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Involvement of brain opiate receptors in the immune-suppressive effect of morphine

(intracerebroventricular injection/naltrexone/natural killer cells/*N*-methyl morphine)

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ABSTRACT **We previously reported that a single systemic injection of a high dose of morphine (≥20 mg/kg) transiently suppresses splenic natural killer cell cytotoxicity in rats. The present study examined the possibility that the immune-suppressive effect of morphine is mediated by opiate receptors in the brain. Supporting this hypothesis, we found that morphine (20 or 40 μg) injected into the lateral ventricle suppressed natural killer cell activity to the same degree as a systemic dose higher by three orders of magnitude. This effect was blocked by an opiate antagonist, naltrexone. Natural killer cell activity was unaffected by systemic administration of *N*-methylmorphine, a morphine analogue that does not cross the blood–brain barrier. These data implicate opiate receptors in the brain in morphine-induced suppression of natural killer cell cytotoxicity.**

Considerable evidence suggests that the nervous system and endocrine system can regulate immune function. Organs and tissues of the immune system, including the thymus, spleen, bone marrow, and lymph nodes, are innervated by the autonomic nervous system (1, 2). Brain stimulation or lesions have profound effects on numbers of thymus and spleen cells and on several immune functions (3, 4). Many hormones and neurotransmitters influence the activity of the immune system. For example, hypophysectomized rats show markedly impaired cell-mediated immunity that can be restored by growth hormone replacement (5). Also, receptors for various hormones and neurotransmitters (e.g., corticosteroids, insulin, prolactin, growth hormone, gonadal hormones, acetylcholine, and opioids) have been found on cells of the immune system (6). Finally, it has been shown that learning (7), and factors such as stress, environmental conditions, early experience, and social dominance can alter immune function (8, 9).

We studied the effects of footshock stress and morphine on the cytotoxic activity of natural killer (NK) cells in the spleen of rats (10, 11). NK cells are a subpopulation of lymphocytes that spontaneously recognize and kill selected target cells (12, 13). We found that exposure to an inescapable form of footshock stress known to cause opioid-mediated analgesia suppressed NK activity, whereas exposure to a slightly different form of footshock stress causing an equipotent analgesia independent of opioid mechanisms did not have this effect. Subcutaneous injection of high doses of morphine (≥20 mg/kg) also suppressed NK cytotoxicity. The immune suppressive effects of the opioid form of footshock stress and of morphine were blocked by an opiate antagonist, naltrexone, suggesting mediation by opiate receptors. Morphine-induced NK suppression manifested tolerance after 14 daily injections, although the stress-induced effect did not (14). In the present study we sought to determine if the NK-suppressive effect of morphine is centrally mediated. To this end, we examined the effects on NK activity of morphine administered directly into the lateral ventricle and of systemic administration of a morphine analogue (*N*-methyl morphine) known not to cross the blood–brain barrier.

MATERIALS AND METHODS

Animals. Fischer 344 male rats (90–120 days old; 250–350 g) were maintained on a 12-hr light cycle (lights on 8 a.m.) with free access to food and water.

Intracerebroventricular Cannulation. A single guide cannula was stereotaxically implanted under pentobarbital anesthesia (55 mg/kg, i.p.). The cannula was aimed 2 mm above the right lateral ventricle using the following coordinates: anterior, 1.5 mm; lateral, 1.0 mm; depth, 3.0 mm; flat skull position, with bregma as the reference. Guide cannulas were made of stainless steel (0.40 mm, o.d.; 0.30 mm, i.d.), were fixed to the skull and to stainless steel anchoring screws with dental acrylic, and were fitted with stainless steel stylets (0.27 mm o.d.) of the same length. Immediately after surgery each animal received 10,000 units of penicillin G benzathine injection (intramuscularly). Animals were then individually housed and allowed 2 weeks for recovery during which they were handled daily.

Intracerebroventricular Injections. Injections were made via cannula extending 2 mm below the guide tip. Stainless steel injection cannula (0.28 mm, o.d.; 0.18 mm, i.d.) was connected by polyethylene tubing to a 5-μl microsyringe (Hamilton, Reno, NV; model 7105 N). All intracerebroventricular (i.c.v.) injections were in a volume of 4 μl infused over 60 sec, and cannulas were left in place 30 sec after the injection. Animals were returned to their cages for 3 hr, then anesthetized with halothane, given an i.c.v. injection (4 μl) of alcyan blue dye, splenectomized, and sacrificed. Only the results from animals with confirmed staining of the lateral ventricle are reported.

NK Cytotoxicity Assay. Spleens were dissociated into a single-cell suspension, and NK activity was measured in a standard 4-hr chromium release assay using radiolabeled YAC-1 murine lymphoma cells as targets. Spleen cells were washed twice in phosphate-buffered saline (40 g of NaCl/1 g of KCl/1 g of KH_2PO_4/5.75 g of Na_2HPO_4, pH 7.3/H_2O to 1 liter) and adjusted to a final concentration of 10^7 cells per ml in complete media [RPMI 1640 media supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 0.1% Genta-

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Abbreviations: i.c.v., intracerebroventricular(ly); NK, natural killer; E/T, effector/target.

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micin, and 1% of each of the following: L-glutamine, nonessential amino acids, and sodium pyruvate]. YAC-1 cells were labeled by incubation with chromium-51 for 2 hr at 37°C, after which their concentration was adjusted to 10^5 cells per ml. Mixtures of 100 μl of spleen cell suspension and 100 μl of labeled target cells were cocultured in 96-well V-bottom microtiter plates at 100:1, 50:1, and 25:1 effector/target (E/T) ratios. After a 4-hr incubation, plates were centrifuged at 900 × *g* for 10 min, and 100 μl of the supernatant was recovered from each well. The amount of radioactivity (measured as cpm) was determined using a γ counter. This count was used to calculate percent specific lysis, using the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Aliquots of 100 μl of supernatant from wells in which only YAC-1 cells were incubated served to determine both spontaneous and maximum release. Maximum release was calculated as 80% of the cpm of 100 μl of supernatant after the YAC-1 cells were remixed with the media.

Data Analysis. Data were analyzed using analysis of variance and post hoc Tukey tests for specific comparisons (15).

RESULTS

In a typical NK assay, percent specific lysis of splenocytes from control rats was 12, 25, and 45 at 25:1, 50:1, and 100:1 E/T ratios, respectively. Spontaneous lysis ranged from 13 to 15% of maximum chromium release.

In the first experiment, we examined the dose-related effect of i.c.v.-administered morphine. Rats were injected with a single i.c.v. dose of morphine (10, 20, or 40 μg) or with saline. At doses of 20 or 40 μg, morphine significantly suppressed the cytotoxic activity of splenic NK cells at E/T ratios of 50:1 and 100:1 compared to the saline control group (Fig. 1*A*). i.c.v.-injected morphine had no effect at a dose of 10 μg.

To examine whether the effect of i.c.v.-injected morphine on NK cells could be blocked by naltrexone treatment, in the next experiment rats were injected systemically with either naltrexone (10 mg/kg, s.c.) or saline. Ten minutes later, half the rats of each drug treatment group were injected i.c.v. with morphine (40 μg); the other rats were injected i.c.v. with saline. Although i.c.v.-injected morphine once again significantly suppressed NK cytotoxicity in animals pretreated with saline, this effect was blocked in animals pretreated with naltrexone (Fig. 1*B*). Naltrexone alone had no significant effect on NK cytotoxicity.

We next sought to determine if i.c.v.-administered naltrexone blocked the NK-suppressive effect of morphine given systemically at doses previously shown to be effective (30 and 50 mg/kg, s.c.) (10). Rats were injected i.c.v. with naltrexone (0.5, 1.0, and 25 μg) both 10 min before and 80 min after systemic injection of morphine. Other rats were treated with naltrexone systemically (2, 4, and 10 μg/kg, s.c.) both 10 min before and 80 min after systemic injection of morphine. Rats injected with saline (i.c.v., s.c., or both) served as controls.

Morphine was completely ineffective in reducing NK cytotoxicity in rats given 25 μg of naltrexone i.c.v. Even at doses of naltrexone as low as 0.5 and 1.0 μg i.c.v., the effect of morphine was attenuated and no longer statistically different from control values. Spleen cells from rats given

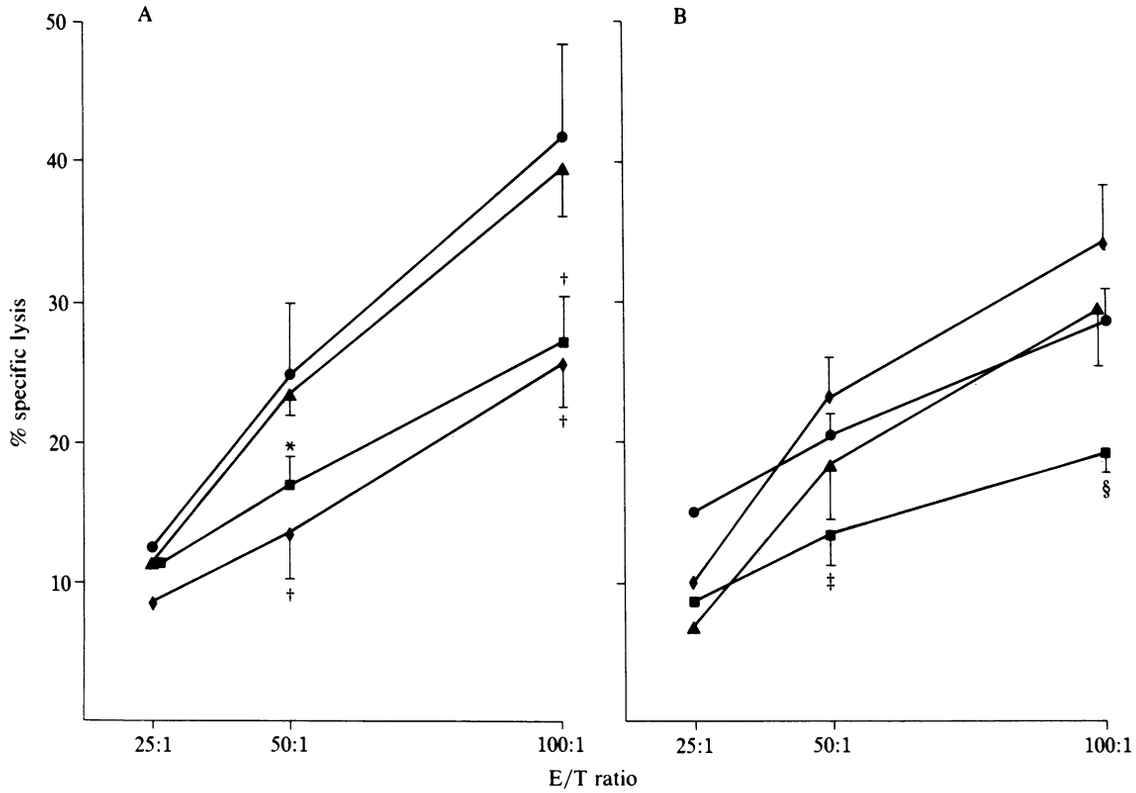

FIG. 1. (*A*) Effect of i.c.v.-administered morphine [10 (▲), 20 (■), and 40 μg (◆)] on NK cell cytotoxicity expressed as percent specific chromium-51 release at three E/T cell ratios. Vertical bars indicate the standard error of the mean; $n = 6$ rats per group. (*B*) Effects of morphine and naltrexone on NK cell cytotoxicity. Rats were injected subcutaneously with either naltrexone ($n = 14$) (10 mg/kg) or saline ($n = 14$). Ten minutes later half the rats from each drug treatment group were injected i.c.v. with morphine (40 μg); the remaining rats were injected with i.c.v. saline. ◆, group injected first with morphine then with naltrexone; ▲, group injected first with saline then with naltrexone; ●, group injected twice with saline; ■, group injected first with morphine then with saline. *Significantly different ($P < 0.05$) or †($P < 0.01$) compared to saline-treated (●) controls or to rats treated with 10 μg of morphine. ‡Significantly different ($P < 0.01$) compared to groups injected only with saline or with morphine and naltrexone. §Significantly different ($P < 0.01$) compared to other three groups.

roughly equivalent doses of naltrexone systemically (2, 4, and 10 μg/kg, s.c.) also failed to show significant NK suppression to systemically administered morphine.

Finally, we tested the effect of systemic administration of a quaternary derivative of morphine, *N*-methyl morphine, known not to cross the blood–brain barrier (16, 17). Using a previously described procedure (18), we first determined that a dose of 40 mg/kg (s.c.) of *N*-methyl morphine inhibited intestinal motility to the same degree as 30 mg/kg (s.c.) of morphine. Groups of rats were then injected with a single dose of morphine (30 mg/kg, s.c.) or *N*-methyl morphine (40 mg/kg, s.c.). Saline-treated animals served as controls. This experiment was replicated on three different days. Percent specific chromium release in the control group (averaged over the three E/T ratios) was 32% on the first day, 32% on the second day, and 15% on the third day. The corresponding values for the morphine group were 27, 24, and 6% and for the *N*-methyl morphine group were 34, 35, and 14%. To correct for daily variations in percent specific chromium release, scores for each animal were transformed to percent of the mean of untreated control values for that day and then pooled.

Systemically administered morphine significantly suppressed the cytotoxic activity of splenic NK cells at all three E/T ratios compared to rats injected with saline (controls) and to rats injected with *N*-methyl morphine (Fig. 2). *N*-methyl morphine had no significant effect on NK activity compared to saline controls.

DISCUSSION

The present findings confirm our previous observation that systemically administered morphine suppresses the cytotoxic activity of NK cells in the rat (10, 11). They also show that morphine injected i.c.v. suppresses NK activity, and the dose necessary to induce the effect is 3 orders of magnitude smaller than the required dose given systemically. The NK suppression induced by i.c.v.-injected morphine is blocked by naltrexone; and *N*-methyl morphine, a morphine analogue that does not cross the blood–brain barrier (16, 17), has no effect on NK cytotoxicity when given systemically. These findings suggest that the immune suppressive effect of morphine is mediated by opiate receptors in the brain.

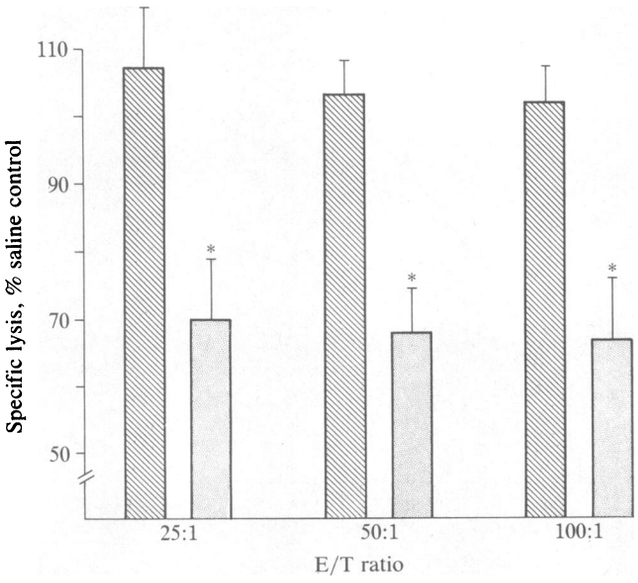

FIG. 2. The effects of subcutaneously administered morphine (stippled bars) (30 mg/kg) or *N*-methyl morphine (hatched bars) (40 mg/kg) on NK cell cytotoxicity expressed as percent of the mean of saline-treated controls. Results are shown at three E/T ratios. Vertical bars indicate the standard error of the mean; $n = 19$ rats per group. *Significantly different ($P < 0.01$) compared to both saline- and *N*-methyl morphine-treated groups.

Several physiological mechanisms could account for this effect of morphine on NK cells. Morphine might suppress NK activity by inducing or modulating the release of certain hormones. For example, adrenocorticotropic hormone and glucocorticoids are known to suppress immune function, including NK activity (19, 20); and 10 μg of i.c.v.-administered morphine has been shown to significantly elevate plasma corticosterone levels in rats (21). Alternatively, morphine could suppress NK activity by stimulating central sympathetic outflow. Centrally administered β-endorphin activates sympathetic outflow to both the adrenal medulla and sympathetic nerve endings (22), and epinephrine and norepinephrine have been shown to suppress immune function (23, 24). In fact, in an experimental paradigm closely resembling ours in regard to dose and post-drug testing time, i.c.v.-injected morphine (30 μg) elevated plasma levels of these catecholamines in rats over a 3-hr observation period (25). Anatomical data indicate that the spleen is innervted by sympathetic nerve fibers that have access to lymphocytes and can modulate lymphocyte function (1, 2). Thus, catecholamines could suppress splenic NK activity either via the bloodstream or after local release from sympathetic nerve endings.

With regard to the underlying immunological mechanisms, the suppression of NK cytotoxicity observed in this study could be attributed to selective emigration of NK cells from the spleen, to less effective target cell recognition and/or binding, to diminished cytotoxic capability, or to changes in the composition of other immunological cells that regulate NK activity.

The present results are consistent with earlier reports demonstrating central nervous system modulation of NK activity. Lesions of the preoptic anterior hypothalamic area in rats (26) or of the tuberoinfundibular region of the hypothalamus in mice (27) suppress NK activity. Similarly, lesions of the left, but not right, cerebral neocortex in mice selectively impair NK cell cytotoxicity and the response of T cells to mitogenic stimulation without affecting B cells or macrophages (28). Hypophysectomy results in profound suppression of NK cells, and this effect is reversed by growth hormone replacement (5, 26). It is interesting to note in this regard that impaired NK activity has been associated with several human neurological disorders, such as multiple sclerosis (29) and Alzheimer disease (30). Shared antigenic determinants between NK cells and nervous tissue have been reported (31), suggesting a possible developmental and functional parallelism.

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