

and poison subunits of any functional homologs. Alternatively, the ETR1 protein may normally be active in the absence of ethylene and negatively regulate the response pathways. Binding of ethylene would inactivate the receptor, resulting in derepression of the response pathways. Mutant receptors that fail to bind ethylene would be locked in the active state, suppressing the response pathways even when wild-type receptors were saturated with ethylene. If true, the latter model must be reconciled with the finding that *Never-ripe* (*Nr*), a mutation in tomato that seems to be attributable to a lesion in a tomato homolog of *ETR1*, shows an increase in *Nr* mRNA abundance in tomato fruits as they become more sensitive to ethylene-induced ripening (18).

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## Identification of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ as the Major HIV-Suppressive Factors Produced by CD8<sup>+</sup> T Cells

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Evidence suggests that CD8<sup>+</sup> T lymphocytes are involved in the control of human immunodeficiency virus (HIV) infection in vivo, either by cytolytic mechanisms or by the release of HIV-suppressive factors (HIV-SF). The chemokines RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were identified as the major HIV-SF produced by CD8<sup>+</sup> T cells. Two active proteins purified from the culture supernatant of an immortalized CD8<sup>+</sup> T cell clone revealed sequence identity with human RANTES and MIP-1 $\alpha$ . RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  were released by both immortalized and primary CD8<sup>+</sup> T cells. HIV-SF activity produced by these cells was completely blocked by a combination of neutralizing antibodies against RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ . Recombinant human RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  induced a dose-dependent inhibition of different strains of HIV-1, HIV-2, and simian immunodeficiency virus (SIV). These data may have relevance for the prevention and therapy of AIDS.

As documented in several viral diseases (1), CD8<sup>+</sup> T lymphocytes are believed to play a critical role in the containment of HIV infection, particularly during the phase of clinical latency and in long-term nonprogressors (2). Activated CD8<sup>+</sup> T cells derived from the peripheral blood of HIV-infected individuals (3), as well as from HIV- or SIV-infected nonhuman primates (4), secrete one or more soluble HIV-suppressive factors (HIV-SF) that may contribute to the control of HIV infection in vivo (5). The production of HIV-SF by CD8<sup>+</sup> T cells isolated in vitro correlates with the disease stage, showing a progressive decline

in parallel with the increasing deterioration of the immune system (6).

We established a sensitive test system for HIV-SF (7), based on a CD4<sup>+</sup> T cell clone (PM1) that has a broad susceptibility to macrophage-tropic and primary HIV-1 isolates (8). The HIV-SF was tested on PM1 cells acutely infected with HIV-1<sub>BaL</sub> (9), a macrophage-tropic isolate with biological properties resembling those of non-syncytia-inducing primary isolates (8, 9). To identify a reproducible source of HIV-SF, we tested (i) three CD8<sup>+</sup> T cell lines immortalized in vitro with human T cell leukemia/lymphotropic virus (HTLV) type I (CD8-UI, CD8-PI, and 67-I), (ii) one HTLV-I-negative CD8<sup>+</sup> T cell line (PF382) and (iii) two CD4<sup>+</sup> T cell lines, one immortalized with HTLV-I (MT-2) and the other with HTLV-II (Vev-II) (Table 1) (10). No effect was seen with the culture supernatant of PF382, which suppresses the differentiation of bone-marrow precursor cells (11). By contrast, all three HTLV-I<sup>+</sup>CD8<sup>+</sup> T cell lines produced extracellular HIV-SF, albeit at different levels. Some HIV-SF activity was also detected in the culture supernatant of MT-2, whereas Vev-II had no significant effect. To ob-

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tain an optimized source of HIV-SF, the two best-producer CD8<sup>+</sup> T cell lines (67-I and CD8-UI) were cloned by the limiting dilution technique, at 0.5 cells per well. Heterogeneity was observed among the clones (Table 1); one of the two clones with the highest HIV-SF activity (FC36.22) was selected for subsequent studies.

Cell-free culture supernatant from FC36.22 was filtered through a 0.22- $\mu$ m membrane and fractionated through a tangential flow filter and centrifugal concentrators (12). The concentrate displaying HIV-suppressive activity in the PM1/HIV-1<sub>BaL</sub> test was further fractionated by weak anion-exchange high-performance liquid chromatography (HPLC). Fractions containing high levels of HIV-SF were pooled and further purified by reversed-phase HPLC (12). Potent HIV-SF activity, in the absence of significant cytotoxic effects, was recovered in two separate fractions, each containing a single major protein peak (Fig. 1). The persistence of the HIV-suppressive activity after reversed-phase HPLC at pH 2.0 was consistent with previous reports that HIV-SF is acid-stable (5). Proteins 1 and 2 were each subjected to proteolytic digestion, followed by sequencing of distinct peptide fragments (three from protein 1 and two from protein 2) (13). The amino acid sequences obtained from tryptic peptides of protein 1 (14) revealed identity

with three different portions of human RANTES, an 8-kD polypeptide belonging to the C-C or  $\beta$ -chemokine subfamily (15). The sequences of tryptic peptides from protein 2 (14) matched those of two different portions of human MIP-1 $\alpha$ , another 8-kD C-C chemokine that is highly related to RANTES (15).

High concentrations of RANTES (139 to 1624 ng/ml) and MIP-1 $\alpha$  (112 to 616 ng/ml) were detected by specific enzyme immunoassay (EIA) in the culture supernatants of all CD8<sup>+</sup> clones tested and their parental cell line (Table 1). In contrast, the only cytokine produced at high levels by the MT-2 cell line was MIP-1 $\alpha$  (340 ng/ml), as recently reported also for MT-4 (16), another HTLV-I-immortalized CD4<sup>+</sup> T cell line. These results confirmed a previous report demonstrating CD8<sup>+</sup>45RO<sup>+</sup> T cells to be the most potent producers of RANTES among blood leukocytes (17). Because of the close similarity to MIP-1 $\alpha$ , we also tested the production of MIP-1 $\beta$ , a third member of the C-C chemokine subfamily (15). Moderate to high levels of MIP-1 $\beta$  were detected in all the culture supernatants tested (9 to 112 ng/ml), except in MT-2 (Table 1).

We next investigated the effects of specific polyclonal goat immunoglobulin G (IgG) neutralizing antibodies (NAb) against these chemokines on the FC36.22-derived

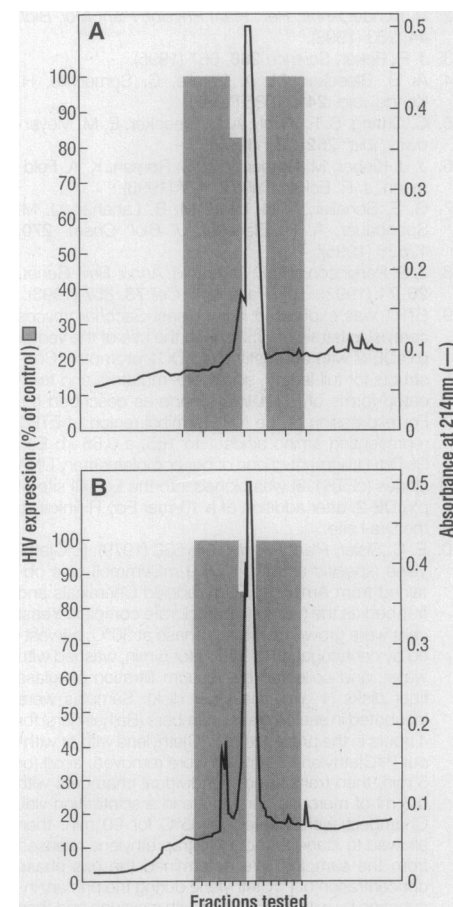
HIV-SF activity. Nonimmune goat IgG, used as a control, had no effect. When the NAb were used alone, only anti-RANTES demonstrated a partial blocking activity. However, the combination of NAb against all three chemokines totally abrogated the HIV-suppressive effect of the FC36.22 culture supernatant (Fig. 2A). Thus, the HIV-SF activity of clone FC36.22 cannot be ascribed to the effect of a single chemokine, but rather to their combined action.

Moderate to high levels of RANTES (6 to 95 ng/ml), MIP-1 $\alpha$  (28 to 255 ng/ml), and MIP-1 $\beta$  (37 to 191 ng/ml) were detected in culture supernatants from activated CD8<sup>+</sup> T cells of HIV-infected patients (Table 1) (18). Lower amounts of all three chemokines were produced by unfractionated peripheral blood mononuclear cells (PBMC), stimulated in the same fashion, from a normal donor. No significant levels of chemokines were produced by unstimu-

**Table 1.** Production of HIV-SF and C-C chemokines by CD4<sup>+</sup> CD8<sup>+</sup> cells; not tested.

Cell type* (phenotype)	Treatment	Extracellular release† of:			
		HIV-SF (ED <sub>95</sub> )	RANTES (ng/ml)	MIP-1 $\alpha$ (ng/ml)	MIP-1 $\beta$ (ng/ml)
<b>Cell lines</b>					
PF-382 (CD8 <sup>+</sup> )	None	-	nt	nt	nt
CD8-UI (CD8 <sup>+</sup> )	IL-2	+ (10%)	nt	nt	nt
CD8-PI (CD8 <sup>+</sup> )	IL-2	+ (20%)	nt	nt	nt
67-I (CD8 <sup>+</sup> )	IL-2	+ (5%)	138.6	111.9	42.4
FC36.4 (CD8 <sup>+</sup> )	IL-2	+ (5%)	189.9	441.4	111.7
FC36.5 (CD8 <sup>+</sup> )	IL-2	+ (1%)	598.0	121.7	15.2
FC36.7 (CD8 <sup>+</sup> )	IL-2	+ (5%)	296.0	148.3	17.9
FC36.12 (CD8 <sup>+</sup> )	IL-2	+ (0.2%)	1623.6	173.8	9.2
FC36.22 (CD8 <sup>+</sup> )	IL-2	+ (0.2%)	810.2	615.8	107.1
Vev-II (CD4 <sup>+</sup> )	IL-2	-	nt	nt	nt
MT-2 (CD4 <sup>+</sup> )	None	+ (50%)	5.3	339.6	2.7
<b>Primary cells</b>					
Normal PBMC	OKT3 + IL-2	nt	2.6	12.3	16.8
CD8.Pt1	None	nt	<1	<1	<1
	OKT3 + IL-2	+	95.1	255.0	187.4
CD8.Pt2	None	nt	<1	<1	<1
	OKT3 + IL-2	+	10.7	28.4	42.2
CD8.Pt3	OKT3 + IL-2	+	29.6	150.0	190.6
CD8.Pt4	OKT3 + IL-2	+	6.5	37.5	134.4
CD8.Pt5	OKT3 + IL-2	+	5.7	28.1	37.5

\*The FC36 series includes representative clones obtained by limiting dilution cloning of the 67-I cell line, performed at 0.5 cells per well in 96-well round-bottom microtiter plates, in the presence of exogenous IL-2. Purified patient CD8<sup>+</sup> T cells (CD8.Pt) from asymptomatic HIV-infected people were cultured for 3 days at 10<sup>6</sup> cells per milliliter in the presence or absence of OKT3 and rIL-2. Normal, unfractionated PBMC, obtained from a healthy blood donor, were stimulated in a similar fashion. †ED<sub>95</sub> = 95% effective dose [percent of culture supernatant (v/v) inducing suppression of HIV-1 p24 antigen release equal to or greater than 95%, compared to untreated controls]. Chemokine production was tested by specific EIA (R&D Systems) in cell-free culture supernatants collected at the stage of maximal cell confluence for cell lines and at day 3 after in vitro stimulation for primary cells. nt, not tested.



**Fig. 1.** Reversed-phase HPLC profile and HIV-suppressive activity of two HIV-SF-containing fractions purified from serum-free culture supernatant of clone FC36.22. (A) Protein 1. (B) Protein 2. The PM1/HIV-1<sub>BaL</sub> system was used for screening the activity of the fractions tested at the concentration of 1% (v/v). At this concentration, no cytotoxic effects were detected. Controls (at least four for each test) were cultured in complete culture medium without supplements.

lated cells from two of the patients.

HIV-SF derived from CD8<sup>+</sup> T cells of four patients tested (CD8.Pt1, 2, 3, and 5) induced a dramatic inhibition of HIV infection (Fig. 2B). Pretreatment with the anti-RANTES NAb alone had only a limited blocking effect (less than 20%) on CD8.Pt1 and virtually no effect on CD8.Pt3. Similarly, anti-MIP-1 $\alpha$  or anti-MIP-1 $\beta$  NAb alone had no effects. However, in three of the four patients tested, the combination of NAb to all three chemokines completely blocked the HIV-SF activity; in the fourth case (CD8.Pt2), the activity was blocked by more than 80%. Thus, the HIV-SF activity produced by in vitro-activated CD8<sup>+</sup> T cells of HIV-infected patients is mostly, if not exclusively, a result of the combined effects of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ .

The HIV-suppressive effect of C-C chemokines was further investigated with recombinant human (rh) proteins, pro-

duced in *Escherichia coli*. A dose-dependent inhibition of the extracellular release of HIV-1 p24 antigen was observed in the PM1/HIV-1<sub>BaL</sub> system with rhRANTES, rhMIP-1 $\alpha$ , or rhMIP-1 $\beta$ , but not with rhMCP-1, a related C-C chemokine (Fig. 3). RANTES was the most effective inhibitor, with the dose inducing  $\geq 95\%$  suppression of HIV p24 release (ED<sub>95</sub>) between 3.12 and 6.25 ng/ml; MIP-1 $\alpha$  and MIP-1 $\beta$  showed ED<sub>95</sub> values of 12.5 to 25.0 and 6.25 to 12.5 ng/ml, respectively. The cellular viability was markedly higher in cultures treated with effective doses of the chemokines, compared to untreated controls (19). Consistent with previous observations with crude HIV-SF (5), the expression of HIV-1 RNA was totally suppressed by treatment of infected PM1 cells with either RANTES (20 ng/ml), MIP-1 $\alpha$  (100 ng/ml), or MIP-1 $\beta$  (50 ng/ml) (20).

We then analyzed the effects of the chemokines on different HIV-1 strains grown

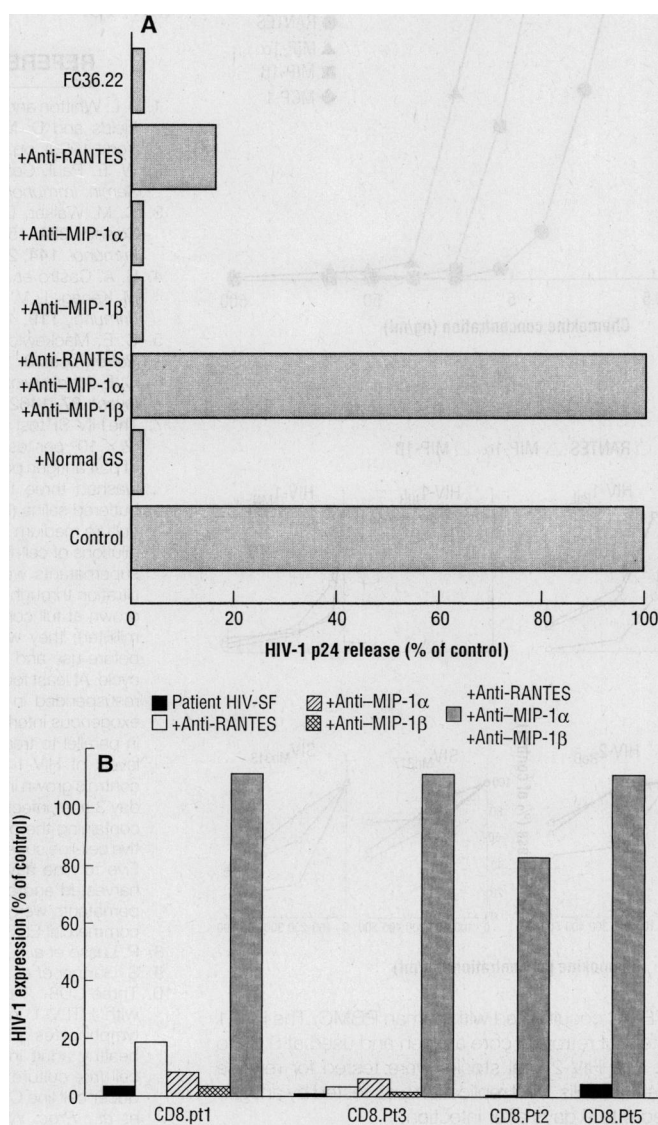
in primary PBMC that had been activated in vitro with phytohemagglutinin (PHA) (21). Two primary isolates (HIV-1<sub>573</sub> and HIV-1<sub>BaL</sub>), never previously passaged in continuous cell lines, and two laboratory isolates (HIV-1<sub>IIIB</sub> and HIV-1<sub>MN</sub>), grown in the CD4<sup>+</sup> T cell line H9, were tested. Treatment with rhRANTES, rhMIP-1 $\alpha$ , or rhMIP-1 $\beta$  induced a dose-dependent inhibition of infection by HIV-1<sub>573</sub>, HIV-1<sub>BaL</sub>, and HIV-1<sub>MN</sub>, albeit with different ED<sub>95</sub> values, whereas HIV-1<sub>IIIB</sub> was virtually insensitive (Fig. 4A). Similar results were obtained with culture supernatant of clone FC36.22 (19). The ED<sub>95</sub> of the three chemokines in primary PBMC was higher than in the PM1/HIV-1<sub>BaL</sub> system. This phenomenon was not dependent on the viral strain used.

The rhRANTES, rhMIP-1 $\alpha$ , and rhMIP-1 $\beta$  induced a dose-dependent inhibition of infection by two HIV-2 and two SIV isolates in primary human PBMC (Fig. 4B) (22). Similar results were obtained with the FC36.22 culture supernatant (19). Neither the C-C chemokines nor the FC36.22 supernatant were able, at the doses tested, to inhibit infection by herpesviruses, either human herpesvirus (HHV)-6 subgroup A (strain GS), HHV-6 subgroup B (strain Z29), or HHV-7 (strain AL), in human cord blood mononuclear cells (23). Similarly, the level of replication of HTLV-I was not affected by treatment with either rhRANTES or the FC36.22 culture supernatant (24). Although these results would suggest a specificity for lentiviruses, a wider panel of viruses and primary patient isolates should be tested.

To rule out that the antiviral activity of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  could be due to a negative effect on cellular proliferation, we tested the proliferative response of primary human PBMC to stimulation with either PHA or monoclonal antibody OKT3 in the presence or absence of these chemokines (25). Only MIP-1 $\alpha$  exerted a dose-dependent inhibitory effect, albeit very limited, on [<sup>3</sup>H]thymidine incorporation induced by OKT3 (24.4, 14.1, and 2.0% inhibition at 500, 100, and 20 ng/ml, respectively), while having no effect on PHA-stimulated cells. All the other chemokine-treated cultures displayed values of [<sup>3</sup>H]thymidine incorporation between 87 and 105% of the control. None of the chemokines, at the doses used, had stimulatory effects on resting cells.

This study demonstrates that chemokines can mediate antiviral effects and identifies RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  as the major HIV-SF produced by CD8<sup>+</sup> T cells. We found that higher concentrations of all three chemokines were required to inhibit HIV in PBMC than in the PM1 cell system. For example, the ED<sub>95</sub> for RANTES

**Fig. 2.** Neutralization of the HIV-SF activity produced by clone FC36.22 (A) or by activated CD8<sup>+</sup> T cells from HIV-infected patients (B) after pretreatment with polyclonal goat IgG NAb against RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$ . The PM1/HIV-1<sub>BaL</sub> test system was used. The neutralization test was performed by incubating the HIV-SF-containing culture supernatants with the NAb, alone or in combination, for 30 min at room temperature. Control, untreated culture supernatants were handled in parallel and kept for 30 min at room temperature. Further controls included cells treated only with the NAb at the same doses and combinations as those used in the neutralization tests. Anti-RANTES, anti-MIP-1 $\alpha$ , and anti-MIP-1 $\beta$  NAb (R&D Systems) were used at 200, 50, and 100  $\mu$ g/ml, respectively, both when used alone and in combination. The IgG fraction purified from a nonimmune goat serum (normal GS) was used as a control at 200  $\mu$ g/ml. Subsequently, both treated and untreated supernatants were added to infected PM1 cells in 48-well plates. The proportion of patient CD8<sup>+</sup> T cell culture supernatant used was 20% (v/v) for patient 1, 40% for patient 3, and 75% for patients 2 and 5.



against HIV-1<sub>BaL</sub> was between 3.12 and 6.25 ng/ml in PM1 and between 50 and 200 ng/ml in PBMC. However, many of the biological activities of RANTES (in terms of Ca<sup>2+</sup> influx, chemotactic responses, basophil and eosinophil activation, and T cell signaling) have been found between 40 and 8000 ng/ml (26). Similarly, several physiological effects of MIP-1 $\alpha$  and MIP-1 $\beta$ , including Ca<sup>2+</sup> influx, chemotaxis, enhancing and suppressive effects on bone marrow progenitor cells, and eosinophil activation, occur in the dose-range between 10 and 1000 ng/ml (26, 27). The experimental conditions used in our PBMC tests (synchronous polyclonal activation with a strong plant-derived mitogen and acute infection with exogenous HIV at relatively high multiplicity) are the most difficult for

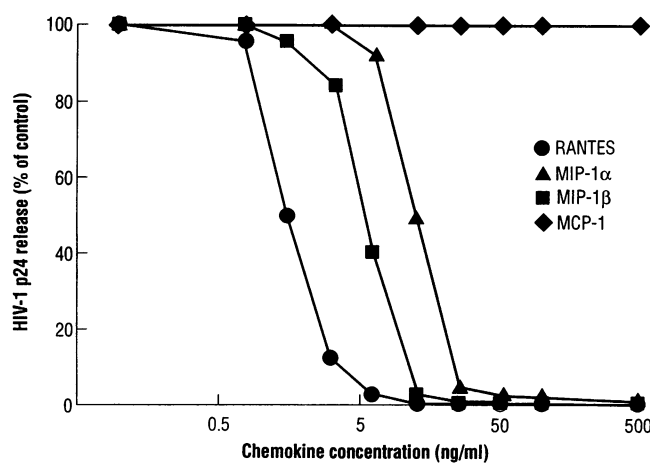
the evaluation of inhibitory factors and are of unlikely physiological relevance. Moreover, the documented propensity of C-C chemokines to form large aggregates at physiological pH may significantly reduce the bio-available levels of these peptides, complicating the interpretation of efficacy studies (15).

Chemokines are actively produced at sites of inflammatory processes and exert proinflammatory effects (15). Chemokine-mediated control of HIV may occur either directly, through their inherent anti-lentiviral activity, or indirectly, through their ability to chemoattract T cells and monocytes in proximity of the infection foci. However, this latter mechanism may also have the opposite effect of providing new, uninfected targets for HIV

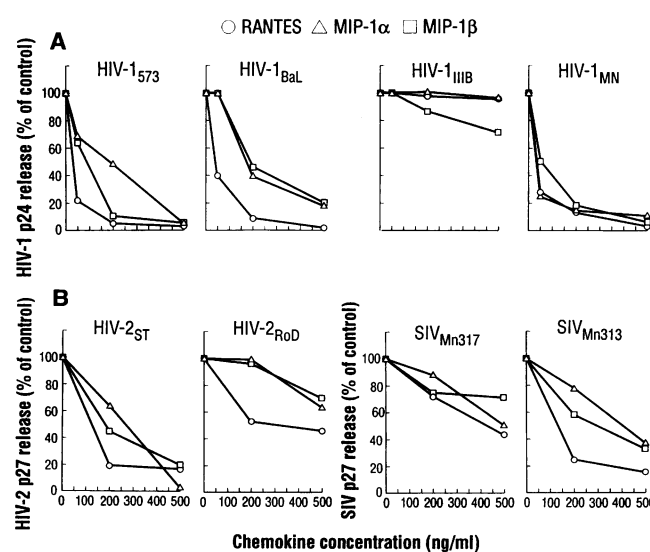
infection. Clinical studies on the production of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  in vivo will be critical to define their role in the natural history of HIV infection. In particular, it will be important to determine whether high levels of these chemokines are associated with a delayed progression of HIV disease. Chemokine levels may also provide a reliable correlate of protection in monkeys treated with experimental vaccines (28). In addition, it is possible that some of the known difficulties with neutralizing antibody assays for HIV are due to the variable amounts of these chemokines in different human sera. Finally, the identification of the HIV-suppressive chemokines may open new perspectives for the development of effective therapeutic approaches to AIDS.

*Note added in proof:* We recently obtained the amino acid sequence analysis of a third protein peak that we have purified from the culture supernatant of clone FC36.22 and found it to exactly match the published protein sequence of human MIP-1 $\beta$ .

**Fig. 3.** Dose-dependent inhibition of HIV-1<sub>BaL</sub> infection in PM1 cells by recombinant human C-C chemokines. The cells were infected with HIV-1 and then cultured in the presence of serial dilutions of each chemokine (all from R&D Systems) in 250  $\mu$ l of complete culture medium. After 3 days, 250  $\mu$ l of fresh culture medium, containing the appropriate amount of the respective chemokine, were added to each culture. The level of HIV-1 replication was tested at days 5 to 7 after infection by p24 antigen capture on cell-free culture supernatants.



**Fig. 4.** Effect of rhRANTES, rhMIP-1 $\alpha$ , and rhMIP-1 $\beta$  on infection by HIV-1 isolates (A) or HIV-2 and SIV isolates (B) in activated human PBMC. HIV-1<sub>573</sub> is a primary isolate grown exclusively in primary PBMC (for less than five passages in vitro); HIV-1<sub>BaL</sub> was passaged several times exclusively in primary adherent macrophage cultures derived from adult peripheral blood; HIV-1<sub>MN</sub> and HIV-1<sub>IIB</sub> are long-term laboratory-passaged isolates, grown in H9 cells. HIV-2<sub>ROD</sub> and HIV-2<sub>ST</sub> were grown in the continuous CD4<sup>+</sup> T cell line SupT1. The two SIV isolates (SIV<sub>Mn313</sub> and SIV<sub>Mn317</sub>) were directly obtained from in vitro-activated monkey PBMC cocultivated with human PBMC. The HIV-1 and SIV viral stocks were previously tested for their content of retroviral core antigen and used at 0.25 to 1 ng of p24 (for HIV-1) or p27 (for SIV) per 10<sup>6</sup> cells. The HIV-2 viral stocks were tested for reverse transcriptase (RT) activity and used at 4000 RT counts per 10<sup>6</sup> cells. Viral replication was tested by antigen capture ELISA on cell-free culture supernatants collected 4 to 9 days after infection.



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7. The HIV-SF test was performed as follows: PM1 cells ( $2 \times 10^5$  per test) were infected with HIV-1<sub>BaL</sub> (4 ng of p24 antigen per  $10^6$  cells) for 2 hours at 37°C, then washed three times with pre-warmed phosphate buffered saline (PBS) and resuspended in complete culture medium (250  $\mu$ l per test) containing different dilutions of cell-free culture supernatant. The culture supernatants were collected by centrifugation and filtration through a 0.2- $\mu$ m membrane from cell lines grown at full confluence ( $2.0$  to  $2.5 \times 10^6$  cells per milliliter); they were often stored frozen at  $-70^\circ\text{C}$  before use and discarded after one freeze + thaw cycle. At least four untreated PM1/HIV-1<sub>BaL</sub> controls, resuspended in complete medium with or without exogenous interleukin-2 (IL-2), were always handled in parallel to treated cultures. No differences in the levels of HIV-1 replication were observed among controls grown in the presence or absence of IL-2. At day 3 after infection, 250  $\mu$ l of fresh culture medium containing the original concentration of the respective cell-line supernatant were added to each culture. Five to nine days after infection, the cultures were harvested and centrifuged to remove cells; the supernatants were tested for HIV-1 p24 antigen by a commercial ELISA test (Coulter).
8. P. Lusso et al., *J. Virol.* **69**, 3712 (1995).
9. S. Gartner et al., *Science* **233**, 215 (1986).
10. Three CD8<sup>+</sup> T cell lines were immortalized in vitro with HTLV-1 in our laboratory. Human CD8<sup>+</sup> T lymphocytes, purified from the peripheral blood of healthy adult individuals, were either exposed to cell-free culture supernatant from the HTLV-1-producer cell line C91/PL [for cell line 67-1; A. DeRossi et al., *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4297

- (1986)] or cocultivated with the HTLV-I-producer cell line MT-2 [I. Miyoshi *et al.*, *Nature* **294**, 770 (1981)], previously irradiated with 10,000 rad (for cell lines CD8-PI and CD8-UI; P. Lusso *et al.*, unpublished data). For CD8-PI, the CD8<sup>+</sup> T cells were activated *in vitro* with purified PHA (Wellcome) at 1 μg/ml, prior to cocultivation with MT-2. All the HTLV-I<sup>+</sup>CD8<sup>+</sup> T cell lines depended on exogenous IL-2 for their growth. Either semipurified IL-2 (Boehringer Mannheim) or recombinant IL-2 (R&D Systems) was used. The HTLV-II-infected CD4<sup>+</sup> T cell line Vev-II, derived spontaneously from *in vitro*-activated peripheral blood leukocytes of an HIV-1/HTLV-II coinfecting patient [P. Lusso, F. Lori, R. C. Gallo, *J. Virol.* **64**, 6134 (1990)], was also IL-2-dependent. In contrast, MT-2 grew without the addition of exogenous IL-2.
- L. Pegoraro *et al.*, *J. Natl. Cancer Inst.* **75**, 285 (1985); G. Bellone *et al.*, *Leukemia* **1**, 603 (1987).
  - Four liters of cell-free culture supernatant of clone FC36.22, grown to full confluence in serum-free medium (HB-101, Irvin Scientific) supplemented with rIL-2 (10 ng/ml), were concentrated to 250 ml with a Millipore prep/scale tangential flow filtration cartridge (0.09 M<sup>2</sup>) with a 30-kD nominal molecular weight exclusion limit. The concentrate was then applied to Centrprep-50 centrifugal concentrators with a 50-kD nominal molecular weight exclusion limit. The Centrprep-50 concentrate (30 ml) was diluted twofold with PBS and reapplied to the Centrprep-50 concentrator. The concentrate was then applied to a Centrprep-100 concentrator with a 100-kD nominal molecular weight exclusion limit. The concentrate obtained was diluted twofold with PBS and reapplied to the Centrprep-100 concentrator to yield a final concentrate of 15 ml. Portions of all the concentrates and filtrates were tested, at various dilutions, for HIV-SF activity. A significant activity was detected in the final concentrate from Centrprep-100. The concentrate (15 ml) was clarified by centrifugation at 100,000g for 60 min at 4°C, diluted threefold in 10 mM tris-HCl, pH 8.0, and applied to a 250 × 10 mm inside diameter weak anion exchange HPLC column (Synchropak, Phenomenex), equilibrated in 10 mM tris-HCl, pH 8.0. Proteins bound to the column were eluted with a linear gradient of 0 to 1 M NaCl in 10 mM tris-HCl, pH 8.0, at a flow rate of 1 ml/min. The fractions obtained, the original material loaded onto the column, and the column flow-through fraction, were tested at various dilutions for HIV-SF activity. The HIV-SF-containing fractions, recovered between 0.3 and 0.4 M NaCl, were pooled, brought to pH 2.0 by addition of trifluoroacetic acid (TFA) and subjected to reversed-phase HPLC on a 3.9 × 300 mm μBondapak C-18 column (Waters Instruments), equilibrated in H<sub>2</sub>O containing 0.1% TFA. Proteins bound to the column were eluted with a 5-min linear gradient of aqueous acetonitrile (0 to 25%) containing 0.1% TFA. After 10 min at 25% acetonitrile in TFA, the column was further developed with a 60-min linear gradient of 25 to 65% aqueous acetonitrile in TFA. The flow rate was maintained at 1 ml/min. Two major peaks of activity were recovered and further analyzed. Fractions corresponding to each of the peaks were combined into two separate pools, diluted twofold in H<sub>2</sub>O with 0.1% TFA and reapplied individually to the analytical C-18 column. The column was developed with a 30-min linear aqueous acetonitrile gradient (0 to 65%) containing 0.1% TFA at a flow rate of 1 ml/min. In both cases, the HIV-SF activity coincided with a single peak eluted from the column.
  - The two fractions containing the active proteins were each reduced, S-carboxyamidomethylated, and subjected to digestion with trypsin. The resulting peptide mixtures were fractionated by narrow-bore HPLC on a 2.1 × 150 mm C-18 reversed-phase column (Vydac) on a Hewlett-Packard 1090 HPLC with 1040 diode array detector. Optimum fractions were chosen on the basis of symmetry, resolution, ultraviolet absorbance, and spectra and then further screened for length and homogeneity by matrix-assisted laser desorption mass spectrometry (MALDI-MS) on a Finnigan Lasermax 2000 (Hemel, England). Strategies for peak selection, reversed-phase separation, and Edman microsequencing have been described [W. S. Lane, A. Galat, M. W. Harding, S. L. Schreiber, *J. Protein Chem.* **10**, 151 (1991)]. Tryptic peptides of protein 1 were subjected to automated Edman degradation on a PE/ABD model 477A protein sequencer (Foster City, CA). Tryptic peptide sequences of protein 2 were determined by microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a Finnigan TSQ7000 triple quadrupole mass spectrometer (San Jose, CA), as described [D. F. Hunt *et al.*, *Science* **255**, 1261 (1992)].
  - The amino acid sequences derived from protein 1 were EYFYTSGK, QVCANPEKK, and EYINSLEMS. The sequences obtained from protein 2 were ADTP-TACCFSYTSR and QVCADPSEEWQK. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The NH<sub>2</sub>-terminal sequence of the first peptide from protein 2 does not coincide with a tryptic site, strongly suggesting that, in contrast with previously published sequences [S. D. Wolpe *et al.*, *J. Exp. Med.* **167**, 570 (1988); T. J. Schall, *Cytokine* **3**, 165 (1991)], it corresponds to the actual NH<sub>2</sub>-terminus of MIP-1α produced by our CD8<sup>+</sup> T cell clone.
  - T. J. Schall, *Cytokine* **3**, 165 (1991).
  - R. Bertini *et al.*, *AIDS Res. Hum. Retroviruses* **11**, 155 (1995).
  - K. Conlon *et al.*, *Eur. J. Immunol.* **25**, 751 (1995).
  - Purified populations of CD8<sup>+</sup> T cells were derived from the peripheral blood of asymptomatic HIV-infected donors by negative immunomagnetic selection with magnetic beads (Dynal) and a cocktail of monoclonal antibodies to CD4, CD19, CD20, and CD56 (Becton Dickinson). The cells were cultured at a density of 1 × 10<sup>6</sup> per milliliter in complete culture medium and stimulated with a mitogenic anti-CD3 monoclonal antibody (OKT3; ascites, used at 1:500 v/v) and rIL-2 (Genzyme) used at 50 ng/ml. Cell-free supernatants were collected at day 3, filtered (0.22 μm), and stored frozen at -70°C until use.
  - P. Lusso *et al.*, unpublished data.
  - A. Garzino-Demo, F. Cocchi, P. Lusso, unpublished data.
  - Human PBMC (5 × 10<sup>5</sup> per test), previously activated with purified PHA at 1 μg/ml for 1 to 5 days, were pelleted and exposed to the viral stocks (0.25 to 1 ng per 10<sup>6</sup> cells) at 37°C. After 1 hour, the cells were washed three times with PBS and cultured in complete medium (500 μl) containing rIL-2 (10 ng/ml) in the presence or absence of different concentrations of rRANTES, rMIP-1α, rMIP-1β, or rMCP-1 (all from R&D Systems). Two days after infection, 500 μl of fresh culture medium containing the same original concentration of the respective chemokine and IL-2 were added to each well. Four days after infection, half of the culture supernatant (500 μl) was removed and replaced with fresh culture medium containing the same original concentration of the respective chemokine and IL-2. Cell-free supernatants were tested at days 4 to 9 after infection for their content of HIV-1 p24 by enzyme-linked immunosorbent assay (ELISA).
  - The test used for HIV-2 and SIV in PBMC was similar to that described for HIV-1 (21). A single, cross-reactive commercial ELISA test (Coulter) was used for both HIV-2 and SIV p27 antigen capture. The two HIV-2 isolates, grown in the continuous CD4<sup>+</sup> T cell line Sup-T1, exhibited marked differences in their cytopathic effect induction and growth kinetics (HIV-2<sub>ST</sub> was slow/low, HIV-2<sub>ROD</sub> was rapid/high). The SIV isolates were derived from two pig-tailed macaques (*Macaca nemestrina*) experimentally inoculated with SIV<sub>sm660</sub> [V. M. Hirsch, R. A. Olmstedt, M. Murphey-Corb, R. H. Purcell, P. R. Johnson, *Nature* **339**, 389 (1989)]. At the time when the two isolates were obtained, approximately 1 year after inoculation, one animal (Mn313) still had high numbers of circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells; the other (Mn317) had markedly reduced numbers [P. Lusso *et al.*, unpublished data].
  - The HIV-6 isolates tested belonged to both subgroup A (GS) [S. Z. Salahuddin *et al.*, *Science* **234**, 596 (1986)] and subgroup B (Z29) [J. Lopez *et al.*, *J. Infect. Dis.* **157**, 1271 (1988)]. For HHV-7, isolate AL [P. Lusso *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3872 (1994)] was used. All herpesvirus stocks were grown in purified, umbilical cord blood, CD4<sup>+</sup> T cells activated *in vitro* with PHA. For infection, activated cord blood mononuclear cells (CBMC) were pelleted and exposed to the viral stocks at the approximate multiplicity of infection of 0.01. After 1 hour at 37°C, the cells were washed three times with pre-warmed PBS and cultured in 500 μl of complete medium supplemented with IL-2 (1 ng/ml), in the presence or absence of each chemokine, used at 200 ng/ml, or of culture supernatant from clone FC36.22, used at 60% (v/v). After 2 days, 500 μl of fresh culture medium containing IL-2 and the respective chemokine or FC36.22 supernatant were added to each culture. A test with IL-2 alone (at 3 ng/ml) was also included in order to control for the amount of IL-2 present in the FC36.22 supernatant. The level of HHV-6 and HHV-7 infection was assessed by indirect immunofluorescence analysis on acetone-fixed cells, harvested at day 5 after infection, with monoclonal antibody 9A5D12 [N. Balachandran, R. E. Amelse, W. W. Zhou, C. K. Chang, *J. Virol.* **63**, 2835 (1989)] which recognizes both HHV-6 (A and B) and HHV-7, as described [P. Lusso *et al.*, *J. Exp. Med.* **166**, 1659 (1988)].
  - Human CBMC were activated *in vitro* with PHA and then cocultured at a 10:1 ratio with MT-2 cells that had been irradiated with 10,000 rad in the presence of rIL-2 (10 ng/ml). rRANTES (100 ng/ml) or FC36.22 culture supernatant (50% v/v) were added immediately after cocultivation and every 48 hours thereafter. HTLV-I replication was tested by p24 antigen capture ELISA (ABI) on cell-free supernatants collected at days 2, 4, 6, 8, and 10 after starting the culture. The lack of susceptibility of HTLV-I to chemokine inhibition was also suggested by the fact that, despite high levels of RANTES, MIP-1α, or MIP-1β production (Table 1), all the HTLV-I-immortalized CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines we tested were found to release high levels of extracellular HTLV-I p24 antigen (F. Cocchi *et al.*, unpublished data).
  - PBMC were separated by Ficoll gradient centrifugation and placed in round-bottom 96-well plates (10<sup>5</sup> cells per well), in triplicate cultures, in the presence or absence of RANTES, MIP-1α, or MIP-1β at 500, 100, or 20 ng/ml, respectively. Monoclonal antibody OKT3 (ascites) was used at 1:500, PHA at 1 μg/ml. [<sup>3</sup>H]Thymidine was added at 1 μCi per well 48 hours after establishment in culture; the cultures were harvested and counted 18 hours later.
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