type resorption lacunae to the cell membrane is in any way analogous to the final antigen-presentation route of macrophages.

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10. Thin bone slices were prepared from the cortex of bovine femurs, incubated in phosphate-buffered saline (PBS) containing water-soluble sulfo-*N*-hydroxysuccinimide-biotin (2 mg/ml) (Pierce, Rockford, IL) for 2 hours, washed repeatedly, and rinsed overnight in PBS. Osteoclast cultures (*9*) were fixed at 48 hours in 3% paraformaldehyde for 10 min, and cells were permeabilized in 0.5% Triton TX-100. Biotinylated material was visualized with FITC-conjugated streptavidin (Sigma) for 2 hours at room temperature. After extensive washes, samples were incubated in TRITC-labeled phalloidin (Sigma). Samples from eight independent experiments (each containing 18 bone slices with 200 to 300 osteoclasts per slice) were studied with conventional immunofluorescence microscopy. At 48 hours, 63% of the osteoclasts were resorbing and 37% were nonresorbing. Fifty-one resorbing osteoclasts and 16 nonresorbing osteoclasts were scanned with laser scanning confocal microscopy (Leica Aristoplan CLSM, Leica Lasertechnik, Heidelberg, Germany).
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12. Species-specific polyclonal antibodies (Biogenesis, Poole, England) that recognize bovine but not rat osteocalcin were used (dilution, 1:100, for 60 min at 37°C). Eight independent experiments were performed. Rat osteoclasts were cultured as described for Fig. 1.
13. LF-BON-I antibodies to purified bovine bone osteonectin were a gift from L. W. Fisher, NIH, Bethesda, MD.
14. A growing piglet, aged 4 weeks, received daily intramuscular injections of oxytetracycline (Terramycin/LA, Pfizer) at a dose of 20 mg per kilogram of body weight for 6 weeks. After the pig was killed, thin bone slices were prepared from the cortex of the femurs. Approximately one-fourth of the cortical bone area revealed intensive tetracycline fluorescence, whereas the remaining three-fourths served as a negative control. Four independent experiments were performed, and 16 osteoclasts were analyzed in detail with confocal laser scanning microscopy. Rat osteoclasts were cultured on these bone slices (Fig. 1).
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16. Osteoclasts were fixed in 3% paraformaldehyde, permeabilized with saponin, and blocked in 0.1% bovine serum albumin for 15 min. Streptavidin peroxidase (dilution, 1:100) was applied to cells for 60 min. After intensive washes, the 3,3′-diaminobenzidine (0.5 mg/ml) and H_2O_2 (0.6 mg/ml) reaction was carried out. Cells were fixed in 2.5% glutaraldehyde for 90 min at 4°C and osmicated. Bone slices were then decalcified in sodium cacodylate–buffered EDTA (4.13 mg/ml) containing 0.1% glutaraldehyde for 3 days at 4°C, dehydrated, and embedded in Epon LX-112. Thin sections (100 nm) were stained with lead citrate and observed with a transmission electron microscope (Philips 410, at 60 kV). Twenty individual osteoclasts from three independent cultures were analyzed.

17. Supported by the Finnish Academy of Sciences and by Astra Hässle AB, Sweden. We thank S. Seinijoki and A. Haimakainen for technical assistance.

12 November 1996; accepted 18 February 1997

Differential Regulation of HIV-1 Fusion Cofactor Expression by CD28 Costimulation of CD4$^+$ T Cells

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Activation of CD4$^+$ T lymphocytes from human immunodeficiency virus–type 1 (HIV-1)–infected donors with immobilized antibodies to CD3 and CD28 induces a virus-resistant state. This effect is specific for macrophage-tropic HIV-1. Transcripts encoding CXCR4/Fusin, the fusion cofactor used by T cell line–tropic isolates, were abundant in CD3/CD28-stimulated cells, but transcripts encoding CCR5, the fusion cofactor used by macrophage-tropic viruses, were not detectable. Thus, CD3/CD28 costimulation induces an HIV-1–resistant phenotype similar to that seen in some highly exposed and HIV-uninfected individuals.

Infection of CD4$^+$ T lymphocytes with HIV-1 is initiated by binding of the viral envelope glycoprotein gp120 to the CD4 receptor on the lymphocyte surface, followed by fusion of the virus with the cell membrane (*1*). Virus-cell fusion is mediated by members of the chemokine receptor family, with different members serving as fusion cofactors for macrophage-tropic (M-tropic) and T cell line–tropic (TCL-tropic) isolates of HIV-1 (*2*).

HIV-1 infection is also influenced by the host T cell activation state. Commitment to T cell activation requires T cell receptor (CD3) engagement as well as a costimulatory signal. Interaction of the costimulatory molecule CD28 with its ligands CD80 or CD86 provides a necessary costimulus to induce an immune response (*3*). In addition to promoting the long-term polyclonal proliferation of CD4$^+$ T cells in the absence of exogenous cytokines or feeder cells, activation with immobilized antibodies to CD3 (anti-CD3) and CD28 (anti-CD28) specifically induces a potent anti-HIV effect (*4*). This resistance is due in part to an enhanced production of the β-chemokines RANTES, MIP-1α, and MIP-1β (*5*), which block infection by M-tropic isolates (*6*). However, CD3/CD28-stimulated cells express an additional, cis-acting component of resistance specific to costimulation with immobilized anti-CD28 (*5*). This intrinsic CD3/CD28-specific antiviral effect was examined by comparing the HIV-1 infection process in cells stimulated with either immobilized anti-CD3 and anti-CD28 or with the mitogenic lectin phytohemagglutinin (PHA) and interleukin-2 (IL-2).

Purified CD4$^+$ lymphocytes obtained from uninfected donors were cultured either with PHA and IL-2 or with beads coated with the CD3 monoclonal antibody OKT3

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and the CD28 monoclonal antibody 9.3. The cells were infected with either the M-tropic isolate HIV_{US1} or the TCL-tropic isolate HIV_{NL4-3} (*7*) 3 days after stimulation, and the kinetics of virus replication were assessed by measuring $p24_{Gag}$ antigen production (Fig. 1). Incubation of PHA/IL-2–activated $CD4^+$ cells with either HIV_{US1} or HIV_{NL4-3} resulted in a productive infection. When CD3/CD28-activated $CD4^+$ cells were infected with HIV_{US1}, $p24_{Gag}$ antigen production was virtually undetectable throughout the experiment, in agreement with our previous observation that CD3/CD28-activated cells are resistant to infection with the M-tropic isolate HIV_{Ba-L} (*4*). However, when CD3/CD28-stimulated cells were infected with the TCL-tropic isolate HIV_{NL4-3}, a productive infection ensued (Fig.1).

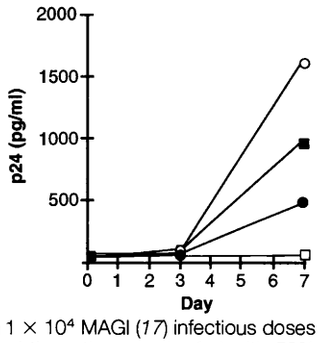

Fig. 1. The CD28 antiviral effect is restricted to M-tropic isolates of HIV-1. Peripheral blood lymphocytes were obtained by apheresis of healthy donors, and $CD4^+$ cells were purified by negative selection as described (*16*). The purified cells were stimulated with DYNAL M-450 beads coated by tosyl conjugation with equal quantities of anti-CD3 [OKT3, mouse immunoglobulin 2a (IgG2a); American Type Tissue Collection], and anti-CD28 (9.3, mouse IgG2a) in medium containing IL-2 (100 U/ml; Boehringer Mannheim) (*4*). Alternatively, purified $CD4^+$ cells were stimulated with PHA [5 μg/ml; Sigma] and IL-2 (100 U/ml; Boehringer Mannheim). Three days after stimulation, 7×10^6 $CD4^+$ cells stimulated with anti-CD3/anti-CD28 (open symbols) or PHA/IL-2 (filled symbols) were infected with 1×10^4 $TCID_{50}$ (median tissue culture infectious dose) of HIV_{US1} (squares) or with 1×10^4 MAGI (*17*) infectious doses of HIV_{NL4-3} (circles). After 2 hours at 37°C, the cells were washed three times and refed with 50% conditioned medium to a final concentration of 1×10^6 cells per milliliter. At the designated time points, cleared supernatant was analyzed for the presence of $p24_{Gag}$ antigen by enzyme-linked immunosorbent assay (Coulter). Each experiment was done at least five times, and representative values are shown.

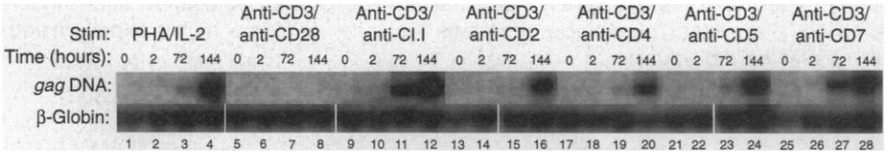

Fig. 2. Resistance of $CD4^+$ cells to infection by M-tropic HIV isolates requires costimulation with immobilized antibodies to CD28. Purified $CD4^+$ cells were stimulated with either PHA and IL-2 (lanes 1 to 4) or with beads coated with equal quantities of anti-CD3/anti-CD28 (lanes 5 to 8), anti-CD3/anti-MHC class I (lanes 9 to 12), anti-CD3/anti-CD2 (lanes 13 to 16), anti-CD3/anti-CD4 (lanes 17 to 20), anti-CD3/anti-CD5 (lanes 21 to 24), and anti-CD3/anti-CD7 (lanes 25 to 28) in medium containing IL-2 (*5*). Three days after stimulation, 5×10^6 $CD4^+$ cells stimulated by each method were infected with 1×10^4 $TCID_{50}$ of HIV_{Ba-L}. Cells (1×10^6) were harvested immediately after virus addition (hour 0), after virus washout (hour 2), and at designated time points thereafter. HIV-1 *gag* DNA sequences present in crude cell lysates were quantitated by means of a previously described PCR-based assay (*18*). Quantitative results are shown (*9*). All exposures were for 1 hour.

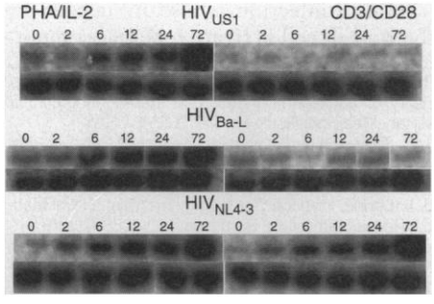

Fig. 3. Infection of CD3/CD28-costimulated cells by M-tropic HIV-1 isolates is blocked at or before the initiation of reverse transcription. Purified $CD4^+$ cells were stimulated with PHA/IL-2 (left) or anti-CD3/anti-CD28 (right) as described in Fig. 1. Three days after stimulation, 5×10^6 cells were infected with 1×10^4 $TCID_{50}$ of the M-tropic isolates HIV_{US1} and HIV_{Ba-L} or 1×10^4 MAGI infectious doses of the TCL-tropic isolate HIV_{NL4-3}, as described in Fig. 1. Virus stocks were treated with deoxyribonuclease (Boehringer Mannheim) before harvesting to degrade contaminating viral DNA. Cells were harvested immediately after infection (lanes marked 0), as well as 2, 6, 12, 24, and 72 hours after infection, as indicated. HIV DNA was detected as described in Fig. 2. Early reverse transcription (strong stop) products were amplified by use of the following primers: 5′-GGCTAACTAGGGAACCCACTG-3′ (sense) and 5′-CTGCTAGAGATTTTCCACACTGAC-3′ (antisense) (*10*). Products were detected by liquid hybridization with an end-labeled oligonucleotide probe (5′-CCGTCTGTTGTGTGACTCTGGTAACTAGAG-3′). The small amount of strong stop DNA present in time zero samples most likely represents reverse transcription products initiated within the virion (*19*). Input cell equivalents were standardized by amplification of human β-globin DNA sequences. Amplified β-globin DNA sequences are shown immediately underneath the HIV DNA panels.

CD28-mediated resistance to M-tropic viruses requires costimulation with bead-immobilized anti-CD28 because stimulation of $CD4^+$ cells with bead-immobilized anti-CD3 and soluble anti-CD28, or with bead-immobilized anti-CD3 and IL-2, renders the cells sensitive to infection with M-tropic viruses (*4*). To further investigate the specificity of the CD28-mediated antiviral effect, we prepared beads containing anti-CD3 in combination with antibodies to the cell surface coreceptors CD2, CD4, CD5, CD7, and major histocompatibility complex (MHC) class I. Binding of antibodies to these coreceptors in conjunction with anti-CD3 treatment increases cellular proliferation (*8*). Purified CD4 cells were stimulated with beads coated with anti-CD3 and antibodies to the various surface receptors. Interleukin-2 was added to all cultures to ensure that all combinations of immobilized antibodies resulted in equivalent cell proliferation (*9*). Only cells stimulated with immobilized anti-CD3/anti-CD28 were resistant to infection with the M-tropic isolate $HIV\text{-}1_{Ba-L}$ (*7*) (Fig. 2). Quantitative analysis indicated that the resistance was robust (*9*).

To determine the nature of the M-tropic–specific block, we examined early events in the viral replication cycle by monitoring reverse transcription in HIV-1–infected CD3/CD28- and PHA/IL-2–stimulated $CD4^+$ cells (Fig. 3). DNA polymerase chain reaction (PCR) was performed with oligonucleotide primers designed to detect reverse transcription products synthesized before the first-strand switch ["strong stop" DNA (*10*)]. Cells stimulated with anti-CD3/anti-CD28 or PHA/IL-2 were infected with HIV_{Ba-L}, HIV_{US1}, or HIV_{NL4-3}. In PHA/IL-2–treated cells, strong stop DNA was detectable shortly after infection with all three viruses, and the level increased for the duration of the experiment. Furthermore, in CD3/CD28-stimulated cells infected with HIV_{NL4-3}, strong stop DNA products accumulated rapidly, confirming that the CD28 antiviral effect was restricted to M-tropic viruses. In contrast, little or no strong stop DNA was detected in anti-CD3/anti-CD28–treated cells infected with either HIV_{Ba-L} or HIV_{US1}.

The failure of M-tropic HIV-1 isolates to initiate reverse transcription in CD3/CD28-stimulated $CD4^+$ cells suggested that a prior event in the replication cycle, such as viral binding or entry, was impaired. Because CD3/CD28-stimulated cells and PHA/IL-2–stimulated cells express equivalent levels of surface CD4 (*4*), the ability of activated $CD4^+$ T cells to

support membrane fusion by envelope glycoproteins from different viral isolates was analyzed with the use of a β-galactosidase reporter gene–based cell fusion assay (*11*). PHA/IL-2–stimulated $CD4^+$ cells fused with cells expressing either TCL-tropic or M-tropic HIV-1 envelopes. In contrast, whereas CD3/CD28-activated cells fused efficiently with cells expressing TCL-tropic envelopes, they failed to fuse with cells expressing M-tropic envelopes. This experiment demonstrated that the block in the ability of M-tropic viruses to enter CD3/CD28-stimulated cells was at the level of envelope-mediated membrane fusion. The high level to which CD3/CD28-stimulated cells fused with cells expressing the LAV envelope is consistent with the susceptibility of CD3/CD28-activated cells to infection by TCL-tropic isolates.

The recent identification of CXCR4/Fusin and CCR5 as fusion cofactors for TCL-tropic and M-tropic HIV-1 isolates, respectively (*2*), prompted us to examine $CD4^+$ cells stimulated with PHA/IL-2 or anti-CD3/anti-CD28 for transcripts encoding these chemokine receptors (Fig. 4). RNA was harvested from cells 24 to 72 hours after stimulation with either anti-CD3/anti-CD28 or PHA/IL-2, as well as from unstimulated cells. The RNA was analyzed by Northern blotting with probes specific for CXCR4/Fusin and CCR5. CXCR4/Fusin transcripts (1.8 kb) were detected at low abundance in unstimulated $CD4^+$ cells, and stimulation with either anti-CD3/anti-CD28 or PHA/IL-2 induced a rapid increase in CXCR4/Fusin transcript levels (Fig. 4). Transcripts encoding CCR5 were not detected in unstimulated cells. Although 4.0-kb CCR5-specific transcripts were detected shortly after PHA/IL-2 stimulation of $CD4^+$ cells, no CCR5 transcripts were detected at any point in CD3/CD28-stimulated cells.

These observations suggest that the susceptibility of CD3/CD28-stimulated cells to TCL-tropic viruses results from up-regulation of CXCR-4/Fusin mRNA expression, consistent with the high level of fusion between CD3/CD28-stimulated cells and cells expressing TCL-tropic envelope glycoproteins. Furthermore, the resistance of CD3/CD28-activated cells to infection by M-tropic viruses and primary isolates of HIV-1 correlates with the absence of detectable CCR5 mRNA expression. This result is consistent with the inability of CD3/CD28-stimulated cells to fuse with cells expressing M-tropic envelope glycoproteins.

Although the mechanism by which CCR5 expression is inhibited in CD3/CD28-stimulated $CD4^+$ cells is unknown, CD28 costimulation exerts many effects on gene expression in general and cytokine expression in particular (*3*, *12*). CD28-induced down-regulation of β-chemokine receptors may be a general feature in T cells, as Loetscher and colleagues recently reported that costimulation of $CD4^+$ cells with anti-CD3/CD28 induced the down-regulation of CCR1 and CCR2 (*13*). In recent studies we have found that CD3/CD28-stimulated $CD4^+$ cells produced high levels of β-chemokines in comparison to lectin-stimulated cells, and that the levels are similar to those in cells stimulated with a variety of costimuli such as CD3/CD5 (*5*). Thus, high levels of β-chemokines are not sufficient to mediate down-regulation of CCR5. Together, these results indicate that chemokine receptor expression is regulated by distinct forms of T cell activation and that chemokine receptor expression is not a consequence of T cell activation in general.

The progression to acquired immunodeficiency syndrome (AIDS) is associated with a shift from an M-tropic to a TCL-tropic viral phenotype (*14*). Although the selective forces driving this phenotypic transition are not well defined, T cell activation itself could be a selective force. CD3/CD28-stimulated $CD4^+$ cells may exert selective pressure in favor of TCL-tropic isolate production through the combination of high levels of β-chemokine production (*5*) and lack of CCR5 expression. Presumably antigen/B7-stimulated CD4 T cells have a similar M-tropic resistance phenotype; however, further studies will be required to establish this. At least early in infection, selective forces other than the susceptibility phenotype of $CD4^+$ target cells may be dominant, as individuals who are homozygous for a defective allele of CCR5 remain resistant to infection and heterozygotes are suggested to have a delayed progression of infection (*15*). These findings may have important consequences for immune reconstitution or gene therapy initiatives in HIV-infected individuals.

Our findings reveal a paradox. CD4 cells stimulated in vitro with anti-CD3 and anti-CD28 on a bead as an "artificial antigen-presenting cell" become resistant to HIV infection, whereas antigen presentation in vivo, as for example after vaccination, leads to enhanced viral replication. Further investigation will be required to establish the relevance, if any, of our findings to the in vivo pathogenesis of HIV infection.

Stimulation of cells with immobilized antibodies to CD3 and CD28 permits the large scale ex vivo expansion of primary $CD4^+$ cells, thus removing one of the largest obstacles to gene therapy or immune replacement therapy for HIV-1–infected individuals (*4*). Indeed, persistent increased CD4 counts and a lack of spikes in viral load have been noted in a clinical trial that is currently in progress to test the CD28 antiviral effect in patients with intermediate stage HIV infection. Our results demonstrate that lymphocyte proliferation and HIV-1 fusion cofactor expression can be unlinked. Such cellular activation regimes could permit the development of new therapeutic approaches for HIV-1–infected individuals.

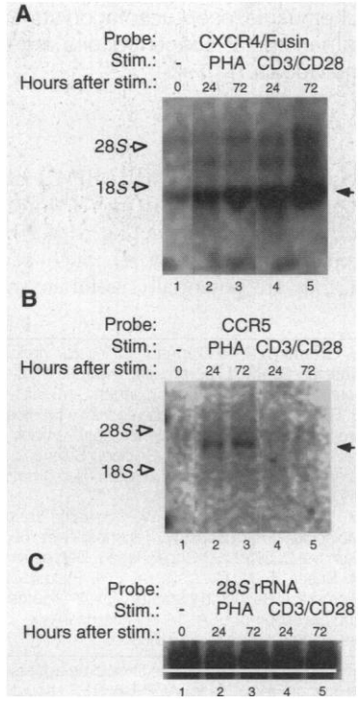

Fig. 4. Expression of chemokine receptor transcripts is differentially regulated in CD3/CD28-stimulated $CD4^+$ cells. RNA was isolated from 5×10^7 unstimulated $CD4^+$ cells (lane 1) or from CD4 cells stimulated with PHA/IL-2 (lanes 2 and 3) or anti-CD3/anti-CD28/IL-2 (lanes 4 and 5) at the indicated times after stimulation with RNAstat (Teltest). Total RNA (20 μg) was separated on agarose-formaldehyde gels and transferred to Zeta-probe membranes (Bio-Rad). The membranes were hybridized initially with an end-labeled oligonucleotide probe specific for CCR5 (5′-CTTGATAATCCATCTTGTTCCACCCTGTGC-3′) (*20*). The sequence of the CCR5-specific oligonucleotide probe was chosen to distinguish between CCR5 and the closely related transcripts encoding CCR2A and CCR2B (*2*). The blots were stripped and then rehybridized with a random-primed 1.3-kb Eco RI CXCR4/Fusin gene fragment (*21*). The membranes were then stripped and hybridized with an end-labeled oligonucleotide probe specific for 28*S* ribosomal RNA (rRNA) (Clonetech) to ensure that equivalent amounts of RNA were loaded in each lane. Transcripts were visualized by means of a Molecular Dynamics PhosphorImager. The positions of 18*S* and 28*S* rRNA are indicated by open arrows, whereas probe-specific bands are indicated by closed arrows. The image in (**A**) was obtained with a 2-hour exposure, and the image in (**C**) with a 10-min exposure, whereas the image in (**B**) was obtained with a 48-hour exposure.

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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.
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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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22. We thank J. Cotte, N. Craighead, T. Francomano, S. Perfetto, and D. Kim for assistance; N. Michael for review of the manuscript; D. Birx and D. Burke for stimulating discussions and for continued support and encouragement; and B. Chesebro for the Hela-CD4-LTR-β-Gal (MAGI) cell line. We acknowledge the National Institutes of Health AIDS Research and Reference Reagent Program for providing the cells. Supported by ARMY DAMD17-93-V-3004 and by the Navy Medical Research and Development Command. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, Navy, Department of Defense, or the U.S. government.

3 December 1996; accepted 6 February 1997

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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