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Morphine Induces Gene Expression of CCR5 in Human CEM x174 Lymphocytes*

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All HIV-1 strains studied to date use CCR5, CXCR4, or both receptors to enter cells. Simian immunodeficiency virus (SIV) infection of non-human primates has served as a useful model for understanding AIDS pathogenesis in humans. Research on several genetically divergent SIV isolates has revealed that SIV uses CCR5, and not CXCR4, for entry. CEM x174, a human lymphoid cell line, has been routinely used to cultivate and maintain various SIV strains. However, questions have arisen about how CEM x174, which reportedly was unable to express detectable amounts of CCR5 transcripts, efficiently supports the growth of SIV. In searching for an answer, we resorted to a sensitive competitive reverse transcriptase-polymerase chain reaction procedure in an attempt to detect as well as quantify the amount of CCR5 expression. Here we present our findings, which indicate that CEM x174 indeed expresses CCR5 and that the amount of CCR5 is increased in cells pretreated with morphine. These results correlate well with our previous observations that morphine treatment causes CEM x174 cells to be more susceptible to SIV infection. Similar morphine effect was not observed on CEM x174 cells infected with simian retroviruses, which do not depend on CCR5 for entry. These findings suggest a plausible mechanism whereby opiate drug users render themselves more susceptible to HIV infection, thereby explaining the vast prevalence of HIV infection among endemic drug use populations.

Opiate users constitute a large portion of the patient population contracting AIDS. The feasibility and success of human studies have always been hampered by the complexity of an individual's history of intravenous drug use. Thus, rhesus monkey treated with opioids and infected with simian immunodeficiency virus (SIV)[1] provides an excellent animal model for studying drug abuse and AIDS under a controlled manner (1). Using this animal system we have previously found that monkeys dependent upon opioid administration and subsequently infected with SIV will have a faster rate of viral replication in comparison with drug-naive, virus-infected monkeys (1). CEM x174 is a human lymphoid cell line (2) commonly used to co-cultivate SIV isolated from infected monkeys. The syncytium formation of SIV-infected CEM x174 cells was found to be significantly enhanced in the presence of morphine sulfate, with a concomitant increase in the activity of cellular reverse transcriptase and the expression of SIV p27 core antigen (3). Chemokines are small 6–10-kDa polypeptides that are synthesized in response to infection and that function mainly as chemoattractants for phagocytic cells, recruiting monocytes and neutrophils from the vascular system to sites of infection (4). Further studies on the effects of opioids on immune cells revealed that the addition of opioids to the chemokines interleukin-8, RANTES, or MIP-1β reduces the ability of monkey neutrophils and monocytes to migrate toward these chemokines (5). The reaction occurs instantaneously, without the inclusion of a cell opioid preincubation step (6). This suggests that the presence of opioids during SIV/HIV infection immediately disrupts the body's first line of defense against harmful external pathogens by disrupting the chemotaxis ability of immune cells toward harmful pathogens. Such observations may provide an indirect mechanism to explain why primates or humans dependent upon intravenous drug administration have a higher probability of developing into a full-blown disease than non-drug users when exposed to a viral challenge (1, 7).

Chemokines act on receptors that belong to the G protein-coupled receptor family whose members contain seven transmembrane domains (4). Activation, desensitization, and resensitization of receptor proteins are thought to involve the activity of receptor-specific G protein-coupled receptor kinases and arrestins (8). Chemokines such as RANTES, MIP-1α, and MIP-1β have been implicated in the pathogenesis of HIV disease; they may be selectively secreted from infected individuals and induce inhibition of different strains of HIV-1, HIV-2, and SIV (9). It was later found that chemokine receptors (especially CCR5 and CXCR4) are coreceptors for HIV or SIV entry (10–13). However, regions in CCR5 or CXCR4 required for ligand (chemokine) binding and coreceptor activity are not identical and only partially overlap (10). It was further established that in addition to blocking viral entry through steric hindrance, cognate ligand interaction with chemokine receptors has been shown to result in receptor down-regulation for CCR5 and CXCR4 (14).

SIVmac239 replicates most efficiently in the human transformed lymphoid cell line CEM x174 (15). In fact, this cell line is routinely used for preparing high titered stocks of this virus

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[1] The abbreviations used are: SIV, simian immunodeficiency virus; RANTES, regulated upon activation, normal T expressed and secreted; MIP-1β, macrophage inflammatory protein 1β; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; RT, reverse transcriptase; bp, base pair(s); FACS, fluorescence-activated cell sorter; SRV, simian type D retrovirus.

(11). It has been reported that SIVmac239 may use CCR5, BOB (GPR15), or BONZO (STRL33) as a coreceptor for entry into various cell types, including 293T.CD4 (16), 3T3.CD4 (16), and Cf2Th.CD4 (17). In all of these studies, CCR5 was a preferred coreceptor over BOB or BONZO; cells with CCR5 produced a greater amount of virus than cells with BOB or BONZO. However, CCR5 has not been identified in CEM x174 (11–13). In the course of a study to quantify virus production in CEM x174 cells, we serendipitously found that addition of morphine sulfate to CEM x174 cell cultures significantly increases the replication of SIVmac239 (3). The present study determines which coreceptor is responsible for the observed morphine effect on CEM x174 cells.

EXPERIMENTAL PROCEDURES

Cell Line—The CEM x174 cell line, a hybrid of the human B cell line 721.174 and human T cell line CEM (2), was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum, 2 mM L-glutamine, 25 mM HEPES, and penicillin and streptomycin. GHOST Parental Cell Line and GHOST Hi-5 (a GHOST cell transfectant with high CCR5 expression) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 500 μg/ml G418, 100 μg/ml hygromycin, and penicillin and streptomycin. For GHOST Hi-5, the medium also contained 1 μg/ml puromycin. All cells were grown at 37 °C in a CO_2 incubator.

Morphine or Naloxone Treatment—CEM x174 cells in culture were diluted 1:3 with fresh medium every 3–4 days. At the time of dilution, morphine sulfate, naloxone HCl, or H_2O (as controls) was added, and incubation was continued for the indicated time. When naloxone was used together with morphine in an experiment, cells were first treated with naloxone HCl for 30 min at 37 °C followed by morphine treatment.

Construction of Competitor Molecules—A PCR fragment of CCR5 (1114 bp), BONZO (797 bp), or BOB (563 bp) was cloned into pCRII (Invitrogen), yielding plasmid CCR5/pCRII, BOB/pCRII, or BONZO/pCRII, which was then sequenced to prove identity (Fig. 1*a*). To construct competitor molecules, small internal deletions were introduced into the inserts. CCR5/pCRII was deleted 96 bp from bases 298 to 393 (inclusive), and BONZO/pCRII was deleted 125 bp from bases 145 to 269 (inclusive) using the Exo mung bean deletion kit (STRATAGENE). BOB/pCRII was deleted 63 bp from bases 404 to 466 (inclusive) after double digestion with *Hinc*II and *Nde*I (Fig. 1*a*).

Competitive RT-PCR—Total RNA was isolated from opioid-treated or control CEM x174 cells using TRIZOL Reagent (Life Technologies, Inc.). In both the reverse transcription and the PCR steps, all the reaction reagents were prepared as master mixes and then aliquoted to each tube to provide uniform reaction conditions and minimize inter-tube variations. To confirm the detection range of competitive RT-PCR, the relationship between the amount of cDNA generated and the initial concentrations of total RNA used were determined: reverse transcription was performed on 0.5, 1, and 2 μg of total RNA, and the reaction was for 30 min at 42 °C using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The reaction mixture also contained 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 10 units of RNase inhibitor (Promega), 1 mM of each dNTP, and 7.5 nM random hexamers in a final volume of 20 μl. For PCR amplication, 20 μl of PCR Master Mix containing 0.5 μM of each primer, 0.5 unit of *Taq* polymerase (Promega), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, and 1.5 mM $MgCl_2$ were dispensed into tubes. To the PCR Master Mix 2 μl of the reverse transcription reaction product and various concentrations of competitor molecules were added. The PCR was performed in a M. J. Research Thermocycler PTC-200, using the following conditions: after an initial 3-min incubation at 95 °C, PCR amplification was carried out for 20 cycles (BOB), 27 cycles (BONZO), or 31 cycles (CCR5) at 95 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min. The last primer extension step was conducted for 10 min. The primers for BOB were 5′-CATCTGCTCTTTGGTGATG-3′ and 5′-GTATGGCTTATCATCAATCAGC-3′, corresponding to bases 66–85 and 607–628 of the published BOB sequence (16). The primers for BONZO were 5′-CAGGCATCCATGAATGGGTGT-3′ and 5′-CAAGGCCTATAACTGGAACATGCTG-3′, corresponding to bases 269–289 and 1041–1065 of the published STRL33 (or BONZO) sequence (16). The primers for CCR5 were 5′-GGTGGAACAAGATGGATTAT-3′ and 5′-ATGTGCACAACTCTGACTG-3′, corresponding to bases 44–63 and 1139–1157 of the published CCR5 sequence (13). PCR products separated on 2% agarose gel were visualized by ethidium bromide staining and photographed under UV illumination (Fig. 1*b*). The intensity of the bands on the image from Eagle Eye II Still Video System (STRATAGENE) was measured with the public domain NIH Image program (developed at the U.S. National Institutes of Health). For analyzing the results, the log of the ratio of amplified target to competitor products was graphed as a function of the known amount of competitor added to the PCR reaction (Fig. 1*c*). CA-Cricket Graph III (Computer Associates) was used for the regression analysis and calculation of x intercepts. When the log ratio equals zero, the concentrations of the target (originally from the RT reaction) and the competitor are equal (Fig. 1*c*). The amount of cDNA synthesized from 1 μg of the initial RNA sample was calculated from the graph (Fig. 1*c*).

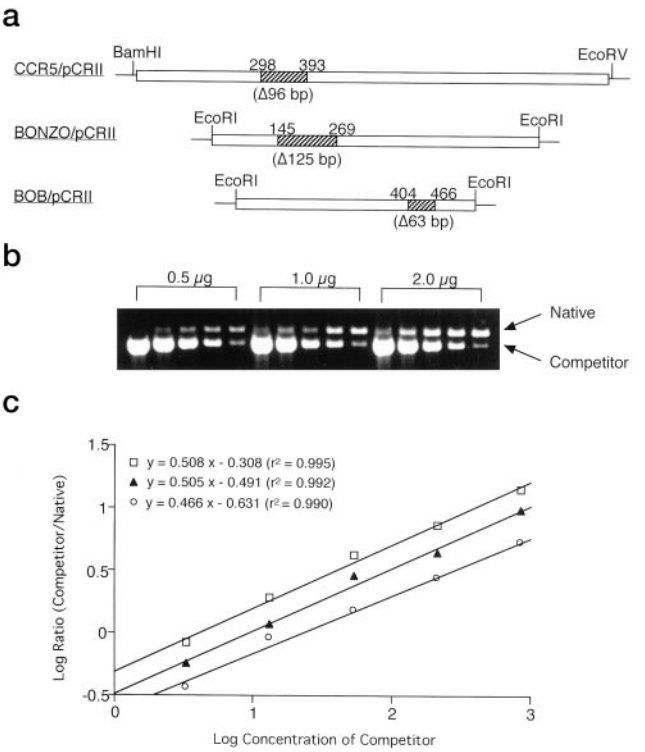

FIG. 1. **Diagram illustration of competitive RT-PCR.** *a*, construction of competitor molecules. A *Bam*HI-*Eco*RV fragment of CCR5 (1114 bp), *Eco*RI-*Eco*RI fragment of BONZO (797 bp), or *Eco*RI-*Eco*RI fragment of BOB (563 bp) was first cloned into the plasmid pCRII. Competitor molecules for RT/PCR analysis were constructed by introducing internal base deletions (*hatched boxes*) into the fragments. The numbers shown on the diagram indicate where the bases were deleted, assuming the first base of each fragment is number 1. *b*, gel analysis. PCR products from each RT-PCR reaction containing native (originally from 2 μl of RT reaction) and one of the five different concentrations of competitor molecules were separated on a 2% agarose gel, visualized by ethidium bromide staining, and photographed under UV illumination. Three concentrations (0.5, 1, or 2 μg) of RNA were used in the initial samples. *c*, calculation of cDNA synthesized. The intensity of each band of the gel portrayed on an Eagle Eye Still video system was measured with an NIH Image program as described under "Experimental Procedures." The ratio of native *versus* competitor of each reaction was obtained from the intensities of the gel bands, and the logarithm of the ratio was graphed against the logarithm of the concentration of the competitor added to the reaction. CA-Criket Graph III was used for the regression analysis and calculation of x intercepts at which the log ratios equaled zero. *c* is a representative figure of the experiment, drawn from data obtained from a RT-PCR analysis of BONZO cDNA synthesized from 0.5 μg(□), 1.0 μg (▲), or 2.0 μg (○) RNA.

Flow Cytometry and Western Blot Analysis—The expression of CCR5 on CEM x174 cells treated with or without morphine was evaluated by FACS. Fluorescein-conjugated mouse monoclonal anti-human CCR5 (clone 45549.111) and fluorescein-labeled mouse IgG2B antibody (clone 20116.11, as a negative control) were obtained from R & D Systems. Procedures for sample staining followed the manufacturer's specifications. After the final wash, cells were fixed in 1% paraformaldehyde before FACS analysis using FACScan (Becton Dickinson, San Jose, CA). The method of Western blotting has been described elsewhere (18). Rabbit anti-CCR5-CT for the Western analysis was obtained from ANASPEC Incorp. (San Jose, CA).

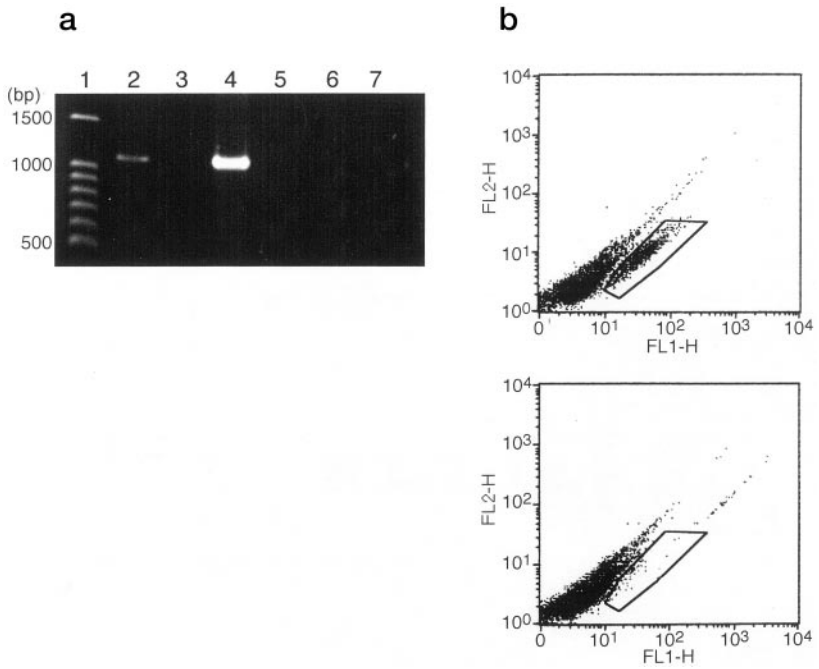

FIG. 2. **CCR5 on CEM x174 as analyzed by RT-PCR (*a*) or flow cytometry (*b*).** *a*, CCR5 mRNA from CEM x174 (*lanes 2* and *3*), GHOST Hi-5 (*lanes 4* and *5*), or GHOST Parental cells (*lanes 6* and *7*) was determined by RT-PCR. *Lanes 3*, *5*, and *7*, RT was omitted in the reactions. *Lane 1*, 100-bp DNA ladder. *b*, cells were stained with either fluorescein isothiocyanate-conjugated mouse anti-human CCR5 monoclonal antibody (*upper panel*) or carboxyfluorescein-conjugated mouse IgG_{2B} isotype control (*lower panel*) and subjected to FACS analysis. *FL1-H*, green florescence; *FL2-H*, red florescence (auto florescence measured in "red" channel). The data were reproducible in three independent experiments.

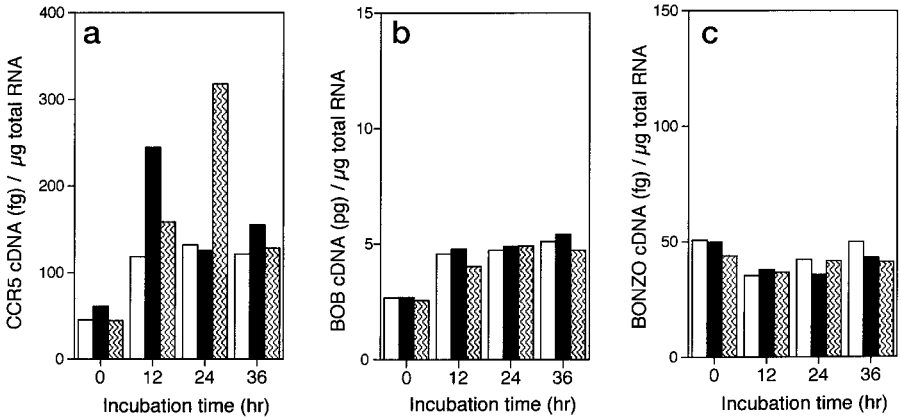

FIG. 3. **Effect of morphine treatment on SIV coreceptor expression.** The amount of CCR5 (*a*), BOB (*b*), or BONZO (*c*) expressed in CEM x174 cells was determined by the competitive RT-PCR after treatment of the cells with 10 μM (*solid column*) or 10 nM (*column with wavy lines*) morphine sulfate for the indicated time. Control (*open column*), H_2O-treated cells. The data were reproducible in at least four independent experiments.

Infection of CEM x174 cells with SIVmac239 or SRV—Methods for the experimental infection of CEM x174 cells with SIVmac239 or SRV and the subsequent assay of the reverse transcriptase activity of the infected cultures were detailed elsewhere (3, 19). SIVmac239 was propagated and titered in CEM x174 cells (3), and titers for SRV serotypes 1–3 were determined by the Raji cell infectivity assay as described previously (19). The Raji cell line was obtained from American Type Culture Collection.

RESULTS

Fig. 2*a* is a representative figure of the results from an RT-PCR analysis of CEM x174 CCR5. Using primer sequences corresponding to bases 44–63 and 1139–1157 of the published human CCR5 sequence (13), CEM x174 cells synthesized a 1114-bp segment (*lane 2*) that was identical in size to the segment synthesized by GHOST Hi-5 (a GHOST cell transfectant encoding CCR5, *lane 4*) and that was not found in GHOST Parental cells (*lane 6*). Using primer pairs corresponding to bases 66–85 and 607–628 of the published BOB sequence (16) and bases 269–289 and 1041–1065 of the published BONZO sequence (16), we also found that CEM x174 cells synthesized cDNA segments of 563 and 797 bp, respectively, in length (data not shown). The cDNA synthesized in each case was sequenced to prove identity. It was found that CEM x174 cells express receptors with gene sequences identical to the published human CCR5, BOB, and BONZO sequences (13, 16) (data not shown).

To further establish that the CCR5 transcripts detected in CEM x174 cells are translated into receptor proteins, we performed both flow cytometry (Fig. 2*b*) and Western blot analysis (see Fig. 4*b*, *inset*) using fluorescein-conjugated mouse monoclonal anti-human CCR5 (for flow cytometry) and rabbit polyclonal anti-human CCR5 (for Western blot). Both procedures confirmed the presence of CCR5 molecules on CEM x174 cells (Figs. 2*b* and 4*b*). Therefore, we demonstrated that in addition to BOB and BONZO, CEM x174 cells indeed express significant amounts of CCR5, the major coreceptor for SIVmac entry.

To determine coreceptor densities on CEM x174 cells, plasmids containing segments of CCR5, BOB, and BONZO, and plasmids containing CCR5, BOB, and BONZO segments with 96-bp (CCR5), 63-bp (BOB), and 125-bp (BONZO) deletions were constructed (see "Experimental Procedures" and Fig. 1*a*). Plasmids with deleted segments were used in quantitative RT-PCR as external controls for quantifying the expression of

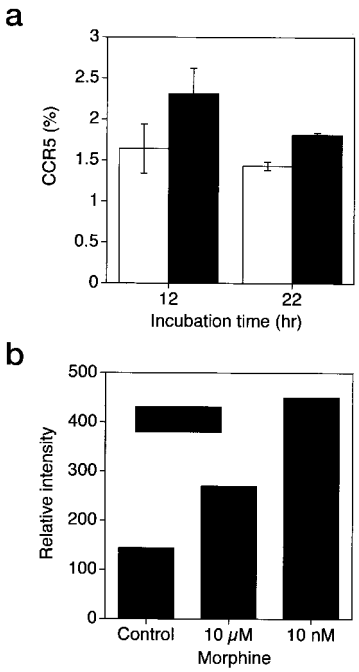

FIG. 4. **Effect of morphine treatment on cell surface CCR5.** *a*, CCR5 expressed on cell surface of CEM x174 cells was analyzed by FACS after treatment of the cells with 10 μM morphine sulfate (*solid column*) or H_2O (*open column*). The *y* axis represents the percentage of total cells being CCR5-positive. *b*, effect of morphine treatment on the expression of CCR5 protein by CEM x174 cells was analyzed by Western blotting. Protein isolated from CEM x174 cells was separated on a 10% SDS/polyacrylamide gel, blotted onto a nitrocellulose filter, and incubated with rabbit anti-CCR5-CT. Morphine treatment was for 24 h. The data were reproducible in three independent experiments.

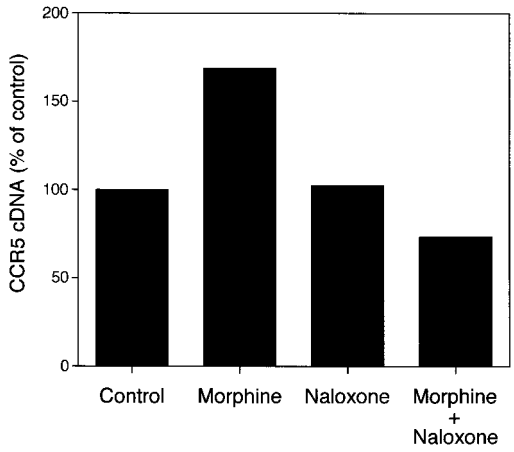

FIG. 5. **Effect of naloxone on morphine-induced CCR5 expression.** The amount of CCR5 expressed in CEM x174 cells was determined by the competitive RT-PCR after treatment of the cells for 12 h with 10 nM morphine, 10 nM naloxone or a combination of naloxone and morphine (10 nM each). The data were reproducible in three independent experiments.

chemokine receptor genes in CEM x174. To select a cycle number to achieve an exponential amplification phase, the densities of amplified fragments from different cyclical amplifications were measured after gel electrophoresis and ethidium bromide staining. Cycle numbers 31, 20, and 27 were selected for experiments on CCR5, BOB, and BONZO, respectively (data not shown). To confirm the detection range of competitive RT-PCR, the relationship between the amount of cDNA generated and the initial concentrations of total RNA used were determined. For each RT reaction of 0.5, 1.0, or 2.0 μg of total RNA, five different concentrations of competitors were used for PCR amplification (Fig. 1*b*). The results showed that the amount of cDNA generated was in proportion to the amount of initial RNA used (Fig. 1*c*). To investigate the effect of morphine treatment on the gene expression of CCR5, BOB, and BONZO, the amount of cDNA amplified by competitive RT-PCR from cells treated with morphine sulfate was compared with that of untreated cells. Morphine treatment, if used, was either 10 μM or 10 nM; these are physiological morphine concentrations in morphine-dependent animals (20). Samples were taken 0, 12, 24, and 36 h post-morphine treatment for analysis. It was found that after the dilution of the cultures at time 0 (see "Experimental Procedures"), the cells synthesized an increasing amount of CCR5 (Fig. 3*a*) or BOB (Fig. 3*b*), with the amount reaching a plateau 12–24 h post-dilution. However, it was also found that in comparison with the control cells at each time point, 10 μM morphine treatment increased CCR5 expression 207% by 12 h post-treatment, whereas 10 nM morphine treatment induced a 240% increase of CCR5 by 24 h post-treatment (Fig. 3*a*). On the contrary, morphine treatment did not affect the expression of BOB (Fig. 3*b*) or BONZO (Fig. 3*c*) in CEM x174 cells. Further experiments showed that the morphine-induced increase in CCR5 expression correlated with the amount of CCR5 proteins on the cell surface, as determined by

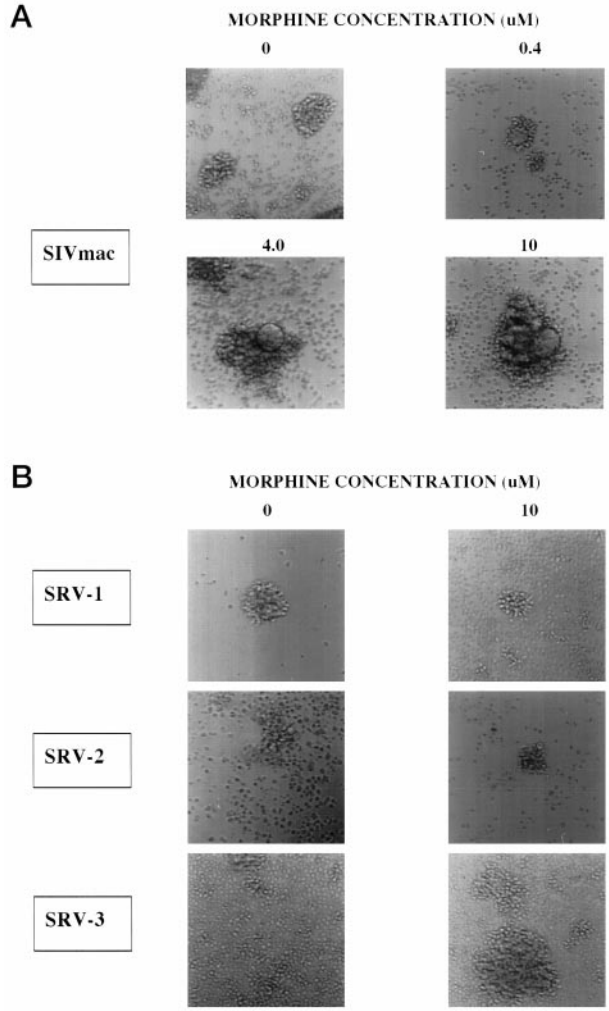

FIG. 6. **Effect of morphine treatment on SIVmac239 (*A*) or SRV (*B*) infection of CEM x174 cells.** CEM x174 cells were infected with SIVmac239 (*A*) or three serotypes of SRV (*B*) in the presence of morphine sulfate at concentrations of 0, 0.4, 4.0, or 10 μM. Syncytium formation was shown as giant protoplasmic mass resulted from fusion of cytopathic cells. The data were reproducible in three independent experiments.

TABLE I
Total number of syncytia formation for SIVmac239

Data were reproducible in three independent experiments.

Day	Morphine concentration			
	0 μM	0.4 μM	4.0 μM	10.0 μM
6	1[a]	3	4	27
8	2	4	6	40
12	39	90	56	75

[a] Total number of syncytia.

TABLE II
Effect of morphine on reverse transcriptase activity of SIV or SRV-infected CEMX174 cells

The reverse transcriptase activity was assayed using standard [^{32}P]dTTP incorporation (19). The mean cpm for the cells alone was 167. The mean cpm for the scintillation fluid was 20. Data were reproducible in three independent experiments.

Virus	Morphine concentration							
	0 μM		0.4 μM		4.0 μM		10.0 μM	
	Day 6	Day 8	Day 6	Day 8	Day 6	Day 8	Day 6	Day 8
SIVmac 239[a]	266 (1.0)[b]	568 (1.0)	603 (2.3)	2310 (4.1)	699 (2.6)	3674 (6.5)	1168 (4.4)	2885 (5.1)
SRV-1[c]	256 (1.0)	454 (1.0)	225 (0.9)	389 (0.9)	228 (0.9)	444 (1.0)	221 (0.9)	338 (0.7)

[a] The p value is ≤ 0.0001 by analysis of variance, considered highly significant.
[b] CPM ratio between sample and no-morphine control.
[c] The p value is > 0.1 by analysis of variance, considered not significant.

TABLE III
Reverse transcriptase activity after 12 days of exposure to morphine in SRV

Virus	Morphine concentration			
	0 μM	0.4 μM	4.0 μM	10.0 μM
	cpm			
SRV-1	370	292	358	354
SRV-2	220	200	194	194
SRV-3	185[a]	194	195	204

[a] The p value is 0.5022 by analysis of variance, considered not significant. Variation between morphine concentration exposure in SRV-3 is not significantly greater than expected by chance. One-way analysis of variance was performed using GraphPad InStat version 3.00 for Mac, GraphPad Software (San Diego, CA).

flow cytometry (Fig. 4*a*) as well as Western blot analysis (Fig. 4*b*) and that the effect was opioid receptor-mediated, because it could be completely abolished when cells were pretreated with naloxone, a μ opioid receptor antagonist (Fig. 5).

To correlate the observed morphine effect on CCR5 up-regulation with viral infectivity, CEM x174 cells with or without morphine treatment were assayed for viral susceptibility after infection with SIVmac239 or with SRV in parallel experiments. SRV infect B cells, T cells (CD4+ and CD8+), macrophages, and epithelial cells. Like SIVmac239, SRV cause an acquired immunodeficiency syndrome in monkeys (19). The receptor for SRV is not CCR5 but a neutral amino acid transporter that has been identified and cloned (21). In this study CEM x174 cells were treated with morphine sulfate at 0, 0.4, 4, or 10 μM and infected with SIVmac239 or three serotypes of SRV (SRV-1, SRV-2, and SRV-3). The cells were observed for syncytia formation and assayed for viral reverse transcriptase activities. Fig. 6 shows that on day 6 post-infection, syncytia appeared in SIVmac239-infected cultures and the number of syncytia formation increased with increasing concentrations of morphine in the culture (Fig. 6*A* and Table I). On the contrary, none of the SRV-infected cultures showed signs of syncytia even in the presence of morphine treatment (Fig. 6*B*). Results of RT assay of the infected cultures indicated a gradual increase of the enzyme activity with time of infection in all cultures (Table II); however, morphine significantly enhanced RT activities of SIVmac239-infected cells but not SRV-infected cells (Table II). For SRV-infected CEM x174 cells, we instead observed a slight decrease in RT activities upon morphine exposure (Table II). Further incubation of SRV-infected cells up to 12 days in the presence of morphine had little effect on, or slightly decreased, viral infectivity, as again shown by RT assays (Table III).

DISCUSSION

Immune cells have been shown to express brain-like opioid receptors (22–24). Similar to chemokine receptors, opioid receptors are also G protein coupled, seven-transmembrane domain receptors (25). Human CEM x174 lymphocytes possess all three subtypes of opioid receptors, μ, κ, and δ (22–24). The current study shows that activation of opioid receptors of human lymphocytes, probably of the μ subtype, by morphine up-regulates the expression of the chemokine receptor CCR5. The downstream molecular mechanisms induced by receptor activation through which morphine affects CCR5 expression awaits further investigation. Morphine, which inhibits chemokine-induced chemotaxis, nevertheless does not perturb chemokine binding to CCR5 (26). It is therefore attractive to propose that morphine, by binding to its own cell surface receptor, initiates a series of G protein-coupled signal transduction pathways (18) that thereby hetero-sensitize (or up-regulate) CCR5. Many facts support this proposal. For instance, morphine has been shown to modulate the expression of other cellular proteins that may induce CCR5 expression. Specifically, morphine reportedly modulates the cellular activation of NFκB and TNF-α in macrophages (27) and interleukin-2 in lymphocytes (28); activation of NFκB, TNF-α, and interleukin-2 has been found to up-regulate CCR5 expression (10, 29). Alternatively, morphine may up-regulate CCR5 by inhibiting chemokine synthesis and thus chemotaxis. Under certain conditions, opiates such as morphine as well as opioid peptides may down-regulate

cytokine synthesis and release (30, 31). Chemokines (chemotactic cytokines) such as RANTES, MIP-1α, and MIP-1β may induce receptor internalization and decrease cell surface expression of CCR5 (14, 32), which ultimately contributes to anti-HIV-1 activity (9, 14). Thus, one yet-to-be-proved mechanism of morphine-mediated up-regulation of CCR5 is through the down-regulation of chemokine synthesis.

SRV are endemic in wild macaques of India and Indonesia and in captive macaques important for medical research (33). Five distinct neutralization serotypes (SRV1–SRV5) have been described, of which three have been molecularly cloned (SRV1–SRV3). The present study shows that unlike SIVmac239 infection, morphine treatment will not increase the infectivity of SRV 1–3 (Fig. 6). Instead, morphine may induce a "protective" effect against SRV infection (Tables II and III). Similar phenomenon has been reported for murine Friend retrovirus infection (34). Like SRV, the Friend retrovirus infection model has been described as relevant to several aspects of AIDS; in particular, there are significant changes in immune function similar to those observed in HIV infection. The receptor for such virus is again not CCR5 but murine cationic amino acid transporter 1 (35), and morphine was found to attenuate the pathological manifestations of the virus in infected animals (34). Therefore, it appears that morphine-induced CCR5 in human lymphocytes facilitates only SIVmac239 infection, for which CCR5 is a co-receptor for viral entry.

This study shows for the first time that lymphocytes express CCR5 at higher levels when treated with morphine sulfate. Previous studies that were unable to detect CCR5 in CEM x174 may have been due to the low levels of CCR5 expression inherent in cells; this level is below the threshold of many conventional detection methods. These low levels of CCR5 expression are significant nevertheless, especially when these low levels of CCR5 expression are augmented by morphine treatment. In addition, recent studies have shown that in cells with large amounts of CD4, a low trace of CCR5 was sufficient for susceptibility to virus infection (36). Therefore, in cells with low levels of CCR5 expression, morphine treatment may bring CCR5 concentrations above threshold levels for maximal infection. Morphine does not affect the gene expression of BOB or BONZO (Fig. 3). In this regard, the induction of the chemokine receptor CCR5 gene expression by morphine may provide a mechanism by which morphine sulfate enhances HIV/SIV infection and hence exacerbates the Simian AIDS or AIDS pathogenesis.

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REFERENCES

1. Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1997) *Addiction Biol.* **2,** 421–430
2. Salter, R. D., Howell, D. N., and Cresswell, P. (1985) *Immunogenetics* **21,** 235–246
3. Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1165–1173
4. Kelvin, D. J., Michiel, D. F., Johnston, J. A., Lloyd, A. R., Sprenger, H., Oppenheim, J. J., and Wang, J. M. (1993) *J. Leukocyte Biol.* **54,** 604–612
5. Choi, Y., Chuang, L. F., Lam, K. M., Kung, H.-F., Wang, J. M., Osburn, B. I., and Chuang, R. Y. (1999) *In Vivo* **13,** 389–396
6. Miyagi, T., Chuang, L. F., Lam, K. M., Kung, H.-F., Wang, J. M., Osburn, B. I., and Chuang, R. Y. (2000) *Immunopharmacology* **47,** 53–62
7. Mazzone, A., Mazzucchelli, I., Fossati, G., Gritti, D., Fea, M., and Ricevuti, G. (1994) *Int. J. Immunopharmacol.* **16,** 959–967
8. Ferguson, S. S., Barak, L. S., Zhang, J., and Caron, M. G. (1996) *Can. J. Physiol. Pharmacol.* **74,** 1095–1110
9. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) *Science* **270,** 1811–1815
10. Lee, B., and Montaner, L. J. (1999) *J. Leukocyte Biol.* **65,** 552–565
11. Chen, Z., Zhou, P., Ho, D. D., Landau, N. R., and Marx, P. A. (1997) *J. Virol.* **71,** 2705–2714
12. Vodicka, M. A., Goh, W. C., Wu, L. I., Rogel, M. E., Bartz, S. R., Schweickart, V. L., Raport, C. J., and Emerman, M. (1997) *Virology* **233,** 193–198
13. Kirchhoff, F., Pohlmann, S., Hamacher, M., Means, R. E., Kraus, T., Uberla, K., and Di Marzio, P. (1997) *J. Virol.* **71,** 6509–6516
14. Trkola, A., Paxton, W. A., Monard, S. P., Hoxie, J. A., Siani, M. A., Thompson, D. A., Wu, L., Mackay, C. R., Horuk, R., and Moore, J. P. (1998) *J. Virol.* **72,** 396–404
15. Park, I. W., Steen, R., and Li, Y. (1991) *J. Virol.* **65,** 2987–2992
16. Deng, H. K., Unutmaz, D., KewalRamani, V. N., and Littman, D. R. (1997) *Nature* **388,** 296–300
17. Choe, H., Farzan, M., Konkel, M., Martin, K., Sun, Y., Marcon, L., Cayabyab, M., Berman, M., Dorf, M. E., Gerard, N., Gerard, C., and Sodroski, J. (1998) *J. Virol.* **72,** 6113–6118
18. Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1997) *J. Biol. Chem.* **272,** 26815–26817
19. Torres, J. V., Werner, L. L., Malley, A., and Benjamini, E. (1991) *J. Med. Primatol.* **20,** 218–221
20. Liu, Y., Blackbourn, D. J., Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1992) *J. Pharmacol. Exp. Ther.* **263,** 533–539
21. Anderson, D., and Torres, J. V. (1999) *Viral Immunology* **12,** 47–56
22. Chuang, L. F., Chuang, T. K., Killam, K. F., Jr., Chuang, A. J., Kung, H. F., Yu, L., and Chuang, R. Y. (1994) *Biochem. Biophys. Res. Commun.* **202,** 1291–1299
23. Chuang, L. F., Chuang, T. K., Killam, K. F., Jr., Qiu, Q., Wang, X. R., Lin, J. J., Kung, H. F., Sheng, W., Chao, C., Yu, L., and Chuang, R. Y. (1995) *Biochem. Biophys. Res. Commun.* **209,** 1003–1010
24. Chuang, T. K., Killam, K. F., Jr., Chuang, L. F., Kung, H. F., Sheng, W. S., Chao, C. C., Yu, L., and Chuang, R. Y. (1995) *Biochem. Biophys. Res. Commun.* **216,** 922–930
25. Law, P. Y., and Loh, H. H. (1999) *J. Pharmacol. Exp. Ther.* **289,** 607–624
26. Grimm, M. C., Ben-Baruch, A., Taub, D. D., Howard, O. M. Z., Resau, J. H., Wang, J. M., Ali, H., Richardson, R., Snyderman, R., and Oppenheim, J. J. (1998) *J. Exp. Med.* **188,** 317–325
27. Roy, S., Cain, K. J., Chapin, R. B., Charboneau, R. G., and Barke, R. A. (1998) *Biochem. Biophys. Res. Commun.* **245,** 392–396
28. Chuang, L. F., Killam, K. F., Jr., and Chuang, R. Y. (1993) *In Vivo* **7,** 159–166
29. Fraziano, M., Cappelli, G., Santucci, M., Mariani, F., Amicosante, M., Casarini, M., Giosue, S., Bisetti, A., and Colizzi, V. (1999) *AIDS Res. Hum. Retroviruses* **15,** 869–874
30. Chao, C. C., Molitor, T. W., Close, K., Hu, S., and Peterson, P. K. (1993) *Int. J. Immunopharmacol.* **15,** 447–453
31. Morgan, E. L. (1996) *J. Neuroimmunol.* **65,** 21–30
32. Franchin, G., Zybarth, G., Dai, W. W., Dubrovsky, L., Reiling, N., Schmidtmayerova, H., Bukrinsky, M., and Sherry, B. (2000) *J. Immunol.* **164,** 2592–2601
33. Rosenblum, L. L., Weiss, R. A., and McClure, M. O. (2000) *J. Virol.* **74,** 3449–3454
34. Veyries, M-L., Sinet, M., Desforges, B., and Rouveix, B. (1995) *J. Pharmacol. Exp. Ther.* **272,** 498–504
35. Davey, R. A., Hamson, C. A., Healey, J. J., and Cunningham. J. M. (1997) *J. Virol.* **71,** 8096–8102
36. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) *J. Virol.* **72,** 2855–2864

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