

## Immunoregulatory Effects of Morphine on Human Lymphocytes

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**It is now well established that parenteral drug abuse is a significant risk factor for contracting human immunodeficiency virus type 1 (HIV-1) infection and subsequently developing AIDS. Earlier studies have shown that morphine can modulate various immune responses and therefore support the premise that morphine is a cofactor in susceptibility to and progression of HIV infection. Dysregulation of interferon (IFN) production, nonspecific apoptosis of T cells, and the immune response to soluble HIV gene products have been associated with potential mechanisms of pathogenesis in HIV disease. The present study was undertaken to examine the immunomodulatory role of morphine on HIV protein-induced lymphocyte proliferative responses, Sendai and Newcastle disease virus-induced alpha IFN (IFN- $\alpha$ ) and IFN- $\beta$  production by lymphocytes and fibroblast cells, respectively, and induction of apoptosis of normal lymphocytes *in vitro*. Our results demonstrate that HIV protein-induced human lymphocyte proliferative responses were significantly inhibited by morphine in a dose-dependent manner. Furthermore, morphine significantly inhibited both IFN- $\alpha$  and IFN- $\beta$  production by normal lymphocytes and fibroblasts but induced apoptosis of normal lymphocytes. Inhibition of IFN- $\alpha$  production by morphine could be reversed by the opiate receptor antagonist naloxone. This suggests that the immunomodulatory effects of morphine are mediated through the opioid receptor. These studies support a role of morphine as a cofactor in the pathogenesis of HIV infection and describe some of the possible pathologic mechanisms which underlie the immunoregulatory effects of morphine.**

Although human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS, there is considerable evidence that cofactors also play a significant role in its pathogenesis. This "cofactor hypothesis" is supported by a number of clinical studies, animal models, and *in vitro* investigations (4, 5, 17, 30, 40, 59). It is well established that parenteral drug abuse is a significant risk factor for contracting HIV-1 infection and subsequently developing AIDS (9, 15, 16, 27). Although morphine and other opioids have been used to alleviate pain and discomfort in various medical situations, abuse of opioid substances has been linked to significant dysregulation of cell-mediated, humoral, and nonspecific immunities (5, 18, 40, 42, 59, 72). These immunosuppressive properties of opioids may potentiate the risk of infection with HIV and the subsequent development of AIDS (4, 17, 58). Previous studies have shown that morphine inhibits T-cell proliferation (4) and alters T- and B-cell responses to various mitogens (6) and alloantigens (39). A number of regulatory effects of morphine and related opioids on the production of various cytokines and on the activation of HIV growth in culture systems and animal models have been reported (8, 11, 28, 67, 69-71). Geber et al. (24, 25) showed that morphine significantly lowered circulating serum interferon levels in a mouse model. However, the effects of morphine on alpha interferon (IFN- $\alpha$ ) and IFN- $\beta$  production by normal lymphocytes has not been described previously.

The selective depletion of CD4<sup>+</sup> lymphocytes is considered to be the major immunologic abnormality in AIDS (21). Since HIV has a specific tropism for CD4<sup>+</sup> cells, active replication of virus leads to the death of infected cells (35). Apoptosis, or programmed cell death, is a physiological mechanism of self-destruction occurring during embryogenesis, hormone-depen-

dent tissue atrophy, and thymic selection. It is assumed to play a pivotal role in normal tissue homeostasis. Recently, it was suggested that apoptosis may be a principal mechanism for the loss of CD4<sup>+</sup> cells during HIV infections (3, 26, 36, 38). We hypothesize that HIV or viral gene products derived therefrom may not be solely responsible for the depletion of CD4<sup>+</sup> cells and that cofactors such as morphine or related opioids which foster HIV replication may potentiate apoptosis of these cells.

Earlier, we showed that intravenous drug users manifest low natural killer (NK) cell activity compared to the NK cell activities of sex- and age-matched control subjects (45). Further studies on the immunological effects of ethanol, another recreational drug, demonstrated that it selectively suppressed HIV protein-induced lymphocyte proliferation (48) and NK cell activity (49). On the basis of these observations, the current investigations were undertaken to determine the biological mechanisms underlying the role of morphine as a cofactor in the pathogenesis of AIDS.

### MATERIALS AND METHODS

**Blood donors.** Peripheral blood from healthy HIV-seronegative adults was drawn into a syringe containing heparin (20 U/ml). All donors were apprised of this study, and informed consents, consistent with the policies of the National Institutes of Health, the State University of New York at Buffalo, and Buffalo General Hospital, were obtained. Healthy donors ages 20 to 40 years who were not taking nonsteroidal anti-inflammatory agents, corticosteroids, or any drugs of abuse at the time of the study were selected for this investigation. Buffy coat samples from healthy blood donors obtained from the Buffalo Chapter of the American Red Cross were also used in the present investigation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood or buffy coat samples by a method modified from that of Boyum (7). Blood was diluted with an equal volume of normal saline and was centrifuged at 400  $\times$  g for 30 min at 18°C. The mononuclear cell band was harvested, washed three times with saline, and resuspended in RPMI 1640 medium containing 25 mM HEPES buffer supplemented with 5% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), 80  $\mu$ g of gentamicin (Schering Corp., Kenilworth, N.J.) per ml, and 300  $\mu$ g of fresh glutamine (complete medium) per ml.

**HIV protein.** The HIV-1 Env-Gag protein is a recombinant fusion product with conserved and antigenic epitopes from the *env* and *gag* regions of the HIV.

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The sequence of the expressed Env-Gag fusion protein used in these studies represents the amino acid sequences from residues 560 to 639 for Env and residues 87 to 276 for Gag, totaling 270 amino acids. Twenty-one extra amino acids are encoded by a vector sequence. The Env-Gag protein is expressed in *Escherichia coli* as a single polypeptide without any evidence of premature termination or internal initiation. Env-Gag was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10 to 20% gradient gel under reducing conditions and represents a protein. Protein was further analyzed by high-pressure liquid chromatography on a TSK 4000 column (Tosohaas, Montgomeryville, Pa.) in sodium phosphate buffer (pH 6.8) in the presence of 10 mM dithiothreitol and produced a single peak. The protein content was ~0.27 mg/ml on the basis of the absorbance at 280 nm, with  $E/mg = 0.487$  (where  $E$  is the extinction coefficient). The protein was found to be highly conserved and antigenic and did not contain any endotoxin as a contaminant. In our experiment Env-Gag was used at concentrations of 1, 10, and 50 ng/ml. We chose to use the Env-Gag protein in these studies for several reasons. First, it is an excellent diagnostic screening reagent, reacting with 100% of HIV-positive sera, as described previously (64). Furthermore, we have demonstrated that Env-Gag is a potent polyclonal activator of T- and B-lymphocyte functions (46).

**Lymphocyte proliferation assay.** PBMCs ( $2 \times 10^5$ ) were cultured in complete medium in sterile U-bottom microtiter plates (Dynatech, Alexandria, Va.). Cultures received HIV-1 Env-Gag peptides at concentrations of 1, 10, and 50 ng/ml and/or morphine at  $10^{-7}$ ,  $10^{-9}$ ,  $10^{-11}$ ,  $10^{-13}$ , and  $10^{-15}$  M concentrations and were incubated for 48 to 120 h at 37°C in a 5% CO<sub>2</sub> atmosphere. [<sup>3</sup>H]thymidine (1 mCi; 1 Ci = 37 GBq) was added to each well during the last 24 h of incubation. The cells were collected by an automated cell harvester, and the incorporation of [<sup>3</sup>H]thymidine into the DNA was determined with a Beckman model LS-700 scintillation counter. Results are expressed as counts per minute per  $2 \times 10^5$  cells. As a control, the T-cell mitogen concanavalin A (ConA; 5 µg/ml; Sigma Chemical Co., St. Louis, Mo.) was also used. A sterile, preservative-free morphine sulfate (Astra Pharmaceuticals, Westborough, Mass.) stock solution was diluted in RPMI 1640 complete medium and was added to the cultures at the desired final concentrations.

**Cells and viruses.** Primary human foreskin fibroblast (BG-9) cells and African green monkey kidney (CV-1) cells were maintained in minimal essential medium containing nonessential amino acids, 10% heat-inactivated fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Cell cultures were maintained in a humidified incubator at 37°C and a 5% CO<sub>2</sub> atmosphere. Sendai virus (Cantell's strain) was grown in 9-day-old embryonated eggs and was titrated for its hemagglutinin activity by using chicken erythrocytes. Newcastle disease virus (NDV) was grown in embryonated eggs and was quantitated by plaque assay on CV-1 cells. Vesicular stomatitis virus (VSV) was grown and quantitated by plaque assay on CV-1 cells. BG-9 cells were used for IFN antiviral assays.

**IFN production.** PBMCs ( $3 \times 10^6$ /ml in RPMI 1640 complete medium) and confluent monolayers of BG-9 cells were exposed to a range of morphine concentrations ( $10^{-5}$  to  $10^{-15}$  M) for 4 h. Cells were washed twice with warm (37°C) medium. The PBMCs were infected with 150 PFU of Sendai virus for IFN-α production, and BG-9 cells were infected with 20 PFU of NDV for IFN-β production. Twenty-four hours after virus infection, the supernatant from respective cultures were collected, first, by low-speed centrifugation ( $400 \times g$  for 10 min) to remove cellular debris, followed by high-speed centrifugation ( $100,000 \times g$  for 3 h) to remove all traces of Sendai virus (10). The antiviral activity of IFN in the supernatant was determined as described previously (10).

**IFN antiviral assays.** The antiviral activities of both IFN-α and IFN-β were assayed with human fibroblast cells by the dye uptake method (10) by using VSV as a challenge virus. Briefly, confluent monolayers of human fibroblast cells in 96-well tissue culture trays were exposed to serial twofold dilutions of IFN preparations. Twenty-four hours later, the monolayers were washed once with warm medium and were then challenged with VSV. When the cells in the control wells were completely lysed (approximately 36 h), the trays were stained with neutral red dye (40 mg/ml). The dye taken up by the residual IFN-protected cells was extracted with a solution containing 50% absolute ethanol, 45% distilled water, and 1% glacial acetic acid and was quantitated spectrophotometrically at 540 nm. All titers were expressed as international reference units. Reference standard IFN was kindly provided by the National Research Resource Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

**DNA extraction.** For apoptosis studies, DNA was extracted by the method of Collins et al. (14) from 0-, 24-, and 60-h-old lymphocyte cultures (complete medium) treated with different concentrations of morphine. Briefly, the cells were pelleted by centrifugation and the supernatant was removed and labeled the S fraction. TENS lysis buffer (0.75 ml; 10 mM Tris, 0.1 EDTA, 150 mM NaCl, and 1% sodium dodecyl sulfate [pH 8.0]) was added to the pellet, and the tube was vortexed and centrifuged at 13,000 rpm for 15 min. The resultant supernatant was placed in a clean tube and labeled fraction T (top). TENS (0.75 ml) was added to the pellet, and the pellet was labeled the B (bottom) fraction. To each tube (S, T, and B fractions), proteinase K (250 µg/ml) and RNase (5 µg/ml) were added, and the tubes were incubated overnight at 37°C. After incubation, 0.75 ml of phenol-chloroform (equal volumes) was added to each tube, and the tubes were vortexed and then centrifuged at 13,000 rpm in a Beckman model J2-21M centrifuge. The top layer of each tube was removed and placed in a clean tube without disturbing the interface, and an equal volume of chloroform was added and extracted as described above. DNA was precipitated from the aqueous layer

TABLE 1. HIV-1 Env-Gag-induced lymphocyte proliferative response<sup>a</sup>

Protein (concn)	cpm	P value
Cell control	1263 ± 250	
Env-Gag (1 ng/ml)	7,421 ± 1,010	<0.0005
Env-Gag (10 ng/ml)	12,725 ± 3,210	<0.0001
Env-Gag (50 ng/ml)	13,740 ± 2,320	<0.0001
ConA (5 µg/ml)	38,258 ± 4,785	

<sup>a</sup> Total peripheral blood mononuclear leukocytes (PBMCs) ( $2 \times 10^5$  cells) were cultured for 48 h with 1 to 50 ng of Env-Gag per ml, and proliferative responses were measured as a function of the incorporation of [<sup>3</sup>H]thymidine. Values represent the mean ± standard deviations of three separate experiments performed in triplicate. ConA was used as a positive control.

by adding 1/10 volume of sodium acetate, vortexing, adding 2.5 volumes of ethanol, vortexing, and incubating for at least 30 min at -70°C. The DNA was pelleted by centrifugation at 13,000 rpm for 5 min in a Beckman model J2-21M centrifuge. The supernatant was removed, and the pellet was washed with 0.4 ml of 70% ethanol, resuspended, incubated for 10 min at 70°C, and spun at 13,000 rpm for 5 min. The wash step was repeated twice. After the last wash, the supernatant was poured off, the tubes were inverted for 10 to 15 min on absorbent paper, and the inside rim of the tube was dried with a cotton-tipped applicator. After 1 h of air drying, the pellet was resuspended in TE (Tris-EDTA) buffer, and the DNA was stored at -20°C until it was analyzed by agarose gel electrophoresis.

**Agarose gel electrophoresis for DNA laddering.** DNA was analyzed for apoptosis by running samples on an agarose gel and looking for the presence of the characteristic ladder pattern which indicates endonuclease activity, the hallmark of apoptosis. Briefly, 5-µg samples of the T fraction described above were loaded onto a 1.8% agarose gel containing ethidium bromide. The gel was run at 90 V for 3 h and was photographed with a Polaroid camera by using UV light to visualize the DNA bands. A low-molecular-weight DNA standard (ϕX174/HaeIII; Promega) was run along with the experimental samples to aid in determining the sizes of the observed fragments. Fractions S and B did not demonstrate fragmented DNA, in contrast to fraction T, which did.

## RESULTS

**Morphine suppresses lymphocyte proliferative responses to HIV-1 envelope protein.** Morphine and related opioids may act as cofactors in susceptibility to HIV-1 infections and progression to clinical AIDS by reducing the immune responses of the infected host to HIV-1 infection, potentially enhancing disease progression to clinical AIDS (57, 59). Our previous studies demonstrated that alcohol inhibits cellular immune responses in AIDS patients (47, 48). In the experiments described here, we investigated the effects of morphine on the proliferative responses of PBMCs to the HIV-1 Env-Gag peptide. The data presented in Table 1 demonstrate a dose-response effect of Env-Gag on inducing the proliferation of normal PBMCs. Env-Gag at 1, 10, and 50 ng/ml caused the optimum proliferative response compared to that for the control culture. As positive controls, PBMCs stimulated with 5 µg of ConA per ml showed substantial proliferative responses compared to those of the control cultures. The data presented in Table 2 demonstrate the effect of morphine on the Env-Gag-induced lymphocyte proliferative response. PBMCs preincubated with Env-Gag (10 ng/ml) showed significant proliferation ( $P < 0.0001$ ), as determined by [<sup>3</sup>H]thymidine incorporation, compared to that for the control cultures. However, Env-Gag-induced proliferation was significantly suppressed by morphine in a dose-dependent manner. Morphine at  $10^{-17}$  or lower concentrations produced no inhibitory effects on Env-Gag-induced lymphocyte proliferative responses (data not shown). As controls, PBMCs stimulated with 5 µg of ConA per ml showed substantial proliferative responses, and cells treated with different concentrations of morphine alone also produced proliferative responses similar to those of control cultures (data not shown).

TABLE 2. Effect of morphine on HIV-1 Env-Gag-induced lymphocyte proliferative response<sup>a</sup>

Protein (concn)	cpm	P value
Cell control	1,287 ± 33	
Env-Gag (10 ng/ml)	12,573 ± 3,309	<0.0001
Env-Gag + morphine (10 <sup>-7</sup> M)	5,074 ± 2,219	<0.0009
Env-Gag + morphine (10 <sup>-9</sup> M)	5,936 ± 2,285	<0.002
Env-Gag + morphine (10 <sup>-11</sup> M)	5,975 ± 2,621	<0.002
Env-Gag + morphine (10 <sup>-13</sup> M)	8,336 ± 3,566	<0.07
Env-Gag + morphine (10 <sup>-15</sup> M)	8,616 ± 3,441	<0.07
ConA (5 μg/ml)	42,768 ± 7,890	

<sup>a</sup> Total peripheral blood mononuclear leukocyte (PBMCs) (2 × 10<sup>6</sup> cells) were cultured for 48 h with 10 ng of Env-Gag per ml alone or with different concentrations of morphine, and proliferative responses were measured as a function of incorporation of [<sup>3</sup>H]thymidine. Values represent the means ± standard deviations of three separate experiments performed in triplicate. Different concentrations of morphine were added to the control culture to examine whether morphine affects the background or baseline proliferative response without Env-Gag peptide, and the results indicated that morphine does not significantly affect the baseline response (data not shown).

**Morphine suppresses IFN-α and IFN-β production.** Dysregulation of the production and activity of IFN has been reported in subjects with HIV infections (43, 62). Previous studies also demonstrate that morphine modulates IFN production by lymphocytes (37). Because of the proposed relationship between opioid-associated dysregulation of IFN production and HIV disease progression, we investigated the effects of morphine on IFN-α and IFN-β production by PBMCs and fibroblasts, respectively. The data in Table 3 indicate that normal PBMCs infected with 150 hemagglutination units of Sendai virus alone produced a mean value of 10,500 IU of IFN-α. Morphine at different concentrations produced a bimodal inhibition curve of Sendai virus-induced IFN-α production. Morphine at 10<sup>-17</sup> M or lower concentrations produced no significant inhibition of IFN-α production by lymphocytes (data not shown). Control fibroblast cultures infected with NDV alone produced a substantial level of IFN-β, and morphine at different concentrations (10<sup>-10</sup> to 10<sup>-15</sup> M) significantly inhibited IFN-β production. However, morphine concentrations of 10<sup>-17</sup> M or lower were not examined for their

TABLE 3. Effect of morphine on human IFN-α and IFN-β production<sup>a</sup>

Morphine concn (M) <sup>b</sup>	IFN-α titer <sup>c</sup>	% Inhibition by IFN-α	IFN-β titer <sup>d</sup>	% Inhibition by IFN-β
0	10,500		1,530	
10 <sup>-5</sup>	4,800	54	450	72
10 <sup>-7</sup>	750	93	80	95
10 <sup>-9</sup>	1,100	90	275	82
10 <sup>-11</sup>	1,400	87	ND <sup>e</sup>	
10 <sup>-13</sup>	2,500	76	175	89
10 <sup>-15</sup>	4,000	62	95	94

<sup>a</sup> Results are presented as mean of duplicate determinations from a representative experiment, and four other experiments produced similar results. IFN antiviral activity was determined on human foreskin fibroblast cells by the dye uptake method by using VSV as a challenge virus.

<sup>b</sup> Both PBMCs and fibroblasts were exposed to a range of morphine concentrations, as indicated, for 4 h. Cells were washed with warm medium and infected with appropriate viruses for IFN-α or IFN-β induction. Control cultures which did not receive morphine were treated similarly.

<sup>c</sup> PBMCs isolated from normal healthy individuals were exposed to 150 hemagglutination units of Sendai virus for IFN-α production.

<sup>d</sup> Primary human fibroblast cells were infected with 20 PFU of NDV for IFN-β production.

<sup>e</sup> ND, not determined.

TABLE 4. Blocking effects of the opiate antagonist naloxone on morphine-induced IFN-α suppression<sup>a</sup>

Naloxone concn (M)	% Inhibition <sup>b</sup>
0	83.0
10 <sup>-7</sup>	11.5
10 <sup>-11</sup>	12.5
10 <sup>-17</sup>	24.8

<sup>a</sup> PBMCs (3 × 10<sup>6</sup>/ml) isolated from healthy individuals were exposed to naloxone at 10<sup>-7</sup>, 10<sup>-11</sup>, and 10<sup>-17</sup> M concentrations and incubated for 4 h. After that, morphine (10<sup>-7</sup> M) was added to the culture, and the culture was incubated again for an additional 4 h. The cultures were washed to remove both morphine and antagonist and were exposed to 150 hemagglutination units of Sendai virus to induce IFN-α production. After 24 h, the culture supernatants were removed and the interferon antiviral activity was determined on human foreskin fibroblast cells by the dye uptake method by using VSV as a challenge virus.

<sup>b</sup> Results are expressed as mean percent inhibition (blocking effect) of morphine-induced IFN-α suppression by naloxone, calculated on the basis of the results for the control culture, which received only morphine. Results are from a representative experiment performed in duplicate, and two other experiments produced similar results.

ability to modulate IFN-β production. To examine whether morphine-induced regulatory effects are opioid receptor mediated, we examined whether the μ-receptor antagonist naloxone could block the morphine-mediated suppression of IFN-α production by PBMCs (Table 4). PBMCs treated with morphine alone at 10<sup>-7</sup> M produced significant inhibition of IFN-α production compared to that for a control culture, and this inhibition was significantly reduced by naloxone in a dose-dependent manner.

**Morphine induces apoptosis of lymphocytes.** We earlier demonstrated that the HIV-1 envelope protein gp120 and cortisol at concentrations that did not induce apoptosis of PBMCs when they were used individually induced significant apoptosis of PBMCs when they were used in combination (51). In this investigation, we examined whether morphine could induce nonphysiologic apoptosis of normal PBMCs in vitro. The data presented in Fig. 1 indicate that PBMCs cultured with morphine at concentrations of 10<sup>-7</sup> M (lane B) and 10<sup>-8</sup> M (lane C) produced significant DNA fragmentation compared to PBMCs in untreated control cultures, which did not show any sign of DNA fragmentation (lane A). PBMCs cultured with cortisol (0.2 μg/ml), used as a positive control for apoptosis, produced significant DNA ladder formation characteristic of apoptosis (lane D). Thus, our data support a role for morphine as a

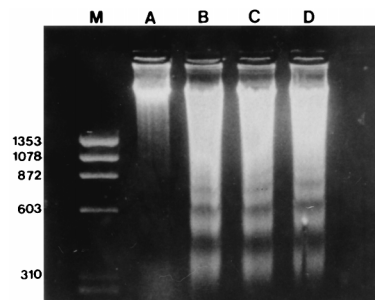


FIG. 1. Morphine induces apoptosis of human PBMCs. PBMCs were cultured alone or with 10<sup>-7</sup> and 10<sup>-8</sup> M concentrations of morphine for 60 h, and total DNA was extracted and electrophoresed on a 1.8% agarose gel in the presence of ethidium bromide. Arrows indicate fragmented DNA. Lane A, control culture, lanes B and C, morphine concentrations of 10<sup>-7</sup> and 10<sup>-8</sup> M, respectively; lane D, culture treated with cortisol at 0.2 μg/ml (positive control); lane M, molecular weight marker. This experiment was repeated twice, with similar results each time.

cofactor in the pathogenesis of HIV infections and suggest that morphine and HIV proteins may act independently and/or synergistically to induce immunosuppression, perhaps through the activation of apoptosis of functional lymphocytes.

## DISCUSSION

Peterson and colleagues (58, 59) showed that morphine and cocaine amplify HIV-1 replication in cocultures of human PBMCs and in chronically HIV-1-infected cell lines. Increased replication of HIV-1 induced by heroin and morphine in primary cultures of infected human Kupffer cells and human neuroblastoma cells also has been reported (28, 63, 65). Morphine also has been shown to potentiate the release of lipopolysaccharide-induced tumor necrosis factor and transforming growth factor  $\beta$  production by microglial and astrocyte cell cultures (59). Using an HIV-1 transactivator of transcription (*tat*) transgenic mouse model, Garza et al. (23) showed that morphine could cause inhibition of interleukin-2 production. Other studies showed that transforming growth factor  $\beta$  potentiated HIV-1 replication, suggesting that this cytokine plays a significant role in the progression of HIV infection (11, 59). Furthermore, when cytomegalovirus was used as an activating signal in the presence of morphine, significant amplification of HIV-1 replication in lymphocytes also was noted (59). Although the precise mechanism underlying how morphine promotes HIV-1 replication is unclear, a recent study showed that morphine increases NF- $\kappa$ B levels in PBMCs (8), and NF- $\kappa$ B, in turn, binds to the HIV long terminal repeat to increase viral transcription and subsequent replication. Although previous studies demonstrated inhibitory effects of morphine on host immune responses in both in vitro and in vivo models, it is known that immunostimulation also is required for productive HIV-1 replication. Thus, there is an apparent paradox wherein morphine as an immunosuppressant could potentially inhibit the immunostimulation required for HIV replication. However, it is likely that these two effects are in a balance and can be shifted by still other factors such as intercurrent opportunistic infections and consequent cytokine production. Thus, the balance between immunostimulation produced by activating signals and drug-induced immunosuppression could, under the appropriate circumstances, increase the viral load even when the immune system is suppressed by external factors such as drugs of abuse. This is similar to the another potential paradox wherein interleukin-2 may be therapeutic for HIV infections (33), even though it is a known inducer of viral replication (34).

Earlier studies showed that morphine is capable of suppressing IFN- $\gamma$  production by ConA- and specific antigen (varicella-zoster virus)-activated lymphocytes (56). Our results showing the suppression of both IFN- $\alpha$  and IFN- $\beta$  production by morphine may be consistent with the premise that morphine may play a significant role in the modulation of various immune responses via dysregulation of cytokine production, leading to immunodeficiency in narcotic addicts.

HIV-1 infection eventually progresses to severe deficiency of various immunological functions, especially during the latter stages of the disease. Since the extent of immunodeficiency observed in HIV-1-infected patients does not necessarily correlate with the number of HIV-infected peripheral blood lymphocytes (29), it was reasonable to assume that circulating, noninfectious products of HIV might contribute to progression of the immunodeficiency state. Using an in vitro model system, we showed that several different HIV-specific proteins could stimulate significant proliferative responses by naive PBMCs from HIV-negative donors (46). The proteins used included

Env-Gag, a recombinant fusion product of the gp41 envelope gene and the p24 core or group-specific antigen (*gag*) genes and other synthetic sequences corresponding to various conserved regions of the HIV envelope. Furthermore, we have shown that Env-Gag can induce de novo polyclonal immunoglobulin synthesis and suppress pokeweed mitogen-activated immunoglobulin production by HIV-negative lymphocytes in vitro (46). Env-Gag also selectively suppressed the NK activity of lymphocytes from AIDS patients, in contrast to the situation for HIV-seronegative blood donors (50, 61). This observation supports the premise that certain soluble HIV proteins secreted into the circulation during HIV infection can inhibit various immune functions critical for host defense against viruses and other pathogens. Others also have demonstrated other significant immunoregulatory activities of HIV peptides in normal and HIV-infected PBMCs (54, 60, 68). Our results indicate that morphine can suppress the host's immune responses to HIV, thus supporting the premise that morphine may be a cofactor in HIV infections.

Earlier studies have shown that apoptosis may be one of the mechanisms of CD4<sup>+</sup>-cell depletion in HIV-infected subjects (22, 26). In vitro studies also showed that cross-linking of the CD4 molecules of lymphocytes from HIV-infected subjects or normal CD4<sup>+</sup> lymphocytes with gp120-anti gp120 immune complexes prepares the cell for programmed cell death that occurs when a major histocompatibility complex class II molecule in complex with specific antigen or superantigen binds to the T-cell antigen receptors (2, 44). Further studies have shown that apoptosis in CD8<sup>+</sup> cells from HIV-infected subjects occurs spontaneously in vitro, even in the absence of anti-gp120 antibodies or superantigen (26, 44).

The Fas/Apo1 antigen (designated CD95) is a cell surface molecule belonging to the nerve growth factor-tumor necrosis factor receptor family (30, 31). Evidence indicating that apoptosis is not limited to immature or transformed cells and can be triggered in mature peripheral blood lymphocytes has recently accumulated (19, 20, 32). Fas antigen, a marker for apoptosis, is constitutively expressed in various cells, including activated T and B lymphocytes. Expression of Fas has been associated with the abnormally high levels of lymphocyte apoptosis seen in HIV-infected subjects (26, 66). Apoptosis also was observed after ligation of CD4 and before signaling through the T-cell receptor (52). Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule was reported recently (12), and studies have shown that opioids like morphine and methadone can induce apoptosis in human lung cancer cells (41). Chuang et al. (13) reported that PBMCs from morphine-treated rhesus monkeys demonstrated a lower degree of apoptosis during the early stage of infection with simian immunodeficiency virus. In the present investigation, we have examined DNA laddering of PBMCs cultured with pharmacologic concentrations of morphine ( $10^{-7}$  and  $10^{-8}$  M). Our data indicated that normal PBMCs undergo apoptosis when cultured with morphine alone. Treatment of PBMCs with  $10^{-7}$  and  $10^{-8}$  M morphine for 24 h did not result in apoptosis, whereas treatment for 60 h resulted in cleavage of DNA into multiples of nucleoside-size fragments (~180 bp), a DNA laddering pattern specific for apoptosis, whereas no significant ladders were formed for untreated control cultures.

Opioids are known to mediate their effects by interacting with  $\mu$ ,  $\delta$ , and  $\kappa$  opioid receptors (55). In the present study we examined the ability of the  $\mu$ -receptor antagonist naloxone to block the inhibition of IFN production by morphine. Our results indicated that naloxone significantly blocked the morphine-induced inhibition of IFN- $\alpha$  production by PBMCs in a dose-dependent manner (Table 4). This demonstrates that

morphine acts on lymphocytes through the  $\mu$  receptor. Furthermore, when naloxone was removed from the culture by washing after treatment of the cells, morphine was still capable of inhibiting IFN- $\alpha$  production, demonstrating that the naloxone effect was reversible (data not shown). Further studies are needed in order to determine the specific opioid receptor types involved in morphine-induced apoptosis of normal PBMCs. Since previous studies have shown that apoptosis may be mediated through different pathways such as induction or modulation of Fas (12), Nur77 (53), and Bcl-2 (1), studies are needed to investigate the specific pathways of morphine-induced apoptosis of PBMCs. The phenotypes of lymphocyte subpopulations undergoing morphine-induced apoptosis were not examined in this study. Investigations of different proteins, protein kinases, free radicals, protooncogenes, and specific blocking factors and different metabolites of morphine that may play a role in morphine-induced apoptosis of normal lymphocytes will be useful in the design of novel strategies to reuse cells from morphine-induced nonspecific apoptosis of lymphocytes in complications of narcotic addiction and other clinical situations.

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