PEDIATRIC AIDS

CCR5 and β-chemokines in HIV-1 infected children

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The duration from initial infection with HIV-1 to CD4 lymphocyte depletion and progression to AIDS varies among infected individuals. Despite treatment with highly active antiretroviral therapy (HAART), patients still show different stages of disease progression. We examined the role of β-chemokines and its receptor, CCR5 in HIV-1 infected children in order to define determinants of HIV progression among treated individuals. Population was divided in two groups: Group 1-Long Term Non Progressors (LTNP) includes 10 patients with B1-B2 CDC disease classification and with a less aggressive therapy (only 2 in HAART); Group 2-Rapid Progressors (RP) includes 9 patients with C3 disease classification. All the patients had a CCR5 wild type (wt) genotype indicating that they do not have the 32 base-pair deletion associated with slower progression. There was an increased production of MIP 1-β in 8/10 LTNP but only in 4/9 Progressors (Paired t-test/Wilcoxon Sign test, p-value < 0.05). The change in the levels of MIP-1β after PHA stimulation was statistically significant in both groups. The levels of RANTES increased in LTNP and RP and the change of the levels after mitogen stimulation was statistically significant for both groups included. The production of RANTES and MIP-1β in response to stimulation between both groups was not statistically significant. The production of MIP-1α was variable in both groups and the difference in the levels after mitogen stimulation between the groups was not statistically significant. These results suggest that β-chemokines do not play an important role in HIV-1 progression in children undergoing HAART.

Keywords: Human immunodeficiency virus-1, Highly active antiretroviral therapy, Disease progression, β-chemokines (RANTES, MIP-1β and MIP-1α), CCR5.

HIV infection does not inevitably lead to the development of Acquired Immunodeficiency Syndrome (AIDS) (1). While the majority of HIV individuals progress to AIDS, a minority of HIV-infected patients does not show signs of disease and maintains a stable CD4 count for at least 10 years after infection. These individuals are known as Long Term Non Progressors (LTNP) (2). The LTNP have one or more of the following characteristics: they maintain a very low viral load both in plasma and peripheral blood mononuclear cells (PBMC); the HIV strain present is usually a less cytopathic or relatively non-virulent virus type; antibodies to the HIV strain present in the individual do not enhance infection; they have a predominant T-helper type 1 cell profile and their cellular CD8 antiviral response is strong (3).

The CD4 receptor is a cell surface glycoprotein present in lymphocytes and monocytes and is important for their activation and participation in the immune response against antigens. It is also the main cell surface receptor for HIV viral entry into the cell (4). However, several studies have determined that the CD4 receptor alone is neither sufficient nor the sole means for viral entry (5). Some human cells expressing high levels of CD4 protein were not susceptible to HIV infection suggesting the need for one or more additional cellular receptors or co-receptors (5). The chemokine receptor CCR5 is one of such co-receptors and is the principal co-receptor for macrophage tropic (M-tropic) strains with a non-syncytium inducing (NSI) phenotype (6).

CCR5 appears not to be essential; there are natural mutations that prevent expression of the native protein. One such mutation is a 32 base-pair deletion in the CCR5

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Supported by NIH MBRS S06GMO8224 and RCRII 1P20 RR 11126

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gene that produces a truncated protein that is not expressed on the plasma membrane (7). The CCR5 protein plays a regulatory role in HIV-1 infection as well as in the rate of disease progression (8). Individuals homozygous for the 32 base-pair deletion in CCR5 appear to have a reduced risk of HIV infection due to the absence of a functional co-receptor. Heterozygous individuals (in which only one of the alleles has the 32 base-pair deletion) can be infected but they show a slower progression to AIDS.

Chemokines are a family of structurally related proteins that induce migration of specific subsets of leukocytes to sites in the body and are involved in the generation of cellular inflammation (9). It has been hypothesized that RANTES (regulated on activation, normal T cells expressed and secreted) and other β-chemokines produced by activated T cells are major HIV-1 suppressive factors (10). The actual role of chemokines *in vivo* in the course of HIV infection may be two sided. They can act as mediators of inflammation favoring the accumulation of activated immune cells at the foci of HIV replication and thereby exposing them to viral infection. Chemokines also can exert a protective effect against virus entry (11). Certain chemokine receptors might be down regulated from the cell surface in a coordinated manner upon chemokine treatment (12). β-chemokines can block HIV infection at an early binding step in the infection process, presumably by competitive inhibition (13).

In this study we determined genetic (CCR5 genotype) and an immunological factor (β-chemokines production) in disease progression in HIV-1 infected children. This study is important for two reasons: a) Previous studies have been done only on adults, these studies did not show differences in the production of β-chemokines by PBMC at different stages of disease and b) there is a need to understand progression in the presence of HAART.

Materials and Methods

Study subjects. Nineteen HIV-1 infected children through vertical transmission who attend to the University of Puerto Rico Pediatric HIV Program (Gamma Project-ACTU) and undergoing highly active antiretroviral therapy (HAART) were selected for this study. They were classified into clinical category A, B or C and immune category 1, 2 and 3 on the basis of Centers for Disease Control guidelines(CDC, Atlanta, GA) defined criteria for HIV classification (14). The children were classified in LTNP or RP based on CD4 percentage, viral load and response to therapy as described: ten of those subjects were identified as LTNP and had a mean age of 11 years, B1-B2 disease classification, high levels of % CD4, mean viral load of 1500 copies/ml and were with a less aggressive therapy (only 2 in HAART) (Table 1). The other nine subjects were RP with a mean age of 4 years, C3 disease classification, variable CD4 percentages (but lower than LTNP) and viral load over 50,000 copies/ml (Table 2).

Table 1. Clinical and Immunological Profiles of Long Term Non Progressors

Patient	Classification	% of CD4	Viral Load (copies/ml)	Therapy *
1	B2	27	24,997	3TC ZDV
2	B1	33	22,030	3TC ZDV
3	B2	35	N.D†	d4T IDN
4	B2	25	704	d4T ddI
5	B2	21	9,633	d4T
6	B2	20	2,604	d4T ddI
7	B2	28	N.D†	ZDV RTV 3TC
8	B1	30	706	3TC ZDV
9	B2	26	430	d4T 3TC NFV
10	B1	31	15,595	d4T 3TC

Abbreviations:

*Therapy: 3TC (Lamiduvine); ZDV (Zidovudine); d4T (Stavudine); IDN (Indinavir); ddI (Didanosine); RTV (Ritonavir); NFV (Nelfinavir)
†Less than 400 copies/ml

DNA isolation. DNA was isolated from 1 X 10^6 frozen PBMC (1 mg of DNA), using the Quiagen Blood DNA Extraction Kit (Quiagen, Valencia CA). Procedures were done as indicated by the manufacturer. DNA isolations were performed in a laboratory where there is no handling of amplified DNA. Standards precautions to avoid PCR contamination were used. These included: separation of the DNA sample, preparation, amplification and analysis areas; use of UV irradiation of all plasticware and pipettors; use of positive displacement pipettes or aerosol resistant tips; and handling a small number of samples per amplification experiment.

CCR5 typing polymerase chain reaction. Genomic DNA was isolated from patients PBMC using Quiagen Blood Extraction Kit (Quiagen Valencia CA) according to manufacturer instructions. Upstream and downstream oligonucleotide primers for amplifying the CCR5 gene corresponding to the second extracellular region of CCR5

Table 2. Clinical and Immunological Profiles of Rapid Progressors

Patient	Classification	% of CD4	Viral Load	Therapy*
1	C3	7	750,000	NFV RTV NVP ddI
2	C3	27	367,857	AMP ddI d4T
3	C3	25	9,629†	3TC NFV d4T
4	C3	8	91,126	NVP NFV ddC RTV
5	C3	28	217,038	ddI d4T AMP
6	C3	4	750,000	NFV d4T 3TC
7	C3	19	56,533	d4T ddI NFV
8	C3	29	52,254	d4T ddI AMP

Abbreviations:

*Therapy: NFV (Nelfinavir); RTV (Ritonavir); NVP (Nevirapine); ddI (Didanosine); AMP (Amprenavir); d4T (Stavudine); ddC (Zalcitabine); 3TC (Lamiduvine)

†The viral load of this patient was higher than 50,000 copies/ml when he was enrolled in the study although at the moment of the sample collection it was lower due to in HAART.

were used. Sequences of the primers has been described (15) and are as follows: 5'-primer GAAGTTCCTCATTA CACCTGCAGCTCTC; 3'-primer: CTTCTTCTCATTTC GACACCGAAGCAGAG. Using this set of primers, the wild type CCR5 allele will give rise to a PCR fragment of 174 bp, whereas the deleted allele will be 142 bp (that is 32 bp less than the wild type). The amplification was performed in reaction mixtures containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 μM of each primer and 1 μg of genomic DNA for each PCR reaction. Genomic DNA was first denatured at 95 °C for 5 minutes, followed by the addition of 2.5 units of Taq polymerase and amplification of genomic DNA by PCR for 5 cycles at (94°C, 45 seconds; 55°C, 45 seconds and 72°C, 45 seconds) and 35 cycles (94°C, 45 seconds; 62°C, 45 seconds and 72°C, 30 seconds). The reaction products were analyzed on a 4% Nusieve GTG agarose gel and DNA bands stained by ethidium bromide. The 123 bp and 50 bp DNA ladder were used as markers.

β-chemokine production. 1 X 10^6 of PBMC were cultured and stimulated with phytohemaglutinin (PHA) for 24 hours. Unstimulated cells were used as controls. Cell free supernatant was collected by centrifugation and stored at -85°C until assayed. β-chemokine levels in culture supernatants were quantitated with commercial enzyme-linked immunosorbent assay (ELISA) kits from R & D Systems (Minneapolis, MN) according to the manufacturer's protocol.

Statistical analysis. Analysis of covariance (ANCOVA) was used to measure the difference between LTNP and RP groups after adjusting for centralized age (x_{age}-μ_{age}) for both analyses. Paired t-test or Wilcoxon Sign-Test was used, when appropriate, to measure the difference before and after PHA stimulation in chemokines (MIP-1α, MIP-1β and RANTES)(16). Because the distribution of the difference in the values of β-chemokines production between unstimulated and PHA stimulated PBMC was highly skewed, a logarithmic transformation was applied prior to statistical analysis in order to normalize the data. A constant value was added in this transformation due to negative difference values ($\ln(x_{chemokine}+constant)$). The statistical analysis was performed with the SAS Software System Version 6.12 (17).

Results

CCR5 genotype examination in nineteen HIV-infected patients revealed that none of them had the mutation for CCR5 as determined by the presence of a band of approximately 174 bp that is characteristic of a wild type genotype (Figure 1 lanes 4-8). The gel electrophoresis shown in the figure is representative for the results obtained on all the patients.

The production of MIP-1β, MIP-1α and RANTES by PBMC was examined in both groups of patients. The mean production of MIP-1β, MIP-1α and RANTES by

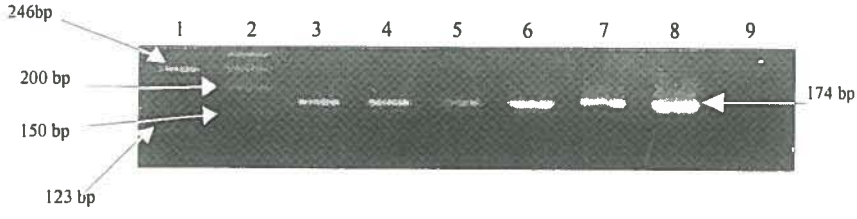

Figure 1. Agarose Gel (4.0%) for the analysis of CCR5 genotype. Lane 1: DNA marker 123bp, Lane 2: DNA marker 50bp, Lane 3: Healthy donor, Lane 4-8: HIV-1 infected patients, Lane 9: Negative Control (No DNA was added)

unstimulate PBMC was higher (8107 ± 13100 pg/ml; 15,306 ± 22061 pg/ml and 657 ± 438 respectively) among RP as compared to LTNP (3123 ± 2432 pg/ml; 7915 ± 5752 pg/ml; 483 ± 168 pg/ml respectively) however the difference between both groups was not statistically

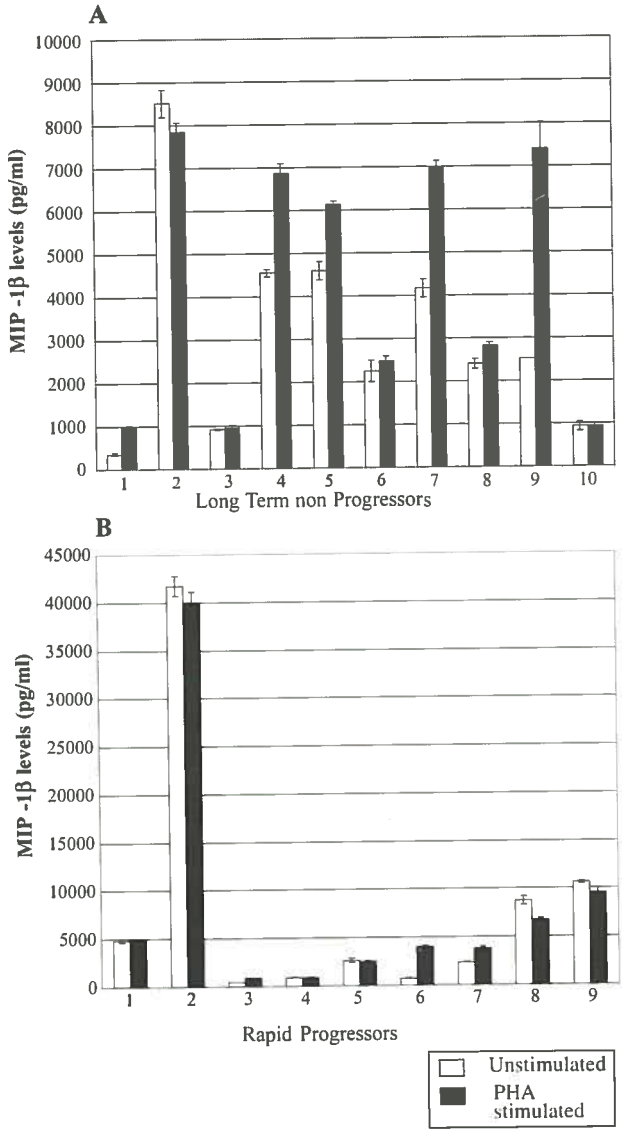

Figure 2. MIP-1β production by PBMC from LTNP(A) and RP(B). Abbreviations: MIP-1β (Macrophage inhibitory protein 1β); LTNP (Long Term Non Progressors); RP (Rapid Progressors)

significant. After PHA stimulation, the mean production of MIP-1β, MIP-1α and RANTES was higher (8152 ± 12253 pg/ml; 17917 ± 16416 pg/ml and 850 ± 457 pg/ml) among RP as compared to LTNP (4345 ± 2949 pg/ml; 10315 ± 8605 pg/ml and 824 ± 290 pg/ml). The mean production between both groups was not statistically significant.

The statistical analysis of MIP-1β and RANTES values from LTNP and RP was done using paired t-test after logarithmic transformation as described in Materials and Methods. A significant increased production of MIP-1β was observed in 8/10 LTNP ($p < 0.05$, Figure 2, Table 3) but in only 4/9 RP ($p < 0.05$, Figure 2, Table 3). The production of RANTES was also significantly increased in all the patients ($p < 0.05$, Figure 3, Table 3).

The statistical analysis of MIP-1α values from LTNP and RP was done using Wilcoxon Sign-test. The production of MIP-1α after stimulation was variable for both groups (Figure 4) and the difference in the levels after stimulation with PHA was not statistically significant for neither of the groups studied ($p > 0.05$, Table 3).

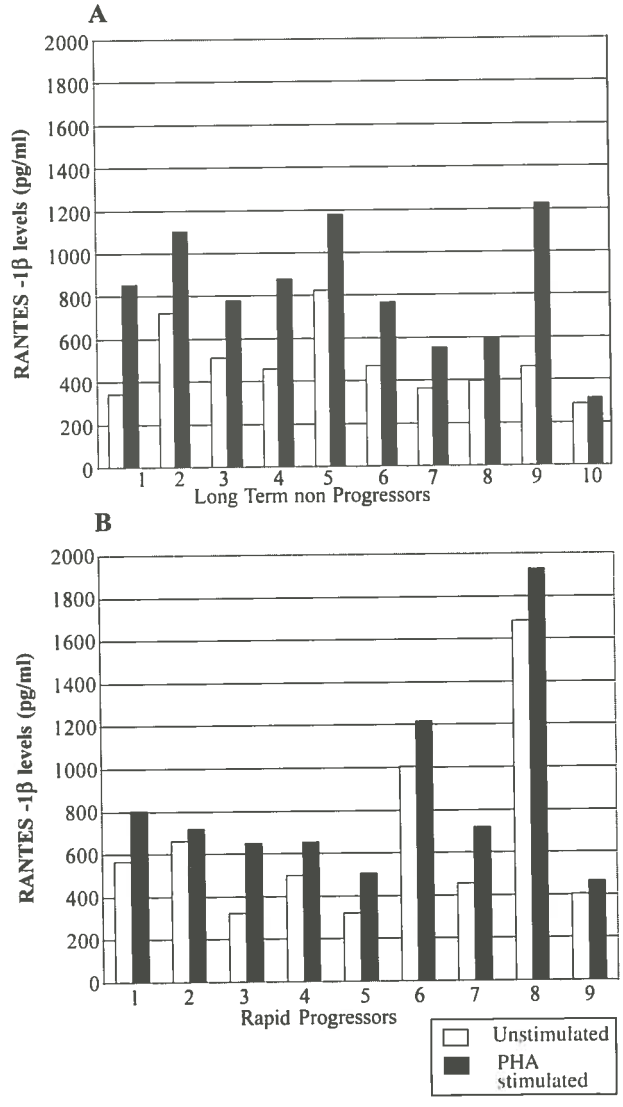

Figure 3. RANTES production by PBMC from LTNP(A) and RP(B). Abbreviations: RANTES (Regulated on Activation, Normal T cells expressed and secreted); LTNP (Long Term Non Progressors); RP (Rapid Progressors)

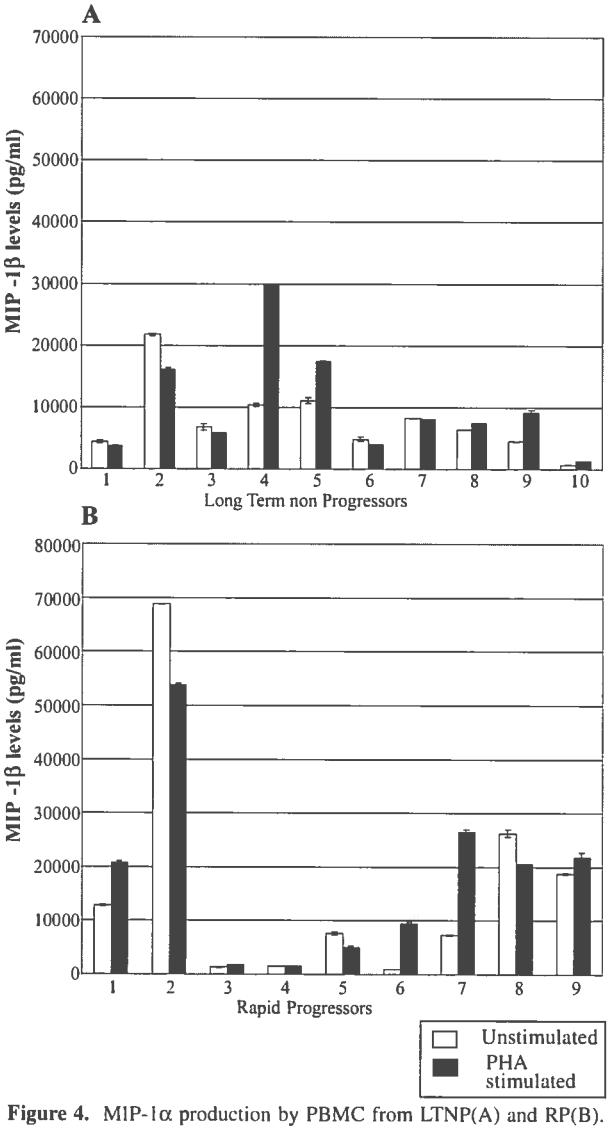

Figure 4. MIP-1α production by PBMC from LTNP(A) and RP(B). Abbreviations: MIP-1α (Macrophage inhibitory protein 1α); LTNP (Long Term Non Progressors); RP (Rapid Progressors)

Comparison of the differences in the production of RANTES and MIP-1β in response to stimulation with PHA between both groups of patients was not statistically significant ($p > 0.05$, Table 3).

Table 3. Statistical Analysis of β-chemokine production in LTNP and RP

Chemokines	LTNP Unstim. vs PHA stim p-value*	RP Unstim. vs PHA stim p-value*	LTNP vs RP p-value**
MIP-1β	0.001	0.002	0.202
MIP-1α	0.278	0.285	0.976
RANTES	< 0.001	< 0.001	0.065

* Paired t-test/Wilcoxon Sign-test
** ANCOVA (Analysis of covariance adjusted by centralized age (X age-μ age)

Discussion

The importance of CCR5 in HIV-1 transmission has been underscored by the observation that certain individuals who were repeatedly exposed to HIV-1 but remained uninfected had a defect in CCR5 expression (8,19). These non-infectable individuals were found to be homozygous for a defective CCR5 allele that contains an internal 32 bp deletion (CCR5 D32). Individuals who are heterozygous for a deletion have a slower progression to AIDS than wild type homozygous individuals suggesting that CCR5 expression may be altered in these individuals and that this affects HIV-1 binding and replication *in vivo* (8,19). In our study, all the patients had a CCR5 wild type genotype indicating the abscence of the 32 bp deletion associated with slower progression. The absence of a mutated allele may be related to the fact that the Δ32 heterozygous genotype can be found among 15% of whites (caucasians) and the Δ32 homozygous genotype is found in less than 1% of whites (caucasians) but in much lower frequency in other ethnic groups. (8, 20).

Regarding the role of β-chemokines in HIV progression, we found no significant differences in β-chemokines production by PBMC from LTNP and RP groups despite differences in the β-chemokines means between both groups. This could be due to the high variability in β-chemokine values of the RP. In this study we found that indeed the levels of β-chemokines in children were higher when compared to adults (21). However, both studies show no significant differences in β-chemokines production between cells from RP and those from LTNP despite being in HAART. In both studies there is no clear correlation between the production of RANTES, MIP-1α, MIP-1β and disease progression.

We also studied the correlation between the percentage of CD4 lymphocytes with the production of β-chemokines (RANTES, MIP-1α and MIP-1β). For RANTES it was notable the fact that all the nineteen patients investigated increased its production after PHA stimulation. There was a direct correlation between CD4 percentage and the levels of RANTES in PBMC cultures since the patients with a better immunological status (higher CD4 percentage) responded better to stimulation with PHA. For MIP-1α and MIP-1β we were unable to establish any type of correlation because patients with similar immunological status (similar percentages of CD4) showed different patterns of response to PHA stimulation. According to these results, only the levels of RANTES showed a direct correlation with the immunological status of the patient since it was the only one of the three β-chemokines studied that had a defined pattern.

Scala and coworkers (1997) were unable to observe differences in the production of β-chemokines when PBMC from subjects at different stages of disease (adults with a range of age from 24 to 42 years) were studied (22). Clerici and coworkers (1996) found that *in vitro* stimulated β-chemokines production did not correlate with disease progression (23).

The importance of our study is that, in contrast to the aforementioned studies, ours was done on children and adolescents undergoing HAART. The overall β-chemokines levels was not a prognostic marker for disease progression because the levels of β-chemokines in RP were much variable than the levels found in LTNP, making the difference between both groups non-significant. Is possible that the protective role of β-chemokines is more effective during initial infection by competitive inhibition and surface downregulation of the chemokine receptors for virus binding. It remains to be studied if additional factors such as the strain of virus, enhancing antibodies or decreased CD8 antiviral response are associated with disease progression in this group of children.

Resumen

La duración del periodo desde la infección inicial con HIV-1 hasta la pérdida masiva de linfocitos CD4 y progresión a SIDA varía entre los individuos infectados. Nosotros examinamos el genotipo del receptor de quimokinas CCR5 y la producción de β-quimokinas por células mononucleares de la sangre periferal de adolescentes infectados con HIV-1 con el propósito de definir determinantes de progresión. La población se dividió en dos grupos: Grupo 1 (Progresores lentos) incluyó 10 pacientes con una clasificación de enfermedad B1-B2 y con una terapia menos agresiva (solo 2 de ellos en HAART). El Grupo 2 (Progresores rápidos) incluyó 9 pacientes con una clasificación de enfermedad C3. Todos los pacientes poseen un genotipo "wild type" para el receptor CCR5 lo que es indicativo de que no poseen la deleción de 32 pares de bases asociada con progresión lenta. Para MIP-1β hubo aumento en su producción en 8/10 progresores lentos pero solo en 4/9 progresores rápidos. El cambio en los niveles de MIP 1-β luego de la estimulación con PHA fue estadísticamente significativo para ambos grupos. Los niveles de RANTES aumentaron en ambos grupos y el cambio en sus niveles luego de la estimulación con PHA fue estadísticamente significativo tanto para los progresores lentos como para los progresores rápidos. Al comparar las diferencias en la producción de RANTES y MIP-1β en respuesta a estimulación entre ambos grupos esta no fue estadísticamente significativa. La producción de MIP-1α fue variable en ambos grupos y la diferencia en los niveles luego de la estimulación con PHA no fue estadísticamente significativa para ninguno de los grupos. Estos resultados sugieren que las β-quimokinas no aparentan jugar un rol importante en la progresión a SIDA en los niños y adolescentes estudiados.

Acknowledgment

The authors want to thank Miguel Arroyo, BS from the Department of Microbiology and Medical Zoology of the Medical Sciences Campus for his help in DNA extraction and CCR5 genotyping.

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