

Rapid Elevation of Plasma Interleukin-6 by Morphine Is Dependent on Autonomic Stimulation of Adrenal Gland

RICHARD A. HOUGHTLING and BARBARA M. BAYER

Departments of Pharmacology and Neuroscience, Georgetown University Medical Center, Washington, DC

Received May 9, 2001; accepted September 25, 2001 This paper is available online at <http://jpet.aspetjournals.org>

ABSTRACT

Several studies have demonstrated that opioids regulate a number of immune cell functions either through direct mechanisms or through the modulation of central nervous system outputs. It has been previously shown that morphine increases serum interleukin-6 (IL-6) levels; however, the mechanism by which this effect is produced is unknown. In the present study, experiments were designed to address the potential role of central opioid receptors, peripheral autonomic ganglia, and activation of the adrenals in the elevation of plasma IL-6 after morphine administration. A rapid and significant (2-fold) increase in plasma IL-6 was observed after morphine administration (10 mg/kg s.c.) to rats. This effect of morphine peaked within 30 min and remained elevated for at least 2 h. Central microinjection of morphine (10 μ g/2 μ l i.c.v.) mimicked the

effects of peripherally administered morphine and was completely blocked by naltrexone (10 mg/kg s.c.) pretreatment. Pretreatment with a ganglionic blocker, chlorisondamine (0.5 mg/kg i.p.), also blocked the elevation of IL-6 by morphine, suggesting a role of the autonomic nervous system. In adrenalectomized animals, morphine administration did not increase IL-6 levels, whereas in adrenal demedullated animals, the effect of morphine remained intact. Thus, the adrenal cortex may be a potential source of IL-6, because IL-6 mRNA has been localized in the adrenal gland. Collectively, these data suggest a unique mechanism by which stimulation of central opioid receptors results in the elevation of plasma IL-6 through autonomic activation specifically of the adrenal cortex.

It is well known that opioids have many diverse effects on the immune system. Interestingly, many of these effects are indirect and appear to be mediated via the central nervous system, suggesting that alterations in opioid neurotransmission can alter the efficacy of the immune response. Our laboratory and others have demonstrated that central morphine administration decreases mitogen-induced blood lymphocyte proliferation (Hernandez et al., 1993; Mellon and Bayer, 1998) or splenocyte proliferation (Lysle et al., 1996), decreases NK cell activity (Weber and Pert, 1989; Moeniralam et al., 1998), as well as increases macrophage nitric oxide release (Fecho et al., 1994). Studies have also indirectly demonstrated a modulatory role of central opioid receptors in the stimulatory effect of interleukin-1 β on serum interleukin-6 (IL-6), which was prevented by naloxone pretreatment (De Simoni et al., 1993). Direct evidence of opioids modulating IL-6 release was obtained by both peripheral and central

routes of administration (Bertolucci et al., 1996; Zubelewicz et al., 1999).

Interleukin-6 is a 26-kDa protein that is produced by both immune and nonimmune tissues and thus has many different biological effects. For example, circulating IL-6 leads to the release of acute phase proteins from the liver (for review, see Heinrich et al., 1990), elicits a febrile response, and activates the hypothalamic-pituitary-adrenal axis (Lenczowski et al., 1999). As such, administration of opioids via illicit or therapeutic use could lead to a dysregulation of circulating IL-6 levels. Likewise, during times of stress and adaptation the release of endogenous opioids may alter the homeostasis of an organism to respond to bodily injury or inflammation. The observations of Bertolucci et al. (1996) suggest that opioids may regulate peripheral levels of this cytokine. However, the characteristics and mechanism(s) by which opioids induce this elevation in IL-6 still remain largely uncharacterized.

To gain a more clear understanding of opioid-induced immunoregulation as measured by the elevation of plasma IL-6 levels, studies were designed to characterize the dose, time, and receptor dependence of morphine administration to rats. In a previous study, only a single time point and dose of morphine were used to determine the effect of opioids on sera

These studies were supported by National Institute on Drug Abuse Grants DA04358 (to B.M.B.) and DA05849 (to R.A.H.). Some preliminary studies were presented at the 4th International Congress of the International Society for Neuroimmunomodulation Meeting in Lugano Switzerland, October 2-5, 1999, and were included in the following invited citation: Houghtling RA, Mellon RD, Tan RJ, and Bayer BM (2000) Acute effects of morphine on blood lymphocyte proliferation and plasma IL-6 levels. *Ann NY Acad Sci* 917:771-777.

ABBREVIATIONS: IL-6, interleukin-6; rIL-6, recombinant rat interleukin-6; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; ADX-CORT, adrenalectomized animal with corticosterone replacement; AD-DM, adrenal demedullated animal.

IL-6 (Bertolucci et al., 1996). Furthermore, because the mechanism of this effect has yet to be determined, studies were completed to elucidate whether the autonomic nervous system (ANS) or activation of the adrenal glands was involved. Previous studies from our laboratory have demonstrated a role of the ANS in the effect of morphine to decrease mitogen-stimulated lymphocyte proliferation (Flores et al., 1996; Mellon and Bayer, 1999; Mellon and Bayer, 2001). However, it remains to be determined whether activation of the autonomic nervous system is required for the effects of morphine on plasma IL-6 levels. To test this possibility, the bisquaternary nicotinic ganglionic receptor antagonist chlorisondamine was used to effectively inhibit peripheral autonomic neurotransmission (Clarke et al., 1994).

Finally, it has been previously determined that the adrenal gland may be a potential source of IL-6. Interleukin-6 mRNA has been observed in the adrenal gland by *in situ* hybridization (Gonzalez-Hernandez et al., 1994; Gadiant et al., 1995). Additionally, the adrenal cortex and medulla have been implicated in the release of IL-6, because primary cultures of adrenal cells produce IL-6 when stimulated with several neurotransmitters, hormones, and cytokines (for review, see Nussdorfer and Mazzocchi, 1998). To determine the potential involvement of the adrenal gland in this response to morphine, adrenalectomized and adrenal demedullated animals were used. The results suggest a unique mechanism for central opioid regulation of a peripheral cytokine, which is distinct from the mechanisms reported for the suppressive effects of opioids on lymphocyte proliferation and NK cell activity.

Materials and Methods

Animals. Male Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY), weighing 200 to 225 g upon arrival. Animals were group housed (three per polypropylene cage with microisolator tops) and were provided free access to food (Purina Rat Chow; Purina, St. Louis, MO) and water *ad libitum*. The animals were housed in a thermoregulated ($23 \pm 1^\circ\text{C}$) and light (12-h light/dark)-controlled room for 1 week to allow for acclimation to this environment before experimentation. Animals that underwent surgery were allowed to recover for an additional week before experimentation. All animal studies have been approved by the Georgetown University Animal Care and Use Committee in accordance with the guidelines adopted by National Institutes of Health.

Drugs. Morphine sulfate was generously provided by the National Institute on Drug Abuse (Research Triangle Park, NC). Chlorisondamine chloride (Ecolid) was a gift from Novartis (Summit, NJ). Recombinant mouse IL-6 and rat IL-6 (rrIL-6) were purchased from Genzyme (Cambridge, MA) and Peprotech (Rocky Hill, NJ), respectively. Naltrexone hydrochloride and all other drugs were purchased from Sigma Chemical (St. Louis, MO). All vehicle and drug solutions were made using sterile, pyrogen-free 0.9% saline.

Surgical Procedures. For all surgeries, a proper and sterile surgical environment was maintained. Specifically, all surgical instruments were autoclaved before and soaked in Novalsan solution during surgical procedures. Incision sites for either central cannula placement or bilateral adrenalectomy were prepared by shaving the skin and cleaning with Novalsan solution and ethyl alcohol (70%). The incision was made using either a sterile surgical blade or sterile surgical scissors. After the completion of the surgical procedure, the wound site was either sutured or stapled as described below. To minimize possible infection after the surgical procedure, each animal was injected intramuscularly with 0.1 ml of gentamicin sulfate (40 mg/ml).

Intracerebroventricular Cannulation and Microinjection Procedures. Animals were anesthetized with equithesin (3 ml/kg *i.p.*). Equithesin was prepared by dissolving chloral hydrate (4.25 g) and magnesium sulfate (2.13 g) in a solution of propylene glycol (26.6 ml), pentobarbital (18 ml), and deionized water (43 ml). Animals were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and a single 5.0-mm guide cannula was directed into the lateral ventricle using the following coordinates relative to bregma: -0.9 mm anterior-posterior, -1.6 mm mediolateral, and -3.1 mm dorsoventral obtained from Paxinos and Watson (1997). The guide cannula was fixed with dental acrylic and jewelers' screws and fitted with an inert 28-gauge cannula to maintain patency. Animals were allowed to recover for at least 1 week before experimentation.

Rats were briefly individually housed for microinjection of vehicle or drug. A 28-gauge injection cannula, which extended 1 mm below the guide cannula, was connected to a Hamilton syringe by polyethylene tubing filled with saline or morphine. A total volume of $5 \mu\text{l}$ was infused over a 45-s period at a rate of $2.7 \mu\text{l}/\text{min}$ by using a model 101 kD Scientific microinfusion pump (New Hope, PA). Verification of drug delivery was monitored by the movement of an air bubble separating saline and drug solutions within the cannula. The injection cannula remained in place for an additional 75 s to allow for drug dispersal. The rat was returned to its home cage for the remainder of the experiment. Animals were sacrificed by decapitation at the times indicated in the figure legends. Upon completion of each experiment, the brains of cannulated rats were injected with 1% fast green dye ($2 \mu\text{l}$) through the injection cannula. Blunt dissection of the brain was used to verify cannula placement within the ventricle.

Adrenalectomy and Adrenal Demedullation. Animals were anesthetized with equithesin. Bilateral incisions were made 1 cm below the rib cage. Underlying muscle was cut with sterile surgical scissors and the adrenal gland was isolated and removed. The musculature was sutured with polyglycolic acid sutures 4-0 (American Cyanamid Co., Wayne, NJ) and the skin wound was stapled. Post-operative care for animals included a bolus subcutaneous injection of sucrose (20%). Adrenalectomized animals were provided saline (0.9%) drinking solution supplemented with corticosterone 21-acetate ($25 \mu\text{g}/\text{ml}$ in 0.2% ethanol) to restore basal corticosterone levels (Jacobson et al., 1989; Zhou et al., 1993). Sham-operated animals were provided with drinking water containing 0.2% ethanol. All animals were allowed at least 1-week recovery from surgery before experimentation. To verify the completeness of the adrenalectomy, plasma corticosterone levels were measured. Any plasma corticosterone level that was ≥ 75 ng/ml was considered incomplete and that animal was eliminated from data analysis.

Sham-operated and adrenal demedullated animals were purchased from Taconic Farms. Animals were housed for 1 week before experimentation and were provided food and water *ad libitum*.

Plasma Corticosterone Measurement. Plasmas collected at the time of sacrifice were stored at -20°C and assayed for corticosterone levels using a solid phase ^{125}I radioimmunoassay purchased from ICN Pharmaceuticals (Costa Mesa, CA).

Plasma IL-6 Measurement. Interleukin-6 activity was determined using the murine splenocyte hybridoma cell line 7TD1 (American Type Culture Collection, Manassas, VA). The cells were maintained in culture medium (RPMI-1640 with glutamine) supplemented with 10% heat-inactivated fetal calf serum, $50 \mu\text{M}$ β -mercaptoethanol, and 10 units/ml recombinant mouse interleukin-6. IL-6 activity was measured as previously described (Van Snick et al., 1986; LeMay et al., 1990) except that rrIL-6 was used instead of recombinant human IL-6 in the standard curve. Briefly, 7TD1 cells were washed three times in the culture medium described above without IL-6 and centrifuged at 1100 rpm (DuPont Sorvall, Newtown, CT). Experiments were completed using 96-well plates containing 100 μl of control media, serially diluted rat plasma (2- to 4096-fold) or rrIL-6 (0.1–1000 U/ml), and 100- μl aliquots of 2×10^3 cells/well were cultured for 72 h. During the last 6 h, the cultures were pulsed with 0.5 μCi of [^3H]thymidine (PerkinElmer Life Sci-

ences, Boston, MA), harvested onto glass fiber filters by using a 96-well harvester (Brandel Inc., Gaithersburg, MD), and radioactivity was counted on a Betaplate scintillation counter (Wallac, Gaithersburg, MD).

Plasma IL-6 activity was determined by comparing the effect of serial dilutions of rat plasma on 7TD1 cell proliferation to that of a standard curve generated by rrIL-6. The standard curve was analyzed using nonlinear regression and fit a sigmoidal dose-response model. The EC_{50} of the 7TD1 cell proliferation response (cpm) of the standard curve was defined as 1 U of IL-6 activity. To determine the amount of IL-6 activity in individual plasma samples, a single dilution of rat plasma was selected. The dilution was chosen based on the criterion that the 7TD1 cell proliferation (cpm) of the sample most closely approximated the EC_{50} (cpm) of the standard curve. Plasma IL-6 activity (units/milliliter) was calculated using the following equation: $U/ml = (\text{plasma (cpm)} \div EC50 \text{ (cpm)}) \times (\text{dilution factor} \div 0.1 \text{ ml of plasma})$. The sensitivity of this assay was determined to be 6.5 ± 1.7 U/ml. Data are expressed as the mean \pm S.E.M. IL-6 activity (units/milliliter) or when data from individual experiments are combined, as the mean \pm S.E.M. percentage of IL-6 activity of saline-injected controls. Because recombinant human IL-11 and oncostatin M have been shown to also stimulate 7TD1 cell growth (Schwabe et al., 1996), the plasma IL-6 activity from morphine-treated animals was validated by using both a polyclonal neutralizing anti-murine IL-6 antibody (R & D Systems, Minneapolis, MN) and an ELISA (Biosource International, Camarillo, CA). The specificity of the polyclonal neutralizing anti-murine IL-6 antibody for recombinant rat IL-6 was confirmed by Western blotting. Pretreatment of serially diluted plasmas for 1 h with this neutralizing antibody (2 μ g/ml) completely blocked the plasma IL-6 activity measured in the 7TD1 bioassay. In addition, using an ELISA specific for rat IL-6 (Biosource International) it was determined that 1 U/ml IL-6 activity was equivalent to 1 pg/ml rrIL-6. Moreover, using this ELISA the increase in IL-6 activity due to morphine was confirmed.

Statistical and Data Analysis. Plasma IL-6 activity and corticosterone data were analyzed using a one-way ANOVA with Newman-Keuls for post hoc comparisons. Values of plasma IL-6 that were more than 2 S.D. from the mean of the treatment group were considered outliers at 95% confidence intervals and omitted from data analysis. For the experiments presented in these studies, plasma IL-6 values for 12 of 245 (5%) animals met this criterion and were omitted. Adrenalectomized animals received exogenous corticosterone therapy and values of plasma corticosterone levels <75 ng/ml were considered complete. Using this criterion, 1 of 24 (4%) adrenalectomized animals was excluded due to elevated corticosterone levels (147 ng/ml). In adrenalectomized animals receiving corticosterone replacement therapy, the range of plasma corticosterone values for saline- and morphine-treated animals was 25 to 69 and 25 to 41 ng/ml, respectively. In adrenal demedullated animals, plasma corticosterone levels were monitored to determine that the remaining cortex was intact. Statistical significance was accepted at a level of $p \leq 0.05$.

The ED_{50} of morphine-induced elevation of plasma IL-6 activity was determined using nonlinear regression analysis. The data were best fit to a sigmoidal dose-response model with variable slope (four-parameter logistic equation).

Results

Time Course of Morphine-Induced Plasma IL-6 Activity. To determine the time course of morphine-induced elevation of plasma IL-6, animals received subcutaneous treatments of either saline (1 ml/kg) or morphine (10 mg/kg) and were sacrificed 30, 60, or 120 min after drug treatment. As shown in Fig. 1, saline-injected rats were sacrificed 30 min after treatment and basal IL-6 activity was observed. After 30 min of morphine treatment, plasma IL-6 activity

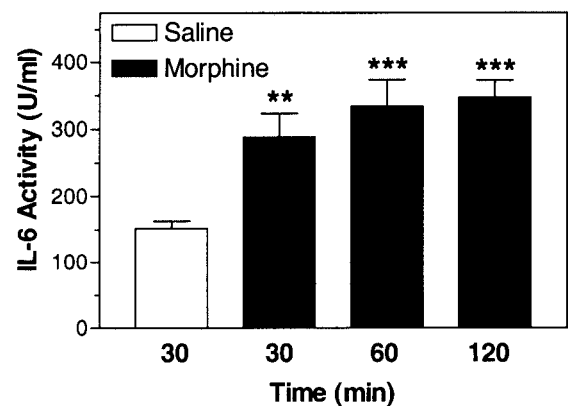


Fig. 1. Time course of morphine-induced elevation of plasma IL-6 activity. Animals were subcutaneously injected with either saline (1 ml/kg) or morphine (10 mg/kg) and sacrificed 30, 60, or 120 min after drug treatment. Plasma IL-6 activity was measured using the 7TD1 bioassay (see *Materials and Methods*). Data are combined from two independent experiments and expressed as the mean \pm S.E.M. IL-6 (units/milliliter) for groups of 8 to 10 animals. **, $p < 0.01$ or ***, $p < 0.001$ compared with saline-injected controls (one-way ANOVA, Newman-Keuls).

increased 2-fold compared with the saline-injected controls. In additional studies, saline treatment for either 1 or 2 h had similar plasma IL-6 levels (data not shown). Likewise, 60- and 120-min treatment with morphine significantly elevated plasma IL-6 activity, as well. For all subsequent studies, plasma IL-6 activity was observed 1 h after morphine treatment.

Dose-Dependent Effect of Morphine on Plasma IL-6 Activity. To determine whether the elevation of plasma IL-6 levels was dose-dependent, increasing doses of morphine were subcutaneously injected into groups of rats. Animals received either saline (1 ml/kg) or morphine (0.1–30 mg/kg), and plasma IL-6 activity was determined 1 h after drug treatment. Lower doses (0.1 and 1 mg/kg) were not significantly different from saline-injected controls (Fig. 2). Doses of morphine greater than 10 mg/kg led to significant elevations (5- to 14-fold) in IL-6 activity. These effects were dose-dependent with approximately 500, 1100, and 1400% increases in plasma IL-6 with 10, 20, and 30 mg/kg doses, respectively.

To determine an ED_{50} value for morphine-induced elevation of plasma IL-6 activity, the data were analyzed by nonlinear regression analysis. As shown in Fig. 2, inset, the data were best represented by a sigmoidal dose-response model with variable slope (four-parameter logistic equation). An ED_{50} value of systemic morphine on plasma IL-6 activity was determined to be 13.8 mg/kg.

Effect of Central Administration of Morphine on Plasma IL-6 Activity. To determine whether the activation of central opioid receptors also increased IL-6 levels, morphine was administered directly into the lateral ventricle. As shown in Fig. 3, intracerebroventricular microinjection of morphine (10 μ g/5 μ l) led to a significant (3-fold) increase in plasma IL-6 activity compared with the saline-injected (5 μ l) control.

Effect of Naltrexone Pretreatment on Morphine-Induced Plasma IL-6 Activity. To determine whether the elevation of plasma IL-6 by morphine was mediated through activation of opioid receptors, the animals described above were pretreated with the opioid receptor antagonist naltrexone. Animals were pretreated with a subcutaneous injection

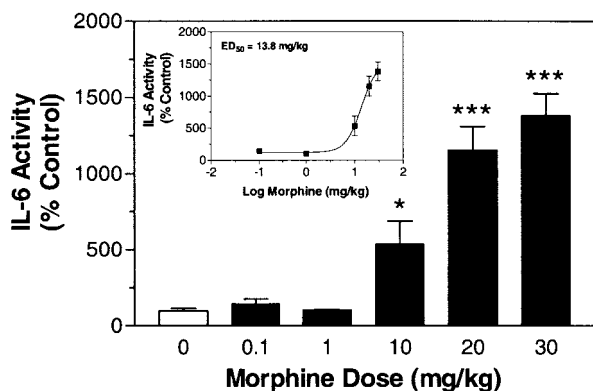


Fig. 2. Dose-dependent effect of morphine on plasma IL-6 activity. Animals were subcutaneously injected with either saline (1 ml/kg) or morphine (0.1–30 mg/kg). One hour after drug treatment, animals were sacrificed and plasma IL-6 was determined. Data are expressed as the percentage of saline-injected controls (mean \pm S.E.M.). The data were obtained from two separate experiments with overlapping doses. In the first experiment, saline and morphine at doses 0.1, 1, 10, and 20 mg/kg were examined. In the second experiment, IL-6 responses to saline and morphine at doses 10, 20, and 30 mg/kg were measured. The data represent groups of six rats for each of the low doses of morphine (0.1 and 1 mg/kg) and groups of 10 to 12 rats for each of the higher doses of morphine (10, 20, and 30 mg/kg). *, $p < 0.05$ compared with saline-treated animals and ***, $p < 0.001$ compared with both saline and morphine (0.1, 1, and 10 mg/kg)-treated animals (one-way ANOVA, Newman-Keuls). Nonlinear regression analysis of this morphine dose response on plasma IL-6 activity was presented in the inset. An ED_{50} value for the morphine-induced IL-6 activity was approximated at 13.8 mg/kg.

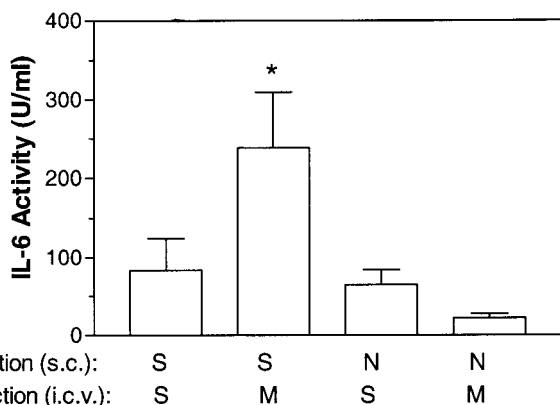


Fig. 3. Central morphine increased plasma IL-6 activity through opioid receptor activation. One week before experiments, animals were surgically implanted with a guide cannula in the lateral ventricle. On the day of the experiment, animals were pretreated for 30 min with a subcutaneous injection of either saline (S, 1 ml/kg) or naltrexone (N, 10 mg/kg) followed by microinjection into the lateral ventricle of either saline (5 μ l) or morphine sulfate (M, 10 μ g/5 μ l). One hour after the last drug treatment, animals were sacrificed and plasma IL-6 was determined. Data are expressed as the mean units/milliliter \pm S.E.M. for groups of six animals and were representative of two independent determinations. *, $p < 0.05$ compared with all groups (one-way ANOVA, Newman-Keuls).

of either saline (1 ml/kg) or naltrexone (10 mg/kg). Thirty minutes later, saline (5 μ l) or morphine (10 μ g/5 μ l) was microinjected into the lateral ventricle, and animals were sacrificed 1 h after the last drug treatment. Naltrexone pretreatment had no effect on plasma IL-6 activity after saline treatment (Fig. 3). However, naltrexone pretreatment completely blocked the central morphine-induced elevation of plasma IL-6 activity.

Effect of Ganglionic Blockade on Morphine-Induced Plasma IL-6 Activity.

Studies were initiated to determine

whether activation of the autonomic ganglia after morphine administration may be involved in the elevation of plasma IL-6. Animals were pretreated with chlorisondamine, a quaternary neuronal nicotinic receptor antagonist, which has previously been shown to effectively inhibit peripheral autonomic neurotransmission (Schneider and Moore, 1955; Clarke et al., 1994). As shown in Fig. 4, morphine (10 mg/kg s.c.) treatment significantly stimulated plasma IL-6 activity. No effect on plasma IL-6 activity was observed in animals treated with chlorisondamine (0.5 mg/kg i.p.) alone. However, chlorisondamine pretreatment significantly decreased the morphine-induced elevation of plasma IL-6 activity. Based upon the dose-response study (Fig. 2), a higher dose of morphine was used for subsequent studies. Additional studies using a higher dose of both chlorisondamine (5 mg/kg i.p.) and morphine (20 mg/kg s.c.) also demonstrated that chlorisondamine pretreatment was accompanied by a blockade of the elevation of opioid-induced plasma IL-6 activity (data not shown).

Effect of Adrenalectomy on Morphine-Induced Plasma IL-6 Activity. To determine the role of adrenal gland activation in elevation of plasma IL-6, animals were either sham-operated (sham) or bilateral adrenalectomized and a basal level of corticosterone (25 μ g/ml) was supplied in the drinking water (ADX-CORT). To verify the completeness of adrenalectomy, plasma corticosterone levels were measured. As shown in Fig. 5A, sham animals were injected subcutaneously with either saline (1 ml/kg) or morphine (20 mg/kg). Saline-treated shams had basal levels of corticosterone (100 ng/ml), whereas animals treated with morphine had significantly elevated plasma corticosterone levels (approximately 5-fold). Comparison of corticosterone levels in saline-treated sham and ADX-CORT animals were not significantly different, although basal levels in ADX-CORT animals were approximately 3-fold lower. Unlike sham animals, morphine treatment (20 mg/kg s.c.) did not elevate plasma corticosterone in ADX-CORT animals.

As shown in Fig. 5B, sham-operated animals treated with saline produced a basal level of plasma IL-6 that was ele-

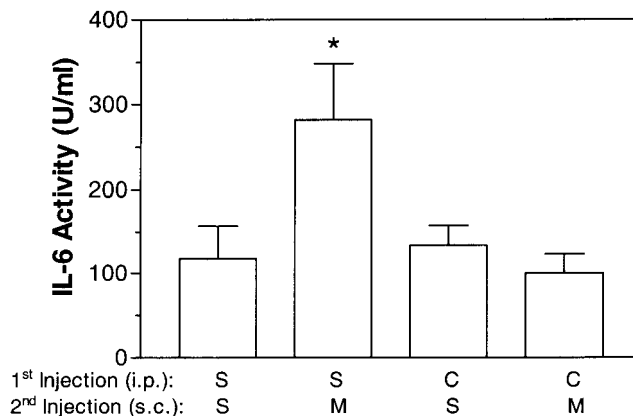


Fig. 4. Blockade of morphine-induced effects by chlorisondamine. Animals were pretreated for 30 min with an intraperitoneal injection of either saline (S, 1 ml/kg) or chlorisondamine (C, 0.5 mg/kg) and then were treated with a subcutaneous injection of either saline (S, 1 ml/kg) or morphine sulfate (M, 10 mg/kg). Animals were sacrificed 1 h after the last drug treatment, and plasma IL-6 was determined. Data are combined from two independent experiments and expressed as the mean \pm S.E.M. IL-6 (units/milliliter) for groups of 10 to 11 animals. **, $p < 0.01$ compared with all groups (one-way ANOVA, Newman-Keuls).

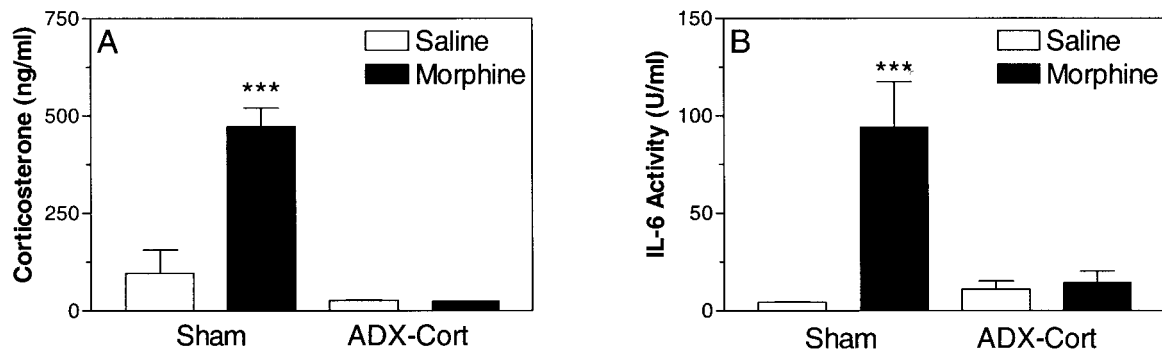


Fig. 5. Blockade of morphine-induced elevation of plasma corticosterone and IL-6 activity by adrenalectomy. One week before experiments, animals were sham-operated or adrenalectomized. Adrenalectomized animals were provided with corticosterone in the drinking fluid as described under *Materials and Methods*. Animals (5–6/group) were treated with a subcutaneous injection of either saline (1 ml/kg) or morphine (20 mg/kg) and sacrificed 1 h after treatment. Data are representative of two independent determinations. A, plasma corticosterone was measured using a radioimmunoassay, and data are expressed as the mean \pm S.E.M. corticosterone (ng/ml). ***, $p < 0.001$ compared with all groups (one-way ANOVA, Newman-Keuls). B, plasma IL-6 activity is expressed as the mean \pm S.E.M. IL-6 (units/milliliter). ***, $p < 0.001$ compared with all groups (one-way ANOVA, Newman-Keuls).

vated approximately 16-fold after morphine treatment. In contrast to sham animals, morphine treatment did not elevate plasma IL-6 activity in ADX-CORT animals.

Effect of Adrenal Demedullation on Morphine-Induced Plasma IL-6 Activity. Because adrenalectomy prevented morphine-induced plasma IL-6 release, studies were initiated to elucidate whether the adrenal cortex or medulla was involved in the effect of morphine. Animals were either sham or adrenal demedullated (AD-DM) and were subcutaneously injected with either saline (1 ml/kg) or morphine (20 mg/kg). One hour after treatment, animals were sacrificed and IL-6 activity was determined.

To determine whether the adrenal cortex was intact after the surgical procedure, plasma corticosterone levels were measured. As shown in Fig. 6A, saline treatment of both sham and AD-DM animals produced basal levels of plasma corticosterone. As expected, morphine treatment significantly elevated plasma corticosterone (4-fold) in sham animals. Similar to sham animals, morphine treatment also significantly elevated plasma corticosterone to the same magnitude (4.5-fold) in AD-DM animals.

In Fig. 6B, saline treatment of either sham or AD-DM animals produced a similar level of plasma IL-6 activity. Morphine treatment significantly elevated plasma IL-6 levels in both sham and AD-DM animals to the same magnitude (7- and 6-fold, respectively).

Discussion

The studies described here provide the first characterization of the time and dose dependence of an opioid-mediated elevation of plasma IL-6. The results show that the effect of systemic morphine administration on IL-6 release is both rapid and dose-dependent. Plasma IL-6 is elevated 2-fold within 30 min of systemic morphine administration. In addition to being rapid, IL-6 elevation after morphine treatment is prolonged and maintained for at least 2 h. Because the half-life of plasma IL-6 is very short ($t_{1/2} = 1.5\text{--}5$ min) (Castell et al., 1988), the maintenance of a prolonged elevation of circulating IL-6 levels is most likely due to either a continual release of IL-6 or a decrease in the clearance of IL-6 from plasma. Systemic morphine treatment was also shown to elevate plasma IL-6 in a dose-dependent manner, but requires high doses (10–30 mg/kg). These data suggest that morphine acts through a receptor-mediated effect with an ED_{50} value of 13.8 mg/kg. However, this dose is relatively higher than the dose of morphine required to inhibit lymphocyte proliferative responses (Bayer et al., 1992) or to produce analgesic responses (for review, see Martin, 1984). The high-dose requirements suggest the effect of morphine on plasma IL-6 may have greater impact during the clinical use of opioids to produce anesthesia or in the recreational illicit use of these drugs.

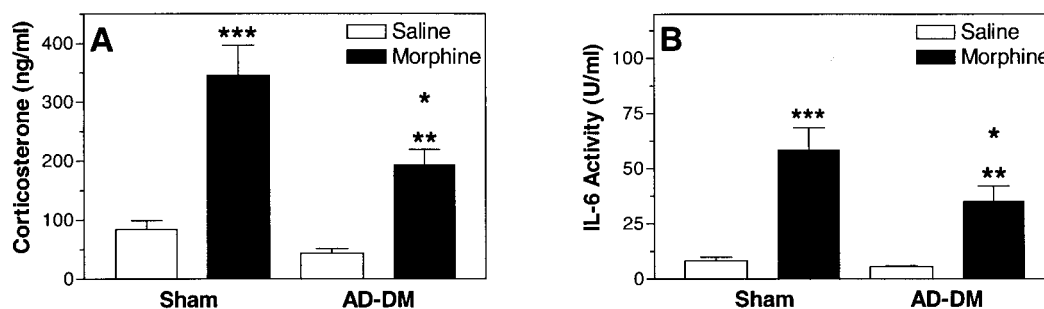


Fig. 6. Morphine elevated plasma IL-6 activity in adrenal demedullated rats. Animals were sham or AD-DM and were treated with either saline (1 ml/kg) or morphine (20 mg/kg). One hour after treatment, animals were sacrificed and plasma corticosterone and IL-6 were determined. The data presented ($n = 11\text{--}13$ /group) are combined from two independent experiments. A, plasma corticosterone was measured using a radioimmunoassay. Data are presented as the mean \pm S.E.M. corticosterone (ng/ml). *, $p < 0.05$ compared with sham morphine (Mor); **, $p < 0.01$ compared with sham saline (Sal) and AD-DM Sal; and ***, $p < 0.001$ compared with sham Sal and AD-DM Sal treatments (ANOVA, Newman-Keuls). B, plasma IL-6 levels were measured and data are presented as the mean \pm S.E.M. IL-6 (units/milliliter). *, $p < 0.05$ compared with sham Mor; **, $p < 0.01$ compared with sham; and AD-DM Sal, and ***, $p < 0.001$ compared with sham and AD-DM Sal treatments (one-way ANOVA, Newman-Keuls).

These studies provide additional evidence for a critical role of central opioid receptors in the regulation of peripheral IL-6 levels. First, comparison of the dose of morphine that was required to produce the elevation of plasma IL-6 after different routes of administration suggested involvement of central opioid receptors. Subcutaneous administration of morphine (10 mg/kg) produced a similar elevation of plasma IL-6 as did microinjection of morphine (10 μ g/5 μ l) into the lateral ventricle. In considering the dose-response study (Fig. 2), if the central dose of morphine (10 μ g) would be administered subcutaneously no effect would be predicted, because the effect of morphine on plasma IL-6 required subcutaneous doses approximately 1000-fold greater. Second, the elevation of plasma IL-6 by central microinjection of morphine was completely antagonized by pretreatment with naltrexone, an opiate receptor antagonist, thus demonstrating for the first time that this effect of morphine is opioid receptor-dependent. Our observations are in agreement with previous studies suggesting a role for central opioid receptors in the elevation of serum IL-6 after administration of IL-1 β (De Simoni et al., 1993). Additional direct evidence for a role of opioids was the observation that intraperitoneal injection of either morphine (15 mg/kg) or etorphin (10 mg/kg) and central administration of morphine (10 μ g) elevated serum IL-6 levels at 2 h after administration (Bertolucci et al., 1996). The results reported here confirm these initial studies and further demonstrate that the central receptor mediating the effect of morphine is naltrexone-sensitive. Taken together, these data suggest that direct stimulation of central opioid receptors by morphine activates opioid pathways that may be involved in altering circulating levels of cytokines, such as IL-6.

The mechanism of opioid-induced IL-6 levels has not been previously examined. In the present studies, it is shown that chlorisondamine pretreatment completely blocked the morphine-induced elevation of plasma IL-6 (Fig. 4). These data are consistent with the fact that central opioid administration has also been shown to modulate the sympathetic nervous system as well as the sympathoadrenal axis. For example, microinjection of morphine into the central nervous system of rats has been shown to increase blood pressure (Conway et al., 1983) and to elevate plasma epinephrine, norepinephrine, and/or dopamine (Van Loon et al., 1981; Conway et al., 1983; Appel et al., 1986). Chlorisondamine is a quaternary neuronal nicotinic receptor antagonist that has been shown to effectively block peripheral ganglionic neurotransmission (Schneider and Moore, 1955; Clarke et al., 1994). High-dose chlorisondamine (10 mg/kg) administration has been reported to access the brain and produce a prolonged blockade of central nervous system nicotinic receptors (Clarke et al., 1994). However, these studies were designed to use a considerably lower dose of chlorisondamine (0.5 mg/kg), which has been previously shown to completely block the effect of epibatidine (a potent nicotinic receptor agonist) on lymphocyte proliferation responses to mitogen (Mellon and Bayer, 1999). In contrast, chlorisondamine (0.5 mg/kg) did not block epibatidine stimulation of the hypothalamic-pituitary-adrenal axis (Mellon and Bayer, 1999), which is mediated by central nicotinic receptor stimulation (Matta et al., 1987). Consistent with a role of the autonomic ganglia to elevate IL-6, pretreatment of animals with chlorisondamine (3 mg/kg) also prevented IL-1 β -stimulated sera IL-6 levels

(Kitamura et al., 1998). Therefore, these data provide the first evidence that stimulation of peripheral ganglionic nicotinic receptors was required for the elevation of plasma IL-6 by morphine.

Because central opioid receptor stimulation also activates the sympathoadrenal axis (Van Loon et al., 1981; Conway et al., 1983; Appel et al., 1986), studies were conducted to determine whether the adrenal glands were involved in this response. The results presented here demonstrated that in adrenalectomized animals, receiving replacement of corticosterone in their drinking water, morphine administration did not elevate IL-6 levels (Fig. 5). Thus, intact adrenal glands were required for this effect of morphine. Additional studies examined the potential of the adrenal cortex or medulla to be involved in this response. In contrast to complete adrenalectomy, surgical removal of the adrenal medulla did not prevent the increase in plasma IL-6 by morphine (Fig. 6). Thus, these data provide the first evidence that morphine-mediated effects on IL-6 are adrenal-dependent and require that the adrenal cortex remain intact.

Although these studies do not directly demonstrate IL-6 is released from the adrenal gland, the adrenal demedullation studies suggest that IL-6 release is dependent on the adrenal cortex. The source of IL-6 in circulation after treatment with morphine is unknown. Interleukin-6 is produced by a variety of cells of both immune and nonimmune origin (for review, see Akira et al., 1993). The results presented here indicate that morphine rapidly induced plasma IL-6 within 30 min and remained elevated for at least 2 h (Fig. 1), required ANS stimulation (Fig. 4), and was adrenal cortex-dependent (Figs. 5 and 6). Based on the adrenalectomy and adrenal demedullation studies, the adrenal cortex may be a potential source of IL-6 release after morphine treatment. The adrenal gland has been demonstrated to be innervated by pre- and postganglionic fibers of the autonomic nervous system (Kesse et al., 1988), and IL-6 mRNA has been detected by *in situ* hybridization in the adrenal gland (Gadient et al., 1995). Because it is currently unknown which cell type releases IL-6 from the adrenal gland, two potential candidates are the resident macrophages and the adrenal cortical cells. Interestingly, primary cultures of adrenal cortical cells have been stimulated to produce IL-6 by a variety of neurotransmitters, hormones, and cytokines (Nussdorfer and Mazzocchi, 1998). Collectively, these data suggest that elevation of plasma IL-6 by morphine may occur via release by adrenocortical cells.

An alternate possibility is that morphine treatment leads to the release of an adrenal-derived factor that elevates plasma IL-6 levels. Several adrenal cortex-derived factors could be potential candidates for the elevation of plasma IL-6. First, there appears to be a correlation between the elevation of plasma corticosterone and plasma IL-6. In both the adrenalectomy and adrenal demedullation studies, the elevation of IL-6 activity mirrored that of plasma corticosterone. Furthermore, the ED₅₀ value of morphine (7.8 mg/kg) to elevate plasma corticosterone has been previously reported by this laboratory (Mellon and Bayer, 2001), which is approximately 2-fold lower than that required to increase plasma IL-6 (Fig. 2), thus suggesting that corticosterone is increased before the elevation of plasma IL-6. Although these two effects of morphine appear related there is evidence that the elevation of plasma corticosterone is independent of the increase in plasma IL-6. The promoter for the IL-6 gene

contains a glucocorticoid response element that negatively regulates IL-6 gene transcription (Ray et al., 1990). Additionally, chlorisondamine pretreatment completely blocked the elevation of plasma IL-6, but did not block the elevation of plasma corticosterone (data not shown). Taken together, the elevation of corticosterone after acute morphine treatment does not appear to be the adrenal cortical factor that increases plasma IL-6. A second potential adrenal-derived factor is the proinflammatory cytokine IL-1 β , which is localized within the adrenal cortex (Schultzberg et al., 1989) and stimulates the release of IL-6 from adrenal cortical cells (Judd and MacLeod, 1995) as well as many other tissues (for review, see Heinrich et al., 1990). Acute morphine treatment to mice has been shown to increase IL-1 β and tumor necrosis factor- α levels released from phytohemagglutinin-stimulated splenocyte cultures (Pacifci et al., 2000), yet it is unknown whether this elevation also occurs in the adrenal gland. Collectively, these findings suggest that an adrenal cortex derived factor such as corticosterone may not be involved in this effect of acute morphine treatment, whereas the cytokine IL-1 β may potentially be involved in this response.

In summary, acute morphine administration rapidly increases plasma IL-6 levels (within 30 min) largely through activation of central opioid receptors. This effect of morphine is prolonged (at least 2 h) and is mediated through stimulation of the autonomic ganglia. The adrenalectomy studies suggest that the effect of morphine to elevate plasma IL-6 is adrenal-dependent. An intact adrenal cortex appears to be largely required for the morphine-induced effect on plasma IL-6, because adrenalectomy, and not adrenal demedullation, completely prevented the morphine-induced elevation of plasma IL-6. This observation is unique, because few responses to date have implicated the autonomic innervation of the adrenal cortex in mediating effects on the immune system. Whether the mechanism of the increased plasma IL-6 levels is due to the release of IL-6 directly from adrenal cortical cells or indirectly via an adrenal cortex derived factor remains to be determined. These data suggest that opioid administration for clinical uses, such as analgesia and/or anesthesia or abuse via illicit use may potentially alter plasma IL-6 homeostasis.

References

- Akira S, Taga T, and Kishimoto T (1993) Interleukin-6 in biology and medicine. *Adv Immunol* **54**:1–78.
- Appel NM, Kiritsy-Roy JA, and Van Loon GR (1986) Mu receptors at discrete hypothalamic and brainstem sites mediate opioid peptide-induced increases in central sympathetic outflow. *Brain Res* **378**:8–20.
- Bayer BM, Gastonguay MR, and Hernandez MC (1992) Distinction between the in vitro and in vivo inhibitory effects of morphine on lymphocyte proliferation based on agonist sensitivity and naltrexone reversibility. *Immunopharmacology* **23**:117–124.
- Bertolucci M, Perego C, and De Simoni MG (1996) Central opiate modulation of peripheral IL-6 in rats. *Neuroreport* **7**:1181–1184.
- Castell JV, Geiger T, Gross V, Andus T, Walter E, Hirano T, Kishimoto T, and Heinrich PC (1988) Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat. *Eur J Biochem* **177**:357–361.
- Clarke PBS, Chaudieu I, El-Bizri H, Boksa P, Quik M, Esplin BA, and Capek R (1994) The pharmacology of the nicotinic antagonist, chlorisondamine, investigated in rat brain and autonomic ganglion. *Br J Pharmacol* **111**:397–405.
- Conway EL, Brown MJ, and Dollery CT (1983) Plasma catecholamine and cardiovascular responses to morphine and D-ala²-d-leu⁵-enkephalin in conscious rats. *Arch Int Pharmacodyn Ther* **265**:244–258.
- De Simoni MG, De Luigi A, Gemma L, Sironi M, Manfredi A, and Ghezzi P (1993) Modulation of systemic interleukin-6 induction by central interleukin-1. *Am J Physiol* **265**:R739–R742.
- Fecho K, Maslonek KA, Coussons-Read ME, Dykstra LA, and Lysle DT (1994) Macrophage-derived nitric oxide is involved in the depressed concanavalin A responsiveness of splenic lymphocytes from rats administered morphine in vivo. *J Immunol* **152**:5845–5852.
- Flores LR, Dretchen KL, and Bayer BM (1996) Potential role of the autonomic nervous system in the immunosuppressive effects of acute morphine administration. *Eur J Pharmacol* **318**:437–446.
- Gadient RA, Lachmund A, Unsicker K, and Otten U (1995) Expression of interleukin-6 (IL-6) and IL-6 receptor mRNAs in rat adrenal medulla. *Neurosci Lett* **194**:17–20.
- Gonzalez-Hernandez JA, Bornstein SR, Ehrhart-Bornstein M, Späth-Schwalbe E, Jirikowski G, and Scherbaum WA (1994) Interleukin-6 messenger ribonucleic acid expression in human adrenal gland *in vivo*: new clue to a paracrine or autocrine regulation of adrenal function. *J Clin Endocrinol Metab* **79**:1492–1497.
- Heinrich PC, Castell JV, and Andus T (1990) Interleukin-6 and the acute phase response. *Biochem J* **265**:621–636.
- Hernandez MC, Flores LR, and Bayer BM (1993) Immunosuppression by morphine is mediated by central pathways. *J Pharmacol Exp Ther* **267**:1336–1341.
- Jacobson L, Akana SF, Cascio CS, Scribner K, Shinsako J, and Dallman MF (1989) The adrenocortical system responds slowly to removal of corticosterone in the absence of concurrent stress. *Endocrinology* **124**:2144–2152.
- Judd AM and MacLeod RM (1995) Differential release of tumor necrosis factor and IL-6 from adrenal zona glomerulosa cells *in vitro*. *Am J Physiol* **268**:E114–E120.
- Kesse WK, Parker TL, and Coupland RE (1988) The innervation of the adrenal gland. I. The source of pre- and postganglionic nerve fibres to the rat adrenal gland. *J Anat* **157**:33–41.
- Kitamura H, Okamoto S, Shimamoto Y, Morimatsu M, Terao A, and Saito M (1998) Central IL-1 differentially regulates peripheral IL-6 and TNF synthesis. *Cell Mol Life Sci* **54**:282–287.
- LeMay LG, Vander AJ, and Kluger MJ (1990) The effects of psychological stress on plasma interleukin-6 activity in rats. *Physiol Behav* **47**:957–961.
- Lenczowski MJP, Bluthé R-M, Roth J, Rees GS, Rushforth DA, Van Dam AM, Tilders FJH, Dantzer R, Rothwell NJ, and Luheshi GN (1999) Central administration of rat IL-6 induces HPA activation and fever but not sickness behavior in rats. *Am J Physiol* **276**:R652–R658.
- Lysle DT, Hoffman KE, and Dykstra LA (1996) Evidence for the involvement of the caudal region of the periaqueductal gray in a subset of morphine-induced alterations of immune status. *J Pharmacol Exp Ther* **277**:1533–1540.
- Martin WR (1984) Pharmacology of opioids. *Pharmacol Rev* **35**:283–323.
- Matta SG, Beyer HS, McAllen KM, and Sharp BM (1987) Nicotine elevates rat plasma ACTH by a central mechanism. *J Pharmacol Exp Ther* **243**:217–226.
- Mellon RD and Bayer BM (1998) Role of central opioid receptors subtypes in morphine-induced alterations in peripheral lymphocyte activity. *Brain Res* **789**:56–67.
- Mellon RD and Bayer BM (1999) The effects of morphine, nicotine and epibatidine on lymphocyte activity and hypothalamic-pituitary-adrenal axis responses. *J Pharmacol Exp Ther* **288**:635–642.
- Mellon RD and Bayer BM (2001) Reversal of acute effects of high dose morphine on lymphocyte activity by chlorisondamine. *Drug Alcohol Depend* **62**:141–147.
- Moerimalam HS, Enderit E, Ackermans MT, Van Lanschot JJB, Sauerwein HP, and Romijn JA (1998) The opiate sufentanil alters the inflammatory, endocrine, and metabolic responses to endotoxin in dogs. *Am J Physiol* **275**:E440–E447.
- Nussdorfer GG and Mazzocchi G (1998) Immune-endocrine interactions in the mammalian adrenal gland: facts and hypotheses. *Int Rev Cytol* **183**:143–184.
- Pacifci R, Di Carlo S, Bacosi A, Pichini S, and Zuccaro P (2000) Pharmacokinetics and cytokine production in heroin and morphine-treated mice. *Int J Immunopharmacol* **22**:603–614.
- Paxinos G and Watson C (1997) *The Rat Brain In Stereotaxic Coordinates*. Academic Press, New York.
- Ray A, LaForge KS, and Sehgal PB (1990) On the mechanism for efficient repression of the interleukin-6 promoter by glucocorticoids: enhancer, TATA box, and RNA start site (Inr motif) occlusion. *Mol Cell Biol* **10**:5736–5746.
- Schneider JA and Moore RF (1955) Electrophysiological investigation of chlorisondamine dimethochloride (EcolidTM). A new ganglionic blocking agent. *Proc Soc Exp Biol Med* **89**:450–453.
- Schultzberg M, Andersson C, Uden A, Troye-Blomberg M, Svenson SB, and Bartfai T (1989) Interleukin-1 in adrenal chromaffin cells. *Neuroscience* **30**:805–810.
- Schwabe M, Cox GW, Bosco MC, Prohaska R, and Kung HF (1996) Multiple cytokines inhibit interleukin-6-dependent murine hybridoma/plasmacytoma proliferation. *Cell Immunol* **168**:117–121.
- Van Loon GR, Appel NM, and Ho D (1981) β -Endorphin-induced stimulation of central sympathetic outflow: β -endorphin increases plasma concentrations of epinephrine, norepinephrine, and dopamine in rats. *Endocrinology* **109**:46–53.
- Van Snick J, Cayphas S, Vink A, Uyttenhove C, Coulie PG, Rubira MR, and Simpson RJ (1986) Purification and NH₂-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. *Proc Natl Acad Sci USA* **83**:9679–9683.
- Weber RJ and Pert A (1989) The periaqueductal gray matter mediates opiate-induced immunosuppression. *Science (Wash DC)* **245**:188–190.
- Zhou D, Kusnecov AW, Shurin MR, DePaolis M, and Rabin BS (1993) Exposure to physical and psychological stressors elevates plasma interleukin 6: relationship to the activation of hypothalamic-pituitary-adrenal axis. *Endocrinology* **133**:2523–2530.
- Zubelewicz B, Braczkowski R, Renshaw D, and Harbuz MS (1999) Central injection of morphine stimulates plasma corticosterone and interleukin (IL)-6 and IL-6 R mRNAs in the pituitary and adrenals in adjuvant-induced arthritis. *J Biol Regul Homeost Agents* **13**:103–109.

Address correspondence to: Dr. Barbara M. Bayer, Department of Neuroscience, Georgetown University Medical School, EG12, NRB, 3970 Reservoir Rd., NW, Washington, DC 20007. E-mail: bayerb@gusun.georgetown.edu