

Stress-Induced Suppression of the Immune System after Withdrawal from Chronic Cocaine

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ABSTRACT

Recent evidence suggests that withdrawal from cocaine shares similarities to the stress response. Here, we examine whether withdrawal from chronic cocaine produces immune system alterations and whether the hypothalamic-pituitary-adrenal axis is involved. Sprague-Dawley male rats received cocaine (10 mg/kg i.p., b.i.d.) or saline, followed by 2 h, 1, 2, 4, 6, and 14 days of withdrawal. Proliferation responses of peripheral blood lymphocytes to concanavalin A were significantly suppressed at the 2-h, 1- and 2-day time points, and persisted for up to 6 days during withdrawal from chronic cocaine. Flow cytometric analysis revealed no significant differences in the immunophenotype of blood lymphocytic populations of T cells, B cells, or monocytes at 2 or 6 days of withdrawal from cocaine. Consistent with the suppression in cellular immunity observed in the in

vitro response, the in vivo delayed-type hypersensitivity response was also significantly decreased in cocaine withdrawing animals. Plasma corticosterone levels were significantly elevated 2 and 24 h after cessation of cocaine but returned to basal values by 2 days of withdrawal. The suppressive effects of cocaine withdrawal were no longer observed in either adrenalectomized animals or those treated with the glucocorticoid receptor antagonist mifepristone (RU486), when administered during the first 2 days of withdrawal. These data argue that repeated exposure to cocaine followed by withdrawal leads to an activation of the neuroendocrine stress response, which alters cellular immunity during the initial withdrawal phase and may contribute to an increased susceptibility to infection.

Cocaine is a potent inhibitor of monoamine reuptake at nerve terminals which is thought to contribute to its reinforcing properties, psychomotor stimulant effects, and its euphoric effects (Self and Nestler, 1995). Past studies have demonstrated that permanent changes occur within the central nervous system after chronic cocaine use (Self and Nestler, 1995; Berke and Hyman, 2000) and after cocaine withdrawal (Kuhar and Pilotte, 1996). Alterations in the neurochemical processes that are affected by the pharmacological effects of cocaine abuse have been related to the "crash", "withdrawal", and "extinction" phases reported after cessation of repeated cocaine use (Gauvin et al., 1997). Cessation of chronic cocaine administration in humans who repeatedly used cocaine has been reported to manifest itself in dysphoria, anergia, general depression, anxiety, and restlessness (Gawin et al., 1989). Likewise, others have shown that

cocaine treated animals demonstrate behavioral symptoms upon withdrawal that suggest the development of cocaine dependence (Sarnyai et al., 1995). Although acute cocaine exposure has been reported to have immune effects (Van Dyke et al., 1986; Klein et al., 1991; Stanulis et al., 1997; Pellegrino and Bayer, 1998), little has been reported on the effects of chronic cocaine and even less has been documented regarding the effects of cocaine withdrawal on the immune response. This is particularly surprising considering reports that cocaine abuse and dependence remains a major public health problem (National Institute on Drug Abuse, 2000).

Release of corticotropin-releasing factor (CRF) and subsequent activation of the HPA axis mediate the endocrine, behavioral, and autonomic effects that occur in response to stressors, and there is considerable evidence to suggest that cocaine simulates these pathways as well (Dunn and Berridge, 1990; Mello and Mendelson, 1997). What is becoming apparent is that drug dependence and withdrawal are acting as "pharmacological stressors", leading to similar increases in the HPA axis (Goeders, 1997). Cocaine activates the CRF system, leading to increased ACTH secretion, causing overstimulation of the adrenal cortex and an elevation of cortisol

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ABBREVIATIONS. CRF, corticotropin-releasing factor; HPA, hypothalamic-pituitary-adrenal; ACTH, adrenocorticotropin; WBC, white blood cell; FACs, flow cytometric analysis; PBS, phosphate-buffered saline; MoAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; FCA, Freund's complete adjuvant; Con A, concanavalin A; ANOVA, analysis of variance; DTH, delayed-type hypersensitivity; IL, interleukin; wdl, withdrawal; CGP37849, (E)-(\pm)-2-amino-4-methyl-5-phosphono-3-pentenoic acid.

in humans and corticosterone in rats (Moldow and Frischman, 1987; Rivier and Vale, 1987; Mendelson et al., 1992; Sarnyai et al., 1992; Baumann et al., 1995). In rodents, the stress-like effect of cocaine has been reported to be mediated by CRF secreted from the hypothalamus, because peripheral or central blockade by CRF antiserum or a CRF receptor antagonist suppressed cocaine-induced ACTH and corticosterone responses (Rivier and Vale, 1987; Borowsky and Kuhn, 1991; Sarnyai et al., 1992). Richter and Weiss (1999) reported that rats experiencing cocaine withdrawal for up to 12 h had 400% greater CRF levels in the amygdala compared with controls.

Although it was originally believed that the immune system was autonomous, an intercommunication between the immune system and the central nervous system has been demonstrated. ACTH and corticosterone are neuroendocrine hormones with multiple effects on the immune response. Chronic HPA axis activation and prolonged elevations of corticosteroids is associated with a decrease in immune cell function and changes in lymphocyte distribution (Parrillo and Fauci, 1979). In vitro administration of glucocorticoids has also been shown to decrease T- and B-cell proliferation (Sandi et al., 1992). In naive normal animals physiological elevations of corticosterone may not produce immunosuppression; however, when animals are chronically treated with cocaine they may develop an altered sensitivity to stress such that cocaine withdrawal may compromise the ability of the immune system to function properly. It has been reported that human peripheral blood lymphocytes and cultured mouse splenocytes when exposed to physiological levels of cocaine in vitro were suppressed in the mitogen proliferation assay (Klein et al., 1991). Immunomodulation by cocaine after human immunodeficiency virus infection could alter disease development, as well as overall resistance to a variety of pathogens found frequently in drug users. The present study was designed to evaluate the effects of cocaine withdrawal on the immune system after chronic cocaine administration in rats and to examine the extent to which the neuroendocrine system is involved.

Materials and Methods

Animals. Pathogen-free adult male Sprague-Dawley rats initially weighing 175 to 200 g upon receipt were obtained from Taconic Farms (Germantown, NY). Animals were group-housed, three per cage, with microisolator tops in a temperature- ($23 \pm 1^\circ\text{C}$) and humidity-controlled vivarium under a 12-h light/dark cycle (6:00 AM on, 6:00 PM off). Food and water were freely available (Purina Rat Show; Ralston Purina, St. Louis, MO). All animals were allowed to acclimate for 1 week before use in an experiment or drug administration. Adrenalectomized animals were purchased from Taconic Farms and provided saline (0.9%) drinking solution supplemented with corticosterone 21-acetate (25 ml/ml in 0.2% ethanol) to restore basal corticosterone levels (Jacobson et al., 1989; Zhou et al., 1993). To verify the completeness of the adrenalectomy, plasma corticosterone levels were measured and outliers were eliminated from data analysis.

Drug Administration. Cocaine hydrochloride, purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in (0.9%) sterile isotonic saline, which also served as the control treatment in these studies. The injection volume for all systemic studies was 1 ml/kg, and the route of administration was i.p. In all experiments, rats received 10 mg/kg cocaine per injection. All acute cocaine or saline administration occurred 2 h before being sacrificed. Cocaine injections

in chronic experiments occurred twice a day (9:00 AM and 5:00 PM). Rats received their first injection the evening of day 1, followed by 6 days (b.i.d.) of administration and their last injection the morning of day 8, 2 h before they were sacrificed. In withdrawal studies, the same dosing paradigm was used with the addition of the withdrawal period after the last cocaine injection. The progesterone/corticosterone antagonist mifepristone (RU486) was dissolved in propylene glycol and administered s.c. at 10 mg/kg b.i.d. for 2 days (Philibert, 1984). Vehicle controls receive propylene glycol (1 ml/kg).

Mitogen-Induced Lymphocyte Proliferation. Rats were sacrificed by rapid decapitation, and trunk blood was collected in 50-ml polypropylene tubes containing heparin (0.1 ml) and immediately placed on ice. Whole blood was diluted 1:5 with cold RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 1% fetal calf serum and gentamicin (20 $\mu\text{g/ml}$). One hundred microliters of each blood suspension was plated into 96-well flat-bottom microtiter plates containing eight concentrations of the T-cell-specific mitogen concanavalin A (100 $\mu\text{l/well}$), incubated for 72 h at 37°C with 8% CO_2 and pulsed with 0.5 $\mu\text{Ci/well}$ of [*methyl*- ^3H]thymidine (6.7 Ci/mmol; PerkinElmer Life Sciences, Boston MA) in a 20- μl volume followed by additional 24-h incubation. Cells were lysed by distilled water using a 96-well cell harvester (Brandel, Inc., Gaithersburg, MD), and labeled DNA was harvested onto glass fiber filters. Radioactivity was quantified via liquid scintillation spectrophotometry (Beta Plate; Pharmacia, Peapack, NJ). Because it has been shown that stress and drug exposure in whole animal studies may be accompanied by alterations in white blood cell (WBC) counts (Keller et al., 1983; Dhabhar and McEwen, 1997), proliferation responses were expressed as cpm/ 10^3 cells to minimize the potential effects of altered cell number on proliferation responses.

Determination of WBCs. Blood was collected into heparinized tubes and then diluted 1:5 with RPMI 1640 medium (1% fetal calf serum). A 20- μl sample was diluted into 10 ml of hematology (Beckman Coulter, Inc. Fullerton, CA). Red blood cells were lysed with Zap-Oglo bin II Lytic reagent (Beckman Coulter, Inc.), and the total white blood cell count was measured using a Coulter counter (Beckman Coulter, Inc.). Blood WBC counts ranged from 5.0 to 7.5×10^6 cells/ml, and mean values of the control and treated groups did not significantly differ in any of the experimental paradigms described.

Determination of Lymphocyte Subpopulations (FACs). Phosphate-buffered saline (PBS, 80 μl) (Beckman Coulter, Inc.) was added to polystyrene tubes. The following mouse anti-rat MoAbs were used: PE anti-CD4 (clone W3/25 R-PE), FITC anti-CD8 (clone OX-8), PE anti-CD45 (clone OX-33), and FITC anti-monocytes (clone R2-1A6a) (Caltag Laboratories, Burlingame, CA). Anti-CD4 MoAb diluted with PBS (1:2) and anti-CD8 MoAb diluted with PBS (1:10) were both added (10 μl each) to one set of tubes containing PBS. Anti-CD45 and anti-monocytes diluted with PBS (1:10) were added (10 μl each) to a second set of tubes with PBS. Rats were sacrificed by rapid decapitation and trunk blood was collected into heparinized tubes. Whole blood samples (100 μl) were added to tubes containing PBS and antibody solutions. Samples were incubated (60 min at 4°C), washed twice with PBS, centrifuged at 1200 rpm, and supernatants were aspirated. Samples were vortexed; immunolyse (1 ml) (Beckman-Coulter, Inc.) was added to each tube and vortexed again. Samples were fixed with 250 μl of formaldehyde 9.25% and vortexed (60 s). Samples were resuspended with PBS, centrifuged (1200 rpm), and supernatants were aspirated. A final volume of 300 μl of PBS was added to each tube and vortexed. As positive controls, cell suspensions were incubated separately with each MoAb. Samples were protected from light and stored at 4°C until analysis using a FACStar flow cytometer (FACS system; BD Biosciences) with a single excitation source (200-mW argon laser). An analysis gate was set to include cells with the forward and side-scatter characteristics of lymphocytes and monocytes.

Plasma Corticosterone Assay. Blood samples were collected when animals were sacrificed, placed on ice, and centrifuged to allow separation of plasma that was collected and stored at -20°C until

needed. Plasma corticosterone was measured using solid phase double antibody ^{125}I radioimmunoassay kits purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Samples were assayed in duplicate, and corticosterone concentrations were expressed as nanograms per milliliter.

Delayed-Type Hypersensitivity. Animals were treated chronically with cocaine for 7 days (10 mg/kg i.p., b.i.d.) followed by 6 days of withdrawal. Primary sensitization with antigen was carried out after 6 days of withdrawal from cocaine treatment. Animals were subcutaneously injected with a 23-gauge needle at the base of the tail with 100 μl of an emulsion consisting of crystallized bovine serum albumin (BSA) and Freund's complete adjuvant (FCA) (Sigma-Aldrich). The emulsion was prepared by vortexing a BSA saline solution (2 mg BSA/ml) with an equal volume of FCA. Seven days after the primary antigen injection, animals were challenged with a 4% solution of heat aggregated BSA dissolved in sterile saline. Heat aggregation was accomplished by heating the 4% solution of dissolved antigen for 1 h in a 70–75°C water bath. Then 100 μl of final solution was injected into the footpad of the animal, and saline was injected into the contralateral paw as a control. Paw swelling was measured 24 and 48 h after challenge injection using a plethysmometer (milliliters). The degree of the reaction was expressed as percentage of increase over control paw [% increase over control paw = (BSA injected paw – saline-injected paw)/(saline-injected paw) * 100%].

Statistical Analysis. In proliferation assays, the mitogen dose response was fitted to a nonlinear regression analysis to generate the best fit curve and determine E_{max} using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). T-lymphocyte function was determined by [^3H]thymidine (cpm) incorporation in cells stimulated by Con A and normalized using total white blood cell counts per 1000 cells. When appropriate, data were expressed as mean \pm S.E.M. For comparison of the means of two groups, statistical significance was assessed by Student's *t* test. One-way analysis of variance (ANOVA) with Newman-Keuls post hoc analysis was conducted for comparison of the mean of three or more groups. Significance was defined as $p < 0.05$. The significance of differences in the mean corticosterone responses (nanograms per milliliter) and to DTH responses was expressed as $E_{\text{max}} \pm$ S.E.M. and where appropriate presented as a percentage of control. For all parameters, any individual value greater than two standard deviations from the mean of the treatment group was omitted.

Results

Assessment of T-Lymphocyte Proliferation in Rats after Acute and Chronic Cocaine Administration. As an initial assessment of cocaine's effects on the immune system, rats were injected with cocaine (10 mg/kg) or saline and sacrificed 2 h after injection. T lymphocyte proliferation from acute cocaine ($n = 6$) and saline ($n = 6$) groups did not significantly differ at any Con A concentrations (Fig. 1A). Plasma corticosterone levels were also measured in this experiment, but there were no significant differences in corticosterone levels at 2 h after an acute cocaine injection (Fig. 1B). In contrast to a single cocaine injection, animals exposed to chronic cocaine ($n = 6$) twice a day for 7 days had significantly decreased T-cell proliferation (333 ± 28) compared with saline-treated controls (460 ± 34) ($n = 6$) 2 h after the final dose of cocaine (Fig. 2A). Maximum responses (E_{max}) were determined from a nonlinear regression analysis using all concentrations of Con A, and significant difference was determined using the Student's *t* test ($p < 0.05$). In addition to the decrease in lymphocyte proliferation in the chronic cocaine group, there was a significant increase in corticoste-

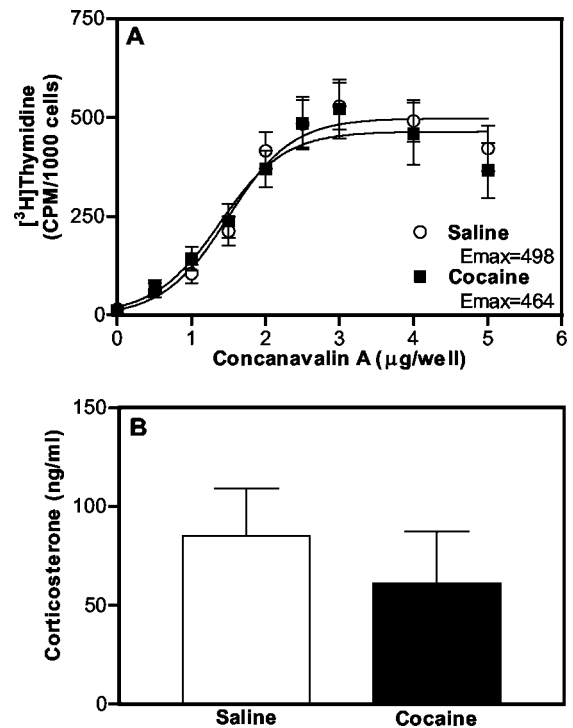


Fig. 1. Effect of acute cocaine administration on lymphocyte proliferation and plasma corticosterone. Animals were injected intraperitoneally with cocaine (10 mg/kg) or saline and sacrificed 2 h after injection. Blood was collected into heparinized tubes, diluted 1:5, and lymphocyte proliferation stimulated by the indicated concentration of Con A. Results are representative of two independent experiments. A, lymphocyte proliferation. Data are expressed as mean \pm S.E.M. [^3H]thymidine (cpm/1000 cells). No significant difference in E_{max} values; saline, 498 ± 24 ; cocaine-treated animals, 464 ± 29 ($n = 6/\text{group}$); were detected ($p > 0.05$). B, plasma corticosterone. Corticosterone was determined as described under *Materials and Methods* and expressed as mean (nanograms per milliliter) \pm S.E.M., and there was no significant difference between cocaine- and saline-treated animals.

rone levels compared with saline control animals determined by Student's *t* test ($p < 0.05$; Fig. 2B).

Chronic Cocaine Withdrawal Alterations in Peripheral Blood Lymphocyte Proliferation. The lymphocyte proliferation was measured in animals exposed to cocaine withdrawal and compared with saline-treated controls. In these experiments, withdrawal is the time period from the last injection of cocaine to the time the animals were sacrificed. A temporal-dependent parameter was added to cocaine treatment to measure the stress of cocaine withdrawal on the immune response. One day after cessation of cocaine treatment ($n = 6$), lymphocyte proliferation responses were decreased by more than 45% compared with saline controls ($n = 6$). This effect was similar in magnitude at 2 days of withdrawal from cocaine treatment (Fig. 3A). Significant differences between E_{max} values were determined between the 1-day (329 ± 27) and 2-day (351 ± 36) withdrawal groups compared with saline controls (687 ± 45) ($p < 0.05$; ANOVA). To further characterize this withdrawal phenomenon, longer withdrawal time points were assessed. More than 75% inhibition was observed in animals in which cocaine treatment was discontinued for 4 and 6 days (Fig. 3B). As previously observed in Fig. 2, chronic cocaine treatment alone resulted in a 25% suppression of T-lymphocyte proliferation. Significant differences were observed in the E_{max} values of both the 4- and 6-day withdrawal groups compared with the chronic

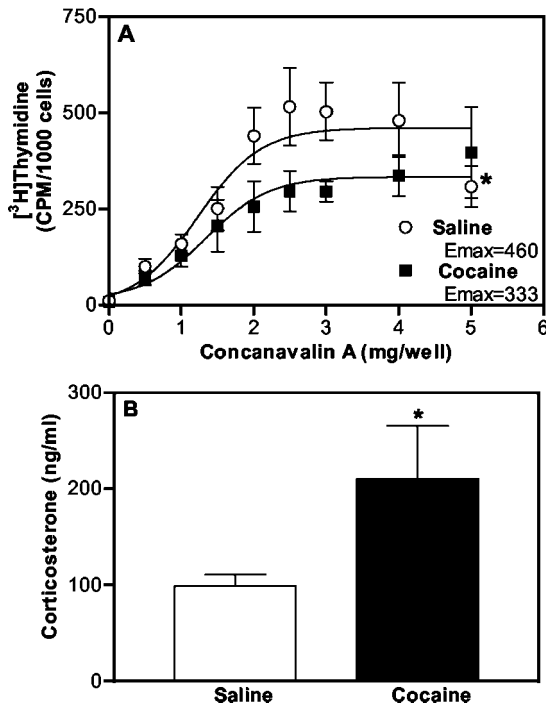


Fig. 2. Effect of chronic cocaine administration on lymphocyte proliferation and plasma corticosterone levels. Animals were injected with i.p. cocaine (10 mg/kg, b.i.d.) or saline for 7 days and sacrificed 2 h after their last injection. Blood was collected into heparinized tubes, diluted 1:5, and stimulated with indicated concentrations of Con A for the lymphocyte proliferation response. Results are representative of two independent experiments. A, lymphocyte proliferation. Data are expressed as mean \pm S.E.M. [*methyl-³H*]thymidine (cpm/1000 cells). *, significant difference ($p < 0.05$) between E_{max} values of the saline-treated controls (460 ± 34) compared with cocaine-treated animals (333 ± 28) was determined by the Student's *t* test ($n = 6$ /group). B, plasma corticosterone. Corticosterone was determined as described under *Materials and Methods* and expressed as mean (nanograms per milliliter) \pm S.E.M. *, significant difference as determined by Student's *t* test, $p < 0.05$.

cocaine alone group and the saline group (saline, 1163 ± 44 ; cocaine, 789 ± 55 ; 4 day, 315 ± 26 ; 6 day, 231 ± 19) ($p < 0.05$; ANOVA, Newman-Keuls). This was not accompanied by a significant alteration in cell viability. Cell viability using trypan blue exclusion was determined in experiments involving the 6-day withdrawal time point, because this was the time point in which the maximal amount of immunosuppression was observed. This effect was also not attributed to an alteration in the number of white blood cells of either the saline or 6 the day cocaine withdrawal group ($6.25 \times 10^6 \pm 8.2 \times 10^4$ versus $5.85 \times 10^6 \pm 1.7 \times 10^5$ cells/ml, respectively). Additional experiments were carried out to determine whether this suppression persisted at 14 days of withdrawal from cocaine. At 14 days of withdrawal, the T-lymphocyte responses in cocaine-treated animals (952 ± 41) returned to levels observed in controls animals (1012 ± 43) (Fig. 3C).

Determination of Lymphocyte Subpopulations (FACs). To account for changes in lymphocyte subtypes between the treatment groups, flow cytometric analysis was implemented. Antibodies to CD4⁺ helper T-lymphocytes clone W3/25 R-PE, CD8⁺ cytolytic T-lymphocytes clone OX-8 FITC, CD 45RA B-lymphocyte clone OX-33 R-PE, and monocytes clone R2-1A6a FITC were used (Fig. 4). The phenotype patterns of the immune cells were not significantly different between animals exposed to 2 (data not shown) or 6 days of

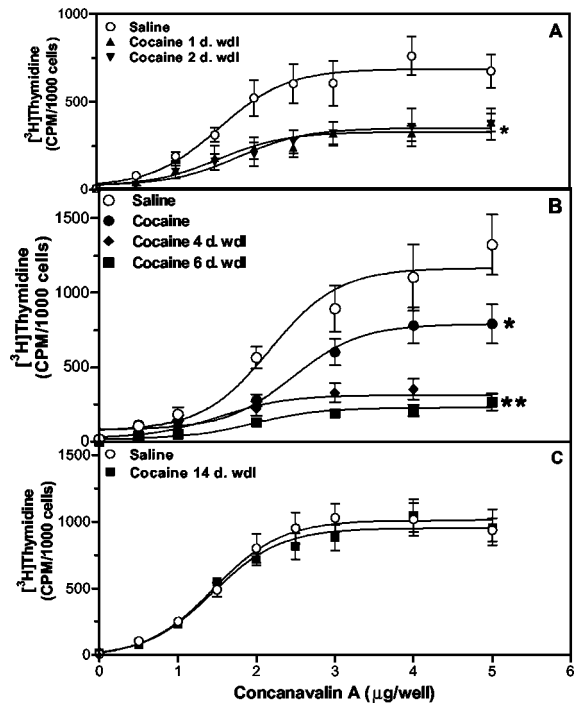


Fig. 3. Time-dependent effects of withdrawal from chronic cocaine on lymphocyte proliferation. Animals were injected i.p. with either cocaine (10 mg/kg, b.i.d.) or saline for 7 days. After their last injection, each group underwent their respective withdrawal period before sacrifice. Blood was collected into heparinized tubes, and lymphocyte proliferation in WB1:5 to Con A was determined. Data are expressed as mean \pm S.E.M. [*methyl-³H*]thymidine (cpm/1000 cells) in saline- and cocaine-treated animals ($n = 6$ /group). A, effects of 1 and 2 days of withdrawal. Results are representative of two independent experiments. Significant differences between E_{max} values were determined between the 1-day (329 ± 27) and 2-day (351 ± 36) withdrawal groups compared with saline controls (687 ± 45) ($p < 0.05$; ANOVA). B, effect of 4 and 6 days of withdrawal. Significant differences were observed in the E_{max} values of both the 4- and 6-day withdrawal groups compared with the chronic cocaine alone and the saline groups (saline, 1163 ± 44 ; cocaine, 789 ± 55 ; 4 day, 315 ± 26 ; 6 day, 231 ± 19) (*, $p < 0.05$ and **, $p < 0.01$; ANOVA, Newman-Keuls). C, effects of 14 days of withdrawal. No significant difference in the cocaine-treated animals (952 ± 41) compared with saline controls (1012 ± 43).

withdrawal from chronic cocaine compared with the saline controls.

In Vivo Assessment of Chronic Cocaine and Withdrawal on Delayed-Type Hypersensitivity Response.

To determine whether the suppression of T-lymphocyte proliferation by cocaine withdrawal was also present in whole animals, the DTH assay was used. Animals were treated chronically with cocaine for 7 days (10 mg/kg i.p., b.i.d.), followed by 6 days of withdrawal, a time point in which the in vitro proliferation response was maximally suppressed (Fig. 3B). After 6 days of withdrawal, animals were appropriately treated for DTH assessment (see *Materials and Methods*). Paw swelling was measured 24 and 48 h after challenge injection (Fig. 5A). Animals that had undergone 6 days of withdrawal from cocaine treatment at the time of primary sensitization had a significantly blunted DTH response at both 24 and 48 h after challenge with BSA compared with saline-treated animals ($p < 0.05$, Student's *t* test; Fig. 5B). These results support the in vitro data, which showed a suppression of the immune response at 6 days of withdrawal from chronic cocaine.

Plasma Corticosterone Effects of Withdrawal from Chronic Cocaine. Plasma corticosterone was assayed in

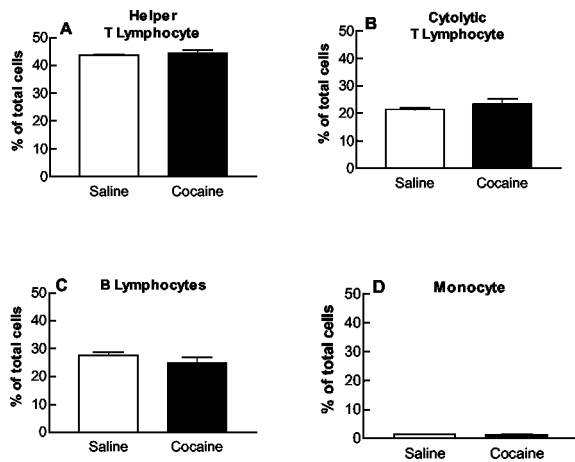


Fig. 4. Effect of chronic cocaine on the immunophenotype of peripheral blood lymphocytic populations after 6 days of cocaine withdrawal. Chronic cocaine (10 mg/kg) or vehicle (saline) was administered under the same conditions and regimen as described in the mitogen proliferation assay. The following mouse anti-rat MoAbs were used: PE anti-CD4 (clone W3/25 R-PE), FITC anti-CD8 (clone OX-8), PE anti-CD45 (clone OX-33), and FITC anti-monocytes (clone R2-1A6a). Anti-CD4 MoAb diluted with PBS (1:2) and anti-CD8 MoAb diluted with PBS (1:10) were both added (10 μ l each) to one set of tubes containing PBS. Anti-CD45 and anti-monocytes diluted with PBS (1:10) were added (10 μ l each) to a second set of tubes with PBS. Rats were sacrificed by rapid decapitation, and trunk blood was collected into heparinized tubes. Whole blood samples (100 μ l) were added to tubes containing PBS and antibody solutions and samples assayed as described under *Materials and Methods*. Effects on CD4⁺ (helper) and CD8⁺ (cytolytic) lymphocytes are shown in A and B, respectively. Effects of CD45 and monocytes are shown in C and D, respectively. Each group ($n = 6$) represents the mean percentage of total cells \pm S.E.M. There were no significant differences of lymphocyte subtypes in any of the treatment groups.

animals exposed to chronic cocaine (10 mg/kg i.p.) twice daily followed by 2 h, 1, 2, 4, 6, and 14 days of withdrawal to measure the stress of cocaine withdrawal on the neuroendocrine system. Figure 6 represents data from multiple experiments with data expressed as a percentage of control. The percentage of control is the combined mean corticosterone values from the saline-treated animals in each experiment. As previously observed, animals that received chronic cocaine had significantly elevated corticosterone levels at 2 h after the last dose of cocaine. To determine whether elevated corticosterone levels were sustained through the withdrawal period, corticosterone was measured at each withdrawal time period. Corticosterone levels continued to be significantly elevated with a 2-fold increase above-saline animals at 24 h after cessation of cocaine treatment (Student's t test, $p < 0.05$). By the 2nd day of cocaine withdrawal, corticosterone levels began to return to baseline values and were no longer significantly elevated at 4, 6, and 14 days of withdrawal.

Adrenalectomy and RU486 Attenuate T-Lymphocyte Suppression in Animals Exposed to Cocaine Withdrawal. After animals arrived, they were immediately given corticosterone replacement in their drinking water (25 μ g/ml) (Jacobson et al., 1989; Zhou et al., 1993) and acclimated for 1 week before receiving any treatment injection. The corticosterone replacement restored basal corticosterone levels, which allowed for 100% animal survival. It is known that complete removal of corticosterone has been shown to result in suppression of a number of immune responses that are restored when basal levels of steroids are maintained (Fleshner et al., 1996). Animals received chronic cocaine (10 mg/kg,

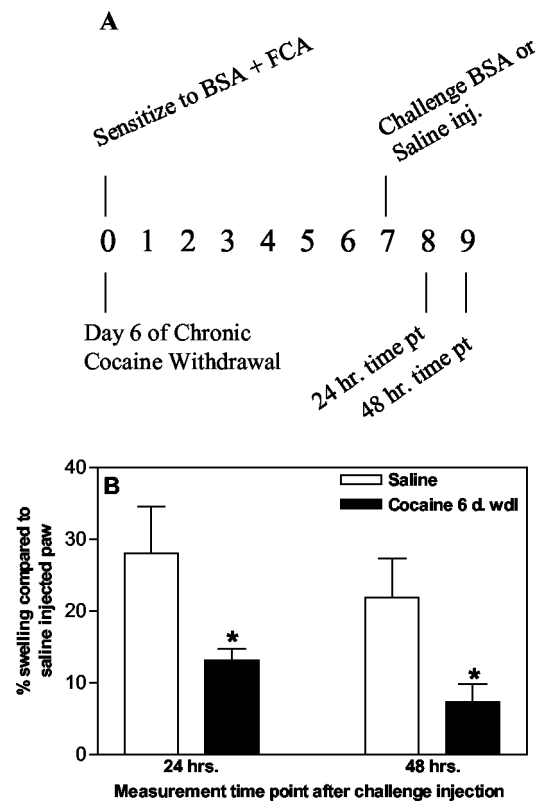


Fig. 5. Cocaine withdrawal is associated with decreased delayed-type hypersensitivity responses. A, dosing and DTH paradigm. Animals were treated for 7 days with saline (1 ml/kg) or cocaine (10 mg/kg i.p., b.i.d.) followed by 6 days of withdrawal. After 6 days of withdrawal (day 0), animals were administered a primary injection of BSA + FCA as described under *Materials and Methods*. On day 7, animals were given a challenged injection of BSA in one hindpaw and saline in the other hindpaw. B, DTH measurements. Swelling responses were measured 24 and 48 h after challenge. Results are representative of two independent experiments. Data presented as mean \pm S.E.M. percentage of increase over control paw ($n = 6$ /group). *, significant difference between saline- and cocaine-treated group in each time point as determined by Student's t test, $p < 0.05$.

b.i.d.) or saline for 7 days, and two groups received either 1 or 6 days of withdrawal ($n = 6$). When plasma corticosterone levels were determined, there were slight but not statistically significant differences between cocaine and saline groups (Fig. 7A). Minimal corticosterone values verified that all animals were adrenalectomized, yet had basal corticosterone levels in their system. In contrast to the intact animals, no significant effect on Con A-stimulated lymphocyte proliferation responses were observed in E_{max} values of adrenalectomized animals from any of the groups (saline, 562 ± 45 ; cocaine, 512 ± 24 ; cocaine 1-day wdl, 545 ± 30 ; cocaine 6-day wdl, 486 ± 31) (Fig. 7B).

To further examine the involvement of the neuroendocrine system on lymphocyte proliferation, the glucocorticoid receptor antagonist RU486 was administered. In these studies, intact animals were treated with either cocaine (10 mg/kg, b.i.d.) or saline for 7 days and were exposed to 6 days of withdrawal. During the first 2 days of withdrawal, the time period when corticosterone levels are elevated, one saline group and one cocaine group received RU486 (10 mg/kg s.c., b.i.d.); the other saline and cocaine groups received vehicle (propylene glycol, 1 ml/kg). This dose has been shown to block corticosterone receptors on lymphocytes in the spleen (Phi-

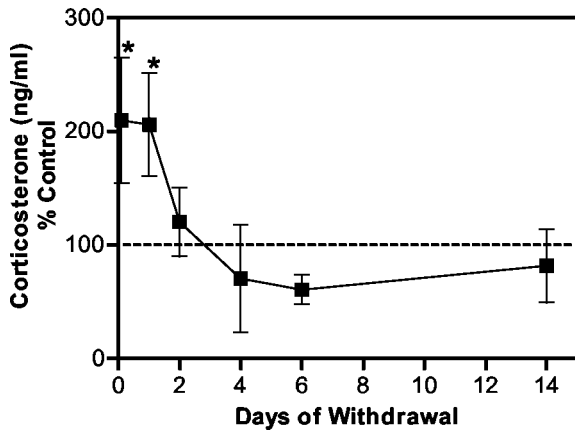


Fig. 6. Time-dependent changes in plasma corticosterone levels during the withdrawal period after cessation of chronic cocaine treatment. Animals were injected i.p. with either cocaine (10 mg/kg, b.i.d.) or saline for 7 days. After their last injection each group underwent its respective withdrawal period before sacrifice. Plasma samples were collected at the time animals were sacrificed, placed on ice, and centrifuged to allow for separation of plasma. Results are representative of at least two independent experiments for 2 h, 1, 2, 6, and 14 days of withdrawal. Each point represents the mean plasma corticosterone level of each group after the designated period of cocaine withdrawal, as measured by radioimmunoassay (nanograms per milliliter) and expressed as percentage of saline control (100%). Saline-treated animals had a mean corticosterone value of 90 ± 11 ng/ml ($n = 15$), which is represented by the dashed line.

libert, 1984). The RU486 treatment during the first 2 days of the 6-day withdrawal period seemed to attenuate the significant lymphocyte suppression of cocaine withdrawal on proliferation (Fig. 8). As expected, the chronic cocaine withdrawal group was still significantly suppressed compared with all other groups (saline, 709 ± 28 ; saline + RU486, 742 ± 46 ; cocaine + RU486, 761 ± 36 ; cocaine, 487 ± 15) ($p < 0.05$; ANOVA). There were no significant differences between the saline-treated animals with and without RU486 postcocaine treatment.

Discussion

Cocaine exposure has been described as a pharmacological stressor due to activation of the HPA axis leading to an increase secretion of glucocorticoids (Sarnyai et al., 1998). In addition to chronic exposure, recent evidence has also demonstrated that the sudden cessation of cocaine administration continues to produce neuroendocrine abnormalities (Richter and Weiss, 1999). Although there are some reports that have demonstrated that chronic cocaine administration produces alterations in a variety of immunological parameters (Van Dyke et al., 1986; Stanulis et al., 1997; Pellegrino and Bayer, 1998), there is comparatively little information on whether the immune alterations persist during the period of cocaine withdrawal. In this article, we demonstrate that withdrawal from chronic cocaine produces a prolonged suppression of cellular immunity through a steroid-dependent mechanism. The sudden cessation of cocaine administration was shown to result in elevation of corticosterone levels that were sustained for up to 2 days. During this period, significant decreases in mitogen-stimulated lymphocyte proliferation responses were also present. Although steroid levels returned to baseline values thereafter, proliferation responses remained significantly suppressed for 6 days after drug cessation. Flow cytometric analysis revealed that there

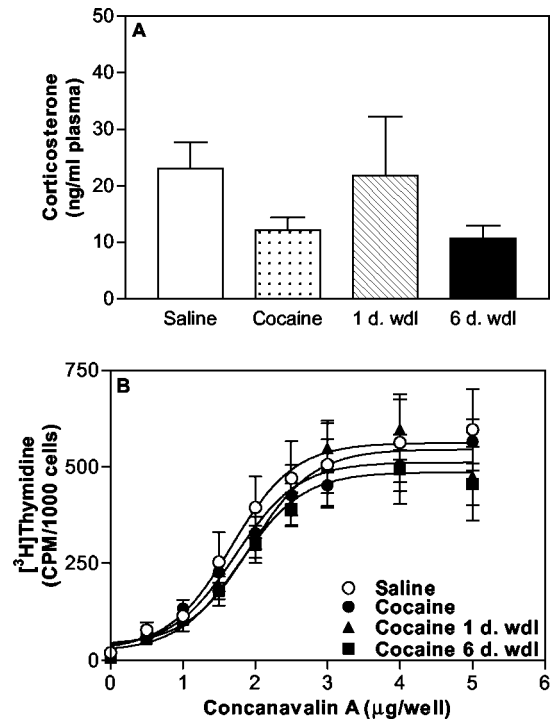


Fig. 7. Adrenalectomy attenuates the effects of cocaine withdrawal on proliferation responses. Adrenalectomized animals were given corticosterone replacement in the drinking water (25 mg/ml) and acclimated for at least 1 week before treatment. Animals were injected with either cocaine (10 mg/kg i.p., b.i.d.) or saline for 7 days. After the last injection animals were either sacrificed or exposed to 1 or 6 days of withdrawal from cocaine before sacrifice. A, corticosterone responses. Data are expressed as mean \pm S.E.M. corticosterone (nanograms per milliliter) in saline- and cocaine-treated animals ($n = 6$ /group). No significant difference was observed, as determined by one-way ANOVA with Newman-Keuls post hoc analysis, $p > 0.05$. B, lymphocyte proliferation responses. Data are expressed as mean \pm S.E.M. [3 H]thymidine (cpm/1000 cells) in saline- and cocaine-treated animals ($n = 6$ /group). No significant difference in E_{max} values between treatment groups on Con A-stimulated lymphocyte proliferation responses of adrenalectomized animals (saline, 562 ± 45 ; cocaine, 512 ± 24 ; cocaine 1-day wdl, 545 ± 30 ; cocaine 6-day wdl, 486 ± 31).

were no significant differences in immunophenotype of peripheral blood lymphocytic populations of T-cell subsets (CD4, CD8), B cells, or monocytes at 2 or 6 days of withdrawal from cocaine compared with saline controls. Therefore, the prolonged suppression of lymphocyte responses after cocaine cessation is more likely due to an altered intrinsic activity of the lymphocytes to proliferate after mitogen stimulation rather than to a decrease in the number of circulating lymphocytes.

To determine whether the decrease in the *in vitro* measure of cellular immunity was predictive of an impaired *in vivo* cellular immune response, the effects of cocaine withdrawal on the delayed-type hypersensitivity response were determined. Animals that were challenged with an antigen during the withdrawal period of cocaine had a significantly blunted DTH response compared with saline controls. This is consistent with the decreased *in vitro* lymphocyte proliferation response also observed at 6 days of withdrawal because primary sensitization with an antigen is the critical exposure for clonal expansion of CD4 T lymphocytes selective for that antigen (Abbas et al., 1997). The decreased DTH response during cocaine withdrawal suggests an alteration in the function of the lymphocyte subtypes that participate in cell-me-

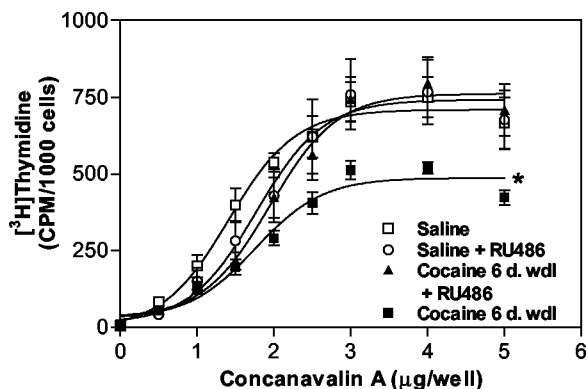


Fig. 8. Effects of the glucocorticoid receptor antagonist RU486 and withdrawal from chronic cocaine on lymphocyte proliferation. Animals were injected with either cocaine (10 mg/kg, i.p., b.i.d.) or vehicle (saline) for 7 days followed by 6 days of withdrawal. After their last injection one saline group and one cocaine group received RU486 (10 mg/kg s.c., b.i.d.). The other saline and cocaine groups received vehicle (propylene glycol, 1 ml/kg) for the first 2 days of the 6-day withdrawal period. Blood was collected into heparinized tubes, and lymphocyte proliferation from WB1:5 to Con A was determined. Data are expressed as mean \pm S.E.M. [*methyl*- 3 H]thymidine (cpm/1000 cells) ($n = 6$ /group). *, significant difference between the E_{max} of the cocaine withdrawal group compared with saline, saline + RU486, and cocaine + RU486 groups (saline, 709 ± 28 ; saline + RU486, 742 ± 46 ; cocaine + RU486, 761 ± 36 ; cocaine, 487 ± 15), as determined by nonlinear regression and one-way ANOVA with Newman-Keuls post hoc analysis, $p < 0.05$.

diated immune responses. A similar decrease in DTH responses has also been reported with either chronic cocaine or corticosterone administration (Stanulis et al., 1997) where the suppressed DTH response due to corticosterone was accompanied by a shift in the cytokine profile from a T helper 1 (IL-2 and interferon- γ) response to a T helper 2 (IL-4 and IL-10) predominant profile (Wilckens, 1995; Stanulis et al., 1997). These observations suggest that the suppression of the DTH response 2 weeks after cocaine withdrawal may be related to the initial elevation of corticosterone at the time of the primary antigen sensitization during the withdrawal period.

It is known that an acute dose of cocaine increases corticosterone levels, and unlike other stressors, tolerance does not develop to the neuroendocrine effects of repeated cocaine injections (Borowsky and Kuhn, 1991). Similarly, we have reported increases in corticosterone levels when animals were sacrificed 30 min after an acute i.v. cocaine injection (Pellegrino et al., 2001). The increase in corticosterone is a transient effect and returns to baseline levels by 2 h. Consistent with our finding that 7 days of chronic cocaine significantly increased basal corticosterone levels 2 h after the last cocaine injection, Sarnyai et al. (1998) showed increases in basal levels of corticosterone after 3 weeks of cocaine administration. The intermittent increases in corticosterone levels over the treatment period as well as the increase in corticosterone after the last injection may explain the decrease in lymphocyte proliferation we observed after chronic cocaine administration. In addition, withdrawal from cocaine resulted in a significant and prolonged elevation in steroid levels for up to 2 days after cocaine administration was discontinued (Fig. 6). It is possible that although there was no further increase in corticosterone levels in the cocaine withdrawal animals compared with the chronic cocaine group, the significant immunosuppression observed in the withdrawal animals can be attributed to the prolonged ste-

roid elevation. These results are consistent with two other studies, which have reported prolonged increases in corticosterone levels after cessation of cocaine (Borowsky and Kuhn, 1991; Levy et al., 1994). More recently, Richter and Weiss (1999) reported that cocaine withdrawal increases CRF release up to 400% above baseline levels in the amygdala between 11 and 12 h postcocaine. Therefore, it may be possible that the observed immunosuppressive effects of cocaine withdrawal seen in this study may be attributed to both the repeated stimulation of the HPA axis from the chronic cocaine administration and the stress of withdrawal.

Cocaine may be affecting the HPA axis and corticosterone levels through central nervous system-dependent pathways. Chronic cocaine exposure has been shown to result in long-lasting alterations in central monoaminergic systems, some of which persist for up to 30 days after the final cocaine injection (Kalivas and Duffy, 1988; Kuhar and Pilotte, 1996). For example, cocaine has been shown to produce permanent and reversible alterations in the function of dopaminergic (Henry and White, 1991), serotonergic (Levy et al., 1992), and noradrenergic (Giralt and Garcia-Sevilla, 1989) neurons and in their respective receptors. The effect of cocaine withdrawal on the immune system may reflect similar changes in central neurotransmitter systems involved in regulating the neuroendocrine response, because stress-induced elevations of ACTH and corticosterone are partially mediated by 5-hydroxytryptamine (Van de Kar et al., 1991), norepinephrine (Maccari et al., 1990), and dopamine (Beaulieu et al., 1987) neurons.

To decipher whether the increase in corticosterone during the repeated cocaine injections, withdrawal, or both were the cause of the observed suppression of lymphocyte responses, studies were carried out in animals that were either adrenalectomized or treated with the steroid antagonist RU486 during withdrawal. Adrenalectomized animals, exposed to 6 days of withdrawal after chronic cocaine exposure, were not immunologically suppressed compared with saline controls. This supports the hypothesis that the immune effects of cocaine withdrawal may be neuroendocrine-dependent. However, it was not clear from these experiments whether the increase in steroids during cocaine exposure, the increase of corticosterone for the first 2 days of withdrawal, or both were responsible for the prolonged immunosuppressive effects. To block the effects of elevated corticosterone during the initial withdrawal period, chronically cocaine-treated animals were administered RU486 during the first 2 days of withdrawal. This treatment was found to completely block the subsequent suppressive effects of cocaine withdrawal on lymphocyte proliferation. These data suggest that the suppression of lymphocyte responses is shown to be dependent on the elevation of corticosterone during the first 1 to 2 days of withdrawal. It is possible that the increases and decreases of HPA axis activation during administration of cocaine may be sensitizing T lymphocytes to the surge of circulating corticosterone during the first 2 days of withdrawal. Together they may alter the ability of the lymphocyte to proliferate at 4 and 6 days of cocaine withdrawal compromising the immune response.

Human exposure to cocaine has been shown to produce similar effects on the HPA axis as reported here in a rodent model. For example in cocaine users, elevation of both ACTH (Mendelson et al., 1992; Sholar et al., 1998) and plasma

cortisol levels (Baumann et al., 1995) have been well documented. We have shown similar observations in our rat model and extended these findings to immunological effects of withdrawal in peripheral blood lymphocytes. Whether immunosuppression is involved in human cocaine withdrawal *in vivo* remains to be seen, but because these systems overlap in regards to the neuroendocrine effects, it is likely that there may be immune effects in humans as well. This report demonstrates that the sudden withdrawal from chronic cocaine produces activation of the HPA axis that results in prolonged effects on the immune systems. It is clear that the first 2 days of withdrawal seems to be a crucial period in the suppression of the immune response. These findings provide a framework that will allow future studies to examine the interaction between cocaine abuse and withdrawal with the concomitants of the neuroendocrine system. This may aid in developing more effective pharmacotherapeutics for treating cocaine addiction and withdrawal.

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