μ-Opioid modulation of HIV-1 coreceptor expression
and HIV-1 replication

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Abstract

A substantial proportion of HIV-1-infected individuals are intravenous drug users (IVDUs) who abuse opiates. Opioids induce a number of immunomodulatory effects that may directly influence HIV-1 disease progression. In the present report, we have investigated the effect of opioids on the expression of the major HIV-1 coreceptors CXCR4 and CCR5. For these studies we have focused on opiates which are ligands for the μ-opioid receptor. Our results show that DAMGO, a selective μ-opioid agonist, increases CXCR4 and CCR5 expression in both CD3+ lymphoblasts and CD14+ monocytes three- to fivefold. Furthermore, DAMGO-induced elevation of HIV-1 coreceptor expression translates into enhanced replication of both X4 and R5 viral strains of HIV-1. We have confirmed the role of the μ-opioid receptor based on the ability of a μ-opioid receptor-selective antagonist to block the effects of DAMGO. We have also found that morphine enhances CXCR4 and CCR5 expression and subsequently increases both X4 and R5 HIV-1 infection. We suggest that the capacity of μ-opioids to increase HIV-1 coreceptor expression and replication may promote viral binding, trafficking of HIV-1-infected cells, and enhanced disease progression.

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Introduction

Intravenous drug users (IVDUs) comprise over 30% of the HIV-1-infected population, and many of these individuals abuse opiates (Donahoe and Vlahov, 1998). The opioid receptors are G-protein-coupled, seven-transmembrane receptors that have been cloned and subsequently grouped into three classes, designated μ, δ, and κ (Chen et al., 1993; Kieffer et al., 1992; Li et al., 1993). The μ-opioid receptor class is associated with the analgesic properties of morphine, based on pharmacological data as well as the observation that morphine-induced analgesia is absent in μ-opioid receptor knockout mice (Matthes et al., 1996). Morphine appears to be the most active metabolite of heroin, and therefore, the effects of morphine on the immune system are important in understanding the ramifications of heroin abuse. The affinity of morphine for the μ-opioid receptor is in the range of 10–20 nM, and during heroin addiction IVDUs can reach levels of up to 2 g per day (Jessop and Taplits, 1991). Patients receiving morphine as therapy for chronic pain, or individuals abusing heroin, can achieve plasma opioid levels of 1 μg/ml or more (Mather and Denson, 1992; Neumann et al., 1982).

Opioid receptors are expressed in the central nervous system (CNS) and on cells of the immune system. Macrophages and T-lymphocytes have been shown to express mRNA for opioid receptors, and binding analysis has revealed surface expression of opioid receptors on these cells (Chuang et al., 1994, 1995; Wick et al., 1996; Wybran et al., 1979). Opioids are known to have several immunomodulatory activities, including inhibition of antibody responses (Bussiere et al., 1993; Wybran et al., 1979), induction of chemotaxis in mononuclear cells (Ruff et al., 1985), altered
macrophage functions (Rojavin et al., 1993; Szabo et al., 1993), and modulation of cytokine production (Chao et al., 1992; Chuang et al., 1993b; Peterson et al., 1987). Opioid use is also associated with enhanced progression of several bacterial and viral infections (Risdahl et al., 1998). Recent in vitro evidence suggests that opioids may also play a role in HIV-1 disease progression. Peterson et al. (1990) have shown that morphine pretreatment of PBMCs, chronically infected with HIV-1 and cocultured with PHA-stimulated PBMCs, induced a three- to fourfold increase in HIV replication. More recently, Peterson et al., (1994) showed that morphine treatment also increased HIV replication in fetal brain cells cocultured with a chronically infected monocyte cell line. The basis for the opioid-mediated increase in HIV susceptibility remains uncertain; however, our laboratory has recently reported that κ-opioid administration induces a significant increase in CC-chemokine receptor (CCR)-2 expression in murine T cells (Zhang and Rogers, 2000). Based on these results, our laboratory investigated whether μ-opioids, including morphine, may elevate the expression of the major HIV-1 coreceptors CXCR4 and CCR5 (Alkhair et al., 1996; Feng et al., 1996). Our data indicate that the expression of the major HIV-1 coreceptors is elevated in CD14+ monocytes and CD3+ lymphoblasts. Furthermore, this increase in CXCR4 and CCR5 expression on the major cellular targets for HIV-1 translates into enhanced replication of X4 and R5 HIV-1 viral strains.

Results

**DAMGO-induced chemokine receptor expression**

To investigate the effect of μ-opioid administration on the expression of CXCR4 and CCR5, normal human PBMCs were treated with the highly selective μ-opioid agonist DAMGO, and chemokine receptor expression was assessed by flow cytometry. PBMCs were first gated for high forward scatter to focus on the monocyte and lymphoblast populations. Our results showed that CXCR4 and CCR5 expression was enhanced in a dose-dependent manner in PBMCs treated with DAMGO (Fig. 1). It should be noted that we observed that basal expression of CXCR4 by nontreated lymphoblasts was typically lower than in the total lymphocyte population, and basal levels can vary as much as fivefold among donors. The induction of CCR5 and CXCR4 by DAMGO was consistent among donors; however, the level of increase varied from two- to sevenfold depending on the basal levels of chemokine receptor expression by donors. We found that DAMGO induced an elevation in CXCR4 and CCR5 expression by 48 h, with a maximal effect at 72 h (data not shown). The percentage of CXCR4-positive cells (Fig. 1; closed circles) increased by two- to fivefold with increasing concentrations of DAMGO up to the maximal effect at 10 nM DAMGO. Cells positive for CCR5 (Fig. 1; closed triangles) increased two- to fourfold after DAMGO administration, with the maximal effect observed at concentrations between 0.1 and 10 nM. These concentrations are physiologically relevant since the binding affinity of DAMGO and morphine is in the nanomolar range for the μ-opioid receptor.

**DAMGO-induced chemokine receptor expression is mediated by the μ-opioid receptor**

Due to the broad expression of CXCR4 on most hematopoietic cells and the sparse expression of CCR5, we wished to focus our studies on the primary target cells for HIV-1 infection which include T-lymphocytes and monocytes (Alkhair et al., 1996; Bleul et al., 1997; Zaitseva et al., 1997). To better define the effect of DAMGO on CXCR4 and CCR5 expression in specific cell populations, we utilized two-color flow cytometric analysis to examine CXCR4 expression on CD3+ T-lymphocytes and CD14+ monocytes. A representative dot plot (Fig. 2) of CD14 and CCR5 expression in the high forward scatter gated population shows that 8.1% of the cells were double-positive without treatment (Fig. 2A). After treatment with DAMGO for 72 h, the CD14+ CCR5+ population increased to 21.6% (Fig. 2B). The induction of CCR5 expression by DAMGO in the CD14+ CCR5+ population was blocked by a 30-min pretreatment with 1 μM CTAP, a μ-selective antagonist (Fig. 2D). Based on two-color analysis, we found that DAMGO significantly increased both CXCR4 and CCR5 expression in CD14+ monocytes (Fig. 3A and B). Pretreatment with the highly selective μ-opioid receptor antagonist CTAP completely blocked the DAMGO-induced increase of both CXCR4 and CCR5 expression. Our results showed that CXCR4 expression was elevated twofold in CD14+ monocytes treated with DAMGO (Fig. 3A). Similarly, the administration of DAMGO also increased CCR5 expression...
twofold in CD14⁺ monocytes (Fig. 3B). Additional studies confirmed that the maximal elevation of CCR5 and CXCR4 was achieved at a concentration of 10 nM DAMGO (data not shown and Fig. 1).

We also carried out an analysis of CD3⁺ T-lymphocytes, and our results showed that DAMGO treatment induced a fourfold increase in CCR5 expression by CD3⁺ lymphoblasts (Fig. 3C and D). In contrast, levels of CCR5 expressed by nonlymphoblast (resting) CD3⁺ T cells were not significantly altered by DAMGO administration (Fig. 3C). Not surprisingly, the expression of CCR5 by nonblast cells was very low, since most of these cells are nonactivated T cells which express low levels of CCR5 (Sallusto et al., 1999). Moreover, the increase in CCR5 expression after DAMGO administration was also blocked by pretreatment with the µ-specific antagonist CTAP (Fig. 3D). Not surprisingly, we found that CXCR4 expression in the CD3⁺ nonlymphoblast population was not altered after DAMGO administration. In contrast, DAMGO induced a twofold increase in CXCR4 expression in the CD3⁺ lymphoblast population (data not shown). The results in our study showed donor variability in the basal levels of chemokine receptor expression. However, most donors fell within a range of two- to fivefold increase in chemokine receptor expression with DAMGO administration.

**DAMGO does not alter CCR1 expression**

We extended our study to determine whether DAMGO administration induced the expression of CCR1, since this receptor is also expressed on both monocytes and T cells. While CCR1 is widely expressed on lymphocytes and monocytes (Su et al., 1996), it does not possess coreceptor activity for HIV-1 (Hill et al., 1997, Rucker et al., 1997). Cells were treated with 10 nM DAMGO, and the expression of CCR1 on CD14⁺ monocytes and CD3⁺ lymphoblasts was determined by flow cytometric analysis. Our results showed that DAMGO administration did not induce a detectable change in CCR1 expression for either CD14⁺ or CD3⁺ cells (data not shown). Our data show that DAMGO administration induces CXCR4 and CCR5 expression, but fails to alter the expression of CCR1.

**HIV-1 p24 levels are enhanced after DAMGO pretreatment**

We tested the possibility that by enhancing HIV-1 coreceptor expression DAMGO may increase the susceptibility to HIV-1 infection. PBMCs were treated with 10 nM DAMGO for 72 h to permit maximal coreceptor expression and then infected with either HIV-1IIIB (X4 strain) or HIV-1JRFL (R5 strain) for 2 h to permit viral binding. The cells were then washed to remove unbound virus and incubated for an additional 72 h, and supernatants were collected to assess viral replication by HIV-1 p24 antigen production. Our results (Fig. 4A) showed that X4 HIV-1 replication was increased twofold in DAMGO-pretreated cells, and this increase was completely reversed in DAMGO-treated cells by CTAP (Fig. 4A). Moreover, susceptibility to R5 HIV-1 infection was also significantly increased in DAMGO-
treated cells. Again, this effect was reversed by pretreatment with CTAP (Fig. 4B). The data in Fig. 4 suggest that the DAMGO-induced elevation in the major HIV-1 coreceptors CXCR4 and CCR5 is coincident with enhanced HIV-1 replication. The effect of DAMGO on HIV-1 susceptibility was consistent and reproducible in that we observed a two-fold or greater increase in p24 levels in a total of 11 of 13 independent experiments, carried out with six donors. Additional experiments showed that levels of HIV-1 p24 remained elevated in the DAMGO-treated cells through at least 120 h (data not shown).

HIV-1 LTR levels are enhanced after DAMGO pretreatment

To further determine whether the DAMGO-induced elevation of HIV-1 coreceptor expression was linked to the elevation of HIV-1 replication by DAMGO, we also performed analysis of HIV-1 long-terminal repeat (LTR) as an early indication of viral replication. Since the presence of HIV-1 LTR is an early indication of acute infection, we measured the production of LTR cDNA in PBMCs 4 h after exposure to viral infection with either X4 or R5 viral strains. Using PCR analysis, we found that DAMGO administration 72 h prior to infection elevated both HIV-1 X4 and R5 LTR cDNA synthesis in PBMCs (Fig. 5). Pretreatment with CTAP was able to block the DAMGO-induced elevation in HIV-1 LTR expression, suggesting that the effect was µ-opioid receptor mediated. These data suggest that DAMGO alters an early event in viral replication, and this leads to increased LTR transcription and viral replication. This is consistent with the capacity of DAMGO to induce an increase in coreceptor expression and accumulation of p24 in culture supernatants. The elevation of HIV-1 LTR was significantly more robust than the effect of DAMGO on HIV-1 p24 levels. The difference in the duration of HIV-1 infection (4 h for HIV-1 LTR and 72 h for HIV-1 p24) may account for the difference in the apparent effect of DAMGO on HIV-1 replication. During the 72-h infection period, differences in the susceptibility of the cells to the infection would be expected to "wash-out" as each cell is eventually infected by the virus. After successful viral binding and internalization, the virus would be expected to replicate at a similar rate in both the DAMGO-treated and the nontreated cell cultures, and this may allow non-DAMGO-treated cells to establish an infection that is not blocked by DAMGOpretreatment.
Morphine augments HIV-1 p24 levels

We wished to extend our studies to determine the capacity of morphine to alter HIV susceptibility, since this opioid agonist is a natural metabolic product of heroin. Morphine primarily interacts with the \( \mu \)-opioid receptor; however, this opioid is less selective for the \( \mu \)-opioid receptor than DAMGO. Our results (Fig. 6) show that pretreatment of PBMCs for 72 h with morphine enhances both HIV-1\( _{HIV} \) (Fig. 6A) and HIV-1\( _{JRFL} \) (Fig. 6B) replication. Morphine also enhances CXCR4 and CCR5 expression, although the effect was much more variable among donors when compared to the highly selective \( \mu \)-opioid agonist DAMGO. Our data indicate that CXCR4 expression in CD3\(^+\) lymphoblasts is elevated from a minimum of 28% to a maximum of 300%, while CCR5 expression in CD14\(^+\) monocytes increases by a minimum of 24% to a maximum of 300% after 10 nM morphine administration. The variability in coreceptor expression may at least partially explain the dissimilarity between donors in the baseline HIV-1 p24 levels following infection (compare untreated cells in Fig. 4B with those in Fig. 6B). The data show that morphine increases HIV co-

receptor expression, and this is associated with an increase in susceptibility to HIV infection.

Morphine and DAMGO enhance CXCR4 and CCR5 mRNA expression

To further determine the effect of opioids on HIV-1 co-receptor expression, we utilized the RNase protection assay (RPA) to determine if \( \mu \)-opioids had an effect on HIV-1 co-receptors at the mRNA level. We found that both DAMGO and morphine had a statistically significant effect on CXCR4 and CCR5 mRNA expression in all donors analyzed; however, STRL33, US28, CCR3, CCR8, GPR1, V28, and CCR2b mRNA expression were not consistently altered (Figs. 7 and 8). Quantitation of the mRNA levels by phosphoimaging showed that following DAMGO administration the level of the minor HIV coreceptor GPR15 was occasionally increased (fewer than half of all donors). Additionally, several other chemokine receptors were analyzed by RPA including CCR1-5, CCR8, and CXCR1-4, and only CCR5 and CXCR4 mRNA expression were consistently elevated following DAMGO administration (Fig. 7 and data not shown). The expression of both CXCR4 and CCR5 mRNA was increased by approximately twofold with DAMGO administration in each donor analyzed (Fig. 7), which is consistent with the increase in protein expression observed for this \( \mu \)-opioid (Fig. 3). Additional studies (Fig. 8) showed that morphine also induced an increase in CXCR4 and CCR5 mRNA levels, and the increase was less robust than with DAMGO administration, but the effect was statistically significant. Taken together, our results show

Fig. 6. Morphine augments PBMC susceptibility to HIV-1 infection. PBMCs were treated with medium only (open bars) or morphine at the designated concentrations (solid bars) for 72 h. Cells were then infected with either the X4 strain HIV-1\( _{HIV} \) (A) or the R5 strain HIV-1\( _{JRFL} \) (B). After 72 h of infection, supernatants were analyzed for HIV-1 p24 by ELISA. Triplet cultures were analyzed, and the results are presented as the mean (± SD) of triplicate determinations. Data are representative of three experiments. *P < 0.01.

Fig. 7. DAMGO administration increases CXCR4 and CCR5 mRNA expression in adherent cells from the total PBMC population. PBMCs were incubated overnight and then incubated with medium alone or DAMGO at the designated concentrations. After 24 h, adherent and nonadherent PBMCs were harvested and analyzed for the expression of CXCR4 or CCR5. A representative RPA is shown along with graphical representation of the samples in triplicate. Data are representative of three experiments. Data are shown as the percentage of the L32 housekeeping gene expression. Lane 1 (0 nM), lane 2 (10 nM) and lane 3 (0.1 nM).
that the induction of CXCR4 and CCR5 is due to effects that appear to be primarily at the level of receptor transcription.

Discussion

Our findings provide a potential mechanism to explain the reported elevation in HIV-1 replication following morphine administration (Peterson et al., 1990, 1994). Our studies suggest that the μ-opioids, including morphine, induce a significant increase in HIV coreceptor expression on both CD3^+ T-lymphoblasts and CD14^+ monocytes. Our data show, moreover, that the induction of coreceptor expression is associated with an increase in both X4 and R5 viral replication. Analysis of LTR reverse transcription, an early event in HIV infection, further corroborates these results by correlating the elevated coreceptor levels with an increase in early HIV replication. Our results are consistent with previous studies showing that upregulation of CCR5 or CXCR4 expression results in elevated HIV-1 replication (Secchiero et al., 1999; Tuttle et al., 1998).

To evaluate the effect of opioid administration on HIV-1 susceptibility, we carried out our experiments without activating agents such as phytohemagglutinin (PHA) or IL-2. Our analysis has shown that PHA itself significantly elevates CCR5 and CXCR4 expression, and this would mask any potential effect on coreceptor expression. Of course, nonactivated cells are less susceptible to HIV-1 infection; however, we were able to achieve consistent and reproducible levels of HIV-1 replication in nonactivated cells. Scales et al. (2001), Shapiro et al. (1999), and Wetzel et al. (2002) found that nonactivated PBMCs or subsets of the nonactivated PBMC population could be readily infected with HIV-1. Also, Chun et al. (1998) found that resting CD4^+ T-lymphocytes from HIV-1^+ individuals expressed HIV-LTR in all patients analyzed.

Our results agree with recent studies which have shown that morphine induces CCR5 expression in the CEMx174 lymphocyte cell line and enhances syncytia formation in these cells after infection with simian immunodeficiency virus (SIV) (Miyag et al., 2000). Chuang et al. (1993a) previously found that rhesus monkeys dependent on morphine have an elevated rate of SIV viral replication. This is in contrast with studies showing that chronic morphine administration may have a protective effect on SIV infection (Donahoe et al., 1993). These conflicting results are likely due, in part, to the differences in the virulence of the SIV strains utilized and the amount of morphine administered. There are also conflicting results arising from epidemiological studies following HIV-infected IVDUs. While studies have shown that CD4^+ counts were decreased and mortality rates were elevated in HIV-infected IVDUs compared to HIV-infected non-IVDUs (DesJarald et al., 1987; Rothenberg et al., 1986), more recent reports have suggested that CD4^+ counts are not altered in HIV-infected IVDUs as compared to those in HIV-infected non-IVDUs (Galai et al., 1995).

The molecular basis for the induction of coreceptor expression by opioids may be related to the well-established capacity of opioids to alter cytokine expression in immune cells. For example, morphine has been reported to elevate the production of IL-2 from rhesus monkey lymphocytes (Chuah et al., 1993b). Recent studies have shown that IL-2 enhances the expression of both CCR5 and CXCR4 by CD4^+ T cells and CD4^+ memory cells, respectively (Jourdian et al., 2000; Weissman et al., 2000). In contrast, morphine treatment has been found to inhibit the production of IFN-γ by activated lymphocytes (Peterson et al., 1987). Interestingly, IFN-γ has been reported to increase the expression of CCR5 and CXCR4 in monocytes (Harsharan et al., 1999; Lee et al., 1999); however, conflicting results have been observed which suggest that CXCR4 expression is reduced in PBMCs and the promonocytic cell line U937 following IFN-γ treatment (Shirazi and Pita, 1998). Finally, at picomolar concentrations, morphine has also been shown to stimulate the production of TGF-β (Chao et al., 1992). It appears that TGF-β induces an increase in the expression of both CCR5 and CXCR4 by dendritic cells (Sato et al., 2000), a population of accessory cells which is believed to be critical for the sequestering of HIV-1 in the thymus and lymph nodes (Granelli-Piperno et al., 1998). It is possible that the opioid-mediated modulation of IL-2, IFN-γ, or TGF-β, alone or in combination, may be responsible for the induction of CCR5 and CXCR4 expression on monocytes and T lymphoblasts. The morphine-induced modulation of cytokine production can be dependent on
both the cell population and the activation state of the cell (Chao et al., 1992; Peterson et al., 1987). This may account for the results observed in our studies which show an induction of chemokine receptors on CD3+ lymphoblasts, but not on the nonlymphoblast population. This creates a complex environment, in which subtle factors may play a pronounced role in controlling the competence of the immune system.

Our laboratory proposes a model for the effects of DAMGO on HIV-1 infection. Engagement of the \( \mu \)-opioid receptor by DAMGO administration triggers events such as cytokine production which can enhance expression of CXCR4 and CCR5, the major HIV-1 coreceptors. The elevation of coreceptor expression in the population of CD3+ T-lymphoblasts and CD14+ monocytes enhances viral binding and fusion of HIV-1 to target cells. This occurs within a relatively short period of time (72 h), allowing HIV-1 to rapidly infect additional target cells. The enhanced expression of HIV-1 coreceptors by activated T-lymphocytes could enhance HIV-1 fusion with cells in a replication competent state, thus allowing for enhanced viral production. Previous studies from our laboratory have shown that DAMGO elevates the expression of MCP-1 (monocyte chemoattractant protein-1), RANTES (regulated upon activation, T cells, expressed and secreted), and IP-10 (IFN-\( \gamma \)-moattractant protein-1), RANTES (regulated upon activation, T cells, expressed and secreted), and IP-10 (IFN-\( \gamma \)-inducible protein-10) by 48 h in PBMCs (Wetzel et al., 2000). The induction of both CCR5 and RANTES (a CCR5 inducible protein-10) by 48 h in PBMCs (Wetzel et al., 2000). Overall, the enhanced HIV-1 coreceptor expression by DAMGO, along with the production of RANTES and viral chemotactic proteins, may create an environment in which DAMGO elevates viral binding and fusion to host cells and also increases trafficking of target cells to sites of infection.

Materials and methods

Cell culture

PBMCs were obtained from whole blood of normal donors and isolated by Ficoll–Paque Plus (Pharmacia Biotech, Piscataway, NJ) density gradient centrifugation. Isolated PBMCs were plated at a cell density of \( 3 \times 10^6 \) cells/ml in 24-well tissue culture plates. Cell cultures were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated (56°C, 30 min) endotoxin-free FBS (HyClone, Logan, UT), 10 \( \mu \)g/ml gentamicin (Life Technologies), and 1 mM L-glutamine (Life Technologies). Cell cultures were maintained at 37°C, 5% CO\(_2\).

Opioid agonist and antagonist treatment

PBMCs were treated with agonist \([\text{D-Ala}^2, \text{N-Me-Phe}^4, \text{Gly-ol}^5]\) enkephalin (DAMGO; Multiple Peptide Systems, San Diego, CA) or morphine at designated concentrations for 72 h. PBMCs pretreated with an antagonist were treated with \([\text{D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH}_2]\) CTAP (Multiple Peptide Systems), at the indicated concentrations, 30 min prior to DAMGO administration.

Flow cytometry and antibodies

PBMCs were harvested using versene to remove both adherent and nonadherent cells. PBMCs were washed with Hank’s balanced salt solution (Life Technologies) with 2% endotoxin-free FBS (Hyclone) (HF), and resuspended in 50 \( \mu \)l HF. Goat serum (Sigma, St. Louis, MO) was added to block nonspecific binding, and cultures were incubated at 4°C for 30 min. Cells were treated with primary antibody, incubated at 4°C for 45 min, and washed, and secondary conjugated antibody was added where necessary. Samples were analyzed with a Coulter Epics XL flow cytometer (Coulter Corp., Hialeah, FL). Primary antibodies were as follows: PE-CXCR4 (12G5), PE-CCR5 (2D7), FITC-CD3 (UCHT1), or FITC-CD14 (M5E2) from PharMingen (San Diego, CA) or Biotin-CCR1 (53504.111) from R&D Systems (Minneapolis, MN). Secondary antibody for Biotin-CCR1 was PE-streptavidin (Sigma). CD3-positive cells were gated by forward and side scatter into lymphoblast (high forward scatter) and resting (low forward scatter) populations for analysis of CCR5 and CXCR4 expression.

HIV-1 viral strains

The viral strains HIV-1<sub>Rbl</sub> (X4) and HIV-1<sub>Rfl</sub> (R5) were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program operated by ERC Bioservices Corp. (Rockville, MD). Virus was purified by pelleting at 110,000 g for 90 min. This procedure produces stock virus between \( 10^6 \) and \( 10^7 \) syncytia-forming units per 0.1 milliliter. The 50% tissue culture infectious dose (TCID<sub>50</sub>) for the R5 virus was determined using PBMCs.

p24 ELISA

HIV-1 p24 analysis was performed on PBMCs infected with either an R5 or X4 HIV-1 viral strain at a multiplicity of infection (m.o.i.) of 0.1, and free virus was washed off after 2 h. Supernatants were collected 72 h post-HIV-1 infection. Supernatants were analyzed for p24 by ELISA using rabbit anti-HIV-1 p24 capture antibody and goat anti-rabbit IgG peroxidase-labeled detector antibody. The capture anti-p24 antibodies were provided on precoated ELISA plates from the AIDS Vaccine Program and the NCI-Fredrick Cancer Research and Development Center. After washing, the capture p24 was detected by developing the peroxidase activity using 3,3',5,5'-tetramethylbenzidine and \( \text{H}_2\text{O}_2 \), and color development was measured by spectrophotometric analysis at 450 nm. As expected, p24 levels
were higher for the X4 viral strain HIV-1_{IIIB} than the R5 viral strain HIV-1_{JRFL}.

**RNase protection assay (RPA)**

The MultiProbe RNase Protection Assay System (PharMingen) was utilized to determine mRNA expression of HIV-1 coreceptors. PBMCs were treated as above and mRNA was extracted from both adherent or nonadherent populations by RNAzol B (Tel-Test, Friendswood, TX). The RiboQuant probe, hCR8, encoding riboprobes for both coreceptors was utilized to determine mRNA expression and hybridized to 10 μg mRNA. The hybridized probe was digested with RNase, and the protected probes were purified and resolved on a 5% denaturing polyacrylamide gel. The gel was then dried and exposed to autoradiogram film to visualize the probe and samples. The gel was analyzed via phosphoimaging screen and using the GS-525 phosphoimager (Bio-Rad, Hercules, CA) to obtain relative values that were normalized to the large ribosomal subunit L32. The data are shown as a percentage relative to L32.

**Polymerase chain reaction (PCR)**

The following primers were utilized to determine both HIV LTR and β-actin DNA expression. The primers for HIV LTR were 5′-AGGCCCTAATAAGCTTGCT-3′ and 5′-CTCCTTGCGCTTAACCCGGAT-3′. The HIV LTR primers were made from a highly conserved region of the LTR which is homologous with both of the viral strains utilized in our experiments. The primers for β-actin were 5′-GGGCGGCCGCCAGCAGCA-3′ and 5′-CTCCTTAAATGTCACGACGATTC-3′. PBMCs were treated with opioids as stated above and infected with either an X4 or a R5 viral strain for 1 h and washed, and 4 h later DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). The PCR reaction was set up as stated above and run on a 1.2% agarose gel, captured using the Kodak 1D Image Analysis Software (Eastman Kodak). The gel was then dried and exposed to autoradiogram film to visualize the probe and samples. The gel was analyzed via phosphoimaging screen and using the GS-525 phosphoimager (Bio-Rad, Hercules, CA) to obtain relative values that were normalized to the large ribosomal subunit L32. The data are shown as a percentage relative to L32.

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