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9. R. Carroll *et al.*, unpublished results. Quantification of the image in Fig. 2 indicated that 1170, 3610, 970, 1220, 570, and 1450 copies of HIV-1 per 10^5 cells were present after 6 days in cells stimulated with PHA, CD3/MHC class I, CD3/CD2, CD3/CD4, CD3/CD5, and CD3/CD7 and that after CD3/CD28 stimulation less than five copies of HIV-1 per 10^5 cells were present. The CD28-induced resistance was robust because prolonged culture to 28 days did not reveal infection, and the resistance was evident after infection of CD4 cells at high and low multiplicities of infection.
10. J. A. Zack *et al.*, *Cell* **61,** 213 (1990).
11. R. Carroll *et al.*, unpublished results. HIV-1 envelope–mediated cell fusion was analyzed by means of a previously described assay [C. C. Broder and E. A. Berger, *Proc. Natl. Acad. Sci. U.S.A.* **92,** 9004 (1995); O. Nussbaum, C. C. Broder, E. A. Berger, *J. Virol.* **68,** 5411 (1994)]. CD4 cells (1×10^7) activated with either anti-CD3/anti-CD28 or PHA/IL-2 were infected with the recombinant vaccinia virus vTF7-3, which expresses bacteriophage T7 RNA polymerase. After an overnight incubation, these cells were mixed for 2 to 3 hours with HeLa cells infected with vaccinia constructs encoding the envelope genes of the TCL-tropic isolate HIV_{LAV} (vCB-41), the M-tropic isolate HIV_{Ba-L} (vCB-43), or the nonfusogenic mutant Unc (vCB-16). Unc was used to measure background fusion levels. The coding sequence of the HIV_{LAV} envelope is identical to that of the HIV_{NL4-3} isolate (7). The envelope-expressing cells were also infected with recombinant vaccinia virus expressing the *Escherichia coli lacZ* gene linked to the bacteriophage T7 promoter (vCB21R-LacZ). All vaccinia virus infections were done in the presence of cytosine arabinoside (40 μg/ml). The extent of fusion was determined by measuring β-galactosidase activity by means of a colorimetric assay. PHA/IL-2–stimulated cells fused with $HIV\text{-}1_{Ba-L}$ envelope-expressing cells at levels ~7-fold over background, whereas they fused with $HIV\text{-}1_{LAV}$ envelope-expressing cells at levels ~22-fold over background. In contrast, fusion of anti-CD3/anti-CD28–stimulated cells with $HIV\text{-}1_{Ba-L}$ envelope-expressing cells did not exceed background levels, whereas anti-CD3/anti-CD28–stimulated cells fused with $HIV\text{-}1_{LAV}$ envelope-expressing cells at levels ~70-fold over background.
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Potent Inhibition of HIV-1 Infectivity in Macrophages and Lymphocytes by a Novel CCR5 Antagonist

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The chemokine receptors CXCR4 and CCR5 have recently been shown to act as coreceptors, in concert with CD4, for human immunodeficiency virus–type 1 (HIV-1) infection. RANTES and other chemokines that interact with CCR5 and block infection of peripheral blood mononuclear cell cultures inhibit infection of primary macrophages inefficiently at best. If used to treat HIV-1–infected individuals, these chemokines could fail to influence HIV replication in nonlymphocyte compartments while promoting unwanted inflammatory side effects. A derivative of RANTES that was created by chemical modification of the amino terminus, aminooxypentane (AOP)–RANTES, did not induce chemotaxis and was a subnanomolar antagonist of CCR5 function in monocytes. It potently inhibited infection of diverse cell types (including macrophages and lymphocytes) by nonsyncytium-inducing, macrophage-tropic HIV-1 strains. Thus, activation of cells by chemokines is not a prerequisite for the inhibition of viral uptake and replication. Chemokine receptor antagonists like AOP-RANTES that achieve full receptor occupancy at nanomolar concentrations are strong candidates for the therapy of HIV-1–infected individuals.

RANTES, MIP-1α, and MIP-1β are β-chemokines that inhibit HIV-1 infection in vitro (*1–4*) by interacting with the HIV-1 coreceptor CCR5 (*3, 4*). Such coreceptor ligands are potentially useful in the treatment of HIV-infected individuals. Unfortunately, although these reagents consistently inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMCs) (*1, 3, 5, 6*), they do not block infection of primary macrophage cultures (*3, 6, 7*), which indicates that their use could fail to influence HIV replication in nonlymphocyte cell types. Moreover, the chemotactic and leukocyte-activating properties of these β-chemokines may result in undesirable inflammatory responses. Recently, Arenzana-Seisdedos *et al.* (*5*) described a chemokine antagonist that inhibited HIV-1 infection of phytohemagglutinin (PHA)-activated PBMCs but lacked chemotactic and leukocyte-activating properties. Here, we tested two other RANTES receptor antagonists for their ability to inhibit HIV-1 infection of different cell types, including primary human macro-

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phage cultures expressing CCR5. Met-RANTES has an additional methionine at the NH_2-terminus and is a potent antagonist of RANTES signaling in a variety of cell types (*8*). Because the side chain of methionine is nearly isosteric with the *n*-pentyl group, we decided to prepare another RANTES derivative (which we called AOP-RANTES) by first generating an aldehyde-like group at the NH_2-terminus of RANTES and then reacting with aminooxypentane (*9*).

AOP-RANTES could not induce chemotaxis of primary human monocytes (*10*) and inhibited monocyte chemotaxis induced by MIP-1β (Fig. 1, A and B). Because MIP-1β only binds CCR5, this result implies that AOP-RANTES acts as a functional antagonist on primary, CCR5-expressing monocytes. In Fig. 1C, which shows competition of ^{125}I-labeled MIP-1α binding to CCR5-expressing HEK293 cells by RANTES, Met-RANTES, and AOP-RANTES (*11*), RANTES and Met-RANTES both displayed clear two-component displacement curves with a high-affinity site of 0.022 nM and a low-affinity site of 18 nM (for RANTES). Thus, not even at 100 nM could RANTES or Met-RANTES displace all of the MIP-1α bound to the CCR5 receptor. In contrast, AOP-RANTES totally displaced MIP-1α binding at 1 nM, with a median inhibitory concentration (IC_{50}) of 0.072 nM. In addition, its high-affinity competition curve

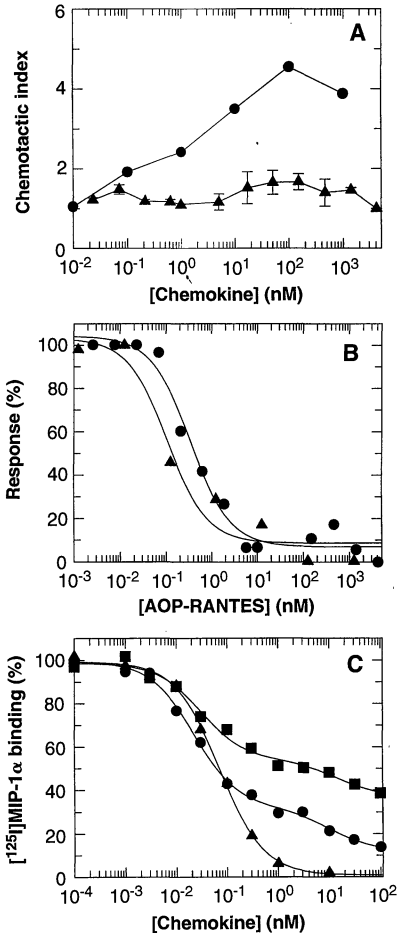

Fig. 1. Chemotactic and binding properties of RANTES, Met-RANTES, and AOP-RANTES. (**A**) Monocyte chemotaxis induced by RANTES (●) and AOP-RANTES (▲). AOP-RANTES elicited no significant migration of monocytes onto the lower surface of the membrane. (**B**) AOP-RANTES was incubated at various concentrations with 100 nM RANTES (●) and 10 mM MIP-1β (▲). Median effective concentration values for these agonists are usually 2 to 5 nM. The inhibition data shown are typical of that seen with five donors. (**C**) Competition of ^{125}I-labeled MIP-1α binding to CCR5. Serial dilutions of RANTES (●), Met-RANTES (■), and AOP-RANTES (▲) were tested for their capacity to compete with MIP-1α binding to CCR5-expressing HEK293 cells.

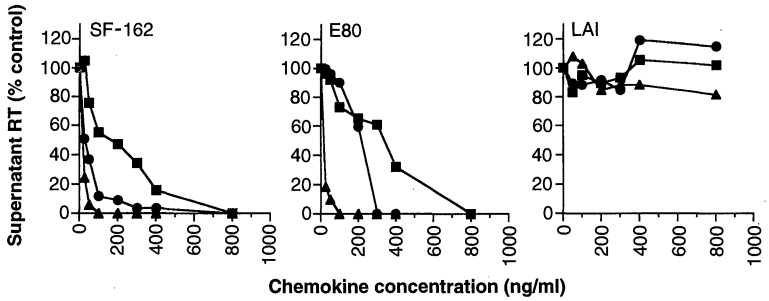

Fig. 2. Inhibition of HIV-1 infection of PBMCs with RANTES (●), Met-RANTES (■), and AOP-RANTES (▲). RANTES, Met-RANTES, and AOP-RANTES inhibited the NSI viruses SF-162 (left) and E80 (center), which use CCR5, but not LAI (right), which uses CXCR4. LAI infectivity, but not SF-162 infectivity, was reduced 50% using stromal cell–derived factor 1 at 3.2 μg/ml.

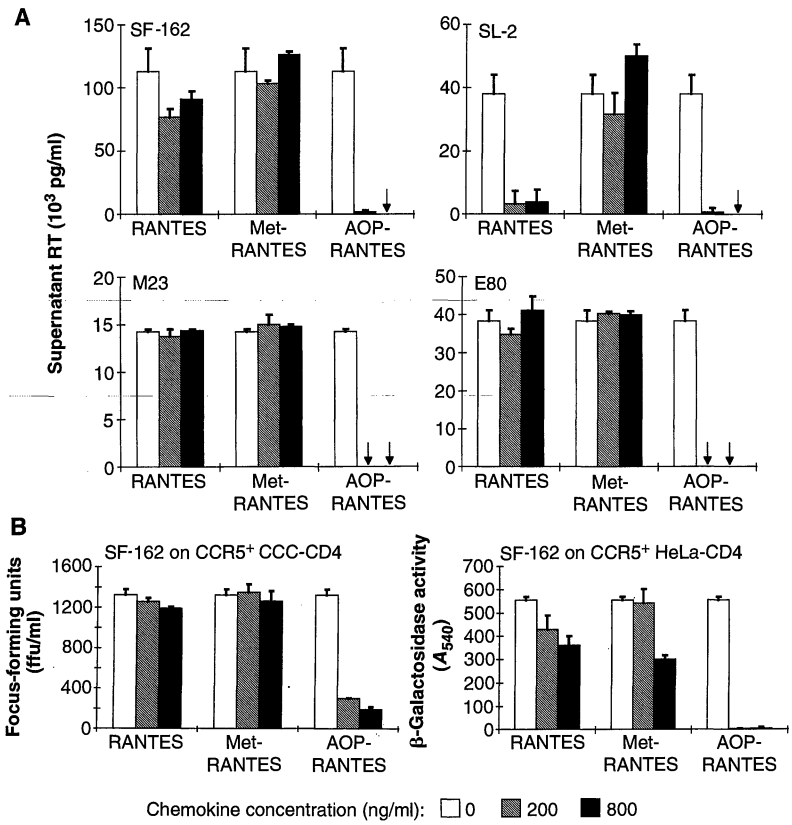

Fig. 3. Effect of AOP-RANTES on HIV-1 infection of primary macrophage cultures and CCR5-expressing CCC-CD4 and HeLa-CD4 cells. (**A**) AOP-RANTES, but not RANTES or Met-RANTES, inhibited SF-162, M23, and E80 infection of primary human macrophage cultures. SL-2 infection was blocked by both RANTES and AOP-RANTES. (**B**) AOP-RANTES, but not RANTES or Met-RANTES, inhibited SF-162 infection of CCR5$^+$ CCC-CD4 and HeLa-CD4 cells. CCR5 is the only coreceptor present on CCC-CD4 and HeLa-CD4 for SF-162, which cannot infect the CCR5$^-$ parental counterparts. Arrows denote columns that do not register on vertical scale. The means of triplicate samples from one experiment are shown; similar results were obtained in additional experiments (*17*).

was classical antagonist-like, with a Hill coefficient of −1.02, which indicates binding to a single site.

We tested RANTES, Met-RANTES, and AOP-RANTES for inhibition of HIV-1 replication in PBMCs stimulated with PHA and interleukin-2 (IL-2) (*12*). The HIV-1 strains first tested were SF-162, E80, and LAI. SF-162 and E80 are primary nonsyncytium-inducing (NSI), macrophage-tropic strains that use CCR5 as a coreceptor (*13*), whereas LAI is a prototype T cell line–adapted, syncytium-inducing (SI) virus that uses CXCR4 as a coreceptor (*14*). Relative to RANTES or Met-RANTES, AOP-RANTES was up to 10 times as potent in blocking infection of PBMCs by both SF-162 and E80 (Fig. 2). Complete inhibition of both strains was achieved with an AOP-RANTES concentration of <100 ng/ml, whereas inhibition with RANTES and Met-RANTES required >200 ng/ml and >400 ng/ml, respectively. None of the three ligands inhibited LAI.

We then compared the effect of RANTES, Met-RANTES, and AOP-RANTES in inhibiting a wider range of HIV-1 strains. Three additional primary NSI macrophage-tropic strains (M23, M53, and SL-2) were blocked by AOP-RANTES, with equivalent concentrations of RANTES and Met-RANTES showing consistent but weaker inhibition. None of these proteins blocked infection by HIV-1 strains that can use CXCR4, including the T cell line–adapted strains LAI and RF (*13, 14*) as well as 2076 and 2044, which are primary viruses that infect macrophages as well as T cell lines (Table 1). Both 2076 and 2044 can use CXCR4, although 2076 can use CCR5 as well (*13*).

Previous studies have reported that RANTES (or a mixture of RANTES, MIP-1α, and MIP-1β) does not block infection of primary macrophage cultures by macrophage-tropic HIV-1 strains (*3, 6, 7*). RANTES, AOP-RANTES, and Met-RANTES were tested for their effect on HIV-1 replication in macrophages. Macrophage cultures were prepared as described (*13, 15, 16*). RANTES and Met-RANTES (200 and 800 ng/ml) had little effect on macrophage replication by the primary NSI macrophage-tropic strains, SF-162, E80, or M23 (Fig. 3A), whereas a fourth strain, SL-2, was blocked by RANTES but not by Met-RANTES. This result indicates that particular strains are likely to be more sensitive to chemokine inhibition than others. In contrast, AOP-RANTES efficiently inhibited macrophage replication of all four strains tested. Similar results were obtained with further macrophage preparations from separate donors (*17*).

It is possible that alternative coreceptors on macrophages could allow HIV to bypass CCR5 and escape inhibition by RANTES and Met-RANTES on macrophages. We therefore tested the effect of RANTES, Met-RANTES, and AOP-RANTES on SF-162 infection of cat kidney CCC cells stably expressing human CD4 (*18*) and transiently expressing transfected CCR5 (*13, 19*), as well as on HeLa cells that stably expressed both CD4 and CCR5 (Fig. 3B) (*20*). CCC and HeLa cells that express CD4 (CCC-CD4 and HeLa-CD4 cells) do not usually express coreceptors for primary NSI viruses (*13*); therefore, when CCR5 is expressed on these cell types, it is the only coreceptor available for such strains. Replication of SF-162 in CCR5-expressing CCC-CD4 cells was efficiently blocked only with AOP-RANTES. Similar results were obtained with HeLa-CD4 cells stably expressing CCR5 (*20*); SF-162 infection was efficiently inhibited by AOP-RANTES, whereas RANTES and Met-RANTES reduced SF-162 infection by <50%. The inability of RANTES and Met-RANTES to efficiently block infection of $CCR5^+$ HeLa-CD4 or CCC-CD4 cells therefore cannot be attributed to the presence of alternative SF-162 coreceptors that do not bind (and cannot be blocked by) RANTES or Met-RANTES.

We currently do not understand why RANTES inhibits HIV-1 NSI infection of PBMCs yet does not reliably block infection of primary macrophage cultures (*3, 6, 7*). Only one of four strains tested here (SL-2) was consistently inhibited by RANTES, although we have observed weak inhibition of other strains if lower amounts of input infectivity are used. We recently observed a similar phenomenon with a CXCR4-specific monoclonal antibody, 12G5 (*21*). This monoclonal antibody inhibited HIV infection and fusion of $CXCR4^+$ RD/CD4 (a rhabdomyosarcoma cell line), yet failed to block infection of most $CD4^+$ T cell lines and infection of at least one cell line expressing CXCR4 as the sole coreceptor (*22*). Together, these results indicate that other factors influence the presentation of coreceptors at the cell surface, and these affect the efficiency with which ligands can inhibit virus infection.

Amino-terminal derivatives of RANTES such as AOP-RANTES and Met- RANTES, which bind with high affinity to the CCR5 receptor yet fail to induce chemotaxis signaling, act as antagonists. Moreover, although Met-RANTES has preserved the two-component interaction mode with the CCR5 receptor (showing even a larger low-affinity component than RANTES itself), the AOP modification has succeeded in creating a classical antagonist with a monocomponent high-affinity binding mode. The total occupancy of CCR5 receptors achieved by the AOP-RANTES antagonist, even at nanomolar concentrations, is probably the reason why AOP-RANTES is a more potent inhibitor of HIV strains that use CCR5 than is RANTES. AOP-RANTES or similarly modified chemokines (or even nonpeptide antagonists) that achieve full re-

Table 1. RANTES and RANTES antagonists inhibit infection of PBMC cultures by NSI, but not SI, HIV-1 strains.

Strain	Supernatant RT (pg/ml)						
	No treatment	RANTES		Met-RANTES		AOP-RANTES	
		200 ng/ml	800 ng/ml	200 ng/ml	800 ng/ml	200 ng/ml	800 ng/ml
NSI viruses							
SF-162	19,071 ± 1,132	1,685 ± 85	<40	9,014 ± 1,998	<40	<40	<40
E80	11,783 ± 143	7,000 ± 407	<40	8,625 ± 525	<40	<40	<40
M23	9,033 ± 543	135 ± 15	<40	2,750 ± 250	140 ± 1	<40	<40
M53	12,750 ± 450	4,850 ± 150	<40	5,150 ± 150	2,000 ± 67	<40	<40
SL-2	33,000 ± 1,080	<40	<40	13,700 ± 1,400	2,900 ± 400	<40	<40
SI viruses							
LAI	8,050 ± 1,150	7,375 ± 25	9,250 ± 650	7,200 ± 123	8,200 ± 154	6,800 ± 200	6,525 ± 175
RF	32,400 ± 2,164	31,833 ± 903	30,933 ± 478	31,933 ± 1,114	31,850 ± 350	30,733 ± 833	32,300 ± 424
2076	25,750 ± 1,250	21,200 ± 1,564	21,933 ± 1,515	25,000 ± 700	23,533 ± 1,826	27,850 ± 2,650	21,300 ± 1,314
2044	34,833 ± 235	33,566 ± 47	33,433 ± 169	34,366 ± 634	34,333 ± 694	33,766 ± 543	33,233 ± 524

ceptor occupancy at low concentrations are therefore suitable candidates for treatment of HIV-infected individuals.

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9. AOP-RANTES was prepared by generating an aldehyde-like group at the NH_2-terminus of RANTES and then reacting with the hydroxylamine derivative C_5H_{11}-O-NH_2 (aminooxypentane) to form the desired oxime, AOP-RANTES. The aldehyde is obtained in a highly site-specific manner by exploiting the susceptibility to very mild periodate oxidation shown only by polypeptides (like RANTES) that have a serine or threonine at the NH_2-terminus [H. F. Gaertner *et al.*, *Bioconjugate Chem.* **3**, 262 (1992)]. The NH_2-terminal oxidation of RANTES was carried out on a 1.2 mg/ml solution that was 60 mM phosphate (sodium, pH 7.0), 12 mM methionine, and 4 M guanidinium chloride. The apparent pH was adjusted to 6.9 by adding 5 M NaOH, and 17.6 μl of 50 mM $NaIO_4$ were added for each milliliter of original solution. The reaction was allowed to proceed at 20°C for 10 min in the dark and was then quenched by adding 6 μl of 6 M ethylene glycol for each original milliliter. After 5 min of quenching, the mixture was exhaustively dialyzed against 1% (v/v) aqueous acetic acid, freeze-dried, and dissolved to a final concentration of 10 mg/ml in 0.1 M sodium acetate adjusted to pH 4.6 with acetic acid, 6 M guanidinium chloride, and 0.1 M methionine. The aminooxypentane was made by reacting terbutoxycarbonyl (BOC)-hydroxylamine with 1-bromopentane in the presence of potassium terbutanolate. Standard methods were used for this synthesis, for the purification of the product by high-performance liquid chromatography (HPLC) (C-18 column in the 0.1% aqueous CF_3COOH/CH_3CN system), and for the removal of the BOC protection. The deprotected aminooxypentane was dissolved to 100 mM in 600 parts (by volume) CH_3CN, 400 parts 0.1% aqueous CF_3COOH, and 7 parts glacial CH_3COOH. The aminooxypentane and protein solutions were mixed in the volume ratio 1:4.36, and the reaction was allowed to proceed overnight at 20°C. The desired derivative was isolated by standard methods of preparative HPLC (C8 column) in the 0.1% aqueous CF_3COOH–CH_3CN system. The derivative was homogeneous on HPLC and by SDS acrylamide electrophoresis. It gave a relative molecular mass of 7901.47 ± 0.58 on electrospray mass spectrometry (calculated, 7901.03).
10. Monocyte chemotaxis was carried out as described [M. Lusti-Narasimhan *et al.*, *J. Biol. Chem.* **270**, 2716 (1995)] with the following modification. The monocytes were purified from the white cell fraction in blood (buffy coats) diluted with 100 ml of phosphate-buffered saline (PBS) before loading onto Ficoll for separation of lymphocytes.
11. HEK293 cells (2×10^5) expressing CCR5 were seeded into each well of a 12-well tray; 24 hours after transfection, the cells were washed in 0.5 ml of binding buffer [50 mM Hepes, 1 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.5% bovine serum albumin (pH 7.2)]. The binding assay was performed in 0.5 ml of binding buffer for 10 hours at 4°C. ^{125}I-labeled MIP-1α (IM258, Amersham) was used at 12 pM throughout and mixed with appropriate dilutions of test chemokines. The reaction was stopped by washing the cells in 0.5 ml of binding buffer and adding 1 ml of lysis buffer (8 M carbamid, 2% Nonidet P40, and 3 M acetic acid) before estimating bound ^{125}I.
12. We prepared virus stocks by infecting PBMC cultures. Virus was harvested when p24 or reverse transcriptase (RT) was detected in the supernatant. Stocks of SF-162 were prepared by first transfecting plasmid DNA directly into PBMCs. Dilutions of RANTES and derivative proteins were mixed with target PHA- and IL-2–stimulated PBMCs and incubated at 37°C for 30 min, and were then exposed to an equal volume of virus supernatant containing 1000 times the median tissue culture infectious dose ($TCID_{50}$), except M23 and SL-2, which were 630 and 800 $TCID_{50}$, respectively, and reincubated at 37°C for 3 hours. Input virus was then washed out before adding growth medium containing appropriate chemokine concentrations. The cultures were incubated at 37°C for up to 12 days with medium changes twice weekly but without further addition of chemokine. Virus production into the supernatant was assessed by measurement of RT activity using a sensitive nonradioactive method (Retrosys RT activity kit; Innovagen AB, Lund, Sweden).
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16. Macrophages were prepared from fresh buffy coats by adherence, as described (*13, 15*). After buffy coats were diluted 1:1 in RPMI 1640, 30 ml was layered onto 15 ml of Ficoll-paque and centrifuged for 30 min at 2500 rpm in a bench centrifuge. The white cells at the interface were harvested and washed once, and 10^8 cells in 20 ml of RPMI 1640 were added to a bacterial culture dish (diameter 140 mm). After 2 hours of incubation, cells that had not adhered were washed away and RPMI 1640 plus 10% human serum was added. After overnight incubation, loosely attached cells were again washed away, RPMI 1640 containing 20% human serum was added, and the cultures were incubated for another 3 days. The day before infection with HIV, cells were treated with versene, gently scraped off using a cell scraper, and reseeded into cell culture dishes. At this stage, >99% of cells expressed the macrophage marker CD14. Chemokine treatment and HIV infection were carried out as described for PBMC cultures (*12*). Culture supernatants were tested for RT activity after 3 weeks.
17. Similar results were obtained from further experiments using macrophages from alternative donors, which confirmed that RANTES and Met-RANTES, at both 200 and 800 ng/ml, did not inhibit SF-162, E80, and M23 infection of macrophages with a virus dose of 1000 $TCID_{50}$ per 10^6 cells, except M23 (630 $TCID_{50}$). In contrast, SL-2 infection (800 $TCID_{50}$) was inhibited by RANTES but was only slightly affected by Met-RANTES. AOP-RANTES inhibited macrophage infection by each of the four strains.
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19. CCC-CD4 cells were transfected with plasmid pcDNA3 containing CCR5 as described (*13*). Twenty-four hours after transfection, the cells were reseeded into 48-well trays at 6×10^4 cells per well. After a further 24 hours, the cells were treated with 75 μl of chemokine at appropriate dilutions in growth medium. After 30 min of incubation, cells were exposed to an equal volume of virus supernatant containing 1000× $TCID_{50}$ and incubated for a further 3 hours. The cells were washed in growth medium and incubated for 4 days in medium containing the appropriate chemokine before fixing and staining in situ for p24 antigen as described [P. R. Clapham, Á. McKnight, R. A. Weiss, *J. Virol.* **66**, 3531 (1992)]. Numbers of stained cells were estimated by microscopy.
20. A subclone of HeLa-CD4-LTRLacZ [T. Dragic, P. Charneau, F. Clavel, M. Alizon, *J. Virol.* **66**, 4794 (1992)] stably expressing CCR5 was used. These cells contain the *LacZ* gene under the control of an HIV-1 long terminal repeat (LTR). Infection of these cells with HIV results in newly synthesized Tat protein that stimulates expression of the *LacZ* gene. Infection of $CCR5^+$ HeLa-CD4-LTRLacZ cells was therefore assessed by estimating LacZ activity 48 hours after infection. Culture medium was removed and the cells were lysed in 100 μl of 0.5% NP-40 in PBS. Cell lysate (50 μl) was mixed with 2× CPRG buffer [16 mM chlorophenol red–β-D-galactopyranoside (Boehringer Mannheim), 120 mM $Na_2HPO_4 \cdot 7H_2O$, 80 mM $NaH_2PO_4 \cdot H_2O$, 20 mM KCl, 2 mM $MgSO_4 \cdot 7H_2O$, and 10 mM β-mercaptoethanol] in a 96-well tray and incubated at room temperature for 2 hours. Absorbance was measured at 540 nm.
21. M. J. Endres *et al.*, *Cell* **87**, 1 (1996).
22. Á. McKnight *et al.*, *J. Virol.* **71**, 1692 (1997).

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