Methadone induces CCR5 and promotes AIDS virus infection

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Abstract Methadone, a regimen for the treatment of opioid dependency, was found to induce the expression of CCR5, a co-receptor for human immunodeficiency virus (HIV)/simian form of HIV (SIV) entry, on human CEM x174 lymphocytes. Both CCR5 mRNA and protein were elevated in methadone-treated cells. A concomitant increase of *mu* opioid receptors was also observed. Upon methadone exposure, SIVmac239-infected CEM x174 cells released greater amounts of virus particles as revealed by both the number of syncytia formation and reverse transcriptase activities. Similar methadone effect was not observed on CEM x174 cells infected with other simian retroviruses that do not depend on CCR5 for cellular entry. These studies raise concerns considering methadone as an innocuous morphine substitute. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Methadone; *Mu* opioid receptor; Chemokine receptor CCR5; Lymphocyte; SIVmac239

1. Introduction

Drug addiction is a major risk factor for human immunodeficiency virus (HIV) infection worldwide [1,2]. Injection drug use accounts for as many as 50% of new cases of HIV infection in the USA yearly [3]. Methadone is a synthetic opiate receptor agonist that is rapidly absorbed and slowly eliminated from the plasma [4]. Unlike most other modalities where research was not involved, methadone was developed as a maintenance drug for opioid addicts through the initial assiduous research efforts of Dole and Nyswander [5]. Today, for opiate abuse, methadone treatment represents a widely offered and widely accepted modality [6,7]. Approximately 179 000 patients were in treatment in 1998 [7]. Methadone programs have been reported to effectively block withdrawal symptoms and reduce a patient's craving for cocaine [8] and heroin [9]. It was also reported that the likelihood of heterosexual HIV-1 transmission from a seropositive methadone-maintained drug user to his/her seronegative, non-drug user/steady sex partner is low [10]. It has been suggested that heroin addicts undergoing methadone treatment have a better functioning immune system [11,12]. Effective drug abuse treatment such as methadone maintenance has hence been suggested a cost-effective intervention for slowing the spread of AIDS among drug users [1,2,13].

In a previous study, we found that morphine will activate *mu* opioid receptors of human lymphocytic cells [14] and up-regulate cell surface CCR5 expression [15], rendering the cells more susceptible to simian form of HIV (SIV)mac239 infection [15]. Opioid receptor and chemokine receptor CCR5 are both G protein-coupled receptors containing seven transmembrane domains that respond in consensus toward extracellular stimuli [14,15]. SIVmac239, a simian counterpart of HIV, has been used in the infection of rhesus monkeys leading to AIDS-like disease [16]. Using monkey monocytes and neutrophils to study the effect of opioids on immune functions, we have demonstrated that both morphine and methadone inhibit chemokine-mediated chemotaxis of the cells [17]. In a study evaluating LAAM (L-α-acetyl-methadol), a derivative of methadone, we reported that T helper cell functions of rhesus monkeys were significantly impaired if the monkeys received LAAM intramuscularly for the treatment of morphine dependency [18]. It thus appears that methadone may have morphine-like immunomodulatory activities on immune cells. This paper will address the effect of methadone on lymphocytes, with particular interest on its effect on the expression of cell surface *mu* opioid and CCR5 receptors and the subsequent release of viral particles from SIVmac239-infected cells.

2. Materials and methods

2.1. Cell culture

CEM x174 cell line, a human lymphocytic cell line which is highly susceptible to SIVmac239 infection and routinely utilized by various investigators for the propagation of SIVmac239 [15], was used in this study. CEM x174 was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a CO_2 incubator.

2.2. Methadone or morphine treatment

CEM x174 cells in culture were diluted 1:3 with fresh medium every 3–4 days. At the time of dilution, methadone hydrochloride (Mallinckrodt), morphine sulfate (Mallinckrodt) or H_2O (as control) was added. When naloxone was included in the experiment involving methadone, cells were first incubated with naloxone for 15 min at 37°C before the addition of methadone. The initial cell density was 2×10^5 cells/ml and incubation continued at 37°C for the indicated

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian form of HIV; LAAM, L-α-acetyl-methadol; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRV, simian type D retrovirus

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time. Cells were collected by centrifugation and washed twice with 1×PBS before use.

2.3. Competitive reverse transcriptase-polymerase chain reactions (RT-PCR)

Opioid-treated or control CEM x174 cells were lysed in Trizol reagent (Gibco BRL) and total RNA was isolated by phenol–chloroform extraction and isopropyl alcohol precipitation. Quantitative analyses of gene expression of chemokine receptor CCR5 and *mu* opioid receptors were performed in competitive RT-PCR. The methods for the construction of competitive molecules containing segments of CCR5 or *mu* with internal deletions, the specific RT-PCR conditions, the gel analysis, and the final calculation of the amount of cDNA synthesized using CA-Cricket Graph III (Computer Associates) have been described in detail in previous publications [14,15].

2.4. Western blot analysis

Total protein was isolated from opioid-treated or control CEM x174 cells with lysis buffer (20 mM Tris–HCl, pH 7.4; 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100 and 1% sodium deoxycholate). The method of Western blotting was previously described [14,15,19]. Briefly, samples of 20 μg protein were analyzed by 10% SDS–polyacrylamide gel electrophoresis (PAGE) with prestained SDS–PAGE protein standards (Bio-Rad) running in parallel to reveal the molecular mass of the test proteins. Polypeptide bands resolved in SDS–PAGE gels were electroblotted onto Immobilon-PVDF (Millipore) filters. After blocking, the filters were incubated with primary antibodies against target proteins followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunocomplexes resolved by electrophoresis were visualized by incubation of the filters with the SuperSignal substrate (Pierce) followed by exposure to a Fuji autoradiography film (Fisher). Results were quantified by densitometry and experiments were repeated at least six times. The primary antibodies used in this study were rabbit anti-CCR5-NT (ANASPEC Incorp., San Jose, CA, USA) and rabbit anti-*mu* opioid receptor (MOR)-1 (INCSTAR Corp., Stillwater, MN, USA) for the detection of CCR5 and *mu*, respectively. The horseradish peroxidase-conjugated secondary antibodies used to detect the primary antibodies were purchased from Promega (Madison, WI, USA).

2.5. Infection of CEM x174 with SIVmac239 or simian type D retrovirus (SRV)

Infection of CEM x174 cells with SIVmac239 or SRV and quantitation of viral infectivity by micro-RT assay were performed as described previously [15,20]. SIVmac239 (3×10^3 $TCID_{50}$/ml) was resuspended at a 1:50 dilution. 50 μl of the diluted virus was added to 10^4 CEM x174 cells in a 96-well, flat-bottomed plate, and incubated overnight at 37°C in a 5% CO_2 incubator. On the following day, methadone was added to the corresponding wells at 4.0 and 20 μM final concentrations. On days 6 and 8, supernatant fractions were collected and viral infectivity was determined by the micro-RT assay. The presence of syncytia formation was also evaluated with an inverted microscope. Titers for SRV serotype 2 were determined by the Raji cell infectivity assay and the Raji cell line was obtained from American Type Culture Collection.

2.6. Statistics

Results are presented as the mean ± S.E.M. Statistical significance was evaluated by Student's *t*-test.

3. Results

3.1. Effect of methadone treatment on expression of mu opioid receptors in CEM x174

As previously described, by use of primer sequences corresponding to bases 921–950 and 1332–1361 of the human brain MOR sequence (Mestek et al., accession number L29301) in RT-PCR amplification of CEM x174 RNA, it was found that CEM x174 cells synthesized a 441 bp segment that was 100% homologous to the corresponding segment in human brain MOR [14]. To determine the densities of CEM x174 MOR, plasmids containing CEM x174 MOR with a 62 bp deletion were constructed and used in quantitative RT-PCR analysis [14]. In order to investigate the effect of methadone treatment on the gene expression of CEM x174 MOR, the amount of cDNA amplified by competitive RT-PCR from cells treated with methadone was compared with that of untreated or morphine-treated cells. Samples were taken 12, 24, 36 and 48 h post-treatment for analysis. The results showed that like morphine [14], methadone treatment of CEM x174 cells increased MOR expression (Fig. 1A). By 24 h post-treatment, 10 μM methadone increased MOR expression by 180% and 10 nM methadone increased MOR by 330%.

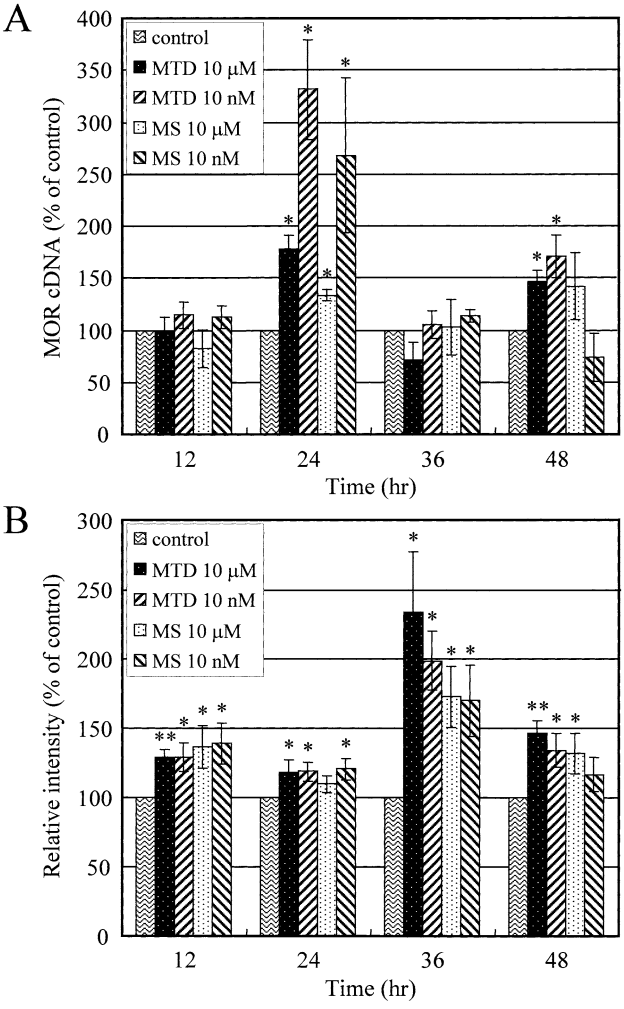

Fig. 1. Effect of methadone on CEM x174 MOR. A: RT-PCR analysis. The amount of MOR transcripts (expressed as cDNA) synthesized in CEM x174 cells was determined by the competitive RT-PCR procedure after treatment of the cells with 10 nM or 10 μM methadone hydrochloride for the indicated time. Experiments with H_2O-treated (as control) and morphine-treated cells were performed in parallel for comparison purposes. B: Western blot analysis. Total protein (20 μg/lane) from 10 nM or 10 μM methadone- or morphine-treated CEM x174 cells was subjected to SDS–PAGE, blotted onto filters, probed with the antibody against neuronal MOR and detected by horseradish peroxidase-conjugated secondary antibody as described in Section 2. Data analyzed by densitometry are presented. The amount of MOR cDNA (A) or MOR protein (B) synthesized from H_2O-treated (control) cells was designated as 100%. MTD, methadone; MS, morphine. Bar, standard error (A, $n=4$; B, $n=6$). $^*P<0.05$, $^{**}P<0.01$.

Subsequent experiments showed that the methadone-induced increase in CEM x174 MOR gene expression correlated

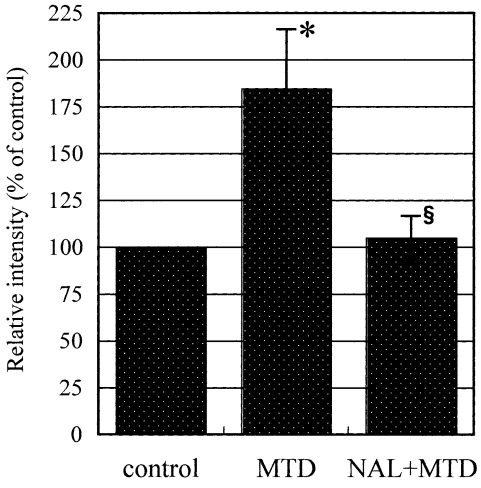

Fig. 2. Effect of naloxone treatment on methadone-induced MOR protein expression. Experiments were performed as in Fig. 1B. When naloxone was included in the treatment, naloxone was added to the cells 15 min before the addition of methadone (see Section 2). Control, H_2O-treated cells. Cells were harvested 36 h post-treatment for analysis and the concentrations of methadone (MTD) or naloxone (NAL) used for treatment were 10 μM each. Bar, standard error ($n=3$). $^*P<0.05$ (compared with the control). $^{\S}P<0.05$ (compared with MTD).

with the amount of MOR protein synthesized in the cells as determined by Western blot analysis. The increase of MOR protein was greatest at 36 h as opposed to 24 h post-treatment (Fig. 1B), indicating that mRNA (Fig. 1A) has a faster turnover rate than protein synthesis. After 36 h treatment with 10 μM or 10 nM methadone, CEM x174 MOR protein was increased by 230 and 200%, respectively (Fig. 1B). The methadone-induced *mu* opioid receptor expression was blocked by pretreatment of the cells with naloxone, a *mu* opioid receptor antagonist (Fig. 2). Preliminary studies showed that like the effect of morphine [19], activation of *mu* opioid receptor by methadone induced a series of intracellular reactions including the stimulation of ERK2 and the 85 kDa mitogen-activated protein kinase (data not shown).

3.2. Effect of methadone treatment on expression of chemokine receptor CCR5 in CEM x174

To determine the effect of methadone treatment on the expression of chemokine receptor CCR5 at the transcriptional level, a competitive RT-PCR analysis of CEM x174 CCR5 was performed. Using primer sequences corresponding to bases 44–63 and 1139–1157 of the published human CCR5 sequence, CEM x174 cells synthesized a 1114 bp segment [15]. To determine CCR5 receptor densities on CEM x174 cells, plasmids containing CCR5 segments with 96 bp deletions were constructed and used in quantitative RT-PCR as previously described [15]. With this method, the effect of methadone on the gene expression of CCR5 was studied and the amount of CCR5 cDNA in methadone-treated cells was compared with that in morphine-treated or control (H_2O-treated) cells. Samples were taken 12, 24, 36 and 48 h post-treatment for analysis. The results showed that as with morphine treatment [15], methadone increased CCR5 gene expression. In comparison with the control cells, 10 μM methadone increased CCR5 expression by 120, 200 and 230% upon 12, 24 and 36 h exposure, respectively (Fig. 3A). Further studies revealed that the methadone-induced increase in CCR5 gene expression could be identified at the protein level by both Western blot analysis (Fig. 3B) and flow cytometry studies (data not shown), although the protein increase was evident at 36 and 48 h post-infection (Fig. 3B), again later than the increase of mRNA expression (Fig. 3A). Dose–response experiments indicated that methadone exerted its optimal effect on inducing CCR5 protein of CEM x174 cells at both 10 μM and 10 nM concentrations (Fig. 4).

3.3. Effect of methadone on viral replication of SIVmac239-infected CEM x174

With the apparent increase in CCR5, a co-receptor for

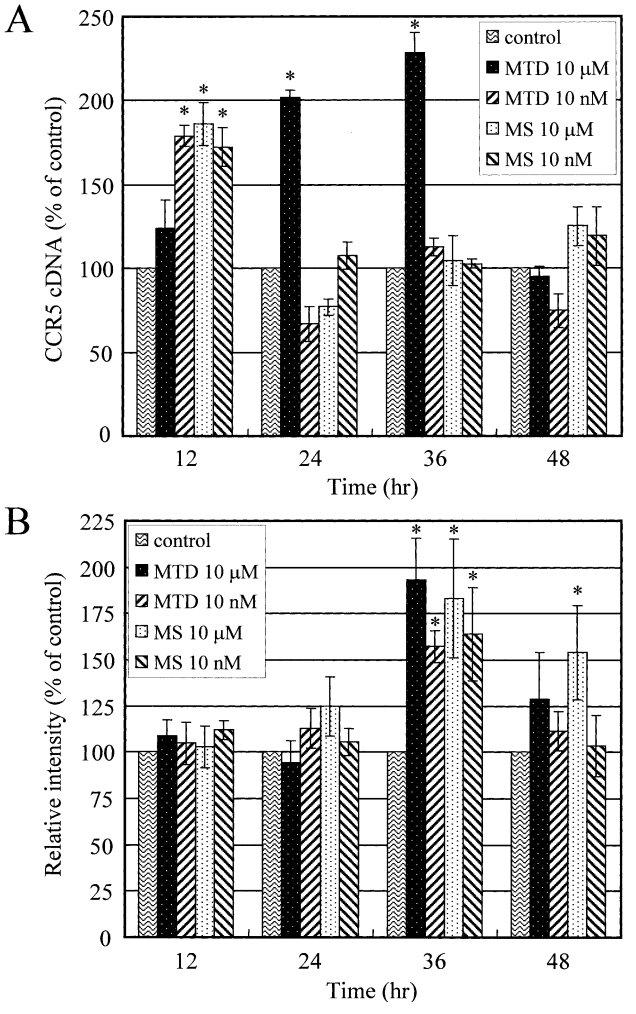

Fig. 3. Effect of methadone on CEM x174 CCR5. A: RT-PCR analysis. The amount of CCR5 transcripts (expressed as cDNA) synthesized in CEM x174 cells was determined by the competitive RT-PCR procedure after treatment of the cells with 10 nM or 10 μM methadone hydrochloride for the indicated time. Experiments with H_2O-treated (as control) and morphine-treated cells were performed in parallel for comparison purposes. B: Western blot analysis. Total protein (20 μg/lane) from 10 nM or 10 μM methadone- or morphine-treated CEM x174 cells was subjected to SDS–PAGE, blotted onto filters, probed with the antibody against CCR5 and detected by horseradish peroxidase-conjugated secondary antibody as described in Section 2. Data analyzed by densitometry are presented. The amount of CCR5 cDNA (A) or CCR5 protein (B) synthesized from H_2O-treated (control) cells was designated as 100%. MTD, methadone; MS, morphine. Bar, standard error (A, $n=2$; B, $n=6$). $^*P<0.05$.

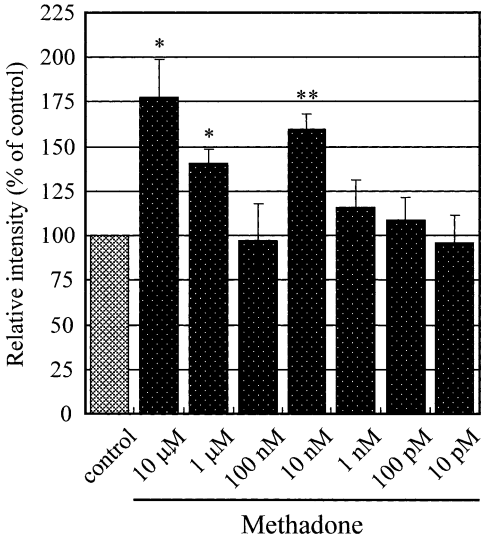

Fig. 4. Effect of methadone on CCR5 protein expression – a dose–response curve. Experiments were performed as in Fig. 3B except that a series of concentrations of methadone was used in the treatment of CEM x174 cells and the treatment was for 36 h. Bar, standard error ($n = 5$). *$P < 0.05$, **$P < 0.01$.

SIVmac entry, CEM x174 cells would be expected to be more susceptible to SIVmac239 infection. CEM x174 cells were infected with SIVmac239 in the presence of methadone and both syncytia formation and viral RT activity were monitored. The results (Fig. 5A, Tables 1A and 1B) show that methadone increased both the number of syncytia and the RT activity of CEM x174 cells. Cells treated with 4 μM methadone for 8 days showed the greatest amount of syncytia and RT activities (Fig. 5A, Tables 1A and 1B). On the other hand, similar infection of CEM x174 cells with SRV, viruses that do not depend on CCR5 for cellular entry, showed no significant difference in virus-induced syncytia formation whether the infection took place in the presence or absence of methadone (Fig. 5B).

Table 1A
Mean number of syncytia formation for SIVmac239[a]

Day	Methadone concentration		
	0 μM	4 .0 μM	20.0 μM
6	0[b]	0	1.2
7	0	0.8	2
8	1.2	7.3	3.2

[a]Data were reproducible in three independent experiments.

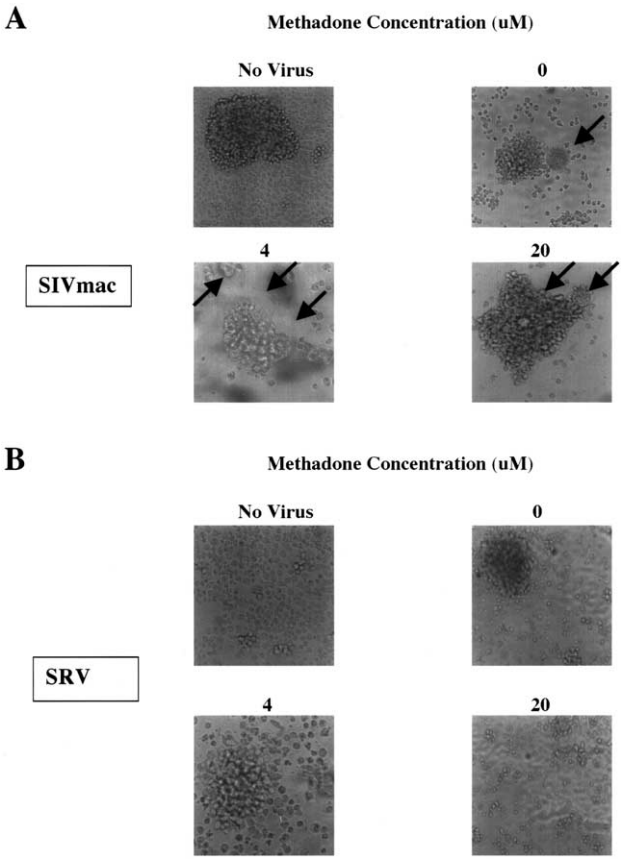

Fig. 5. Effect of methadone on SIVmac239 or SRV infection of CEM x174 cells. CEM x174 cells were infected with SIVmac239 (A) or SRV serotype 2 (B) in the presence of methadone at concentrations of 0, 4 or 20 μM. Syncytia formation was shown as giant protoplasmic mass that resulted from fusion of cytopathic cells. The data were reproducible in three independent experiments.

4. Discussion

The above studies indicate that the effects exerted by methadone on human lymphocytes are likely similar to the effects caused by morphine. Like morphine, methadone activates *mu* opioid receptors in a naloxone-reversible manner (Figs. 1, 2). The activation of *mu* opioid receptors up-regulates CCR5 receptors (Figs. 3, 4). The precise mechanism by which methadone induces CCR5 expression remains to be determined. As postulated [15], methadone may act like morphine and induce CCR5 by activating the release of cytokines (e.g. TNF-α or IL-2) known to stimulate chemokine receptor expression or,

Table 1B
Effect of methadone on RT activity of SIV- or SRV-infected CEM x174 cells[a]

Virus	Methadone concentration					
	0 μM		4.0 μM		20.0 μM	
	Day 6	Day 8	Day 6	Day 8	Day 6	Day 8
SIVmac239[b]	804 (1.0)[c]	881 (1.0)	836 (1.1)	1231 (1.4)	1030 (1.3)	1078 (1.3)
SRV-2[d]	620 (1.0)	780 (1.0)	612 (1.0)	789 (1.0)	631 (1.0)	795 (1.0)

[a]Data were reproducible in three independent experiments.
[b]The P value is ≤ 0.05 by analysis of variance (ANOVA), considered significant. One-way ANOVA was performed using GraphPad InStat version 3.00 for Mac, GraphPad Software, San Diego, CA, USA, www.graphpad.com.
[c]CPM ratio between sample and no-methadone control.
[d]The P value is > 0.1 by ANOVA, considered not significant.
The RT activity was assayed using standard [^{32}P]dTTP incorporation [20]. The mean cpm for the scintillation fluid was 120.

alternatively, methadone may inhibit the synthesis of certain chemokines (e.g. RANTES, MIP-1α or MIP-1β) which cause internalization of CCR5. In as much as CCR5 is a co-receptor facilitating cellular entry of AIDS virus, increasing CCR5 receptors renders the cells more susceptible to SIVmac239 infection. Indeed, methadone treatment of CEM x174 caused the cells to release a greater amount of SIVmac239 virus, but similar treatment had no effect on the virus production of SRV-2, the simian retroviruses that do not use CCR5 for cell entry (Fig. 5, Tables 1A and 1B).

Heroin is a synthetic morphine derivative and a common form of opiate sought by street users. Heroin is injected intravenously because it is poorly absorbed by the oral route. Once in the body, heroin is rapidly (within 10–15 min) converted to 6-acetylmorphine and then to morphine, which has a plasma half life of 2 h [21]. Methadone, a long-acting opioid with a plasma half life of 15–40 h [21], is readily absorbed if administered orally and has been used as the drug of choice for the treatment of heroin addiction [1,2,5]. Little is known about the effect of methadone on the immune functions of treated patients. An earlier study determining the activities of natural killer cells and lymphocyte subsets in methadone maintenance patients in comparison with parenteral heroin abusers suggested that significant abnormalities of cellular immunity in parenteral heroin abusers can be 'normalized' by successful long-term methadone treatment [22]. Accordingly, methadone maintenance therapy for treatment of opiate abuse has given sanguine hope for slowing the spread of AIDS. A low HIV seroconversion rate was reported among injection drug users enrolled in methadone programs of Los Angeles [23] and New York [24], although HIV patients in New York did not experience greater survival [25]. On the other hand, other studies indicated that in Amsterdam, prescription of methadone to illicit drug users is not sufficient to halt the spread of HIV [26]. Likewise, in Thailand, HIV-1 transmission risk remains high among Bangkok drug users despite methadone treatment [27].

The results of the present study which compared the effects of methadone and morphine on cellular receptors of human lymphocytic cells and the subsequent virus production after infection, together with other reports which showed the immunotoxic properties of methadone [28,29], suggest that methadone itself may not countervail the detrimental effects of morphine/heroin on the immune system. Rather, the decline of HIV infection among drug users enrolled in the methadone maintenance programs may be largely attributed to treatment paraphernalia such as reduction in needle use/sharing and other high risk behaviors as well as educational interventions. Furthermore, it was found that consistent participation in a methadone maintenance program facilitated therapy with an antiretroviral regimen [30]. Therefore, it appears that further biological and immunological studies of methadone as well as a more detailed epidemiological surveillance of methadone-treated HIV patients are necessary to establish methadone as the drug of choice for the treatment of opioid addiction.

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