

## OPIOIDS AND THE PROGRESSION OF SIMIAN AIDS

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### 1. ABSTRACT

This review is a concise description of the study undertaken to examine the modulation by opioids of simian acquired immunodeficiency syndrome (SAIDS) induced by inoculation of rhesus monkeys with simian AIDS virus SIVmac239. The study showed that the replication rate of the virus was several times greater in monkeys made dependent on morphine than in those of non-morphine treated monkeys. Further, a significant change in the mutation rate of the infecting virus in morphine-treated monkeys resulted in the production of mutants that were silent to conventional serological screening tests as well as resistant to AZT. In addition, opioid and chemokine receptors involved were identified in immune cells and a full comparative spectrum of the compromise of the immune system was examined allowing subsequent studies to evaluate wherein the modulation of the development of the syndrome could be better characterized. The data gathered to date are unique and germane to furthering our understanding of AIDS in humans and its subsequent treatment thereof.

### 2. INTRODUCTION

AIDS (acquired immunodeficiency syndrome) is a cellular immune disorder found in patients infected by HIV (human immunodeficiency virus). HIV infection is the leading cause of death among Americans ages 25 to 44 (1). Injecting drug users constitute a large portion of the patient population contracting AIDS in the U.S. (2) and worldwide (3, 4). However, among drug users, needle-sharing alone cannot explain the reportedly high levels of infectivity, since the blood volumes transferred by shared needles are rather small (<0.75 µl) according to Gaughwin *et al.*, (5). Opioid addicts who did not share needles also showed substantial rates of seropositivity to HIV (6). Questions thus remain whether opioids themselves may directly influence immune function and AIDS progression.

In earlier *in vitro* studies to assess a possible opiate effect on HIV infection, Squinto *et al.* (7) found that morphine can function to transactivate the HIV/LTR (long terminal repeat) gene of neuroblastoma cells, but not of T cells. Using a human peripheral blood mononuclear cell (PBMC) co-culture assay system in which PBMC were stimulated with phytohemagglutinin (PHA), Peterson *et al.* (8) found that morphine promotes the growth of HIV-1 and that the effect of morphine could be blocked by the opioid receptor antagonist naloxone. Taken together, these findings suggest that morphine enhances HIV infection.

Due to the complexity and heterogeneity of the human system, particularly with the addition of drug use, it has been difficult to systematically characterize the relationship between opioid abuse and HIV infection in humans. The simian immunodeficiency virus (SIV), on the other hand, was isolated from non-human primate hosts and is related to HIV-2 by approximately 75% nucleotide sequence homology (9, 10). Similarities between SIV and HIV infection include CD4+ lymphoid cell and macrophage tropism, CD4+ cell depletion, serological and immunological responses and pathology including neuropathology and opportunistic infections (11, 12). Virus from a molecular clone of SIV, SIVmac239, establishes persistent viremia in experimentally infected macaques (13). Whereas the pattern of the infection of rhesus macaques with SIVmac239 and the resulting clinical syndrome of simian AIDS (SAIDS) are similar to those of HIV infection in man, the onset of SAIDS is much more rapid (within three months of infection), and hence suitable for laboratory investigation (13, 14). Therefore, SIV infection of rhesus macaques is generally considered the best animal model system available for studying AIDS (11, 12, 15) and the effect of drug abuse on AIDS (16).

Through the use of SIV/rhesus monkey model, we found that chronic administration of opioids

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significantly compromises host (both T and PMN cell-mediated and humoral) immune functions, affects the rate of viral replication and mutation, expedites the progression of the clinical disease, and generally shortens the life span of the infected animals. We further demonstrated that opioids act via opioid receptors on immune cells and that opioid receptors and chemokine receptor CCR5 (a coreceptor for SIV/HIV entry) form an oligomeric complex on the membrane of immune cells in effecting receptor functions.

### 3. OPIOID INJECTION ALTERS SIMIAN AIDS PROGRESSION

To determine the effect of chronic opioid use on the development of AIDS, we subjected experimental macaques (*Macaca mulatta*) to opioid injection followed by SIVmac239 infection. Sixteen male rhesus monkeys between 2 and 3 years of age were used for our investigation. All monkeys were initially injected with saline 3 times daily. The monkeys were then divided into 3 groups. Group 1 served as controls and continued to receive saline treatments at regular intervals. Group 2 received morphine sulfate intramuscularly three times daily, in place of saline. Initially, the animals were injected with 1 mg/kg of morphine sulfate. The dosage was then increased to 3 mg/kg, and finally stabilized at 5 mg/kg per injection. Group 3, also, was subjected to morphine treatment. However, after morphine dependence had been established, Group 3 animals received intramuscular LAAM (*L*- $\alpha$ -acetylmethadol) injections once per day, starting at 0.8 mg/kg per injection and increasing to a maximum concentration of 2 mg/kg per injection. All injections were given 7 days per week. LAAM is a long-acting opioid. After both morphine and LAAM dependency had been established, the animals were subjected to SIVmac239 infection. Each monkey received 1 ml of virus containing  $1.58 \times 10^3$  rhesus monkey infectious dosage. The progress of the viremia, the functions of immune cells, and behavioral assessments were monitored.

Results of these studies showed that in addition to weakening the host's T cell and PMN functions, chronic morphine or LAAM treatment of SIVmac239-infected animals caused (a) an increased virus replication rate, (b) an increased rate of viral mutation, (c) a nascent humoral (antibody) response against mutated autologous strains of virus, (d) an alteration of CD8+ cell-mediated immunity toward the mutant virus, (e) an increased tolerance of AZT among the infectious virus, and (f) a shorter life span for the infected animals (16-25). Among these findings, it is particularly interesting to note that in SIV-infected monkeys, morphine may induce mutations in the gag region of SIV genes, with the consequence of these mutations being that the initial infecting virus escapes host immune surveillance (22). AZT- and ddI-resistance could also be identified in the virus isolated from morphine-treated animals (16, 21, 24, 25). This high frequency of genetic variability and the resulting "viral quasispecies" in animals treated with morphine may be attributed to the inability of the immune system of these animals to constrain virus replication. These findings have far-

reaching implications, which range from the possibility of false-negative clinical diagnoses of potential HIV carriers, to supplying sufficient evidence which suggest that these retroviruses can mutate into AZT-resistant strains upon opioid treatment even without exposure to AZT.

Our investigation suggests that chronic administration of opioids alters general fundamental aspects of the AIDS viremia. Studies by others using SIVsmm9, isolated from sooty mangabey monkeys (*Cercocebus atys*), to investigate the effect of morphine on susceptibility of rhesus macaques to a "simian analogue of the AIDS virus" have suggested that morphine has a protective role against virus infections (26, 27). AIDS progression in opiate-dependent monkeys was reported to be slower than expected (26). Questions remain as to whether the different outcomes of investigation from different laboratories relied upon the different strains of the viruses used. According to Dr. Donahoe, the strain SIVsmm9, isolated at Yerkes, is a less virulent viral strain than what is commonly used (28). SIVsmm (commonly known as SIVsm) does not cause clinical disease in sooty mangabeys, its natural African host (14, 29). When SIVsmm9 was injected into rhesus macaques, virus could be isolated from monkey PBMC by co-cultivation and the monkeys seroconverted soon after infection (29). Nevertheless, in 50% of the infected monkeys, virus was eliminated and could not be isolated by co-cultivation from the monkey PBMC several months after infection (29). Also, none of the rhesus macaques infected with SIVsmm9 showed clinical signs of disease at any time during the study (29). For these reasons, SIVsmm9 infection of rhesus monkeys contrasts markedly with SIVmac239 infection of rhesus macaques, and the clinical manifestations that result from these two viral infections (SIV239 vs. SIVsmm9) cannot be readily compared for purpose of studying the effect of drug abuse on AIDS progression.

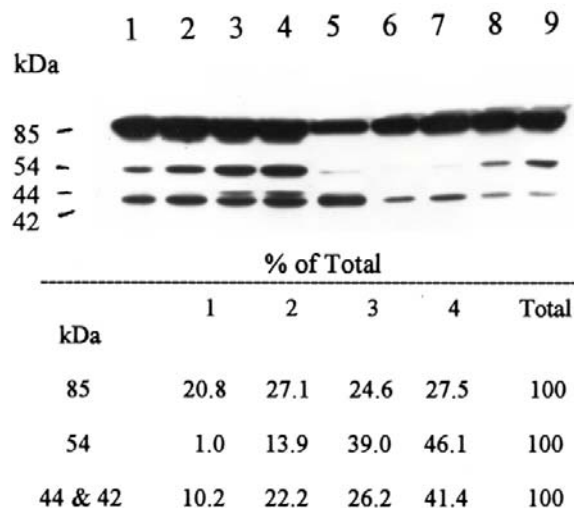
### 4. FINDING OPIOID RECEPTOR TRANSCRIPTS AND PROTEIN IN IMMUNE CELLS

The observations from our animal studies indicating that morphine has a direct effect on immune function prompted us to search for opioid receptors on immune cells. While there is some controversy in the literature (see review, 30), the weight of the evidence suggests such a possibility (30-33). Naltrexone, an opioid antagonist, was found to reverse the analgesic and immunosuppressive effects of morphine in mice (34). Using flow cytometry and fluorescein isothiocyanate (FITC)-labeled naltrexone, opioid binding to rat PBMC was reported (35). At the same time, Lawrence *et al.* (36) identified the binding of kappa opioid receptors on immune cells using high affinity fluorescein-conjugated opioid ligands and indirect immunofluorescence with phycoerythrin fluorophore to amplify the signal. There are also functional studies that suggest the existence of opioid receptors on immune cells. Among these studies are findings that opioid peptides stimulate the cytokine production of murine T cells (37) and morphine suppresses IL-2R expression on rhesus monkey PBMC (38). Still, without evidence showing that immune cells indeed

**Table 1.** Comparison of the lymphocyte *mu* opioid receptor (antagonist binding domain) with corresponding known human brain and placenta opioid receptor sequences

lymph	Y W E N L L K I C V F I F A F I M P V L I	249
hMOR	- - - - - - - - - - - - - - - - - - - -	249
hKOR	W - D L F M - - - - - - - - - - V I - - -	241
hDOR	- - D T V T - - - - - L - - - - V V - I - -	228
lymph	I T V C Y G L M I L R L K S V R M L S G S	270
hMOR	- - - - - - - - - - - - - - - - - - - -	270
hKOR	- I - - - T - - - - - - - - - - L - - - -	262
hDOR	- - - - - - - L - - - R - - - L - - - -	249
lymph	K E K D R N L R R I T R M V L V V V A V F	291
hMOR	- - - - - - - - - - - - - - - - - - - -	291
hKOR	R - - - - - - - - - - L - - - - - - - -	283
hDOR	- - - - - S - - - - - - - - - - G A -	270
lymph	I V C W T P I H I Y V I I K A L V T I P	311
hMOR	- - - - - - - - - - - - - - - - - - - -	311
hKOR	V - - - - - - - - - F I L V E - - G S T S	303
hDOR	V - - - A - - - - - F - - V W T - - D - D R	291
lymph	E T T F Q T V S W H F C I A L G Y T N S C	332
hMOR	- - - - - - - - - - - - - - - - - - - -	332
hKOR	H S - A A L S - Y Y - - - - - - - - - S	324
hDOR	R D P L V V A A L - L - - - - - - A - - S	312
lymph	L N P V L Y A F L D E N F K R C F R E F C	353
hMOR	- - - - - - - - - - - - - - - - - - - -	353
hKOR	- - - I - - - - - - - - - - - - - - - D -	345
hDOR	- - - - - - - - - - - - - - - - - Q L -	333
lymph	I P T S S N I E Q Q N S T R I R Q N T R D	374
hMOR	- - - - - - - - - - - - - - - - - - - -	374
hKOR	F - L K M R M - R - S T S - V - - - V Q	365
hDOR	R K P C G R P D P S S F S - P - E A - A R	354

Lymph human lymphocyte *mu*; hMOR, human brain *mu*; hKOR, Human placenta *kappa*; hDOR, human brain *delta*. "-" indicates amino acids identical to those in lymph. Spaces and gaps have been introduced for alignment



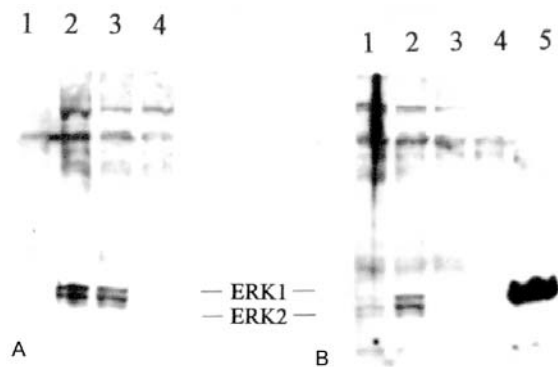
**Figure 1.** Immunoblot analysis using MAP kinase monoclonal antibodies. Anti-pan-ERK from Transduction Laboratories was used. The lanes contained 15  $\mu$ g (lanes 1-4) or 5  $\mu$ g (lanes 6-9) of total protein from human CEM x174 cells treated with H<sub>2</sub>O (lanes 1, 3, 6 and 8) or 10  $\mu$ M morphine sulfate (lanes 2, 4, 7 and 9). Lanes 1, 2, 6 and 7 represent 12 hr post-treatment, and lanes 3, 4, 8 and 9 represent 42 hr post-treatment. Lane 5, human fibroblast cell extract as a positive control. The table at the bottom presents data from densitometry scanning of each of the first four (lanes 1 through 4) protein bands of the gel, expressed as a percentage of the total areas of the four bands.

synthesize opioid receptor mRNA and its corresponding protein, whether cells of the immune system possess opioid receptors in mediating the opiate effect remained speculative. With the availability of the gene sequence of opioid receptor cDNA from brain cells, our laboratory presented evidence, and later supported by others, that opioid receptor genes for all three classes of the receptor, delta, kappa and mu, are actually transcribed and translated in cells of the immune system (39-47). For example, we found that human and non-human primate lymphocytes possess mu in the range of 10<sup>-18</sup> g cDNA/1  $\mu$ g of total RNA (48). The nucleotide and the translated amino acid sequences of the morphine binding domain of the human lymphocyte mu opioid receptor are 100% homologous to the corresponding region of the mu receptor in human brain (Table 1). In addition, it was demonstrated that the amount of mu opioid receptors on lymphocytes increased upon morphine treatment and the increase could be reversed by treating the cells with naloxone (an opioid receptor antagonist) prior to morphine treatment (48). Furthermore, kappa opioid receptor (KOR) of lymphocytes were also shown to be activated in the presence of extracellular morphine or U50,488H, a KOR selective agonist (49). The activation caused an increase in the expression of KOR mRNA, as determined by a quantitative competitive RT-PCR procedure. The observed agonist-induced KOR upregulation could be blocked by treating the cells with either naloxone (a KOR-partially selective antagonist) or nor-Binaltorphimine (a KOR-selective antagonist). Upregulation of lymphocytic KOR by morphine was also evidenced by flow cytometric analysis of phycoerythrin amplification of fluorescein isothiocyanate-conjugated arylacetamide labeling of KOR (49). Although morphine binds primarily to mu-opioid receptors, congruent with the previously reported phenomenon that morphine modulation of immune functions also exists in mu-opioid receptor knockout mice (50), our study suggests that opioids such as morphine may exert its effects through multiple opioid receptor types and that the effects of morphine or endogenous opioids on immune cells could not be simply adduced from the anticipated effects of a synthetic, selective opioid receptor ligand.

It is generally considered that continuous or repeated administration of opioids results in an individual's tolerance, which is characterized as a reduction of response to the same drug. Desensitization (or down regulation) of neuronal opioid receptors occurs such that the effect following continued or subsequent exposure to the same concentration of drug is diminished. Our studies, however, show that unlike that which occurs in neuronal cells, morphine treatment of lymphocytes will increase receptor (both mu and kappa) concentrations (48, 49).

**5. THE SIGNAL TRANSDUCTION OF MORPHINE INVOLVES THE MAP KINASE PATHWAY**

After finding opioid receptors on immune cells, we began to explore the signal transduction pathway morphine uses to modulate immune function after the receptor is activated. We used CEM x174 human lymphocytic cells for our studies and found that addition of morphine to CEM x174 culture media resulted in increased expression of MAP kinase (mitogen-activated protein kinase) cascade proteins inside the cells (Figure 1). Morphine enhanced the cellular levels



**Figure 2.** Immunoblot detection of ERK1 and ERK2 activation in CEM x174 cells using anti-active MAPK polyclonal antibody. (A) A time-dependent phosphorylation. CEM x174 cells were grown in suspension cultures and diluted 1:3 with fresh medium every 3-4 days. 24 hr after dilution, the culture were treated with 10  $\mu$ M morphine sulfate. Cells were withdrawn at 2.5 (lane 1), 9.5 (lane 2), or 19.5 (lane 3) hr for analysis for active MAP kinases by immunoblot detection using anti-active MAPK polyclonal antibody (Promega). The remaining culture was diluted again, and morphine treatment was repeated (to a final concentration of 10  $\mu$ M) for 3 hr before the cells were harvested for analysis (lane 4) of the presence of active MAP kinases. The negative results in lane 4 suggest the importance of the cell growth kinetics for MAPK activation induced by morphine. (B) The naloxone effect. 24 hr after dilution, CEM x174 cells were treated with H<sub>2</sub>O (lane 1), 10  $\mu$ M morphine sulfate (lane 2), 10  $\mu$ M naloxone (lane 3), or 10  $\mu$ M morphine sulfate plus 10  $\mu$ M naloxone (lane 4). The treatment was for 19.5 hr before analysis was made for active MAP kinases. Lane 5, activated human fibroblast cell extract as a positive control. Each gel lane in A or B contained 15  $\mu$ g of total protein.

of ERK1 (44 kDa), ERK2 (42 kDa), a 54 kDa ERK, MEK1 (45 kDa) and MEKK (78 kDa). In particular, a time dependent increase in the activated (Thr and Tyr dually phosphorylated) state of ERK1 and ERK2 was observed (Figure 2). Naloxone, a morphine antagonist, reversed the observed morphine effects, indicating a process mediated by mu opioid receptors (51). These results suggest that MAP kinases are important intermediates in signal transduction pathways initiated by morphine receptors in immune cells. That morphine and other opioids activate MAP kinases has later been demonstrated in human stem cells (52), mice neuronal cells (53), and opioid receptor-transfected cell lines (54).

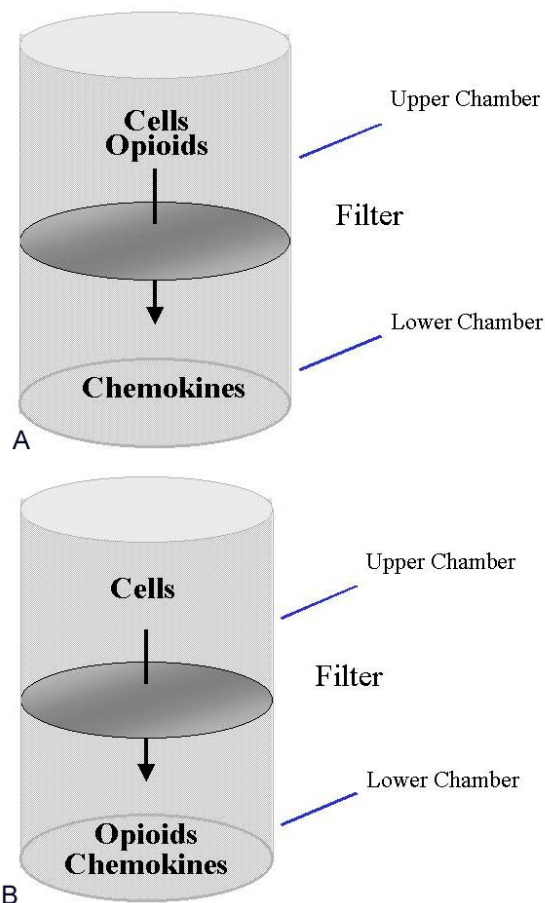
## 6. OPIOIDS SUPPRESS CHEMOKINE-MEDIATED MIGRATION OF NEUTROPHILS AND MONOCYTES

Since chemotaxis and phagocytosis constitute the first line of defense in the immune system in fighting pathogens (e.g. HIV/SIV), and chemokines function mainly as chemoattractants for phagocytic cells, recruiting monocytes and neutrophils to sites of infection, we began

to examine the effect of morphine on the process of chemokine-induced chemotaxis. Using human chemokines IL-8 (interleukin-8), MIP-1 $\beta$  (macrophage inflammatory protein 1 $\beta$ ) and RANTES (regulated upon activation, normal T expressed and secreted) as chemoattractants, we found that all opioids tested had an inhibitory effect on chemokine-induced chemotaxis and the inhibition could be reversed by mu or kappa opioid antagonists (55, 56). To assess the "efficiency" of the inhibition, opioids were either incubated with monkey leukocytes (Figure 3A) or added directly to chemokines without a cell-opioid pre-incubation step (Figure 3B) and the number of cells migrating toward IL-8 (for neutrophils) or RANTES (for monocytes) was scored. Inhibition of chemotaxis by opioids was seen with both assay conditions which indicates the inhibition was mediated instantly by opioid binding to mu and kappa opioid receptors (Figure 4). Opioids did not cause the same inhibitory effect on the chemotactic migration of neutrophils when the complement component C5a or the chemotactic peptide fMLP (N-formyl-MET-LEU-PHE) was used as chemoattractant, showing the specificity of the inhibition (56). These studies suggest that the presence of opioids during SIV infection immediately alters chemokine-mediated immune functions. This may explain why drug abusers seem to be more susceptible to HIV or other viral infections than non-drug users when exposed to a viral challenge. When HIV first enters the body of drug users through perhaps dirty needles, the volume of HIV transferred is very small, almost too small to cause a significant threat. With morphine or other opiates present, however, the first line of defense against this viral pathogen, mainly the chemotactic activities of monocytes and neutrophils which lead to viral engulfing and subsequent stimulation of the cellular and humoral immune response of the immune system, is immediately compromised at the site of infection. Thus, this may be one reason for the high incidence and acceleration of successful HIV infection among drug use populations.

## 7. MORPHINE INDUCES EXPRESSION OF CCR5, A CORECEPTOR FOR HIV/SIV ENTRY

From the above studies, it seems that opioids and chemokines have an intricate relationship that affects the functions of either chemokines or their target cells. Since chemokines attract immune cells with chemokine receptors, we asked the question, "do opioids such as morphine have a role in modulating the expression/activity of chemokine receptors?" Research on several genetically divergent SIV isolates has revealed that most forms of SIV, whether macrophage-tropic or T-cell tropic, syncytium-forming or non-syncytium forming, require chemokine receptor CCR5 for cell entry. We therefore studied the effect of morphine on CCR5 expression in both CEM x174 lymphocytic cells (57) and monkey PBMC (58). It was found that morphine treatment in fact increases CCR5 expression and subsequent SIV production (Figure 5; Table 2). Naloxone, on the other hand, reverses the morphine effect (57). The morphine-induced increase in CCR5 was seen at both the transcriptional level and the protein level, as shown by competitive RT-PCR, FACS, and immunoblot analysis (57, 58). Similar morphine effect was not observed on BOB or



**Figure 3.** A chemotaxis chamber. Migration of cells was from the top well toward the bottom well (which contained chemokines) of the chamber. (A) Opioids were placed in the upper well of the chamber. (B) Opioids were placed in the lower well of the chamber.

BONZO, two other chemokine receptors found on CEM x174 cells but not involved in SIV entry (Figure 5). Likewise, this morphine effect was not observed in CEM x174 infected with SRV (simian retroviruses), which do not depend on CCR5 for cell entry (Table 2).

The phenomenon of elevated CCR5 expression in morphine-treated PBMC (58) correlated with the finding that plasma viremia was increased in morphine-dependent monkeys. In practice, the isolation of HIV or SIV from PBMC of infected humans or animals relies on a cocultivation procedure: PBMC are cultured *in vitro* in the presence of T cells of an immortalized cell line (e.g., Hut 78 or CEM x174); without this cocultivation, the virus load in infected PBMC is below the threshold of detection by conventional assays and unable to sustain continuous replication. In our study, however, it was found that SIV could be isolated from morphine-dependent, SIVmac239-infected animals' PBMC without cocultivation with an immortalized cell line. Conversely, virus titers from PBMC of saline-treated, morphine-naïve SIVmac239-infected animals remained undetectable in the absence of cocultivation (58). These studies further aid in

establishing the unequivocal role of opioids as a risk factor in promoting the propagation of the AIDS virus. The results of our studies and their significance were later confirmed by others who showed that (a) treatment of human astrocyte cultures with morphine enhanced the expression of CCR5 gene (59); (b) morphine enhanced HIV R5 strain infection of macrophages through the upregulation of CCR5 expression (60, 61) and (c) morphine enhanced both CXCR4 and CCR5 expression and subsequently increased both X4 and R5 HIV-1 infection (62).

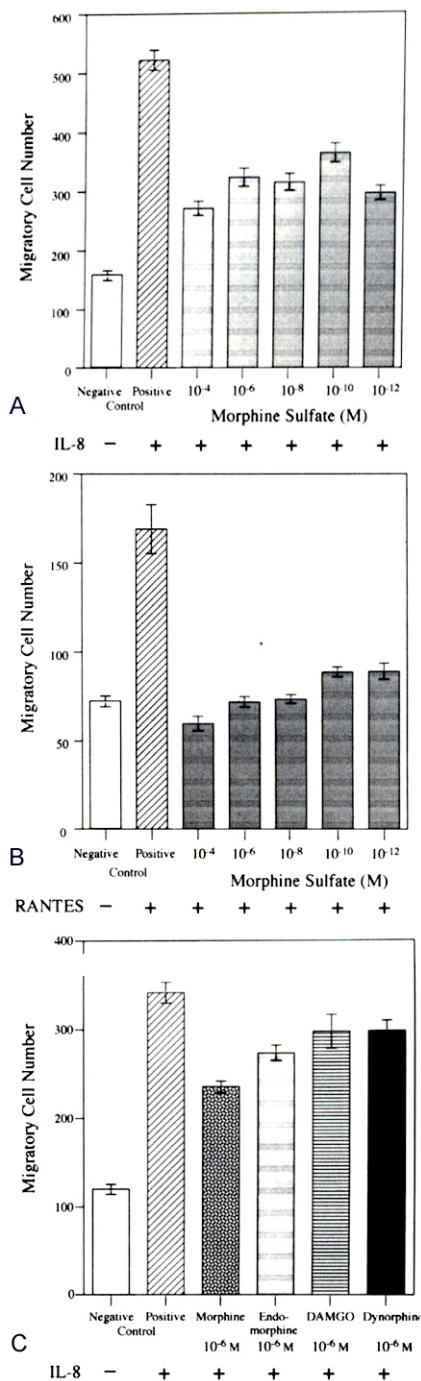
**8. CHEMOKINE RECEPTOR CCR5: POLYMORPHISM AT PROTEIN LEVEL**

Since chemokine receptor CCR5 stands out in our studies - it can be induced by morphine and it serves as an entry receptor for HIV/SIV - we began to analyze the structure and subcellular functions of CCR5. Polymorphisms in CCR5 genes have been implicated in HIV disease progression, resistance or non-progressive infection. For example (63), a 32-bp deletion in the CCR5 coding region (CCR5  $\delta$ 32) protects the CCR5  $\delta$ 32 homozygote from infection and delays disease progression in the CCR5  $\delta$ 32 heterozygote. Multiple CCR5 transcripts and mRNA diversity have also been described. The generation of multiple CCR5 transcripts has consequences for the regulation of CCR5 gene expression. In spite of such findings, multiple forms of CCR5 protein have not been described. In one of our studies (64), we presented evidence to show that two distinct forms of CCR5 protein, 62 kDa and 42 kDa, are present in both human lymphocytic cells and monkey PBMC. The ratio of these two forms of CCR5 protein changes with cell growth. The 62 kDa CCR5 predominates if the electrophoresis sample buffer does not contain reducing agent. However, the 62 kDa and the 42 kDa CCR5 are not inter-convertible. Morphine induces the formation and expression of both forms of CCR5 whereas RANTES, MIP-1 $\alpha$  or MIP-1 $\beta$  inhibits them (64). Localization studies indicated that the 62 kDa CCR5 resides mainly on the cell membrane while the 42 kDa protein resides solely in the cytoplasm (Figure 6, A, B). The notion that the membranes of immune cells possess a unique form of CCR5 was further supported by inclusion of brefeldin A (BFA) in the assay system. BFA is a fungal metabolite which disrupts the structure and function of the Golgi apparatus and has thus been used to monitor protein transport to cell membrane. When we treated cells with BFA and examined membrane proteins up to 36 hours post-BFA treatment we found that BFA treatment gradually reduced the amount of the 62 kDa CCR5 on cell membrane (Figure 6C). Subsequently, we presented evidence to show that the 62 kDa CCR5 (the membrane CCR5) forms an oligomeric complex with opioid receptors on the cell membrane, thereby modulating receptor functions (see "9" below).

**9. THE MEMBRANE CCR5 FORMS AN OLIGOMERIC COMPLEX WITH OPIOID RECEPTORS**

As we described above, activation of opioid receptors by morphine specifically and simultaneously induces the expression of chemokine receptor CCR5, promoting simian AIDS virus entry and subsequent





**Figure 4.** Effect of opioids on chemotaxis when the test opioid was placed in the lower chamber with the chemokines. (A) Effect of various concentrations of morphine on the migration of monkey neutrophils toward IL-8. (B) Effect of various concentrations of morphine on the migration of monkey monocytes toward RANTES. (C) Effect of endomorphine, DAMGO or dynorphin on the migration of monkey neutrophils toward IL-8. Morphine, endomorphine and DAMGO are ligands for mu opioid receptors while dynorphin is a kappa opioid receptor agonist.

replication in immune cells. We began to ask whether a physical association involving opioid receptors and CCR5 exists on the cell membrane. Our studies (65) showed that these two structurally and functionally distinct G-protein-coupled receptors are indeed in close proximity to one another and in fact form an oligomeric complex in the cell membrane so that the activation of one triggers the activity of the other. Both human CEM x174 and monkey lymphocytes were used in our studies and yielded similar results. Immunoprecipitation experiments in the study showed that membrane CCR5 (62 kDa), but neither CD4+ receptor nor Na<sup>+</sup>/H<sup>+</sup> exchanger, coprecipitates with all three subtypes (mu, delta and kappa) of opioid receptors (Figure 7). A single protein band immunoreactive with antibodies against both the CCR5 receptor and the three opioid receptor subtypes was identified after electrophoresis on non-denaturing polyacrylamide gels (Figure 8). Chemical cross-linking experiments using glutaraldehyde or BS<sup>3</sup> indicate that these receptors are closely situated on the surface of the cell membrane with an intermolecular distance of less than 11.4 Å (65). Functional studies revealed that treatment of cells with a combination of morphine, an agonist for mu, and MIP-1β, a ligand for CCR5, suppresses the inhibitory effect of MIP-1β and increases the stimulatory effect of morphine on CCR5 expression (65). These results suggest that oligomerization of chemokine receptor CCR5 with opioid receptors on the cell membrane of human or monkey lymphocytes may indeed modulate receptor functions. This study may explain at a cellular level the higher prevalence of HIV-1 infection among IV drug users.

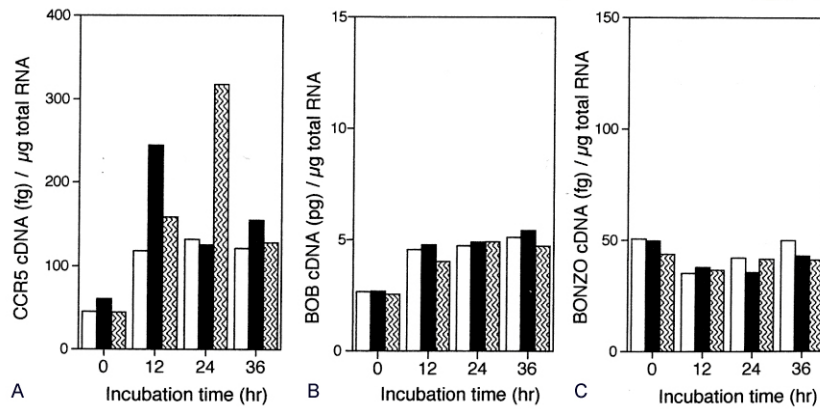
### 10. MORPHINE PROTECTS HUMAN LYMPHOCYTES AND MONKEY PBMC FROM APOPTOSIS

As described in section “5” above, the signal transduction of morphine may involve a MAPK pathway. Are there any other pathways that morphine uses to effect its immuno-modulatory action? To answer this question, we used a reverse transcriptase-polymerase chain reaction-based differential display technique (RT-PCR DD) coupled with a hybridization procedure to compare gene expression patterns of morphine-treated cells with those of control cells. Using this technique, we identified novel specific genes, mRNAs and proteins that we had no previous knowledge of and whose expression in immune cells was regulated by the presence of morphine. Through assiduous screening efforts, ten positive gene segments including two morphine down-regulated genes and eight morphine upregulated genes were identified in CEM x174 lymphocytes (66). Among these genes, two apoptosis/cell growth-related genes (PNAS-133/Krüppel-like factor 7) were revealed. PNAS-133 (apoptosis) gene was found to be down-regulated by morphine whereas Krüppel-like factor 7 (KLF7), a zinc finger growth-stimulating transcription factor, was up-regulated by morphine. The up-regulation of KLF7 by morphine was noted to be present at both the transcriptional and translational levels in a naloxone-reversible manner (66). Cell growth was promoted by morphine up to 72 hours post-treatment (66). These findings suggest a unique role for morphine in the

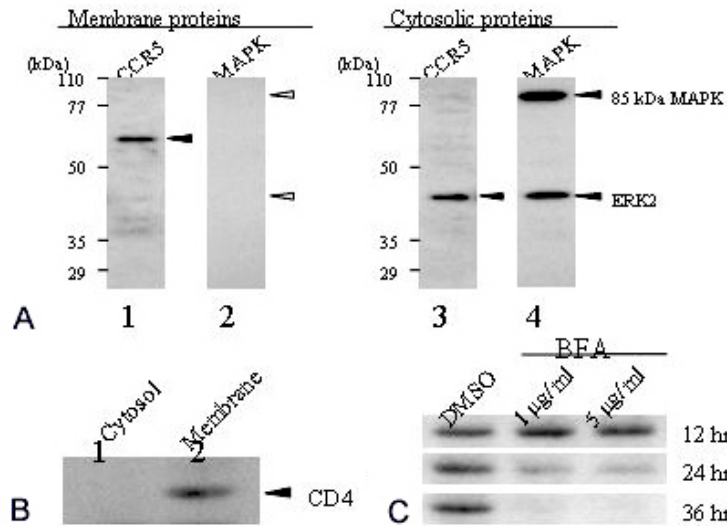
**Table 2.** Morphine enhances the propagation of SIVmac239, but not SRV-1. The reverse transcriptase activity was assayed using standard [<sup>32</sup>P]dTTP incorporation. The mean cpm for the cells alone was 167. The mean cpm for the scintillation fluid was 20. Data were reproducible in three independent 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
SIVmac239 <sup>1</sup>	266 (1.0) <sup>2</sup>	568 (1.0)	603 (2.3)	2310 (4.1)	699 (2.6)	3674 (6.5)	1168 (4.4)	2885 (5.1)
SRV-1 <sup>3</sup>	256 (1.0)	454 (1.0)	225 (0.9)	389 (0.9)	228 (0.9)	444 (1.0)	221 (0.9)	338 (0.7)

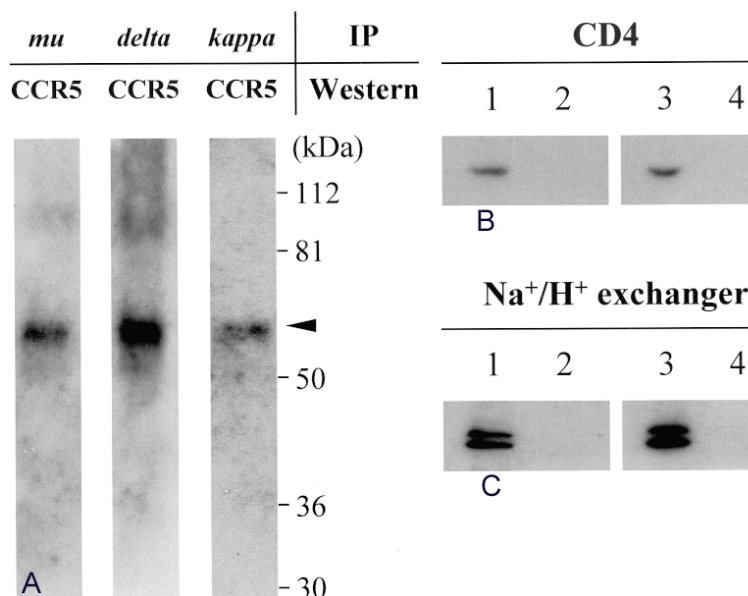
<sup>1</sup> The *p* value is ≤0.0001 by analysis of variance, considered highly significant, <sup>2</sup> CPM ratio between sample and no-morphine control, <sup>3</sup> The *p* value is >0.1 by analysis of variance, considered not significant.



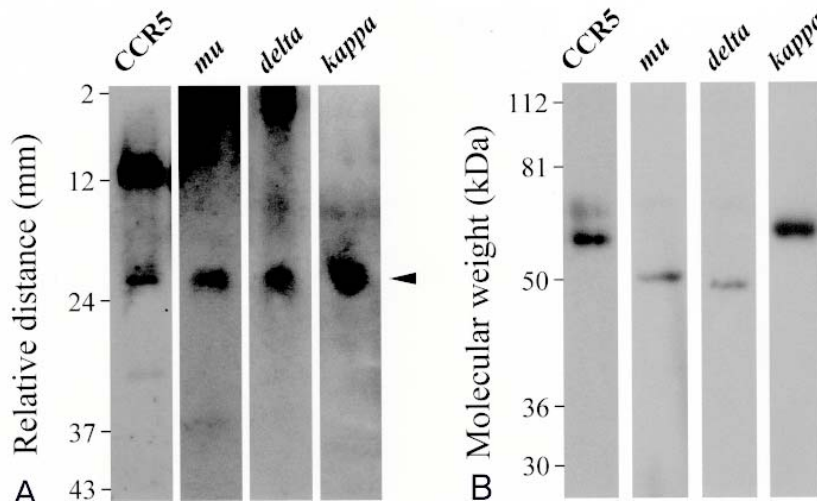
**Figure 5.** Morphine induces the expression of CCR5, but not BOB or BONZO. The amount of (a) CCR5, (b) BOB or (c) BONZO expressed in CEM x174 cells was determined by competitive RT-PCR after treatment of the cells with 10 μM (solid column) or 10 nM (wavy column) morphine sulfate for the indicated time. Control (clear column), H<sub>2</sub>O-treated cells. The data, represented by the amount of cDNA synthesized from 1 μg of the initial RNA sample, were reproducible in at least four independent experiments.



**Figure 6.** Cellular localization of the 42 kDa and the 62 kDa CCR5. (A) Membrane and cytosolic CCR5. Membrane and cytosolic proteins of CEM x174 cells were prepared separately and a 10-μg protein sample was subjected to 10 % SDS/PAGE followed by Western blot analysis using anti-CCR5-NT (lanes 1 & 3) or anti-MAPK (lanes 2 & 4) as immunodetection probe. Closed arrows, immunoreactive signals of CCR5 (lanes 1 & 3), 85 kDa MAPK (lane 4) or ERK2 (lane 4). Open arrows, the expected positions of the 85 kDa MAPK and ERK2. The positions of molecular size markers are indicated on the left. (B) Western blot analysis of cytosolic (lane 1) or membrane (lane 2) CD4 glycoprotein using anti-CD4 antibody. (C) Effect of BFA treatment on the transport of CCR5 to cell membrane. CEM x174 cells were treated with DMSO (as control), 1 μg/ml BFA or 5 μg/ml BFA for the indicated times. Membrane proteins (10 μg) were isolated from the treated cells and subjected to 10% SDS/PAGE followed by Western blot analysis using anti-CCR5-NT. These results represent reproducible data from three independent experiments.

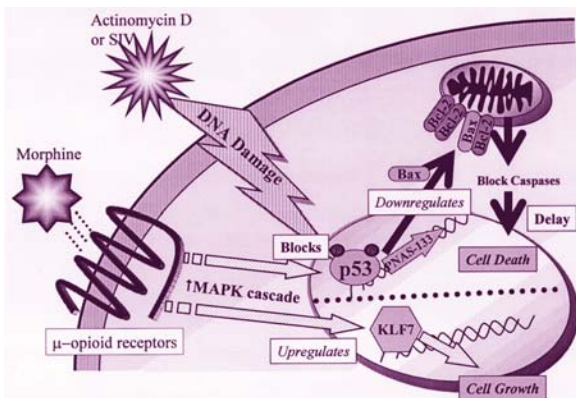


**Figure 7.** Co-immunoprecipitation of CEM x174 membrane protein with opioid receptors. (A) Detection by anti-CCR5 antibodies. Total protein from CEM x174 ( $3 \times 10^7$  cells) was isolated and immunoprecipitated with antibodies against mu (lane 1), delta (lane 2) or kappa (lane 3). The immunoprecipitated materials were subjected to 10% SDS/PAGE and blotted onto a PVDF membrane. Immunodetection was performed on each blot using anti-CCR5-NT as a probe. The arrow indicates the position of a 62 kDa CCR5 band. The positions of SDS/PAGE molecular weight size markers are indicated on the right. (B) Detection by anti-CD4 antibodies. Total protein (20  $\mu$ g, lane 1 or 3) or protein (same amount as in "A") immunoprecipitated with antibodies against mu (lane 2) or against delta (lane 4) was subjected to SDS/PAGE and analyzed using anti-CD4 antibodies as immuno-probes. (C) Detection by anti- $\text{Na}^+/\text{H}^+$  exchanger antibodies. Total protein (20  $\mu$ g, lane 1 or 3) or protein (same amount as in "A") immunoprecipitated with antibodies against mu (lane 2) or against delta (lane 4) was subjected to SDS/PAGE and analyzed using anti- $\text{Na}^+/\text{H}^+$  exchanger antibodies as immuno-probes. The data presented were reproducible in three independent experiments. IP, immunoprecipitation.



**Figure 8.** Detection of a receptor complex by non-denaturing PAGE. (A) Western blot analysis on non-denaturing PAGE showing a single polypeptide band. CEM x174 protein (75  $\mu$ g) was loaded onto a 6% non-denaturing PAGE and electro-transferred onto a PVDF membrane. Western blot analyses with antibodies against CCR5 (lane 1), mu (lane 2), delta (lane 3) or kappa (lane 4) opioid receptors were performed, showing an identical immunoreactive band (arrow). The numbers on the left indicate the relative migration of molecular weight size markers during electrophoresis (millimeters). (B) Extraction and analysis of the immunoreactive protein identified in the non-denaturing gel showing the single band in (A) contains CCR5, mu, delta and kappa. The immunoreactive band (shown by arrow) in Figure 8A was excised and protein extracted. The protein extract was subjected to 10% SDS/PAGE and western blot analysis with antibodies against CCR5 (lane 1), mu (lane 2), delta (lane 3) or kappa (lane 4) opioid receptors. The numbers on the left indicate the positions of SDS/PAGE molecular weight size markers. These results represent reproducible data from at least three independent experiments.





**Figure 9.** A hypothetical model for the role of morphine in SIV-induced cell apoptosis.

regulation of cell proliferation and apoptosis in lymphocytes. We further defined the role of morphine in the cell's apoptotic process by triggering lymphocytes to undergo apoptosis in response to an extracellular signal (e.g. actinomycin D, HIV/SIV, etc.). The addition of morphine was found to suppress the expression of active (Ser<sup>15</sup> phosphorylated) p53 (67), possibly through activation of the MAPK pathway (68). Blockade of p53 phosphorylation/activation was noted to induce downregulation of Bax (an apoptosis-promoter) and upregulation of Bcl-2 (an apoptosis -inhibitor), thus increasing the ratio of Bcl-2 to Bax in mitochondria (67). Hence, the death signaling, possibly through the caspase (also known as CPP32) pathway we previously identified in CEM x174 cells (69), may transiently be suppressed by changes in the ratio of Bcl-2/Bax in mitochondria, leading to increased cell growth and an arrest of apoptosis (67). Our studies have therefore provided a mechanism (as illustrated in Figure 9) to account for the delayed apoptosis and prolonged SIV infection in morphine-treated cells that we had previously observed in both CEM x174 lymphocytic cells (18) and monkey PBMC (18, 58).

Our studies contrast with others who suggest that morphine may promote apoptosis in immune cells (70-72). Morphine was reported to promote T cell apoptosis by inducing the expression of the protein Fas (also known as CD95), a receptor on the cell surface that triggers cell suicide when it binds to its ligand, FasL (71). Because Fas-mediated apoptosis requires FasL, which is expressed by only a very limited set of cells, only a small portion of cells undergo opioid-induced apoptosis under non-pathological conditions (71). This may explain why in the absence of extracellular stimulants (e.g. actinomycin D, SIV, HIV), apoptosis was not detected in our system even in the presence of morphine (67). In the presence of extracellular stimulants, however, morphine may protect cells from apoptotic death, allowing the virus to replicate, persist and survive after infection. It thus appears that morphine may exert diverse mechanisms to promote HIV/SIV infection: on the one hand, morphine may increase entry of HIV/SIV to cells by increasing the entry receptor CCR5 on the surface of the cell membrane (57, 58); on the other hand, after the virus has entered the cells, morphine may delay

apoptosis, augmenting viral replication. This, again, may explain the increased incidence of HIV infection among illicit drug users.

## 11. PERSPECTIVE

A variety of events occur following the establishment of opioid dependency. Our studies using the simian AIDS model presented evidence that opioid administration enhances the pathogenesis of AIDS. It thus appears that laboratory investigation has confirmed the results of epidemiological studies which indicate that opioids have an unequivocal role in causing immune dysfunction and exacerbating the AIDS epidemic. The identification of opioid receptors in immune cells and the discovery of the unique relationship between opioid receptors and chemokine receptor CCR5 are both important and intriguing. Understanding the interactions between opioid receptors and chemokine receptor CCR5 may provide new insights in the development of potential therapeutic targets for the treatment of AIDS among drug users.

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**Key Words:** Opioids, rhesus monkeys, SIV, immune functions, SAIDS, opioid receptors, chemokine receptor CCR5, Review

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