## Morphine Enhances HIV Infection of Neonatal Macrophages

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## ABSTRACT

Perinatal transmission of HIV accounts for almost all new HIV infections in children. There is an increased risk of perinatal transmission of HIV with maternal illicit substance abuse. Little is known about neonatal immune system alteration and subsequent susceptibility to HIV infection after morphine exposure. We investigated the effects of morphine on HIV infection of neonatal monocyte-derived macrophages (MDM). Morphine significantly enhanced HIV infection of neonatal MDM. Morphine-induced HIV replication in neonatal MDM was completely suppressed by naltrexone, the opioid receptor antagonist. Morphine significantly up-regulated CCR5 receptor expression and inhibited the endogenous production of macrophage inflammatory protein-1 $\beta$  in neonatal MDM. Thus, morphine, most likely through alteration of  $\beta$ -chemokines and CCR5 receptor expression expression and inhibited the endogenous production of macrophage inflammatory protein-1 $\beta$  in neonatal MDM. Thus, morphine, most likely through alteration of  $\beta$ -chemokines and CCR5 receptor expression expression expression and inhibited the endogenous production of macrophage inflammatory protein-1 $\beta$  in neonatal MDM. Thus, morphine, most likely through alteration of  $\beta$ -chemokines and CCR5 receptor expression expre

sion, enhances the susceptibility of neonatal MDM to HIV infection, and may have a cofactor role in perinatal HIV transmission and infection. (*Pediatr Res* 54: 282–288, 2003)

#### Abbreviations

MDM, monocyte-derived macrophages
MIP, macrophage inflammatory protein
RANTES, regulated on activation, T cell expressed and secreted
DMEM, Dulbecco's modified Eagle's medium
MLV, murine leukemia virus
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
RLU, relative light units
RT, reverse transcription

Current estimates of perinatal transmission of HIV infection range from lower than 2% to as high as 25 to 40%, particularly owing to high viral load in women in underdeveloped countries where there is limited prenatal and perinatal care and limited resources for standardized treatment regimens (1, 2). Increased risk of perinatal transmission of HIV infection may result from several maternal, placental, and neonatal factors. There is an increased risk of perinatal transmission of HIV infection associated with maternal illicit substance use, perhaps through alteration of maternal immune function, enhanced HIV replication in maternal immune cells, or possible effects on neonatal immune function (2–4). A multicenter study in the United States demonstrated that HIV-infected women who used illicit drugs during pregnancy had a higher risk of transmitting HIV to their infants than did HIV-infected women who did not use

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drugs while pregnant (3). There is an increased risk of vertical HIV transmission and an increased risk of preterm birth if substance use is continued into the second and third trimesters in a study without antiretroviral therapy of the pregnant women (4).

Although little is known about neonatal immune system alterations after morphine exposure, there is substantial evidence that shows morphine alters the function of the adult immune system. Endogenous and exogenous opioids and opiate abuse modulate immune function in both in vitro and in vivo systems (5-8), including a variety of effects on human adult-derived macrophages (7). Opioids alter cytokine production and cell trafficking, enhance susceptibility of immune cells to HIV infection, and increase viral titers in the brain (9). Opioids promote the growth of HIV in adult immune cells in *vitro* (10-12). We and others have recently demonstrated that methadone, a long-acting synthetic opiate that has similar pharmacologic properties to morphine (13), enhances expression of CCR5 (14, 15), a principal coreceptor for HIV entry into macrophage on human immune cells, thus promoting HIV replication in human immune cells. Neonatal cellular immunity is less robust than that of the adult, placing the neonate at

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higher risk for infection. Acute or chronic morphine exposure may further exacerbate defects in the neonatal cellular immune system. Given the factors that there is efficient transfer of opioids across the placenta and fetuses of mothers with illicit opiate use are chronically exposed to opiates, it is possible that opioids alter neonatal immune function in utero and at the time of delivery. In addition, mothers frequently receive systemic opiates for pain control during labor and delivery. These opiates may cross the placenta and affect the neonate during exposure to potentially infectious agents such as HIV. Finally, critically ill newborns, including preterm newborns, frequently receive continuous prolonged courses of i.v. morphine for sedation and pain control. These newborns are the subset of neonates at highest risk of nosocomial infection from bacterial, fungal, and viral organisms. Thus, there is considerable interest in determining whether opiates, through their receptors, compromise functions of the neonatal immune cells that also are the primary targets for HIV, thus increasing the risk of vertical transmission of HIV. In the present study, we determined whether morphine, at clinically relevant doses, affects HIV

#### **METHODS**

infection of neonatal macrophages.

Neonatal monocyte preparation. Cord blood was obtained from the placenta of deliveries of healthy human neonates, after delivery and separation of the infant from the placenta. Informed consent was obtained, and the institutional review board of the Children's Hospital of Philadelphia has approved the present study. The heparinized cord blood was separated by centrifugation over lymphocyte separation medium at 400-500  $\times$  g for 45 min. The mononuclear layer was collected and incubated with DMEM in 2% gelatin-coated flasks for 45 min at 37°C, followed by removal of the nonadherent cells with DMEM. Purified monocytes were detached by EDTA. After the initial purification, at least 97% of the cells were monocytes, as determined by nonspecific esterase staining and flow cytometry using MAb against CD14 and LDL specific for monocytes and macrophages as described previously (16, 17). Cells were plated in 48-well culture plates at a density of  $0.5 \times$ 10<sup>6</sup> cells/well in the DMEM containing 10% FCS to obtain freshly isolated monocytes (within 48 h of isolation), and monocyte-derived macrophage (7-8 d after isolation). Monocyte viability was monitored by trypan blue exclusion and maintenance of cell adherence. In all cases, Limulus amebocyte lysate assay (Pyrochrome, Association of Cape Cod Inc., Falmouth, MA, U.S.A.) with sensitivity of 0.005 endotoxin units per milliliter demonstrated that the media and reagents were endotoxin-free (<0.1 endotoxin units/mL).

**Reagents.** FITC-conjugated antibodies against CD14, CD4, and CCR5, as well as the control IgGs (IgG1, IgG2a, and IgG2b) were obtained from PharMingen (San Diego, CA, U.S.A.). FITC-conjugated anti-CXCR4 antibody was obtained from R&D Systems (Minneapolis, MN, U.S.A.). Morphine sulfate was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ, U.S.A.). Naltrexone was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

*HIV strains.* On the basis of their differential use of the major HIV coreceptors (CCR5 and CXCR4), HIV isolates have been referred to as R5, X4, or R5X4 strains (18). The macrophage-tropic R5 strain (Bal) was obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD, U.S.A.

**Preparation of pseudotyped HIV.** Recombinant luciferase encoding HIV virions were pseudotyped with the envelopes (Env) from macrophage-tropic (ADA) or amphotropic murine leukemia virus (MLV). Human embryonic kidney cell line (293T) was cotransfected with the plasmids encoding either ADA Env or MLV Env and the plasmid containing luciferaseencoding NL4–3 HIV backbone (pNL-Luc-E<sup>-</sup>R<sup>-</sup>). Supernatants were collected as virus stock 48 h later. The plasmids encoding HIV ADA or MLV Env were generously provided by John Moore (Aaron Diamond AIDS Research Center, New York, NY, U.S.A.), and the plasmid with luciferase-encoding NL4–3 HIV backbone was provided by Ned Landau (Aaron Diamond AIDS Research Center). All virus stocks were assayed for p24 antigen and stored at  $-70^{\circ}$ C as cell-free virus after filtration through a 0.22- $\mu$ m-pore-size filter.

*RT* assay. HIV RT activity was determined based on the technique of Willey *et al.* with modification (19). In brief, 10  $\mu$ L of collected culture supernatants was added to a cocktail containing poly(A), oligo(dT) (Pharmacia Inc., Piscataway, NJ, U.S.A.), MgCl<sub>2</sub>, and [<sup>32</sup>P]dTTP (Amersham Corp., Arlington Heights, IL, U.S.A.) and incubated for 20 h at 37°C. Then 30  $\mu$ L of the cocktail was spotted onto DE81 paper, dried, and washed five times with 2× saline-sodium citrate buffer and once with 95% ethanol. The filter paper was then air-dried. Radioactivity was counted in a liquid scintillation counter (Packard Instrument Inc., Palo Alto, CA, U.S.A.).

*Flow cytometry.* To determine whether morphine affects the expression of CCR5 on neonatal monocytes/macrophages, cells were incubated with or without morphine  $(10^{-10} \text{ M})$  for 24 h. The cells were removed from the culture plate and then resuspended in 100  $\mu$ L of PBS. After incubation with 20  $\mu$ L of FITC-conjugated anti-CCR5 antibody for 30 min at 4°C, the cells were washed twice with PBS and fixed with 1% paraformaldehyde in PBS. FITC-conjugated control IgG was isotypematched for anti-CCR5 antibody. Fluorescence was analyzed on an EPICS-elite flow cytometer (Beckman Coulter Electronics, Hialeah, FL, U.S.A.).

**MIP-1** $\beta$  titration. MIP-1 $\beta$  ELISA kits were purchased from Endogen, Inc. (Cambridge, MA, U.S.A.). The assay was performed as instructed in the protocol provided by the manufacturer. In brief, 50  $\mu$ L of supernatants was added to antibodycoated wells and incubated for 1 h at room temperature. The plate was washed with the provided buffer solution and incubated with 100  $\mu$ L of biotinylated antibody reagent for 1 h at room temperature. The plate was washed again, treated with 100  $\mu$ L of prepared streptavidin–horseradish peroxidase solution, and incubated for 30 min at room temperature. After an additional wash, 100  $\mu$ L of tetramethylbenzidine (TMB) substrate solution was added to each well, and color was allowed to develop at room temperature for 30 min. The reaction was stopped by the addition of 100  $\mu$ L of stop solution to each well. The plate was read on a microplate reader (ELX800, Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.).

*Luciferase activity assay.* The luciferase activity was determined using a luciferase assay kit (Promega Biotec, Madsion, WI, U.S.A.). Pseudotyped HIV-infected neonatal MDM (5 ×  $10^5$  cells) incubated with or without morphine (see pseudotyped reporter virus entry essay below) were lysed in 150 µL of 1× reporter lysis buffer (Promega Biotec). Lysates (50 µL) were mixed with an equal volume of luciferase substrate (Promega Biotec) and luciferase activity was then determined in a luminescence counter (PerkinElmer Wallac Inc., Gaithersburg, MD, U.S.A.). Emitted light in each well was measured over a 0.5-s period and designated as RLU.

RNA extraction and reverse transcription. Total cellular RNA was isolated from neonatal MDM (10<sup>6</sup> cells) using Tri-reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). In brief, the total RNA was extracted by a single-step, guanidinium thiocyanate-phenol-chloroform extraction. After centrifugation at 13,000  $\times$  g for 15 min at 4°C, the RNAcontaining aqueous phase was precipitated in isopropanol. RNA precipitates were then washed once in 75% ethanol and resuspended in 30  $\mu$ L of RNase-free water. One microgram of total RNA was subjected to RT using the RT system (Promega Biotec) with specific primers (antisense) for HIV gag gene (5'-TGACATGCTGTCATCATTTCTTC-3') and CCR5 (see below for primer sequences) genes for 1 h at 42°C. The reaction was terminated by incubation of the reaction mixture at 99°C for 5 min and then kept at 4°C. The resulting cDNA was ready for serving as a template for PCR amplification or real-time PCR quantification.

PCR analysis. PCR amplification of CCR5 cDNA was performed for 35 cycles using AmpliTaq Gold (Perkin Elmer-Cetus, Foster City, CA, U.S.A.) in a GeneAmp PCR System 2400 (Perkin Elmer-Cetus). The specific oligonucleotide primers are listed as follows: CCR5 gene primers: 5'-CAAAAA-GAAGGTCTTCATTACACC-3' (sense) and 5'-CCTGTGC-CTCTTCTTCTCATTTCG-3' (antisense); β-actin gene primers: 5'-ATGTGGCACCACACCTTCTACAATGAG-CTGCG-3' (sense) and 5'-CGTCATACTCCTGCTTGCT-GATCCACATCTGC-3' (antisense).  $\beta$ -Actin was used as a control to monitor the amount and integrity of RNA in each sample (Clontech, Palo Alto, CA, U.S.A.). The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.). The PCR reaction mixture contained 0.2 mM dNTPs, 20 pM each of two primers, and 1.5 U of Ampli*Taq* Gold in  $1 \times$  reaction buffer (Perkin Elmer-Cetus). Each of the PCR amplifications consisted of heat activation of AmpliTaq Gold for 9 min at 94°C, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 45 s and further elongation at 72°C for 7 min. PCR-amplified products were electrophoresed on ethidium bromide-stained 3% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, U.S.A.).

**Real-time RT-PCR.** Real-time RT-PCR was performed using ABI Prism 7700 Sequence Detection System (Perkin Elmer-Cetus). For HIV *gag* gene expression, the reaction mixture contained 0.25 mM dNTPs, Ampli*Taq* Gold (1.5 U), 5 mM MgCl<sub>2</sub>, 50 pM each of the two primers (SK38: 5'-ATAATCCACCTATCCCAGTAGGAGAAAT-3'; SK39: 5'-

TTTGGTCCTGTCTTATGTCCAGAATGC-3'), 20 pM molecular beacon probe (SK19: 5'-ATCCTGGGGATTA-AATAAAATAGTAAGAATGTATAGCCCTAC-3') labeled with 6-carboxyfluorescein (FAM) (a fluorophore) at its 5' end and 4-(4'-dimethylaminophenylaso) benzoic acid (DABCYL) (a quencher) at the 3' end. The cycle conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The known amounts of HIV DNA isolated from ACH-2 cells were used as standard controls. All controls and samples were run in duplicate in the same plate. For CCR5 receptor gene expression, the same primer pair used for conventional RT-PCR was used for real-time RT-PCR. The molecular beacon probe for CCR5 gene is as follows: 5'-GCGAGTCCTGC-CGCTGCTTGTCATGGTCCTCGC-3'. The measurement of GAPDH mRNA levels of the samples by real-time RT-PCR performed on the same plate was used as a control to normalize the mRNA contents among the samples tested.

**Pseudotyped reporter virus entry assay.** Seven-day-cultured neonatal MDM in 48-well plates  $(5 \times 10^5 \text{ cells/well})$  were incubated for 24 h with or without morphine  $(10^{-10} \text{ M to } 10^{-8} \text{ M})$  and then infected with 10 ng of P24 Gag antigen equivalent of each pseudotyped HIV per well in the presence of polybrene  $(4 \ \mu \text{g/mL})$ . At 72 h after infection, the cells were lysed in 150  $\mu$ L of 1× reporter lysis buffer (Promega Biotec). Lysate (50  $\mu$ L) was mixed with an equal volume of luciferase substrate (Promega Biotec), and luciferase activity was then determined in a Wallac Trilux Microbeta luminometer (Wallac, Turku, Finland). Data were presented in RLU.

Morphine treatment and HIV infection. Seven-daycultured neonatal MDM (5  $\times$  10<sup>5</sup> cells/well in 48-well plates) were incubated for 12 h with or without morphine  $(10^{-14} \text{ M to})$  $10^{-8}$  M) or naltrexone ( $10^{-8}$  M), or both, before infection with HIV strain (Bal). In the case of combination treatment of cells with morphine and naltrexone, naltrexone was added to the cell cultures 30 min before the addition of morphine. The cells were infected with equal amounts of cell-free HIV on the basis of p24 protein content (20 ng/10<sup>6</sup> cells) for 2 h at 37°C in the presence or absence of the reagents described above. The cells were then washed three times with DMEM to remove unabsorbed virus, and fresh medium containing morphine or naltrexone was added to the cell cultures. The final wash was tested for viral RT activity and shown to be free of residual inocula. Untreated cells severed as controls. The cells were incubated in the presence of the reagents described above every 4 d after infection. Supernatants were harvested 96 h after infection for MIP-1 $\beta$  production as determined by ELISA, and d 8 supernatants were collected for HIV RT activity assay. For HIV gag gene expression, total cellular RNA was extracted from MDM 72 h after infection.

Statistical analysis. When appropriate, data were expressed as mean  $\pm$  SD. For comparison of means of two groups (morphine-treated versus untreated controls), statistical significance was assessed by t test. Calculations were performed using Stata Statistical Software (Stata Corp., College Station, TX, U.S.A.). Statistical significance was defined as p < 0.05.

## RESULTS

Morphine enhanced HIV infection of neonatal MDM. To evaluate whether morphine affects HIV infection of neonatal MDM, 7-d-cultured neonatal MDM were incubated with or without morphine at different concentrations  $(10^{-12} \text{ M to} 10^{-8})$ M) for 12 h and then infected with HIV Bal strain for 2 h. The addition of morphine to the cultures leads to an increase in HIV infection of neonatal MDM (Fig. 1). These effects of morphine were abrogated by the addition of naltrexone (Fig. 1), suggesting the concept that the morphine effect was mediated through interaction with opioid receptors on the cells. MDM in the additional wells of the same experiments were also collected for RNA extraction 72 h after infection and subjected to RT-PCR and real-time RT-PCR analysis using a specific primer pair for HIV gag gene. Increased expression (2- to 2.5-fold) of HIV gag gene mRNA was observed in morphinetreated neonatal MDM in comparison to untreated neonatal MDM (Fig. 2).

Effect of morphine on pseudotyped HIV infection of neonatal MDM. To examine the hypothesis that morphine enhances HIV infection of neonatal MDM by affecting viral entry, we examined the effect of morphine on ADA (CCR5dependent and macrophage-tropic) Env or MLV (HIV receptor independent) Env-pseudotyped HIV infection of MDM. This pseudotyped HIV genome that encodes a luciferase reporter gene allows a quantitative measure of the levels of a single round of infection (20). Neonatal MDM incubated with or without morphine ( $10^{-10}$  M) were infected with recombinant luciferase-encoding HIV particles pseudotyped with ADA Env or MLV Env in the presence of polybrene (4 µg/mL). The cells were lysed and subjected to luciferase activity determination 72 h after infection as described in "Methods." When infected with ADA Env-pseudotyped virus, significant increase of lu-



**Figure 1.** Effect of morphine on HIV Bal infection of neonatal macrophages. Seven-day-cultured neonatal MDM were incubated with morphine (*Mo*) or naltrexone (*Nalt*) at the concentrations indicated for 12 h and then challenged with HIV Bal strain for 2 h in the presence of morphine or naltrexone. When both morphine and naltrexone were used, cells were treated with naltrexone ( $10^{-8}$  M) for 30 min before the addition of morphine ( $10^{-10}$  M). Cultures containing neither morphine nor naltrexone served as the control. Cultures were refed with fresh medium containing morphine every 4 d. Day 8 culture supernatants were collected for HIV RT assay. The results shown are the mean  $\pm$  SD of triplicate cultures and are representative experiments using cells from three different cord blood samples (\*p < 0.05, morphine *vs* control).



**Figure 2.** Effect of morphine on HIV Bal *gag* gene expression in neonatal macrophages. The HIV Bal strain was used to infect 7-d-cultured neonatal MDM with or without morphine at the indicated concentrations for 2 h. Total cellular RNA was extracted from the cell cultures 72 h after infection and then subjected to RT-PCR (*A*) and real-time RT-PCR (*B*) for determination of HIV *gag* gene expression.  $\beta$ -Actin served as the control to monitor the amount and integrity of RNA in each sample. HIV *gag* gene mRNA copy numbers were normalized with GAPDH mRNA. The results are expressed as HIV *gag* mRNA copy numbers per 1,000,000 GAPDH mRNA. One representative of four experiments using four different cord blood samples is shown.

ciferase activity was observed in the morphine-treated neonatal MDM compared with the untreated neonatal MDM (Fig. 3). Morphine, however, had no effect on MLV Env-pseudotyped HIV infection of neonatal MDM (Fig. 3), further confirming the observation that morphine enhances HIV infection of neonatal MDM by affecting steps involved in HIV entry.

Effect of morphine on CCR5 receptor expression. Because morphine promoted ADA Env-pseudotyped HIV but not MLV Env-pseudotyped HIV infection of neonatal MDM, we hypothesized that morphine and its receptor interaction participated in the regulation of the CCR5 receptor expression, a primary coreceptor for HIV R5 strain entry into macrophages. CCR5 receptor expression on neonatal MDM was up-regulated by morphine  $(10^{-10} \text{ M})$  as determined by flow cytometry (Fig. 4). CCR5 mRNA was also up-regulated in MDM by morphine  $(10^{-10} \text{ M} \text{ and } 10^{-8} \text{ M}; \text{ Fig. 5})$ . To determine that the effect of morphine on CCR5 receptor expression in neonatal MDM is specific, we also examined whether monocyte/macrophage marker (CD14) and other HIV entry receptors are affected by morphine. The expression of CD14, CD4, and CXCR4 receptors in neonatal MDM was not altered by morphine (data not shown).

Effect of morphine on MIP-1 $\beta$  production. Because  $\beta$ -chemokines such as MIP-1 $\beta$ , the natural ligands for CCR5 receptor, have been identified as the HIV suppressive factors (21),



**Figure 3.** Effect of morphine on pseudotyped HIV infection of neonatal macrophages. Seven-day-cultured neonatal MDM were treated with morphine (10<sup>-10</sup> M) for 24 h, and then challenged with recombinant luciferase-encoding HIV pseudotyped with either ADA Env or MLV Env. Luciferase activity was quantitated in the cell lysates 72 h after infection. The data are expressed as RLU of morphine-treated cells to that of controls incubated without morphine. The results demonstrated are the mean  $\pm$  SD of triplicate cultures and representative of six experiments using six different cord blood samples (\**p* < 0.01, morphine *vs* control).

we investigated whether morphine affected MIP-1 $\beta$  production in HIV-infected neonatal MDM. Seven-day-cultured neonatal macrophages in 48-well plates were incubated with or without morphine (10<sup>-14</sup> M to 10<sup>-8</sup> M) or naltrexone for 12 h and then challenged with HIV Bal strain. Culture supernatants were collected 96 h postinfection for MIP-1 $\beta$  analysis by ELISA. Morphine significantly inhibited the production of MIP-1 $\beta$ (67.3%, 79.7%, and 71.6% of down-regulation at 10<sup>-12</sup> M, 10<sup>-10</sup> M, and 10<sup>-8</sup> M, respectively) in HIV-infected neonatal MDM (Fig. 6). Morphine at the concentration of 10<sup>-14</sup> M had little effect on MIP-1 $\beta$  production (Fig. 6). The inhibitory effect of morphine on MIP-1 $\beta$  production was reversed by pretreating MDM with naltrexone, although naltrexone alone had no effect on MIP-1 $\beta$  production (Fig. 6).

### DISCUSSION

The transmission of HIV in pregnant women is influenced by "lifestyle cofactors," including substance abuse. There are several reasons why increased HIV transmission from mother-to infant might be associated with maternal drug use. Preterm delivery, a known consequence of maternal drug use, has been reported in some studies to be related to increased risk of perinatal HIV transmission (22-24). A history of continued cocaine and heroin injection drug use after the first trimester of pregnancy is strongly associated with vertical HIV transmission (4). Morphine is commonly administrated to pregnant women during labor and delivery for pain control and to intubated neonates for sedation. Opiates in maternal blood cross the placental barrier very efficiently and selectively accumulate in fetal blood. However, there is little information about neonatal immune system alteration and subsequent susceptibility to HIV infection after morphine exposure. In the present study, we have analyzed the impact of morphine on HIV infection of neonatal MDM. We also explored the poten-



**Figure 4.** Effect of morphine on CCR5 receptor expression on 7-d-cultured neonatal MDM. Seven-day-cultured neonatal MDM were incubated with (*Bottom*) or without (*Top*) morphine at  $10^{-10}$  M for 24 h, and expression of CCR5 on neonatal MDM was determined by flow cytometry. *Shaded histo-gram*, control staining with isotope-matched antibody (IgG2b); *open histo-gram*, CCR5 expression with MAb 2D7. The results are shown as the percentage of CCR5-positive cells and are representative of four experiments.

tial mechanism(s) by which morphine enhances HIV infection of neonatal MDM.

Direct action of opiates on viral replication in the immune cells requires interaction with opioid receptors on the cells. Evidence for the expression of opiate receptors on immune cells, in particular receptors for morphine and the metabolites of heroin, provided a biologic link between opiates and the cells of the human immune system (25, 26). Opioid receptors  $(\mu, \kappa, \text{ and } \delta)$ , as well as nonclassic opioidlike receptors, are present on cells of the human immune system (27-30). Binding sites for the novel morphine receptor designated  $\mu$ 3 are detectable on human peripheral blood isolated monocytes (31). Opioid receptor mRNA is also constitutively expressed in highly purified human microglia (32). To determine whether the modulating effect of morphine on HIV infection and MIP-1 $\beta$  production is opioid receptor specific, we incubated neonatal MDM with or without naltrexone, an opiate receptor antagonist, before morphine treatment. Our data showed that naltrexone blocked the effects of morphine on HIV infection and MIP-1 $\beta$  production, which provides evidence of the presence of opioid receptors on neonatal MDM. Inasmuch as morphine has a high affinity and selectivity for the  $\mu$  receptor



**Figure 5.** Effect of morphine on CCR5 mRNA expression in neonatal macrophages. Seven-day-cultured neonatal MDM were incubated with or without morphine  $(10^{-10} \text{ M} \text{ and } 10^{-8} \text{ M})$ . Total cellular RNA was extracted from the cell cultures at indicated time points (6, 12, and 24 h after morphine treatment) and subjected to real-time RT-PCR using the specific primer pairs and probe for the CCR5 receptor gene. CCR5 mRNA copy numbers in MDM incubated with or without morphine were normalized with GAPDH mRNA. The results are expressed as CCR5 mRNA copies per 10,000 GAPDH mRNA. One representative of four experiments using four different cord blood specimens is shown.

(33–35), the observed morphine effects are most likely through the specific  $\mu$  receptors on the cells.

There are several mechanisms by which opiates may effectively enhance HIV infection (10, 36, 37). We showed in this communication that morphine at clinically relevant concentrations ( $10^{-12}$  to  $10^{-8}$  M) significantly enhanced HIV infection of neonatal macrophages (Figs. 1 and 2). We also demonstrated that morphine up-regulated expression of the CCR5 receptor at both mRNA and protein levels (Figs. 4 and 5). CCR5 is a primary coreceptor for HIV R5 strain infection of macrophages. The  $\beta$ -chemokine receptor CCR5 plays an important role in nonsyncytium-inducing HIV strain infection of macro-



**Figure 6.** Effect of morphine on MIP-1 $\beta$  production in HIV-infected neonatal macrophages. Seven-day-cultured neonatal MDM were incubated with or without morphine (*Mo*) or naltrexone (*Nalt*) at indicated concentrations for 12 h. The cells were also treated with both morphine (10<sup>-10</sup> M) and naltrexone (10<sup>-8</sup> M) or naltrexone (10<sup>-8</sup> M) only. The cells were then infected with HIV Bal strain. HIV-infected (HIV only) and uninfected neonatal MDM (cell only) were used as controls. Culture supernatants were collected 96 h after infection for MIP-1 $\beta$  production as determined by ELISA. The data shown are presented as the mean ± SD of triplicate cultures (\*p < 0.05, morphine vs control) and are representative of six experiments using six different cord blood specimens.

phages (20, 38, 39). The natural ligands (RANTES, MIP-1 $\alpha$ and  $-1\beta$ ) of the CCR5 receptor inhibit HIV infection by interfering with HIV binding to CCR5 (38, 40-42). Morphine significantly inhibits MIP-1 $\beta$  production in HIV-infected neonatal MDM (Fig. 6). Monocytes and macrophages are important in HIV infection during all stages of disease in that they serve as major target cells, reservoirs, vehicles to other tissues, and transmitters of virus to  $CD4^+$  T cells (43). We and others demonstrated that neonatal monocytes/macrophages have increased susceptibility to HIV infection in vitro compared with adult macrophages (44-46). In addition, we have recently demonstrated that morphine, as well as methadone (a synthetic opiate), potentiates HIV infection of adult blood mononuclear phagocytes (14). Thus, our data provide a possible mechanism by which morphine potentiates HIV infection of neonatal macrophages. Our data showing that morphine modulated expression of MIP-1 $\beta$  and CCR5 receptor are supported by several recently reported observations (47-49). Nair et al. (47) recently reported that cocaine selectively down-regulates endogenous MIP-1 $\beta$  secretion and up-regulates CCR5 expression by adult peripheral blood monocytes, suggesting a mechanism by which cocaine increases susceptibility of peripheral blood monocytes to HIV infection. Morphine induces CCR5 expression in a human T-lymphoid cell line (CEMx174) (48). This increased CCR5 expression by morphine was correlated with morphine-enhanced susceptibility of the CEMx174 cells to simian immunodeficiency virus infection (49). These data support our hypothesis that morphine, through modulation of neonatal cell function, enhances HIV infection of these cells.

## CONCLUSIONS

Taken together, our *in vitro* data in association with the clinical and epidemiologic evidence (3) indicate the possibility that morphine may play an important role as a cofactor in neonatal HIV infection. The up-regulatory effects of morphine on HIV infection of both maternal and neonatal immune cells may have a significant impact on perinatal transmission and infection. Further studies are necessary to prove the association between opioid abuse and increased risk of HIV vertical transmission.

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