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Tolerance and Crosstolerance to the Suppressive Effects of Cocaine and Morphine on Lymphocyte Proliferation

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BAYER, B. M., M. C. HERNANDEZ AND X. Z. DING. *Tolerance and crosstolerance to the suppressive effects of cocaine and morphine on lymphocyte proliferation.* PHARMACOL BIOCHEM BEHAV **53**(1) 227-234, 1996.—The effects of acute or daily exposure to either cocaine or morphine on lymphocyte proliferative responses and NK cytolytic activity were determined. Two hours following the IV infusion of cocaine (5 mg/kg), blood lymphocyte proliferative responses were found to be suppressed by 75%. Cocaine had no effect on proliferative responses of thymic or splenic lymphocytes or cytolytic activity of splenic NK cells following acute or 5-day repetitive dosing. Similar to the effects of cocaine, morphine (10 mg/kg) administration was also accompanied by a suppressed blood lymphocyte response, which was no longer apparent 8 days following repeated morphine injections. Animals that had received daily injections of either morphine of cocaine were also found to be resistant to the inhibitory effects of a single dose of morphine or cocaine, respectively. These data suggest repeated exposure to either morphine or cocaine results in the development of an apparent crosstolerant state to further suppression of blood lymphocyte proliferative responses by either drug.

Cocaine Morphine Tolerance Lymphocyte Immunity

THE NATIONWIDE abuse of morphine and cocaine has been shown to be associated with a higher risk for HIV infection (8,38). Therefore, it has been suggested that these drugs may serve as cofactors in the development of AIDS by increasing the susceptibility to infection with the retrovirus. Consistent with this hypothesis was the finding that morphine produced general immunosuppressive effects in both humans and animals (1,15,20,27,32,33). However, relatively few studies have investigated the potential in vivo effects of cocaine on the immune system. Additionally, to our knowledge, there are few studies available that have examined the potential effects of concurrent exposure to both cocaine and morphine on the immune system. This may be particularly relevant, considering the increased popularity of the combined use of these drugs.

In contrast to the well-documented immunosuppressive effects of morphine, studies of the in vivo effects of cocaine on the immune system have often yielded contradictory effects. We have recently reported that acute exposure of rats to cocaine (5 mg/kg; IV) resulted in suppression of blood lymphocyte proliferative responses to mitogen (5). However, daily administration of high doses of cocaine (30–80 mg/kg) to rodents over 1 to 3 weeks were reported to produce only slight changes in antibody titer to *Streptococcus pneumonia* and susceptibility of mice to infection (18,32). These doses have been shown to be accompanied by marked changes in behavior, decreases in body weight, tremors, and death (18,29,34). In contrast to these results, equally high doses of cocaine for 5 days resulted in suppression of the humoral response to sheep red blood cells and cellular immunity as measured by delayed type hypersensitivity response to dinitrofluorobenzene (36). Similarly, decreases in the number of immune cells, macrophage phagocytic activity, and number of plaque-forming cells were accompanied by accelerated tumor cell growth in mice with relatively lower doses of cocaine (5 mg/kg) (24). The discrepancies in the effects of cocaine on the immune system may reflect differences in doses and duration of exposure to cocaine, sex, and species of animals used and the particular immune cell parameter measured (2).

To begin to systematically characterize the potential effects

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of cocaine on the immune system, we chose to administer cocaine IV to rats implanted with indwelling jugular vein cannula. This particular route of administration was chosen for several reasons. First, the pharmacokinetic profile of cocaine following IV and inhalation routes has been demonstrated to be very similar (21) and would, therefore, more closely approximate the human exposure to cocaine. Additionally, recent studies have shown that the neuroendocrine effects of cocaine via the IV route appeared to be more selective and less likely to result in a nonspecific stress effect (30). The irritation produced at the site of injection (6) by other routes of administration may also potentially confound interpretation of whether the effects of cocaine on the immune system are stress or drug related.

In the present studies, we determined the effects of cocaine or morphine on lymphocyte proliferative responses and NK cytolytic activity following either acute or daily administration. Because drug abusers often use cocaine and morphine concurrently, we also examined the effects of the individual drugs on lymphocyte activity in either daily cocaine or morphine treated animals.

METHOD

Animals

Male Sprague-Dawley rats, weighing approximately 200 g on receipt, were purchased from Taconic Laboratories (Germantown, NY). Following receipt, they were allowed to adapt to animal room conditions for at least 5 days prior to surgeries. During this time animals were group housed (four/cage) in polypropylene cages (20 × 16 × 8-½) with metal rung tops fitted with microisolator filter units and food (Purina rat chow) and water available ad lib. The light cycle was automatically controlled (on: 0700 h; off: 1900 h) and room temperature thermostatically controlled to maintain 23°C ± 1°.

Jugular Cannulation and Cocaine Administration

Cannulas were made from 5 cm of vinyl tubing (i.d. 0.5 mm × o.d. 1.0 mm) (Critchley Electronics and Engineering, Dural, N.S.W., Australia) joined to 27 mm silastic tubing (Dow-Corning, Midland, MI) via a 23 gauge (0.75 cm) stainless steel connecting tube. A piece of heat-shrink Teflon tubing (Small Parts, Inc., Miami, FL) was applied and heated over the joint between the silastic and vinyl tubing. To prevent the catheter from being pulled through the skin while taking blood samples, a tygon collar on the polyvinyl tubing end was constructed by making a hole through a piece of tygon tubing (¼″ inner diameter) with a hot 18 gauge needle, threading the vinyl tubing through and securing it with a drop of cyclohexanone (Sigma, St. Louis, MO). Cannulas were prepared at least 24 h in advance to allow the cyclohexanone to set firmly. Prior to all surgeries catheters and surgical instruments were soaked in a 20% Nolvason solution.

Animals were anesthetized with Equithesin (3 ml/kg, IP). A small incision (2–3 cm) was made at the level of the arm and parallel to the midline of the rat. Blunt dissection of the overlaying fascia exposed the jugular vein and two ligatures (4-0 silk) were placed under the vein; the proximal ligature was tied tightly and the distal ligature was looped loosely around the vein. The ends of a slightly curved forceps was placed under the vein, forming a bridge and stopping blood flow. A small incision was made in the vein and the silastic end of the catheter, previously filled with saline, was inserted into the jugular vein and threaded to the level of the atrium. The catheter was flushed with saline (0.2 ml) and blood was withdrawn and flushed back to check for patency. The ligatures were then tightened around the catheter and another ligature secured the catheter to the masseter muscle. The free end of the cannula (vinyl tubing) with the attached tygon collar was passed under the skin and out through the dorsal side of the neck. The cannula was filled with saline containing 1.5% gentamicin (10 mg/ml, Gibco, Grand Island, NY) and the incision over the jugular vein closed with sterile wound clips. Rats were placed in individual cages for 5–7 days before use in an experiment.

On the morning of the experiment, food and water were removed, and each cannula was connected to a 38 cm length of PE-50 tubing threaded through the top of the wire cage cover and attached to a 1 cc syringe filled with heparinized saline. Because the tubing and syringe hung outside the cage, drug infusions were carried out without disturbing the rats. All experiments were initiated between 0700 and 0900 h. Cocaine hydrochloride was obtained from The National Institute of Drug Abuse (Research Triangle Park, NC) and was dissolved in 0.9% sterile saline. A 1 ml solution of cocaine (5 mg/kg) was slowly injected into the jugular cannula in three equal parts (0.33 ml/push) over a 10-min period. A final push of 300 μl of 0.9% saline was delivered to ensure that no residual drug remained in the tubing. Animals were sacrificied by decapitation and trunk blood samples were collected in heparinized tubes at 2 h following cocaine infusion and processed for immune cell measurements as described below.

For chronic studies, animals were similarly injected with either saline or cocaine (5 mg/kg) twice daily (0900 and 1600 h) for 4 days. On day 5, animals received a final injection and were sacrificed 2 h later.

Morphine Administration

Morphine sulfate was obtained from the National Institute of Drug Abuse (Research Triangle Park, NC). For SC injections, morphine was dissolved in sterile saline and administered twice a day at 0800 and 1500 h at the following doses:

	Morphine (mg/kg)	
Treatment Day	a.m.	p.m.
1	10	10
2	10	20
3	20	20
4	30	30
5	30	40
6	40	40
7	40	40
8	10	

Animals were sacrificed 2 h after the last dose on day 8. Control animals were similarly injected with equal volumes of saline twice a day.

Blood, Splenic, and Thymic Lymphocyte Proliferation Assay

Whole blood was diluted 1 : 10 with cold culture media (RPMI 1640; Gibco, Grand Island, NY) containing 1% fetal bovine serum (FBS, Biofluids, Rockville, MD) and gentamicin (20 μg/ml, Gibco). Triplicate samples of diluted whole blood (100 μl) were added to 96 well microtiter plates containing 100μl of concanavalin A (Con A) (Sigma) at submaximal and maximal concentrations (1.0, 2.0, or 4.0 μg/culture). The total

volume of each culture was 200 μl. Following incubation for 72 h at 37°C with 5% CO_2, [^{3}H]-methylthymidine (6.7 Ci/mmol, ICN) (0.5 μCi/20μl/culture) was added to the cell cultures, which were then incubated for an additional 24 h. Labeled DNA was collected on glass fiber filters using a 96-well cell harvester (Brandel), and radioactivity was determined by liquid scintillation spectrophotometry (Beta Plate, L.K.B. Pharmacia).

To prepare splenic or thymic lymphocyte suspensions, the organs were removed and placed in cold RPMI-1640 containing 1% FBS and gentamicin (20 μg/ml). Cells were gently teased apart and passed through a fine nylon mesh (Nitex: 40 μm mesh) to remove all aggregates and connective tissue. Following two washes with media, cells were resuspended at a concentration of 5×10^6 cells/ml and added in triplicate to 96-well microtiter plates in 100 μl aliquots. Con A (100 μl) was added at submaximal (0.05 μg/culture) and maximal (0.125 and 0.25 μg/culture) concentrations. Following incubation for 48 h at 37°C with 5% CO_2, ^{3}H-methylthymidine (0.5 μCi/well) was added and cultures incubated for an additional 24 h. Cells were harvested and radioactivity was counted as described for whole blood cultures.

Data were expressed as the mean counts per minute (cpm) per culture of the maximum response of lymphocytes from control and drug-treated animals.

White Blood Cell Number

Total white blood cell (WBC) counts and splenic lymphocyte number were determined by diluting 20 μl of each sample with Hematall (Fisher Scientific Co.) counting buffer (10 ml). Red blood cells were lysed with 100 μl LAS (Fisher Scientific Co.) and samples were then immediately counted using a model ZBI Coulter counter.

Measurement of Natural Killer Activity of Spleen Lymphocytes

Splenic natural killer cytolytic activity was measured by the chromium release assay. Splenic lymphocytes (effector cells) were adjusted with RPMI-1640 containing 5% FBS, to the following concentrations: 1.25, 2.5, 5, and 10×10^6 cell/ml. Triplicate aliquots (100 μl) of each cell concentration were added to 96-well round-bottom microtiter plates. YAC-1 cells, a murine lymphoma, served as the target cell line. This cell line was maintained in suspension culture in RPMI-1640 with 5% FBS at 37°C and 5% CO_2. To label cells, YAC-1 cells were washed in serum-free medium, adjusted to 10×10^6 cells/ml, and 0.3 ml of the suspension was incubated for 1 h with 100 μCi chromium-51 (Na_2-$^{51}CrO_4$) (ICN Radiochemicals, Irvine, CA). Cells were then washed twice in RPMI-1640

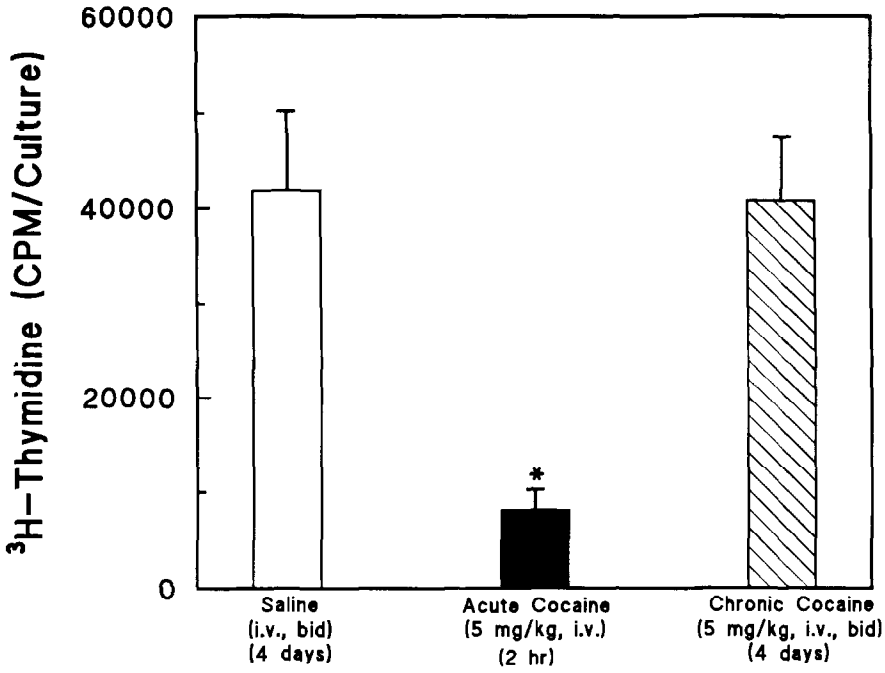

FIG. 1. Development of tolerance to the suppressive effects of cocaine on blood lymphocyte proliferative responses. Animals were implanted with indwelling jugular cannula as described in the Method section. Approximately 5 days following surgeries the animals were divided into three groups. Two groups were slowly infused (10 min) with a saline solution (1 ml), and the third group was administered cocaine (5 mg/kg; 1 ml) twice a day (0900 h and 1600 h) for 4 days. On day 5, one saline-treated group received another infusion of saline (saline), the second saline treatment group received 5 mg/kg cocaine (acute cocaine), and the cocaine-treated group received another dose of 5 mg/kg cocaine (chronic cocaine). Two hours later, blood samples were collected in heparinized tubes. Maximum lymphocyte proliferative responses to Con A were determined as described in the Method section. Results are expressed as the mean ± SEM [^{3}H]thymidine cpm/culture of four to six animals per group. *Significantly different from saline-treated controls, $F(2, 16) = 10.37, p \leq 0.05$.

with 5% FBS and resuspended to a final volume of 0.1×10^6 cells/ml in the same medium. Triplicate samples of labeled YAC-1 cells (100 μl) were counted by liquid spectrophotometry to determine total cpm. Labeled YAC-1 cells (100 μl) were added to plates containing the spleen cell suspension as described above. To determine the amount of chromium released by YAC-1 cells alone (spontaneous cpm), labeled cells (100 μl) were incubated with RPMI-1640 with 5% FBS (100 μl). After a 4-h incubation at 37°C with 5% CO_2, 100 μl supernatant was collected and counted by liquid scintillation spectrophotometry. Results are expressed as mean % specific lysis according to the following formula:

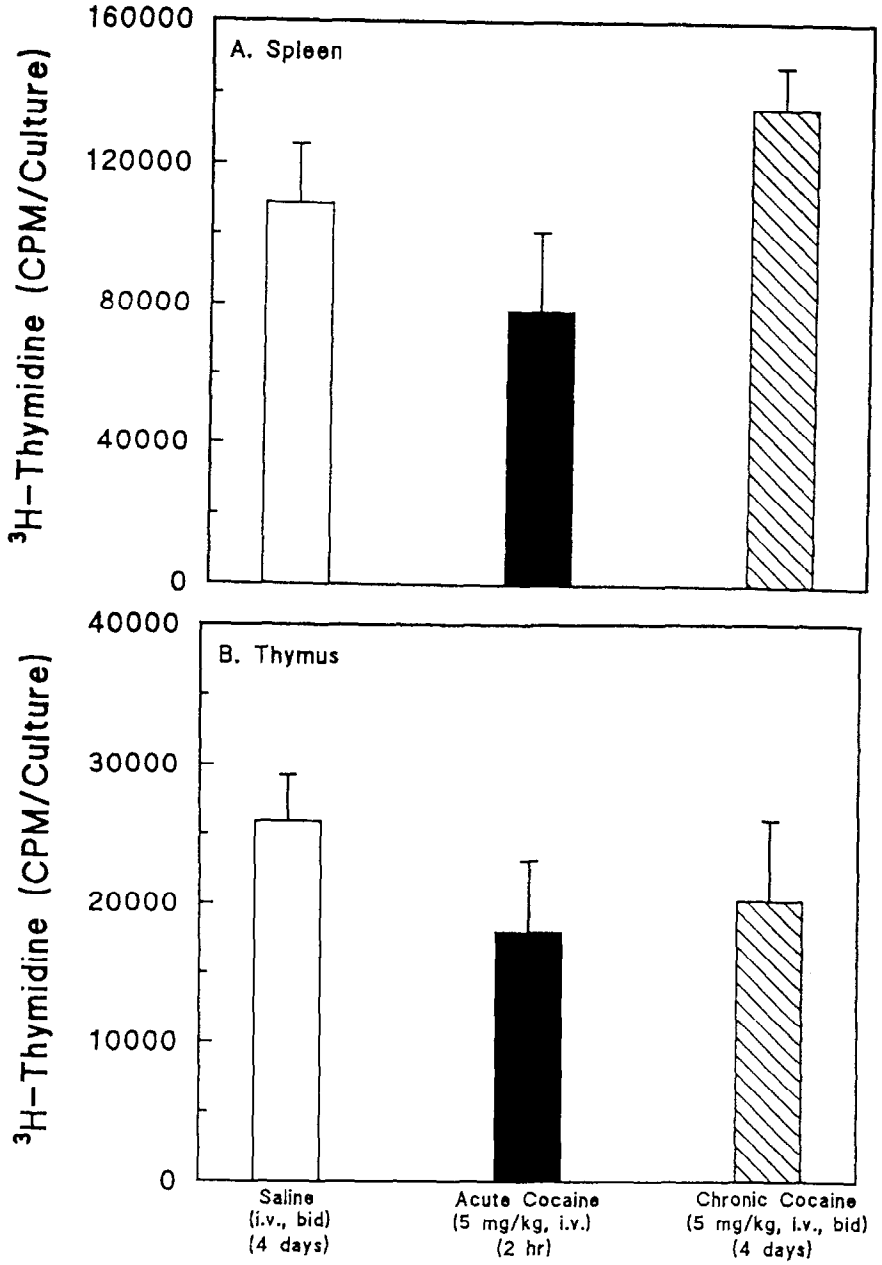

FIG. 2. Lack of an effect of acute or chronic cocaine exposure on splenic or thymic lymphocyte proliferative responses. Animals were treated as described in the legend to Fig. 1. Two hours following the last injection, splenic (A) and thymic (B) lymphocyte proliferative responses to Con A were determined as described in the Method section. Results are expressed as the mean ± SEM [^{3}H]thymidine cpm/culture of five to eight animals per group.

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

Statistical Analysis of Data

All data were expressed as the mean values ± SEM of each treatment group of animals. Each experiment was repeated at least two times. Differences between more than two means were analyzed by either a one- or two-way variance (ANOVA) with replication and individual comparisons were made using a post hoc Newman-Keuls test. The criterion for significance in all comparisons was $p \leq 0.05$.

RESULTS

Effects of Acute and Chronic Cocaine Administration on Proliferative Responses of Lymphocytes From Different Lymphoid Tissues

Animals with indwelling jugular cannulas were infused with cocaine (5 mg/kg) and sacrificed 2 h later. Blood lymphocyte proliferative responses to Con A were found to be inhibited by greater than 75% in cocaine-treated animals compared to responses of saline injected animals (Fig. 1). When cocaine (5 mg/kg) was administered twice daily for 4 days, a challenge dose of cocaine (5 mg/kg) on day 5 had no significant effect on blood lymphocyte response (Fig. 1).

In contrast to the effects on blood lymphocyte responses, acute exposure (2 h) to cocaine was without significant effects on the proliferative responses of splenic (Fig. 2A) or thymic (Fig. 2B) lymphocyte mitogenesis. Similarly, 2 h following the last injection of cocaine to daily treated animals, no significant effects on lymphocyte proliferation were observed in spleen (Fig. 2A) or thymus (Fig. 2B).

Lack of Effects of Acute or Chronic Administration on Splenic NK Cytolytic Activity

The effects of acute and chronic cocaine administration on splenic NK cell activity were also determined. As shown in Fig. 3, neither acute (2 h) nor daily exposure to cocaine resulted in a significant effect on NK cytolytic activity at any effector:target cell ratios examined.

Lack of Effects of Cocaine or Morphine in Daily Cocaine-Treated Rats

To begin to examine the potential interactions between cocaine and morphine on the immune system, the effects of acute morphine exposure in daily cocaine-treated animals were determined. Saline or cocaine (5 mg/kg) was injected twice a day for 4 days, as previously described. On day 5, the saline- and cocaine-treated animals all received a SC injection of saline (Saline) or cocaine 5 mg/kg (Acute cocaine). The daily treated cocaine animals were also divided into two groups; each received a final challenge dose of cocaine (5 mg/kg) and either SC saline (Chronic cocaine) or morphine (10 mg/kg) (Chronic cocaine + morphine). Blood responses of saline-treated animals that received cocaine (5 mg/kg) were again

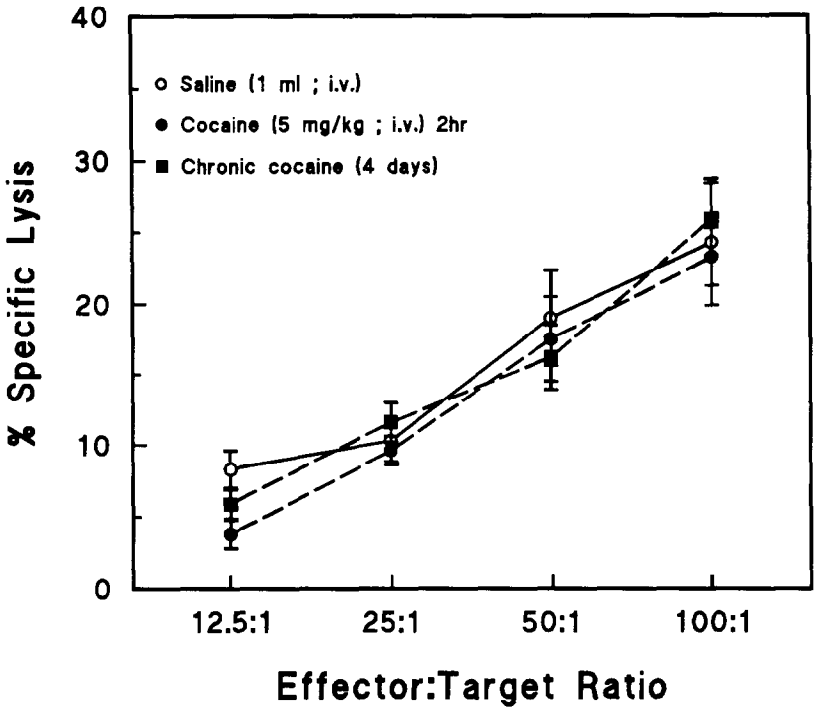

FIG. 3. Lack of an effect of acute or chronic cocaine treatment on splenic NK cytolytic activity. Animals were intravenously injected with saline or cocaine (5 mg/kg) as described in the legend of Fig. 1. Splenic NK cytolytic activity was determined as described in the Method section. Results are expressed as the mean ± SEM percent specific lysis of saline and cocaine-treated animals for six animals per group.

significantly suppressed (Figs. 1 and 4). Also, as shown in Fig. 1, blood lymphocyte responses were no longer inhibited in daily treated cocaine animals and were similar in magnitude to the responses of saline-treated animals (Fig. 4). Previous studies have shown that a single injection of morphine (10 mg/kg) resulted in a 70–90% inhibition of blood lymphocyte proliferative responses (3,4). However, in daily cocaine-treated rats, this dose had no effect on proliferative responses (Fig. 4). A significant difference was observed between the responses of the acute cocaine treatment group and the chronic cocaine group receiving morphine (Fig. 4).

Lack of Effects of Cocaine or Morphine in Daily Morphine-Treated Animals

In the next experiment, the effects of acute and daily exposure to morphine on blood lymphocyte responses were examined. The potential interaction between cocaine and morphine was also evaluated in daily morphine-injected animals. All animals within the study were implanted with jugular cannula prior to the onset of the chronic injections. As described in the Method section, animals were injected subcutaneously with either saline or increasing doses of morphine twice a day for 7 days. On the day of the experiment, the saline- and morphine-treated animals were divided in groups containing six to seven rats per group. The saline-treated animals all received an IV injection of saline and a SC injection of either saline (Saline) or 10 mg/kg morphine (Acute morphine). The daily morphine-treated group was similarly divided into two groups; each received a final challenge dose of morphine (10 mg/kg) and either IV saline (Chronic morphine) or cocaine (5 mg/kg) (Chronic morphine + cocaine). As previously reported (3), 2 h following the injection of morphine, lymphocyte proliferative responses were inhibited by 60%. However, following 7 days of morphine treatment, lymphocyte responses were no longer suppressed by this dose (Fig. 5). Furthermore, the administration of cocaine (5 mg/kg) to daily morphine-treated animals had no effect on blood lymphocyte proliferative responses (Fig. 5).

DISCUSSION

The results in the present study demonstrated that 2 h following an IV infusion of cocaine in rats, blood lymphocyte proliferative responses were significantly suppressed by greater than 60%. However, upon repetitive, intermittent exposure to cocaine (5 mg/kg) over 4 days, blood lymphocyte responses were no longer significantly suppressed. The inhibitory effects were unique to lymphocytes from peripheral blood, since neither splenic nor thymic lymphocyte responses were altered by either acute or repetitive cocaine exposure. Additionally, cytolytic activity of splenic NK cells was also not inhibited following cocaine treatment.

The reasons for the apparent selective effect of cocaine within 2 h on blood lymphocyte proliferative responses are not clear at the present time. Similar differences in sensitivity

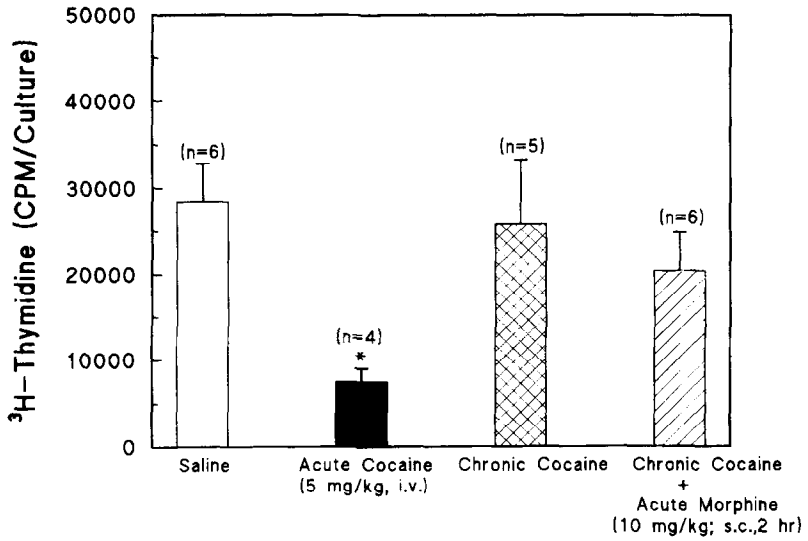

FIG. 4. Development of tolerance and morphine crosstolerance to the immunosuppressive effects of cocaine. Animals were implanted with jugular catheters and allowed to recover for approximately 5 days. Rats were divided into two treatment groups: one receiving saline, and the other cocaine (5 mg/kg) IV injections twice a day for 4 days. On day 5, half of the saline-treatment group received another infusion of saline (saline) and the other half received 0.5 mg/kg cocaine (acute cocaine). Half of the chronic cocaine treatment group received another infusion of 5 mg/kg cocaine and the other half received IV cocaine and a SC injection of morphine (10 mg/kg). Two hours after the last injections blood samples were collected in heparinized tubes. Blood lymphocyte proliferative responses to Con A were determined as described in the Method section. Results are expressed as the mean ± SEM [^{3}H]thymidine cpm/culture of four to six animals per group. *Significantly different from saline-treated controls, $F(3, 20) = 4.05, p \leq 0.05$.

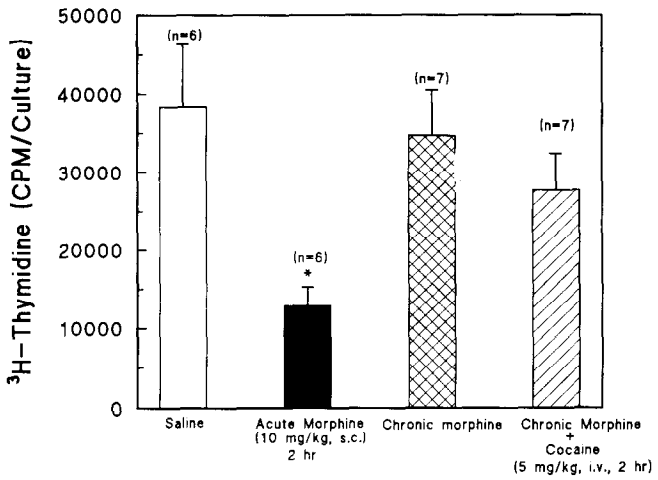

FIG. 5. Development of tolerance and cocaine crosstolerance to the immunosuppressive effects of morphine. Animals were implanted with jugular catheters and divided into two treatment groups receiving either saline or morphine injections twice a day for 7 days as described in the Method section. On the day of the experiment, the saline-treatment group received an IV injection of saline and was then divided into two equal halves. One group received a SC injection of either saline (Saline) or 10 mg/kg morphine (acute morphine). The chronic morphine-treated group was similarly divided into two groups: each received a final challenge dose of morphine (10 mg/kg) and either IV saline or cocaine (5 mg/kg). Blood lymphocyte proliferative responses to Con A were determined as described in the Method section. Results are expressed as the mean ± SEM [^{3}H]thymidine cpm/culture of six to seven animals per group. *Significantly different from saline-treated controls, $F(3, 25) = 4.23, p \leq 0.05$.

to the suppressive effects of morphine on lymphocyte activity have also been shown to be dependent on the tissue source of the lymphocyte (3,4). This may be indicative of an altered sensitivity or a difference in the time required for effects to become significant in lymphocytes from different lymphoid compartments. For example, we have found 4 h following cocaine (5 mg/kg) administration that splenic, but not blood, lymphocyte proliferative responses were significantly suppressed (5). Because cocaine administration is accompanied by both stimulation of the hypothalamic-pituitary-adrenal axis (10,16,26,30) and the sympathetic nervous system (9,22,28), the differences in time required for significant suppression to occur may represent the relative sensitivity of lymphocytes from different compartments to these individual pathways.

In contrast to the suppression of splenic NK cell activity following a single dose of morphine (3,31,37), we report here that cytolytic activity was not altered by either acute or daily cocaine injections. Consistent with this observation was the finding that cocaine had no direct in vitro effect on the activity of either mouse splenic (23) or human blood NK cells. However, it has recently been reported that chronic treatment of mice with cocaine (1 mg/kg/day; 5 days) resulted in a decreased splenic NK activity (17). In humans, a single dose of cocaine was found to rapidly increase NK cell activity due to a rise in the number of circulating NK cells (35). These apparent discrepancies may be related to the considerable differences in experimental design among these studies, including species, dose, duration, and mode of administration of cocaine treatment.

Upon repetitive, intermittent dosing with cocaine (5 mg/kg) we observed the development of an apparent tolerance to the inhibitory effects of cocaine on blood lymphocyte responses. Tolerance to the neuroendocrine, behavioral, neurochemical, and physiological effects of cocaine has also been described (19,22). Using a model very similar to the one used in the present study, single IV injections of cocaine were found to increase ACTH in a dose-dependent (1 to 10 mg/kg) manner, whereas repeated infusions had no effect on plasma concentrations of either ACTH or corticosterone (26). Therefore, should the acute effects of cocaine on blood lymphocytes be the result of elevated plasma corticosterone concentrations, the development of tolerance to these effects may be related to the lack of HPA activation in chronically treated animals. Further studies are warranted to address this possibility more directly.

Similar to cocaine, an apparent tolerance to the suppressive effects of morphine also developed following daily injections. The phenomena of tolerance to other opioid effects has been well documented and thought to involve central mechanisms. We have recently reported that the effects of morphine on lymphocyte proliferative effects also appear to require the activation of central opioid receptors (20). We found that following repetitive morphine exposure, cocaine (5 mg/kg) administration had no significant effect on blood lymphocyte proliferation. Similarly, lymphocyte responses were found to be unaltered to morphine (10 mg/kg) in cocaine tolerant animals. Surprisingly, once tolerance to the suppressive effects of either morphine or cocaine was achieved, animals became crossresistant to the suppressive effects of either drug. The presence of an apparent cross-tolerant state following daily administration of either cocaine or morphine suggests the possibility of a common underlying mechanism for the suppression of blood lymphocyte activity by both drugs.

Interactions between the analgesic and behavioral effects of cocaine and morphine have recently been described. For example, cocaine has been shown to produce analgesia that was antagonized by pretreatment with high doses of naloxone, an opioid receptor antagonist (25). Cocaine has also been shown to mediate some of its effects on immune parameters through endogenous opioid pathways (7). Cocaine abuse was shown to alter T cell E-rosette formation in PBMC of heroin addicts (12,14). This has previously been characterized as an opioid receptor-mediated phenomenon (11,13). Although it has been shown that the immune effects of morphine involve central opioid receptor activation (20), it remains to be determine whether cocaine may also produce immunosuppression through central mediated effects.

Caution should be applied in interpreting that the lack of continued suppression of blood lymphocyte responses in chronically morphine- or cocaine-treated animals represents a normally functioning immune system. For example, chronic use of cocaine has been shown to suppress phagocytic activity (24), decrease T-dependent antibody responses (24), inhibit T suppressor cell activity (2), and suppress delayed-type hypersensitivity reactions (36). Therefore, the absence of drug-induced suppression of lymphocyte activity in daily treated cocaine or morphine animals may be more indicative of an aberrant rather than normal response.

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