22. Mature Th0 subset when: INF- $\gamma$ >309, and IL-4>15; the other cytokines can show normal, decreased, or increased values. Subset Th2 when: IL-4>15; IL-10>22; INF- $\gamma$ <309; and IL-2<176.

#### ***Difference between serum cytokine values and the in vitro cell values from five normal individuals***

Means+2SD of serum levels and from *in vitro* cell supernatant from normal G<sub>c</sub> individuals were: INF- $\gamma$ =340 and 367 (difference 7.3%); IL-2=192 and 223 (difference 13.9%); IL-4=18 and 20 (difference 10.0%); and IL-10=21 and 20 (difference 4.7%). Differences varied between 4.7 and 13.9 %, and although small, the highest was for IL-2.

#### ***Definition of cytokine serum profiles in the study groups***

Table 1 shows medians of serum cytokines, CD4<sup>+</sup> T and CD8<sup>+</sup> T/mm<sup>3</sup> lymphocyte and VL count of the 79 HIV-1 infected patients, and medians of serum cytokines of the 20 normal individuals in relation to cytokine profiles of each group (G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub>, and G<sub>c</sub>). Analysis of this table shows that CD4<sup>+</sup> T and CD8<sup>+</sup> T count were lower in G<sub>2</sub> than G<sub>1</sub>. G<sub>3</sub> patients had CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocyte counts equal to G<sub>2</sub>. VL was 0.5 log lower in G<sub>2</sub> than G<sub>1</sub>, and 4.0 log between G<sub>2</sub> and G<sub>3</sub>; one of the inclusion criteria for G<sub>3</sub> was undetectable VL. Although values of CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocytes and VL in G<sub>1</sub> were lower than in G<sub>2</sub>, INF- $\gamma$  and IL-2 were higher in the latter, IL-4 was equal, and both IL-10 and TNF- $\alpha$  were lower. G<sub>3</sub> was different to the other groups, with slightly lower values of TNF- $\alpha$ , IL-4 and IL-10; the same value of INF- $\gamma$ ; and the highest IL-2 value, higher than the median in G<sub>c</sub>. The TNF- $\alpha$ , INF- $\gamma$ , IL-4, and IL-10 values in G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> were always higher than the normal cytokine values in G<sub>c</sub>. Although IL-2 was the only cytokine in G<sub>3</sub> higher than in G<sub>c</sub>, it was also the only one in G<sub>1</sub> and G<sub>2</sub> that was lower than in G<sub>c</sub>.

**Table 1.** Median of cytokine values, CD4<sup>+</sup> T and CD8<sup>+</sup> T/mm<sup>3</sup> lymphocyte count, and viral load of the 79 HIV-1 infected patients, and medians of serum cytokines of the 20 normal individuals, according to cytokine profile type (subsets Th1; mature Th0; and Th2) (31) of each group (G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub>, and G<sub>c</sub>).

Groups	Variables	TNF- $\alpha$	INF- $\gamma$	IL-2	IL-4	IL-10	CD4 <sup>+</sup> T	CD8 <sup>+</sup> T	CV		Cytokines (subset)
				(pg/ml)			(n/mm <sup>3</sup> )		(copies/ml)	(log)	
G <sub>1</sub>	Variation	346-850	210-720	54-136	29-63	21-71	113-1024	554-2665	80-200000	1.9-5.3	Mature Th0
	Median	628	333	71	48	56	642	1067	32000	4.5	
G <sub>2</sub>	Variation	120-1161	99-938	54-161	15-117	19-111	38-1543	201-1850	400-220000	2.6-5.3	Mature Th0
	Median	397	513	88	48	40	345	897	12000	4.0	
G <sub>3</sub>	Variation	118-910	201-908	49-164	21-79	15-112	82-777	137-1701	0	0	Mature Th0
	Median	384	516	120	41	39	344	939	0	0	
G <sub>c</sub>	Variation	49-143	98-310	63-183	0-15	0-27	-	-	-	-	Normal
	Median	98	213	107	9	0	-	-	-	-	

Variable	Hypothesis	Calculated Statistics	Significance level	Comments
TNF- $\alpha$	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>	H=48.323	p<0.001	(G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> )>G <sub>c</sub>
INF- $\gamma$	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>	H=28.992	p<0.001	(G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> )>G <sub>c</sub>
IL-2	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>	H=6.071	p>0.10	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>
IL-4	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>	H=48.323	p<0.001	(G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> )>G <sub>c</sub>
IL-10	G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> =G <sub>c</sub>	H=47.256	p<0.001	(G <sub>1</sub> =G <sub>2</sub> =G <sub>3</sub> )>G <sub>c</sub>

G<sub>1</sub>=11 patients with HIV-1 without treatment; G<sub>2</sub>=40 patients with HIV-1 on retroviral treatment, 33 with two NRTI and seven on HAART, with VL>80 copies/ml; G<sub>3</sub>=28 patients with HIV-1 on antiretroviral treatment, 23 on HAART, and five with two NRTI and undetectable VL for at least the past 6 months; G<sub>c</sub>=20 normal blood donors (control group).

We were unable to measure IL-4 and IL-10 levels (equal to zero) in some control group (G<sub>c</sub>) individuals. There was no statistical difference between G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub> for INF- $\gamma$ , IL-4, IL-10, and TNF- $\alpha$ . All were statistically different to G<sub>c</sub>. There was no statistical difference in IL-2 between all groups, including G<sub>c</sub>. With regard to cytokine profiles, no group (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) showed subset Th1 profile (31); all three showed mature Th0 subset (31).

The distribution of serum cytokine profiles in the 79 HIV-1 infected patients (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) showed that none had Th1, or naive Th0 subsets (31). Therefore from the 11 G<sub>1</sub> patients, eight (72.73%) were mature Th0 subset, and the other three (27.27%) Th2; in G<sub>2</sub>, 29 (72.50%) were mature Th0 subset and 11 (27.50%) Th2; and in G<sub>3</sub>, 25 (89.29%) were mature Th0 subset and only three (10.71%) Th2. There was no statistical difference between groups ( $\chi^2=3.014$ ; p>0.10; G<sub>1</sub>=G<sub>2</sub>=G<sub>3</sub>).

**Table 2.** Linear correlation between pairs of variables (TNF- $\alpha$ ; INF- $\gamma$ ; IL-2; IL-4; IL-10; CD4<sup>+</sup>/mm<sup>3</sup>; CD8<sup>+</sup>/mm<sup>3</sup>; and viral load – VL), when two different group combinations were considered: G<sub>1</sub> & G<sub>2</sub>; and G<sub>1</sub>, G<sub>2</sub> & G<sub>3</sub>.

Correlation		G <sub>1</sub> & G <sub>2</sub>							
		TNF- $\alpha$	INF- $\gamma$	IL-2	IL-4	IL-10	CD4 <sup>+</sup>	CD8 <sup>+</sup>	VL
G <sub>1</sub>	TNF- $\alpha$		-0.65**	-0.42**	0.33*	0.46**	-0.30*	-0.15	0.07
	INF- $\gamma$	-0.70**		0.52**	-0.26	-0.39**	0.30*	-0.22	-0.13
	IL-2	-0.55**	0.54**		-0.28*	-0.28*	0.39**	-0.22	-0.03
G <sub>2</sub>	IL-4	0.39**	-0.41**	-0.35*		0.10	-0.19	-0.10	0.16
&	IL-10	0.54**	-0.49**	-0.45**	0.38**		-0.20	0.19	-0.20
G <sub>3</sub>	CD4 <sup>+</sup>	-0.26*	0.26	0.27*	-0.16	-0.19		-0.12	-0.44**
	CD8 <sup>+</sup>	-0.16	-0.04	-0.09	-0.16	-0.003	0.004		0.06
	VL	0.08	0.12	-0.23*	0.14	0.03	-0.03	0.08	

**Spearman point correlation:**

G<sub>1</sub>, G<sub>2</sub> & G<sub>3</sub>: for  $\alpha = 0.05$   $r = 0.23(*)$ ; for  $\alpha = 0.01$   $r = 0.30 (**)$

G<sub>1</sub> & G<sub>2</sub>: for  $\alpha = 0.05$   $r = 0.27(*)$ ; for  $\alpha = 0.01$   $r = 0.35 (**)$

G<sub>1</sub>: 11 patients with HIV-1 infection without treatment;

G<sub>2</sub>: 40 patients with HIV-1 infection on antiretroviral treatment, 33 with NRTI, and seven on HAART, with 80 copies/ml VL;

G<sub>3</sub>: 28 patients with HIV-1 infection on antiretroviral treatment, 23 on HAART, and five with two NRTI and undetectable VL for at least the last 6 months.

**Antiretroviral therapy**

From the 68 under treatment, 40 were G<sub>2</sub>, of which 33 (82.5%) received two NRTI and the other seven (17.5%) two NRTI and one PI; from the 28 G<sub>3</sub>, 18 (64.28%) received two NRTI and one PI, and the other five (35.72%) two NRTI and one NNRTI. There was statistical difference between G<sub>2</sub> and G<sub>3</sub> ( $\chi^2_1 = 27.932$ ;  $p < 0.001$ ;  $G_3 > G_2$ ); the triple regimen with PI (HAART) predominated in G<sub>3</sub> patient treatment.

### ***Linear Correlation between pairs of variables***

Table 2 shows the linear correlation between the pairs of variables considering two study groupings: all 79 patients (G<sub>1</sub>, G<sub>2</sub> and G<sub>3</sub>), and the detectable VL groups (G<sub>1</sub> and G<sub>2</sub>), 51 patients. There was a strong positive correlation for all three groups between TNF- $\alpha$  and IL-4; TNF- $\alpha$  and IL-10; INF- $\gamma$  and IL-2; and IL-4 and IL-10. There was also strong positive correlation for the two detectable VL groups between TNF- $\alpha$  and IL-10; INF- $\gamma$  and IL-2; and IL-2 and CD4<sup>+</sup>. There was weak positive correlation for all three groups between IL-2 and CD4<sup>+</sup>. There was also weak positive correlation for the two detectable VL groups between TNF- $\alpha$  and IL-4; and INF- $\gamma$  and CD4<sup>+</sup>. There was strong negative correlation for the three groups between TNF- $\alpha$  and INF- $\gamma$ ; TNF- $\alpha$  and IL-2; INF- $\gamma$  and IL-4; INF- $\gamma$  and IL-10; IL-2 and IL-4; and IL-2 and IL-10. There was also strong negative correlation for the two VL detectable groups between TNF- $\alpha$  and INF- $\gamma$ ; IL-2 and TNF- $\alpha$ ; and INF- $\gamma$  and CD4<sup>+</sup>. There was weak negative correlation for the three groups between INF- $\gamma$  and CD4<sup>+</sup>; and IL-2 and CV. There was also a weak negative correlation for the two VL detectable groups between TNF- $\alpha$  and CD4<sup>+</sup>; IL-2 and IL-4; and IL-2 and IL-10. Considering the correlations together, that is, analyzing the results obtained both in the three groups and the two detectable VL groups, it was possible to establish which variables showed the highest number of positive and negative, and strong and weak correlations suggesting a marker hierarchy; IL-2 showed 11 correlations, TNF- $\alpha$ : 10; INF- $\gamma$ : 8; IL-10: 7; IL-4: 6; CD4<sup>+</sup>: 6; VL: 2; and CD8<sup>+</sup>: none. The study of correlations allowed us to evaluate both quantitative and qualitative aspects. We can therefore show that IL-2 has positive correlation with INF- $\gamma$  and CD4<sup>+</sup>, and negative with VL. This is due to IL-2 and INF- $\gamma$  corresponding to Profile 1, and the higher the value of CD4<sup>+</sup> count and the lower the viral load (undetectable), the more competent the patient's immune response.

## **DISCUSSION**

The destruction of CD4<sup>+</sup> T lymphocytes has generally been accepted as a direct consequence of the immune-pathogenesis in HIV-1 infection. Also that viruses indirectly control non-infected CD4<sup>+</sup> cell mechanisms driving them to apoptosis and destroying them after antigenic activation (11). Many authors have found a reduction in IL-2, IL-12, and INF- $\gamma$ , and an increase in IL-4 and IL-10 production during the evolution of HIV-1 infection (1,6,15). Other

authors have reported that combined HAART causes a reduction in viral replication, reverses IL-2 and INF- $\gamma$  suppression, and decreases IL-4 and IL-10 production (4,11,12,13,19,31). However, the increase in CD4<sup>+</sup>, as a result of the antiretroviral treatment, is complex. It is in part due to the blood trafficking these cells, which were sequestered in the lymphatic compartment (24), also to the increased production rate of circulating lymphocytes resulting from the expansion of peripheral T cells (10), apoptosis suppression, and new T cell production (9). To help interpret patient immune response during antiretroviral therapy, mainly HAART, and verify if immunity regeneration is occurring, other progression markers should be used with CD4<sup>+</sup> cell and VL count. Haase (12) reported that immunity regeneration by treatment is slow, variable, and partial. In fact there is not always an increase in CD4<sup>+</sup> cells, and when there is, it may be due to recirculation of cells retained in the lymphatic system and not naive or memory cells. Also when VL becomes undetectable and remains so in later controls, it cannot be used to evaluate immunity recovery. This is a cross-sectional study with HIV-1 patients in three groups according to antiretroviral therapy: one group without treatment, and the other two with treatment; VL in one group being equal or higher than 80 copies, and in the other undetectable. A fourth group of normal uninfected individuals were used as controls to establish normal serum values, define profiles, and types of cytokines (31). In the HIV-1 groups, we also considered CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocyte count, and VL.

There is no international normal standard for serum cytokines; they are produced by activated T cells. It is possible to determine cytokine levels in activated cell supernatant or serum using ELISA as in this study (18,19,31). These methods measure the quantity of cytokines produced by T cell population, as it is impossible to estimate the quantity of cells that produced them (18). In this study, we compared serum and *in vitro* cytokines collected from five randomly selected control group individuals. There was no significant difference in “1”, “mature 0”, and “2” profile cytokine definer values (INF- $\gamma$ , IL-2, IL-4, and IL-10). There was significant difference only for TNF- $\alpha$ , which is not a profile definer. The levels of profile definer cytokines were therefore equivalent by both methods in this study.

CD4<sup>+</sup> and CD8<sup>+</sup> cell count was higher in the non-treated group (G<sub>1</sub>) than the treated groups (G<sub>2</sub> and G<sub>3</sub>), which were equivalent independent of VL detection and without differences in behavior. These observations do not invalidate the efficacy of antiretroviral therapy but in a way agree with Haase (12) that immunity regeneration under this treatment is slow, variable, and partial, especially when naive cells (CD45RA) and memory cells (CD45RO) are considered. Gougeon et al (11) agreed that immune system restoration is quantitatively and qualitatively partial despite having shown an increase in these cell populations together with a

reduction in apoptosis after 18 months of treatment. In other words, when study groups are not classified according to CD4<sup>+</sup> T/mm<sup>3</sup> cell numbers, this surrogate marker does not always show significant variation between groups, and as a consequence, therapeutic efficacy may not be so visible when considering immunity recuperation.

VL however displayed different behavior between groups with a drop of 0.5 log from G<sub>1</sub> to G<sub>2</sub> and 4.0 log from G<sub>2</sub> to G<sub>3</sub>; it was used for group classification, especially as a surrogate marker for G<sub>2</sub> and G<sub>3</sub>. In this study, when analyzed, it had less meaning despite agreeing with Haase (12) and Carcelain et al (4).

Several studies have reported the effect of HAART in AIDS patient's immunity reconstitution (13,31,33); treatment significantly inhibited viral replication and the consequent reduction of IL-2 suppression and INF- $\gamma$  production, while promoting IL-4 and IL-10 decrease. In this study, we observed that TNF- $\alpha$ , INF- $\gamma$ , IL-4, and IL-10 were statistically higher than in the control group, but there was no statistical difference between all HIV-1 groups. IL-2 was the only cytokine that was not statistically different between all groups including controls. In absolute values, IL-2 increased with the lowest values in non-treated patients (G<sub>1</sub>). It was also lower in treated detectable VL (G<sub>2</sub>) patients than controls. The highest value was in undetectable VL (G<sub>3</sub>) patients; its absolute value was higher than the control group median and, when considered in absolute values, showed a behavior suggesting immunity reconstitution.

According to the Spellberg and Edwards-Jr (31) definition of cytokine profile behavior, and considering median cytokine values in each group, all patient groups (G<sub>1</sub>, G<sub>2</sub>, and G<sub>3</sub>) had mature Th0 subset. On the other hand, considering each patient from each group, none of them showed Th1 or naive Th0 subset profile. This latter is defined by IL-2 production. In contrast, the highest proportion in each group was mature Th0, and the lowest Th2. Despite no statistical difference between groups, the group with the lowest proportion of Th2 was treatment with undetectable VL; this is relevant as could be the result of the antiretroviral treatment, mainly HAART, administered to this group (7,27,31).

In relation to ARV treatment in the study groups, most G<sub>3</sub> patients received triple regimen associated with two RITN and one PI (HAART). The patients in this group performed best with undetectable VL, higher increase in IL-2, which was higher than controls; and lower Th2. These results are in agreement with several authors (4,8,11,12,13,24,31,33).

Correlation of pairs of variables using the detectable viral load groups, G<sub>1</sub> (infected, non-treated) and G<sub>2</sub> (infected, treated), against these two groups plus G<sub>3</sub> (undetectable VL) showed IL-2 with the most correlations. IL-2 correlated positively with INF- $\gamma$ , the definer of Th1 subset (5,31), and with CD4<sup>+</sup> T, an immunity recuperation surrogate marker (12); negatively with VL, TNF- $\alpha$ , IL-4, and IL-10. VL is a surrogate marker of disease progress (20,21). According to Gougeon et al (11), the T cells responsible for producing TNF- $\alpha$  are more sensitive for inducing apoptosis than those responsible for IL-2. Apoptosis is the main CD4<sup>+</sup> T reduction mechanism in HIV-1 infection (11). IL-4 and IL-10 are definers of Th2 subset (31). The positive and negative correlations of IL-2 to the above variables make it an important marker of immunity recuperation in HIV-1 infected patients under HAART. In this sense, IL-2 serum determination may be useful in follow-up of these patients together with VL determination and CD4<sup>+</sup> T/mm<sup>3</sup> cell count.

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