

C-C Chemokines, IL-16, and Soluble Antiviral Factor Activity Are Increased in Cloned T Cells from Subjects with Long-Term Nonprogressive HIV Infection¹

Enrico Scala,* Gianpiero D'Offizi,* Rosamaria Rosso,* Ombretta Turriziani,[†] Rosetta Ferrara,* Anna Maria Mazzone,* Guido Antonelli,[‡] Fernando Aiuti,* and Roberto Paganelli^{2*}

A combination of three β , or C-C, chemokines, as well as IL-16, have been shown to inhibit HIV replication in vitro. Cellular antiviral factor is a more potent agent, and acts on all HIV strains. All are mainly, but not exclusively, produced by CD8⁺ T cells, both in HIV⁺ and healthy subjects. We studied the production of these HIV-suppressive factors in patients with HIV infection at different stages of disease. No difference in production by PBMC stimulated with PHA has been observed in asymptomatic HIV⁺, long-term nonprogressors (LTnP), and AIDS patients. When T cell line supernatants from these three groups were studied, no significant difference was found for C-C chemokines or IL-16 production, and viral suppression. However, T cell clones from LTnP secreted higher levels of all three chemokines, IL-16, and exerted a stronger inhibition on HIV replication. CD8⁺ clones showed a higher production than CD4⁺ clones. These clones were able to produce all antiviral factors irrespective of the secretion of type 1 or type 2 cytokines. The antiviral activities were not correlated, implying that viral suppression did not depend solely on C-C chemokines or IL-16. We postulate that all factors are needed to prevent HIV disease progression. *The Journal of Immunology*, 1997, 158: 4485–4492.

Cell-mediated immune responses represent the main bodily defences against viral infections. Among T lymphocytes, CD8⁺ cells are the primary effectors of cellular immunity. Both cytotoxic, specific, lytic mechanisms and noncytotoxic, MHC-unrestricted, nonlytic actions are exerted by CD8⁺ lymphocytes to control spread of viruses (1). The latter have been described in HIV infection by Levy over 10 yr ago (2). The effect is mediated, at least in part, by a soluble factor, which has not been further characterized (1, 3).

Recently, Cocchi et al. (4) demonstrated an inhibitory effect on HIV replication in vitro using a combination of three C-C chemokines (RANTES,³ MIP-1 α , and MIP-1 β), and postulated to have identified the nature of the antiviral soluble factor.

Chemokines are a superfamily of over 30 distinct proinflammatory, inducible, secreted proteins of 6 to 14 kDa, which regulate the traffic of leukocytes in mammalian species (5, 6). Most members of the chemokine family exhibit from 20 to 50% homology in primary amino acid sequences with a conserved four-cysteine

motif. On the basis of the arrangement of the first two of these four cysteines, two subfamilies of chemokines are recognized. The C-C chemokines exert their activity on monocytes, basophils, eosinophils, and activated T lymphocytes, but not on neutrophils (6, 7). Many other cytokines possess chemotactic properties for T lymphocytes, including the recently described C chemokine, lymphotactin (5), and IL-16, produced by CD8⁺ cells. Baier et al. (8) reported that both African green monkey and human rIL-16 suppress HIV replication in vitro. The relationship between the recombinant and the natural product has been questioned (9), but the antiviral activity, although at high doses, seems to be confirmed (3).

Neither C-C chemokines nor IL-16 account in full for the CD8⁺-derived antiviral activity described by Levy et al. (1, 3). Therefore, it appears that these factors may synergize in suppressing HIV replication. In HIV-infected patients, both CAF and C-C chemokine production has been found to correlate with lack of progression in HIV-infected subjects, and of infection in highly exposed subjects, respectively (10, 11).

People with no sign of disease progression, despite several years of infection with HIV, have been designated long-term nonprogressors (LTnP), and found to retain an efficient immunity (12–14). The characteristics of these subjects are very different from those of patients progressing to AIDS, but very similar to asymptomatic HIV-positive subjects who have a shorter time of infection (15). We therefore looked at the production of all the antiviral factors described in LTnP, asymptomatic HIV-positive individuals, and patients who progressed to AIDS. In addition, since a switch from type 1 to type 2 cytokine production has been postulated to determine disease progression (16), we also measured IL-4 and IFN- γ levels in supernatants derived from mononuclear cells and T cell lines (TCL) and clones (TCC) obtained from HIV-infected and noninfected subjects, and compared them with the production of different antiviral factors.

*Department of Clinical Medicine, Chair of Clinical Immunology and Allergy, University La Sapienza, Rome, Italy; [†]Institute of Virology, University La Sapienza, Rome, Italy; and [‡]Department of Biomedicine, University of Pisa, Pisa, Italy

Received for publication September 10, 1996. Accepted for publication January 3, 1997.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported by Grants 9304/96 and 9304/02 of the VIII AIDS Project of Istituto Superiore di Sanità, Ministry of Health, Italy, 1995. This work is part of the NOPHROCO (nonprogressor HIV Rome cohort) study group.

² Address correspondence and reprint requests to Dr. Roberto Paganelli, Dipartimento di Medicina Clinica, Cattedra di Immunologia Clinica e Allergologia, viale dell'Università 37, 00185 Rome, Italy.

³ Abbreviations used in this paper: RANTES, regulated upon activation, normal T cell expressed and secreted; CAF, cellular antiviral factor; LTnP, long-term nonprogressors; TCL, T cell lines; TCC, T cell clones; MIP-1 α , macrophage inflammatory protein-1 α ; MIP-1 β , macrophage inflammatory protein-1 β .

Materials and Methods

Patients

Thirty-one HIV-1 seropositive subjects from the Rome metropolitan area represented our study group. A total of 13 patients (10 males and 3 females) had no symptoms, at least 10 yr of seropositivity, a stable absolute number of CD4⁺ lymphocytes >500/ μ l, and no use of antiretroviral agents. These individuals, defined as long-term nonprogressors (LTnP), ranged in age from 31 to 48 yr; the route of infection was i.v. drug abuse in 6 individuals, 5 males were infected through homosexual sex and 2 women were infected heterosexually. HIV-infected individuals studied as controls were 11 (7 male and 4 female) asymptomatic subjects with a duration of infection of 1.2 to 3.5 years, CD4⁺ lymphocytes stably >500/ μ l, and age range from 24 to 42 yr. The route of infection was in 5 males homosexual sex, 2 were i.v. drug abusers and 4 women were infected heterosexually. Seven AIDS patients were HIV-infected males (age range 35 to 48 years), with CD4⁺ T cell counts below 50/ μ l, all classified, according to Centers for Disease Control and Prevention criteria as group IV C-1. Seven HIV-seronegative subjects (four coauthors of this paper) were also studied as healthy controls.

Not all subjects were analyzed in all assays. In particular, TCL supernatants were available in 4 patients in each group, PBMC were not studied in AIDS patients, and antiviral activity was tested in 15 subjects (9 LTnP and 6 asymptomatic HIV⁺ controls), due to unavailability of other supernatants. TCC supernatants were selected according to high or low chemokine production and Th pattern.

Flow cytometric analysis

For double-fluorescence analysis the cells were stained with the following mAbs, R-phycoerythrin- or FITC-conjugated: anti-CD3 (Leu-4), TCR- $\alpha\beta$, anti-CD45RO (Leu-45RO), anti-CD4 (Leu-3a), and anti-CD8 (Leu-2a; all from Becton Dickinson Immunocytometry System, Mountain View, CA).

A total of 2×10^5 lymphocytes from PBMC, TCL and clones were delivered into the test tube containing 20 μ l of each mAb, and placed in an ice water bath for 20 min. After two washes with PBS containing 2% FCS (Life Technologies, Paisley, Scotland, U.K.) and 0.02% NaN₃, cells were resuspended in PBS. Quantitative analysis for double-color immunofluorescence was carried out by flow cytometry (Cytoson Absolute, Ortho Diagnostic Systems, Raritan, NJ). Five thousand events were acquired and the data were analyzed by the Immunocount software (Ortho), in an IBM-compatible computer. Live lymphocytes were electronically gated by the side and forward light scatters.

PBMC stimulation, TCL, and TCC

Lymphoprep (Nycomed, Oslo, Norway)-separated PBMC were resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with penicillin 100 U/ml and streptomycin 100 μ g/ml (Life Technologies), L-glutamine 2 mM (Life Technologies) and 10% FCS (Hyclone, Logan, UT) (complete medium) at 2×10^6 cells/ml. Lymphocytes were stimulated with PHA-P (1 μ g/ml; Wellcome, Beckenham, U.K.) in 24-well flat-bottom plates (Falcon, Oxnard, CA). After 48 h supernatants were collected and stored at -80°C for cytokine evaluation. rIL-2 (20 U/ml; Boehringer Mannheim, Mannheim, Germany) was then added to the cells at weekly intervals. After 3 wk, viable T blasts were resuspended in complete medium (supplemented with 2-ME 50 μ M; Sigma Chemical Co, St. Louis, MO) and seeded at 0.3 cell/well in U-bottom 96-wells plates (Falcon), as described (17). A total of 2×10^5 irradiated (6000 rad from Cs source) allogeneic mononuclear cells were used as feeders. After 3 wk, growing wells were identified using an inverted microscope, and expanded by weekly splitting and addition of rIL-2 supplemented complete medium. TCL were also cultured in bulk with rIL-2 addition.

Assay of antiviral activity

The antiviral activity of supernatants was evaluated as previously described (18). Briefly, C8166 cells were incubated with HIV-IIIB at multiplicity of infection of 0.01 50% tissue culture infective dose/cell. After 1 h the culture was washed and resuspended in medium containing dilution of supernatants of PHA-activated PBMC, TCL, or clones derived from LTnP and asymptomatic HIV⁺ controls. HIV replication was evaluated after 72 h by measuring the yield of viral Ags (p24 EIA, Abbott Laboratories, Abbott Park, IL) and the reduction in comparison to the control has been calculated. The values reported in *Results* represent the medium $\pm$ SD of the percentage of reduction obtained using supernatants of cells derived from the two groups of patients. HIV strain IIIB has been chosen because it is insensitive to the action of C-C chemokines (1, 4), and therefore it may more appropriately reflect CAF activity.

Table 1. C-C chemokines and cytokines production in PHA-stimulated PBMC cultures^a

	LTnP	Asymptomatic HIV ⁺
No.	11	10
RANTES	4 (3.4–12.8)	4 (3.2–8)
MIP-1 α	24 (3.8–225)	17.4 (4.8–52)
MIP-1 β	14.6 (4.6–207)	9.7 (4.8–36)
IL-4	80 (<15–290)	46 (<15–166)
IFN- γ	1.6 (0.1–12.5)	2.5 (0.1–15.5)

^a Production of C-C chemokines and prototypic type 1 and 2 cytokines by 48-h PHA-stimulated PBMC from LTnP and asymptomatic HIV⁺ subjects. Results are expressed as median (and range) in ng/ml, except IL-4 (pg/ml).

Cytokine measurement

Supernatants of PBMC (11 from LTnP and 10 from HIV⁺ controls), TCL (4 from each group of subjects, after 3-wk culture) and clones (at the second month of culture) were collected 48 h after stimulation for PBMC, or restimulation for TCL and TCC, with PHA-P (1 μ g/ml). Cytokine content was determined in supernatants using the following kits: human IFN- γ , IL-4, RANTES, MIP-1 α , and MIP-1 β ELISA kits (Quantikine, R&D Systems, Minneapolis, MN) and human IL-16 capture assay (Immunokontact, Frankfurt-am-Main, Germany). All samples were tested in duplicate and thawed only once. Measurements were performed according to the manufacturers' instructions, and results interpolated from the standard reference curve provided with each kit. Supernatants were diluted 1:5 for IFN- γ measurement, 1:200 for RANTES, MIP-1 α and - β , 1:10 for IL-16, and undiluted for IL-4.

Statistical methods

We applied Wilcoxon's rank sum test for mean differences of cytokines levels, χ^2 for proportions in disease groups, *t* test for antiviral activity, and linear regression analysis when applicable. A *p* value <0.05 was accepted as significant.

Results

PBMC and TCL cytokines production

Cytokines production was studied in PHA-stimulated PBMC from 11 LTnP and 10 asymptomatic HIV⁺ subjects. Both IFN- γ and IL-4 were synthesized from all but 2 LTnP and one asymptomatic patient, who produced IFN- γ but no detectable IL-4. All PBMC cultures produced intermediate to high amounts of all three chemokines studied, and no significant differences between LTnP and controls were seen even though a slight, not significant increase was observed in MIP-1 α and MIP-1 β levels (Table 1). IL-16 production by PBMC was not investigated.

Four TCL from each group (healthy donors, LTnP, asymptomatic HIV⁺ subjects, and AIDS patients) were established. After culture for 3 wk with rIL-2, the phenotype of T cells grown differed among the groups, with 65 to 78% CD4⁺ cells in healthy subjects, 15 to 20% in the asymptomatic persons, 30 to 35% in LTnP, and none in AIDS patients. The corresponding percentages of CD8⁺ T lymphocytes accounted for most other cells, with 2 to 5% double-negative (CD4⁻CD8⁻) $\alpha\beta$ ⁺ T cells in seronegative controls and AIDS cases, and 2 to 3% double-positive (CD4⁺CD8⁺) $\alpha\beta$ ⁺ cells in LTnP.

All TCL studied secreted IFN- γ , but no IL-4 was detectable (i.e., <15 pg/ml). Levels of IFN- γ in TCL supernatants decreased from LTnP to asymptomatic HIV⁺ subjects and healthy donors, and last, to TCLs from AIDS patients (data not shown). MIP-1 β (above 1 ng/ml) was found in TCLs from two healthy individuals, and RANTES was measurable at a low level in only one; none secreted MIP-1 α above 1 ng/ml. Moderate to high levels of all three C-C chemokines were found in TCLs from both LTnP and asymptomatic subjects. TCLs obtained from AIDS patients produced very low amounts (<1 to <5 ng/ml) of MIP-1 α , MIP-1 β ,

Table II. Production of IL-16 and C-C chemokines in T cell lines^a

	Healthy Subjects	Asymptomatic HIV ⁺	LTnP	AIDS
RANTES	1 (<1–3)	5.2 (<1–82)	7 (4–18)	1 (<1–4.8)
MIP-1 α	1 (<1–1)	20 (1–250)	12 (2.6–27)	2.5 (<1–4.4)
MIP-1 β	35 (1–36)	48 (14–203)	36 (7–52)	1.5 (1–4.6)
IL-16	1.3 (<1–3.6)	4 (<1–7.5)	3.9 (2.4–4.1)	3 (1–3.1)

^a C-C chemokines and IL-16 secreted by TCL (four in each group) after culture for 3 wk in rIL-2 and restimulation with PHA. Results (in nanograms per milliliter) are reported as median (and range). All T cell lines produced IFN- γ , but no IL-4.

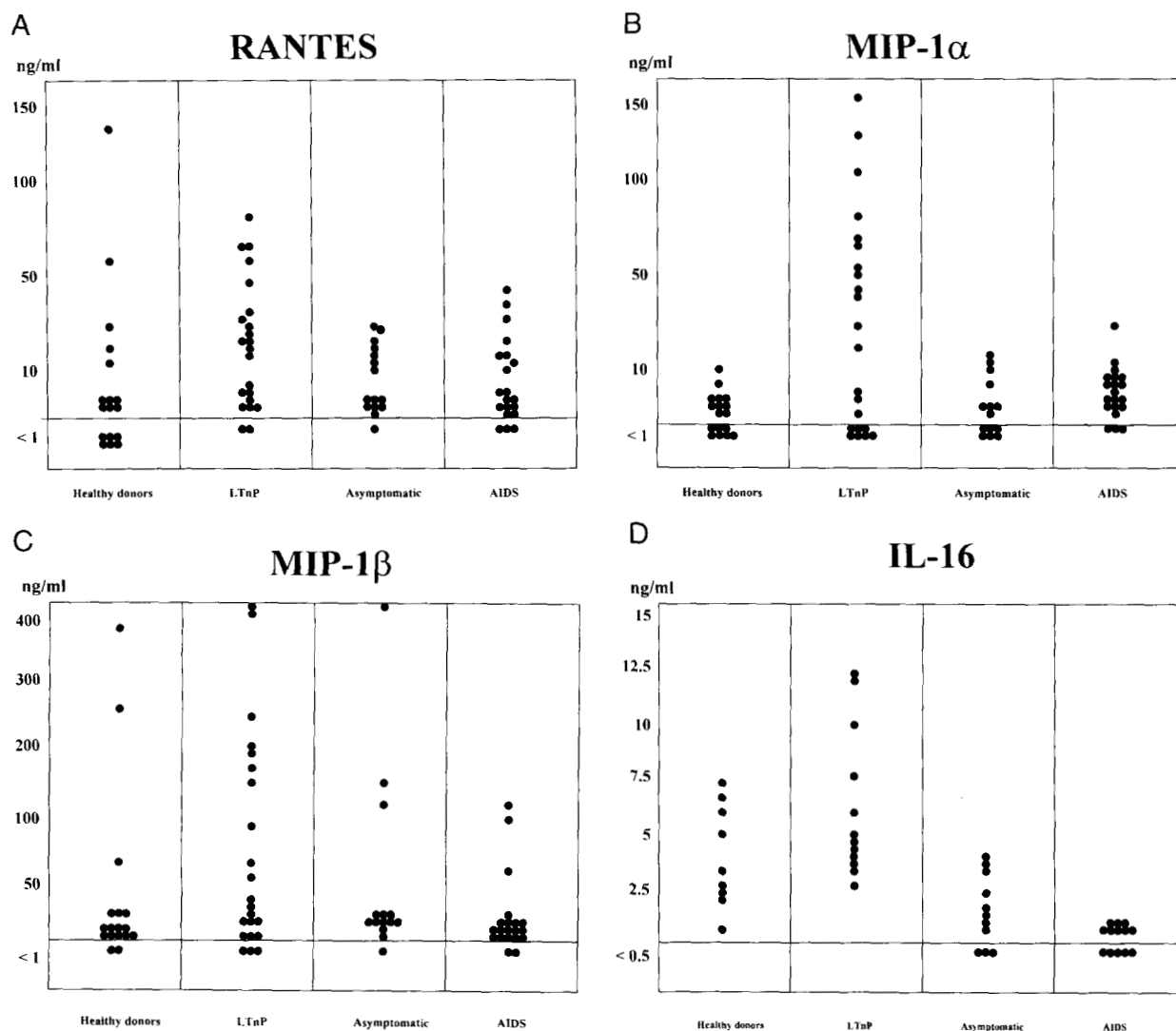


FIGURE 1. A to D, Production of C-C chemokines and IL-16 in CD8⁺ TCC from HIV⁺ patients (LTnP, asymptomatic HIV⁺, AIDS) and healthy subjects. Levels below 1 ng/ml (0.5 for IL-16) were undetectable.

and RANTES. These chemokines were never synthesized together in TCLs from AIDS patients and in one TCL no chemokine was detected. IL-16 was found to be present in most TCL supernatants from all subjects. Levels were lower in TCLs from healthy seronegative individuals, and were similar (3 to 4 ng/ml) in all HIV⁺ groups studied (Table II).

TCC cytokine production

A total of 101 TCR- $\alpha\beta$ ⁺ CD45RO⁺ TCC (19 CD4⁺, 73 CD8⁺, 6 CD4[−]CD8[−], 3 CD4⁺CD8⁺) from patients and controls were cho-

sen to study the production of IL-16, RANTES, and MIP-1 α and - β , on the basis of their Th profile, according to secretion of IFN- γ and IL-4. A total of 15 TCC were IL4⁺IFN- γ [−] (type 2), 35 IL-4⁺IFN- γ ⁺ (type 0), and 47 IL-4[−]IFN- γ ⁺ (type 1). No CD4⁺ clones were obtained from AIDS patients. We analyzed 25 TCC from healthy subjects, 33 TCC from LTnP individuals, 19 from asymptomatic HIV⁺ patients, and 24 from AIDS patients. Results of CD8 clones are reported in Figure 1.

The simultaneous production of the three C-C chemokines was more often found in CD8⁺ TCC, in comparison with CD4⁺ TCC,

Table III. Chemokines and IL-16 production by $\alpha\beta$ TCR⁺ double-positive or double-negative TCC^a

	Clone No.	Cytokine Pattern	RANTES	MIP-1 α	MIP-1 β	IL-16
Healthy donors						
CD4 ⁺ CD8 ⁻	LV 29	Th1	119.5	<1	298.4	6.4
CD4 ⁻ CD8 ⁻	RR 04	Th1	49	5	14.2	5.2
LTnP						
CD4 ⁺ CD8 ⁺	UN101 35	Th1	10	10.8	57.4	3.3
CD4 ⁺ CD8 ⁺	UN102 119	Th1	170	12.8	23.2	1.8
CD4 ⁺ CD8 ⁺	UN102 135	Th0	<1	8.4	240.6	27.4
AIDS						
CD4 ⁺ CD8 ⁻	CD35	Th0	31.5	57.6	20.8	ND
CD4 ⁺ CD8 ⁻	MF 11	Th1	27.5	20.6	10	9
CD4 ⁺ CD8 ⁻	CD13	Th0	15	48.4	63.6	2.6
CD4 ⁺ CD8 ⁻	MF 41	Th2	12.5	42.4	21.8	ND

^a C-C chemokines and IL-16 secreted by DP or DN TCC after 48 h of PHA-P restimulation. Individual results are reported in nanograms per milliliter.

irrespective of the patient's or control group ($p < 0.002$, $p < 0.02$, and $p < 0.006$ for RANTES, MIP-1 α , and MIP-1 β , respectively). Seven out of nine double-positive or -negative TCC produced all three chemokines (Table III).

No relationship was found between the type 1 or 2 cytokine pattern and C-C chemokines production, but higher amounts of all three chemokines were found in Th1/Th0 clones (Fig. 2, A to D). MIP-1 β production was significantly more represented in Th1 and Th0 clones (χ^2 test: $p < 0.03$), producing IFN- γ .

IL-16 production was found to be higher in CD8⁺ compared with CD4⁺ TCC supernatants (Table IV and Fig. 1D). However, CD4⁺ clones from LTnP exhibited levels of IL-16 comparable to, or even higher than, those of other groups' CD8⁺ clones. This finding was associated with the observation that all 20 clones studied in LTnP secreted IL-16 (>0.7 ng/ml), but they decreased to 12/15 in healthy controls, 10/16 in asymptomatic HIV⁺ subjects, and 7/13 in AIDS patients, with mean levels decreasing in the same order.

RANTES production. A total of 20/22 CD8⁺ clones tested from LTnP, 13/14 from asymptomatic stage II, and 17/20 from AIDS cases produced RANTES, whereas only 11/17 from HIV⁻ controls (χ^2 test: $p < 0.04$ vs LTnP). This chemokine was detectable, at low levels, in 7/19 CD4⁺ TCC (1/6 from controls, 2/8 from LTnP, and 4/5 from asymptomatic HIV⁺, Table IV). Double-negative and 2/3 double-positive TCC produced RANTES.

The highest levels of RANTES were found in LTnP CD8⁺ TCC, significantly more in comparison with CD8⁺ TCC from healthy donors ($p < 0.03$), asymptomatic HIV⁺ ($p < 0.002$), and AIDS patients ($p < 0.005$). No differences were found among CD8⁺ TCC obtained from HIV-negative controls, asymptomatic HIV⁺, and AIDS patients (Fig. 1A).

MIP-1 α production. A total of 15/22 CD8⁺ clones tested from LTnP, 8/14 from stage II, 17/20 from AIDS, and 10/17 from controls, produced MIP-1 α (Fig. 1B). This chemokine was also found in all CD4⁺ TCC from HIV⁺ subjects but only in 2/6 CD4⁺ TCC from controls. Double-positive and all but one double-negative TCC produced detectable levels of MIP-1 α (Table III).

The highest levels of MIP-1 α were found in LTnP CD8⁺ TCC, significantly different in comparison with CD8⁺ TCC from healthy donors ($p < 0.002$), asymptomatic HIV⁺ ($p < 0.002$), and AIDS patients ($p < 0.03$). MIP-1 α levels synthesized by CD8⁺ TCC from AIDS patients were significantly higher when compared with healthy donors ($p < 0.03$).

All CD4⁺ TCC from both LTnP and stage II patients, but only 2/6 from healthy donors, produced MIP-1 α (χ^2 test: $p < 0.03$).

MIP-1 β production. High levels of MIP-1 β were found in all CD8⁺ TCC, both from HIV⁺ or HIV⁻ seronegative subjects, but in LTnP they were significantly higher compared with asymptomatic HIV⁺ ($p < 0.02$) or AIDS patients ($p < 0.01$) (Fig. 1C). A total of 4/6 CD4⁺ TCC of normal subjects, 6/8 of LTnP, and 3/5 of HIV⁺ asymptomatic individuals secreted this chemokine (Table IV).

It is noteworthy that none of the Th2 CD4⁺ TCC produced MIP-1 β , despite the fact that Th2-like CD8⁺ clones secreted this cytokine, although to a lesser extent than Th1/Th0-like clones ($p < 0.03$).

Interleukin-16. All T cell clones from LTnP produced this cytokine at levels above 0.7 ng/ml, in contrast to 7/11 CD4⁺ clones and 22/33 CD8⁺ clones of the other groups. Moreover, the amount of IL-16 was significantly higher both in CD4⁺ and CD8⁺ TCC from LTnP, compared with other control or HIV-infected groups (Table IV and Fig. 1D). Asymptomatic HIV⁺ subjects did not differ from seronegative healthy controls for CD4⁺ clones' production of IL-16, but their CD8⁺ clones secreted less of this cytokine with respect to normal persons ($p < 0.02$), and did not differ from those of AIDS patients.

Seven DN or DP TCC from normal seronegative controls, LTnP, and AIDS patients produced detectable or even high amounts of IL-16 (Table III).

Antiviral activity

We have preliminarily examined the ability of supernatants of PHA-activated PBMC or TCL derived from LTnP and HIV⁺ asymptomatic controls (PBMC: nine LTnP and six HIV⁺ controls; TCL: four LTnP and four controls) to reduce HIV-1 replication. The results show that the degree of inhibition of replication is generally moderate and highly variable, being between 2.8 and 98%. No significant differences were observed between antiviral activity (expressed as the average mean of percentage reduction compared with the control value) of supernatants from LTnP and that of supernatants derived from HIV⁺ controls (LTnP_{PBMC} 80.2 $\pm$ 12.0% vs HIV⁺ control_{PBMC} 65.4 $\pm$ 19.6% p = NS; LTnP_{TCL} 47.8 $\pm$ 41.3% vs HIV⁺ control_{TCL} 47.6 $\pm$ 42.9%, p = NS). The above values represent the results obtained in two different experiments in which the supernatants were diluted 1:5.

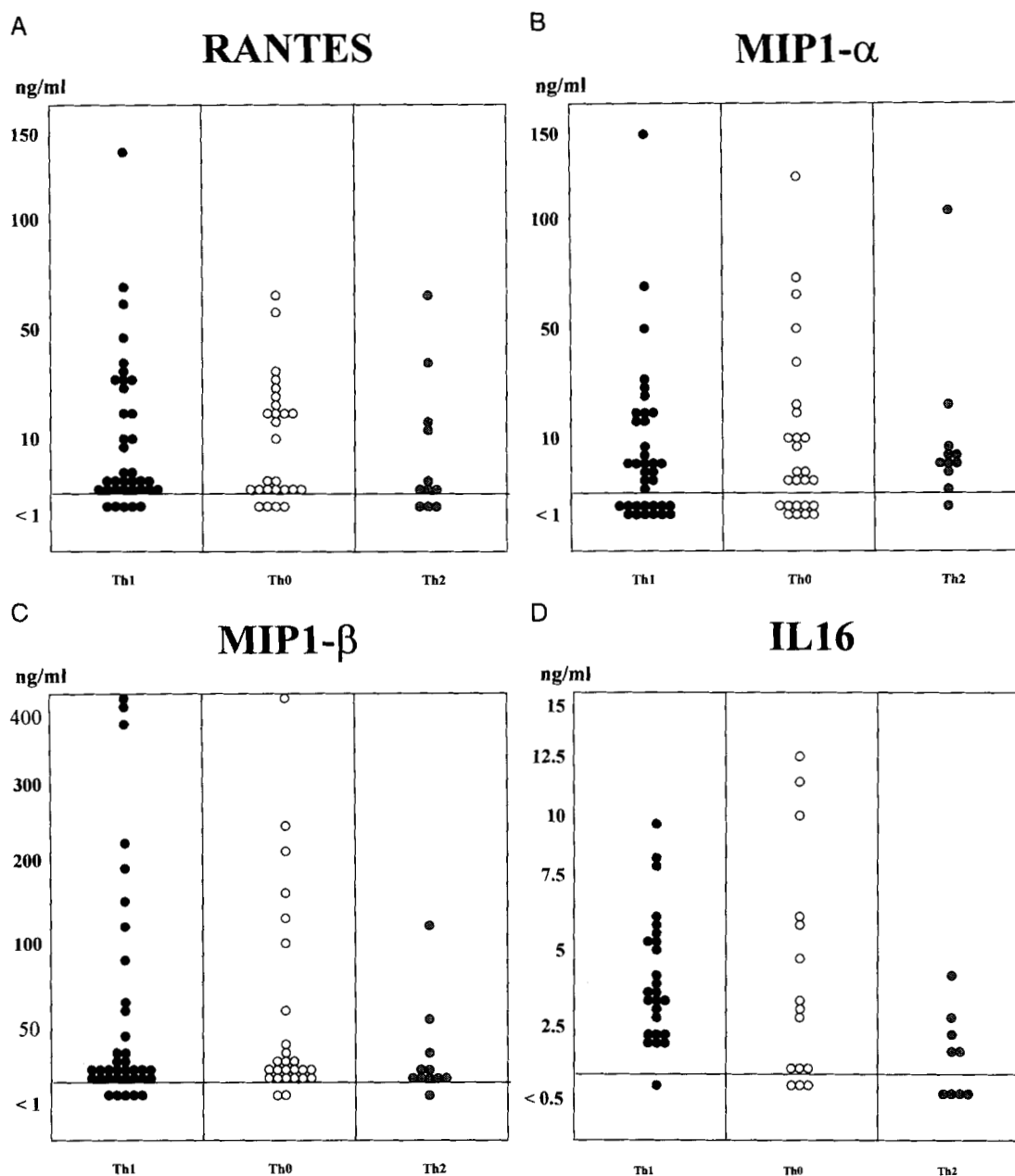


FIGURE 2. A to D, Production of C-C chemokines and IL-16 in CD8⁺ TCC according to Th pattern of cytokine production.

Supernatants of CD8⁺ TCC from 10 patients (four LTnP and six controls) were also examined for antiviral activity. Interestingly, supernatants derived from LTnP had a higher activity to reduce HIV-1 replication compared with those derived from controls (LTnP_{clone} $63.1 \pm 27.3\%$ vs HIV⁺ control_{clone} $18.3 \pm 15.7\%$; $p < 0.05$). Attempts to correlate the presence of C-C chemokines or IL-16 with antiviral activity have been made but no significant association has been found between the degree of antiviral activity and the amount of any cytokines present in various supernatants (Table V).

Discussion

Our study examined the putative role of antiviral factors (C-C chemokines, IL-16, and soluble HIV-suppressive activity) secreted by T cells in the progression of HIV disease, and determined their lack of association with the production of type 1 or type 2 cyto-

kines. We were unable to observe differences in the production of such factors when PBMC or TCL from HIV-infected subjects at different stages of disease were studied, as has been described by others (3). However, when TCC were used as sources of factors, both CD4⁺ and CD8⁺ clones from LTnP showed a higher production of chemokines and IL-16, as well as a stronger inhibition on HIV-IIIB replication (a chemokine-insensitive strain) compared with both AIDS patients and asymptomatic HIV⁺ individuals.

The different results obtained may be explained with the cellular composition of cell types generating the supernatants studied: in PBMC from asymptomatic persons and LTnP, a large proportion of cells were CD4⁺, also producing C-C chemokines and IL-16, thus far thought to be a product of CD8⁺ T lymphocytes only (8), whereas TCL from all groups were more homogeneous for the cytokine pattern, being all IFN- γ ⁺ producers, but with CD8⁺ T

Table IV. Cytokine production by CD4⁺ T cell clones^a

Th Phenotype	RANTES (ng/ml)	MIP-1 α (ng/ml)	MIP-1 β (ng/ml)	IL-16 (ng/ml)	IL-4 (pg/ml)	IFN- γ (pg/ml)
Healthy individuals						
Th1	<1	6	7, 2	1	0	265
Th1	<1	<1	6	0.8	0	125
Th0	<1	<1	3, 6	1.1	33	110
Th0	25	11	68	2.07	1360	135
Th2	<1	<1	2	<0.5	74	0
Th2	<1	<1	2	2.05	49	0
LTnP patients						
Th1	<1	4, 4	2	6.24	0	>5000
Th1	<1	7, 2	93, 6	3.99	0	3475
Th1	10	8, 8	14, 8	5.93	0	5000
Th1	11	23, 8	40, 8	3.92	0	4370
Th0	<1	4, 8	14, 4	2.57	50	3330
Th0	<1	4	3, 4	3.49	508	>5000
Th0	<1	5	5, 2	2.53	>2000	3315
Th0	<1	4, 4	2	9.58	665	4924
Asymptomatic HIV ⁺						
Th1	9	5, 2	2, 8	0.94	0	271
Th0	<1	4	4	<0.5	714	345
Th0	10	8, 2	7, 6	1.27	513	190
Th2	14	3, 6	<1	1.38	2000	0
Th2	12, 5	3, 1	<1	1.13	144	0

^a Cytokines were measured in supernatants collected after 48-h restimulation with PHA-P. All CD4⁺ clones are represented.

cells in the order of >90% in AIDS patients, 80% in asymptomatic HIV⁺ patients, 65% in LTnP, down to 30% or less in noninfected healthy subjects. Therefore, no CD4⁺ TCC seeded from TCLs were grown from patients with AIDS. Only the use of single cell clones revealed differences between recently infected asymptomatic and long-term asymptomatic HIV⁺ subjects. If one assumes that clones grown are representative of the frequency of cells in the original population, they represent a more powerful tool of analysis than mixed populations where only some cells contribute to the phenomenon studied. Moreover, T cell clones gave us the possibility to gain information about the correlation between type 1 and type 2 cytokines and IL-16 or chemokines production (5). The antiviral activity of the supernatants tested was often weak, and it needs to be emphasized that a huge variability was recorded between samples from patients belonging to the same groups. Nevertheless we found that supernatants from different subjects, although containing significantly different amounts of C-C chemokines or IL-16, did not significantly differ in their ability to reduce viral replication. It is possible that both the variability and the low antiviral activity of some supernatants may be due to the presence of different factors (i.e., content of susceptible CD4⁺ cells, activation-induced cytokines and PHA itself) affecting both the viral replication and the cellular activation, which is known to modulate viral replication, as well as in vitro consumption or binding of antiviral factors. This suggests that the antiviral activity might be masked by the presence of other (activating) factors. Indeed, when supernatants from clones, which are supposed to contain lower amount of "disturbing" factors, were examined, higher antiviral activity was observed in those from LTnP, despite <1 log of reduction on average, but no association was found between the amount of chemokines or IL-16 and degree of antiviral activity.

Furthermore, it is possible that the virus and the cellular system used in this study are not suited for evaluating the antiviral activity of such factors. Indeed, it has been reported that HIV-III_B is insensitive to the antiviral activity displayed by chemokines (4), and it has been selected because of this reason to evaluate the full antiviral potential

of T cells. However, we have obtained similar findings using another viral strain (HIV-MN) and, more importantly, when present (>80% of reduction in five LTnP clones), the antiviral activity has been promptly and accurately measured in both systems, thus indicating that the experimental procedure is suitable to detect such type of activity. However, we cannot rule out the possibility that other viruses and/or other cells can allow detection of more reproducible results and, above all, higher degrees of inhibition, and we are currently developing new test systems.

IL-16, a CD4-binding lymphocyte chemoattractant cytokine (8), was found to be produced by both CD4⁺ and CD8⁺ T cells, with very high synthesis in cells from LTnP. The nonexclusive pattern of production, within the limits of the test used (showing, however, a higher inducible secretion by CD8⁺, compared with CD4⁺, cell clones, with again the exception of CD4⁺ cells from LTnP subjects), represents a novel finding of this study. Moreover, we found no correlation between IL-16 and anti-HIV-III_B activity, although in CD8⁺ TCC supernatants from LTnP IL-16 levels appeared to rise in parallel to the content of RANTES and MIP-1 α , but not MIP-1 β . Therefore we must look forward to other details addressing the antiviral function of this cytokine, which seems also to be unrelated to the type 1 or 2 pattern of other cytokines. Its mode of action certainly does not account for the CD8-derived CAF activity (1, 3).

C-C chemokines are produced by both CD4⁺ and CD8⁺ T cells, as well as by other cell types (5). In HIV⁺ subjects, and also in healthy seronegative controls, CD8⁺ T lymphocytes were the major producers, but this applied also to IFN- γ production.

MIP-1 α was produced at lower levels both in TCL and clones from healthy donors compared with HIV⁺ subjects. MIP-1 α chemoattracts activated CD8⁺ and NK cells, whereas MIP-1 β is a potent attractant for CD4⁺ lymphocytes (19, 20). Levels of this latter chemokine are not significantly different between healthy subjects and asymptomatic and symptomatic HIV⁺. MIP-1 α production is up-regulated in HIV infection, and LTnP show a fivefold increase of production compared with all other groups.

Table V. Comparison between chemokine production and antiviral activity

	RANTES (ng/ml)	MIP-1 α (ng/ml)	MIP-1 β (ng/ml)	IL-16 (ng/ml)	% of Viral Ag Reduction
PBMC					
LTnP-1	3.4	9.4	8.0		86.6
LTnP-2	4	64.5	53.8		79
LTnP-3	3.8	6.8	7.8		67.1
LTnP-4	4.6	23.8	4.8		67.1
LTnP-5	6	25.8	36.2		73.1
LTnP-6	4.6	224	207		73.1
LTnP-7	3.8	54	33.6		98.6
LTnP-8	12.8	3.8	4.6		98.6
LTnP-9	3.8	9	14.6		79
HIV ⁺ control 1	8	13.8	11.4		50.7
HIV ⁺ control 2	3.6	10.6	8.8		79
HIV ⁺ control 3	4	21.8	10.4		56.7
HIV ⁺ control 4	3.2	4.8	5.6		59.7
HIV ⁺ control 5	6.8	21	4.8		47.7
HIV ⁺ control 6	3.4	11.2	8.4		98.6
TCL					
LTnP-1	4	2.6	46.6	4.1	0
LTnP-2	7	19.8	24	2.4	28.5
LTnP-3	18.2	27.4	51.8	3.8	91.4
LTnP-4	7.1	12	7	3.9	71.4
HIV ⁺ control 1	<1	40	203	<1	0
HIV ⁺ control 2	4.2	1	48	3.9	28.5
HIV ⁺ control 3	82.6	250	48.8	7.5	99.2
HIV ⁺ control 4	6.2	1	1.4	3.9	62.8
$\alpha\beta^+$CD8⁺ Clones					
LTnP-A (Th1)	60.5	146	412	3.9	99.1
LTnP-B (Th2)	64.5	100.4	48.4	3.9	66.0
LTnP-C (Th1)	35.5	70.2	185.4	9.5	53.5
LTnP-D (Th0)	14.5	62.8	54.6	11.9	33.9
HIV ⁺ control A (Th0)	29	46.8	37.8	1.2	47.3
HIV ⁺ control B (Th1)	10	11.6	11.2	<0.5	19.2
HIV ⁺ control C (Th2)	10.5	6	8	1.2	12.1
HIV ⁺ control D (Th1)	25.5	11.6	111.2	2.3	17.8
HIV ⁺ control E (Th0)	13	4.4	3.4	<0.5	0
HIV ⁺ control F (Th1)	20	6.2	56.8	<0.5	13.4

The production of the C-C chemokines is not related to the type 1 or 2 cytokine pattern of CD4⁺ and CD8⁺ cells, with the notable exception of MIP-1 β , which is preferentially expressed in IFN- γ -producing clones. Our data on TCC from HIV⁺ subjects demonstrate that C-C chemokines and IL-16 secretion by T cells appears to be independent of IL-4 and IFN- γ production. If we assume that the hypothetical switch from Th1 to Th2 type of response underlies the progression to AIDS (16), this model would not account for a protective role of chemokines, because the two events are unrelated. Since we found that all antiviral activities are significantly increased at the clonal level in cells from LTnP, and they are expressed irrespective of the other cytokine types, it is difficult to reconcile the different roles of these factors in HIV disease progression. The evidence provided that both C-C chemokines, IL-16, and HIV-antiviral factor activity are independently present in higher quantity in T cell clones of LTnP may constitute an indication that all are needed to prevent progression of HIV infection to overt disease. It is necessary to emphasize that most immune parameters differ between asymptomatic HIV⁺ and patients with signs of progression (3, 12–15), whereas little changes have been found between LTnP in comparison with asymptomatic HIV⁺; however, our study indicates that long-term nonprogressors also have a quantitative increase in noncytolytic HIV-suppressive factors in respect to asymptomatic and immunologically balanced HIV⁺ with a shorter time of infection, most of whom are likely to

develop symptoms within 10 yr (21). A clinical and immunologic follow-up of these patients is currently being made.

References

- Levy, J. A., C. E. Mackewicz, and E. Barker. 1996. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8⁺ T cells. *Immunol. Today* 17:217.
- Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy. 1986. CD8⁺ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* 234:1563.
- Clerici, M., C. Balotta, and M. Galli. 1996. Soluble HIV suppressive factors: more than one Holy Grail? *Immunol. Today* 17:297.
- Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 α and MIP-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 270:1811.
- Hedrich, J. A., and A. Zlotnik. 1996. Chemokines and lymphocyte biology. *Curr. Opin. Immunol.* 8:343.
- Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* 9:617.
- Paxton, W. A., S. R. Martin, D. Tse, T. R. O'Brien, J. Skurnick, N. L. VanDevanter, N. Padian, J. F. Braun, D. P. Kotler, S. M. Wolinsky, and R. A. Koup. 1996. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposures. *Nat. Med.* 2:412.

12. Buchbinder, S. P., M. H. Katz, N. A. Hessel, P. M. O'Malley, and S. D. Holmberg. 1994. Long-term HIV-1 infection without immunologic progression. *AIDS* 8:1123.
13. Cao, Y., L. Quin, L. Zhang, J. Safrit, and D. D. Ho. 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* 332:201.
14. Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiore, J. M. Orenstein, C. Fox, L. K. Schrager, J. B. Margolick, S. Buchbinder, J. V. Giorgi, and A. S. Fauci. 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N. Engl. J. Med.* 332:209.
15. Aiuti, F., G. D'Offizi, and the NOPHROCO Study Group. 1995. Immunologic and virologic studies in long-term non-progressor HIV infected individuals. *J. Biol. Regul. Homeostatic Agents* 9:82.
16. Clerici, M., and G. M. Shearer. 1994. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol. Today* 15:12.
17. Paganelli, R., E. Scala, I. J. Ansotegui, C. M. Ausiello, E. Haiapi, E. Fanale-Belasio, G. D'Offizi, I. Mezzaroma, F. Pandolfi, M. Fiorilli, A. Cassone, and F. Aiuti. 1995. CD8⁺ T lymphocytes provide helper activity for IgE synthesis in HIV-infected patients with hyper-IgE. *J. Exp. Med.* 181:423.
18. Dianzani, F., R. Capobianchi, G. Antonelli, P. Amicucci, and F. De Idarco. 1989. Susceptibility of human immunodeficiency virus to antiviral agents measured by infectious virus yield reduction. *Antiviral Res.* 11:299.
19. Taub, D. D., K. Conlon, A. R. Lloyd, J. J. Oppenheim, and D. J. Kelvin. 1993. Preferential migration of activated CD4⁺ and CD8⁺ T cells in response to MIP1- α and MIP1- β . *Science* 260:355.
20. Schall, T. J., K. Bacon, R. D. Camp, J. W. Kaspar, and D. V. Goeddel. 1993. Human macrophage inflammatory protein alpha (MIP-1 α) and MIP-1 β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* 177:1821.
21. Baltimore, D. 1995. Lessons from people with nonprogressive HIV infection. *N. Engl. J. Med.* 332:259.