The Effects of Cocaine Injections on Mouse Thymocyte Population (44085)

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Abstract. **C57 BL mice were injected daily with either saline or varied doses of cocaine (5–50 mg/kg), and thymocyte subpopulations were analyzed 4 hr after the fifth injection. Mice injected with either 25 or 50 mg/kg of cocaine showed a decrease in the percentage of $CD4^+8^+$ cells and increase of $CD4^-8^-$, $CD4^+$, and $CD8^+$ cells. The absolute numbers of each subpopulation, calculated by multiplying the percentage of each subpopulation with the total cell number, revealed an extensive decline in $CD4^+8^+$, a decrease in $CD8^+$, an increase in $CD4^-8^-$, and no change in the $CD4^+$ subpopulation. Flow cytometric analysis of thymocytes and electrophoresis of the thymocyte DNA revealed a dosage-dependent increase in cells undergoing programed cell death with apoptosis. Culturing of thymocytes from control or drug-treated mice demonstrated an inverse relationship between cell viability and cocaine concentrations, suggesting that *in vivo* cocaine, or its biological products, may damage thymocytes. Incubation of normal cells with cocaine showed a dose-dependent decrease of viability with identical patterns of the alteration of cell subpopulations observed *in vivo*. A dose-dependent increase of apoptosis was also observed. In summary, we demonstrate a selective *in vivo* cocaine-induced alteration of the thymocyte subpopulations and identified programed cell death with apoptosis as the likely mechanism mediating this thymic atrophy. The comparable findings observed *in vivo* and *in vitro* support the concept that cocaine may directly affect some features of thymocyte biology, and suggest the usefulness of the *in vitro* system in studying cocaine effects on thymocyte biology.** [P.S.E.B.M. 1997, Vol 214]

The thymus is the site of T-lymphocyte selection and maturation. Newly migrated from the bone marrow, the $CD4^-8^-$ T cells account for about 2% of the thymocytes in an adult mouse. These cells develop into CD4 and CD8 double-positive immature cells, comprising 85% of the thymus cell numbers. The majority of the double-positive cells ($CD4^+8^+$) die due to a selection process of programed cell death (PCD) with apoptosis. The remaining cells develop into mature, functional $CD4^+$ or $CD8^+$ lymphocytes. Mature lymphocytes migrate to the secondary lymph organs, such as the spleen and the lymph nodes (1) to carry on immune functions. Abnormalities in thymocyte populations may result in improper immune functioning (2–5).

There have been a number of reports regarding the effects of cocaine on the immune systems of experimental animals and cocaine abusers (6–10), as well as on the association between cocaine and disease (11–15). Despite the thymus's crucial role in normal immune function, there are few reports concerning the effect of cocaine on this organ. Significant concentrations of cocaine were found in the thymuses of cocaine-injected mice (16). Chronic long-term cocaine injections for 11 weeks induced a statistically significant reduction in the number of $CD8^+$ cells (17). Recently, we reported initial findings that daily injections of mice for 5 days with cocaine reduced thymus weight, altered cell populations, and increased apoptosis (18). In this continuing study, the effects of cocaine on thymus cell populations was further examined both *in vivo* and *in vitro*.

Materials and Methods

Animals. Male C57BL mice (8–10 weeks old), housed in the animal care facilities of VA West Side Medi-

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cal Center in accordance with the FDA guidelines on Animal Care, were provided free access to food and water.

Thymocyte Preparation. C57BL mice were randomly divided into the required groups with two mice per group, and injected intraperitoneally (ip) with 0.2 ml of saline (control) or cocaine (5, 10, 25, or 50 mg/kg) daily for 5 days. Four hours after the last injection, thymuses were collected asceptically and thymocytes obtained by gently squeezing cells into 5 ml of saline. The cells were washed once with RPMI 1640 and adjusted to a concentration of 1 $\times$ 10^7 cell/ml in RPMI 1640 medium containing 5% of heat-treated fetal bovine serum (FBS, complete medium; Sigma Co., St. Louis, MO). This stock cell suspension was used in the following assays.

Cell Culture. Thymocyte suspension (1.2 ml) was added to a 25-ml culture flask. Twenty microliters of saline (control) or freshly prepared cocaine solution of varying concentrations were added. The flasks were incubated in a 5% CO_2 incubator at 37°C. After 24, 48, or 72 hr, viable cells were counted manually in a hemocytometer by trypan blue (0.4%) exclusion and the cell suspension was used for analysis.

Flow Cytometric Analysis of Surface Markers. Cell suspensions were adjusted to give the desired viable cell concentrations (10^6/35 μl) in PBS containing 5% FBS and 0.1% NaN_3, and aliquoted (35 μl) into 1.5 ml vials. Four microliters of antibody solution (Becton Dickinson Co., San Jose, CA), containing 1 μg each of either CD4 (L3T4, PE; rat IgG2b antimouse CD4 antigen), CD8 (Lyt-2, FITC; rat IgG2a antimouse CD8 antigen), or CD4 plus CD8, were added to each tube. In control tubes, antibody solution was replaced by 4 μl of PBS. The tubes were incubated on ice for 30 min and then washed twice with 1 ml of PBS before resuspending in 400 μl of PBS buffer containing 0.13% of propidium iodide. Within 1 hr, the percentage of each surface marker was measured with a FACScan flow cytometer (Becton Dickinson). Cells were gated by generating a forward-versus-scatter plot. The population of cells with forward scatter and negligible side scatter were gated electronically. Nonimmune mouse IgG1 or IgG2 tagged with FITC and PE were used for control staining. Cells were analyzed using the Lysys II software. Percentages of positive cells for each marker or double positive cells were read off the computer printout from the flow cytometer.

Flow Cytometric Analysis of Apoptosis. For flow cytometric analysis a cell suspension containing 5 million cells was centrifuged at 200*g* for 10 min. Five milliliters of 70% ethanol (EtOH) were added to the pellet. The suspension was incubated on ice for 4–5 hr, and then centrifuged. After removing the supernatant, the cell pellet was washed once with PBS buffer (phosphate buffer in 0.1% of NaN_3, pH 7.4) and resuspended in 1 ml of propidium iodide solution (50 μg/ml of propidium iodide, 0.1% of sodium citrate, 0.1% of Triton X-100). The tubes were kept at 4°C and analyzed with FACScan flow cytometer within 1 hr. A gate was created by drawing a polygon on a dot plot with the FL2-W (horizontal axis) versus FL2-A (vertical axis). These two parameters allowed us to discriminate against doublets. This gate includes all cells binding PI dye to their DNA. Apoptotic cells appear as a distinctive peak to the left of the G0/G1 peak (19).

DNA Electrophoresis. To determine the nature and extent of DNA fragmentation, 500–800 μl of the cell suspension was pelleted. The cell pellet was resuspended in 200 μl of TES buffer (20 m*M* of Tris-HCl, pH 8.0, 200 m*M* of EDTA, and 1% of SDS) and RNase A (Sigma) was added to a final concentration of 20 μg/ml. After incubation at 37°C for 1 hr, proteinase K was added to a final concentration of 100 μg/ml and the mixture was incubated at 55°C overnight. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the solution vortexed for 1 min, and centrifuged 12,000*g* in a microcentrifuge at 4°C for 10 min. The aqueous phase containing the DNA was transferred to a new tube, mixed with 1.5 volume of ammonium acetate/isoamyl alcohol (1:5) and kept overnight in a −20°C freezer. The DNA pellet was collected by spinning at 12,000*g* in a microcentrifuge for 10 min, washed with 100 μl of 70% EtOH and resuspended in 100 μl of TE buffer. The concentration of DNA was determined by UV spectrophotometry at OD260 with 1 OD unit = 50 μg DNA/ml. To perform DNA electrophoresis, 10 μl of the DNA preparation was mixed with 5 μl of bromophenol blue:glycerin (1:1), loaded onto a 1.2% agarose gel containing ethidium bromide (1 μg/ml) and electrophoresed at 60 V (6 V/cm).

Statistical Analysis. All experiments have been repeated several times. Three typical experiments were analyzed. A one-way analysis of variance (ANOVA) was performed to assess significant differences between the groups. *Post hoc* analysis by the Tukey procedure (20) was used to examine all pairwise group comparisons with overall α levels held at 0.05 and 0.01. Significant differences were $P < 0.05$ or $P < 0.01$.

Results

Cocaine Injection Altered the Thymocyte Cellular Composition. Four different dosages of cocaine were injected daily for five days and thymuses removed 4 hr after the last injection for flow cytometric analysis of cell surface markers. Table I shows that statistically significant changes in the percentage of each subpopulation occur at or above 25 mg/kg of cocaine. When the percentage of each subpopulation in mice injected with 25 or 50 mg/kg of cocaine was multiplied by the total cell number, which was significantly decreased, it was evident that cocaine selectively altered the composition of the thymocyte subpopulation (Fig. 1). The total number of cells in $CD4^+8^+$ and $CD8^+$ subpopulations decreased and $CD4^-8^-$ cells increased. No significant change was found for the $CD4^+$ subpopulation. The effects were most noticeable in the $CD4^+8^+$ subpopulation, with about a 60% decrease for a 25 mg/kg cocaine

Table I. The Effect of Different Dosages of Cocaine Injection on the Percentages of Thymocyte Subpopulations

Cocaine dosage (mg/kg/day)	% of subpopulations			
	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD8^+CD4^-$
0	3.0 ± 0.3	86.4 ± 1.2	7.5 ± 1.0	3.2 ± 0.7
5	4.0 ± 3.1	84.0 ± 3.1	8.2 ± 0.8	3.7 ± 0.1
10	5.0 ± 0.9	80.5 ± 4.6	9.8 ± 2.6	4.5 ± 1.4
25	4.9 ± 1.1[a]	77.6 ± 1.7[a]	12.7 ± 1.1[b]	4.9 ± 0.7[b]
50	6.0 ± 0.8[a]	70.5 ± 8.2[a]	17.2 ± 6.5[a]	6.0 ± 1.4[a]

Note. Mice were randomly divided into the required groups (two mice per group), and injected ip with saline oir different dosages of cocaine for 5 days. Four hours after the last injection, thymus cells were removed and stained with anti-Lyt 2, anti-L3T4, or a combination of both antibodies, and assayed by flow cytometry. Values are means ± SD of three separate experiments.
[a] $P < 0.01$, [b] $P < 0.05$, compared with control.

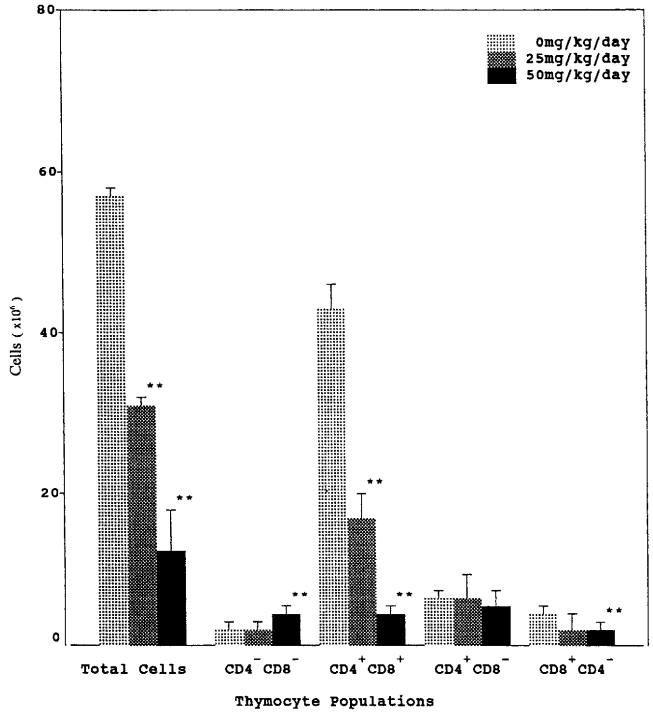

Figure 1. Mice were randomly divided into the required groups (two per group) and injected ip with saline or different dosages of cocaine for 5 days. Four hours after the last injection, thymus cells were removed and stained with anti-Lyt 2, anti-L3T4, or a combination of both antibodies, and assayed by flow cytometry. The number of cells was calculated by multiplying the total number of thymocytes by the percentage of each subpopulation. Values are means ± SD of three separated experiments. $^{**}P < 0.01$, compared with control.

injection and a 90% decrease when 50 mg/kg of cocaine was used.

Cocaine Injections Induced Apoptosis. Flow cytometric analysis of PI-treated cells demonstrated a typical diploid DNA peak in the control thymocytes (Fig. 2A). However, when analyzed 4 hr after the last cocaine injection, an additional population of hypodiploid cells was present (Fig. 2B). This result is consistent with the occurrence of apoptosis. The data in Figure 3, representative of three separate experiments (two mice per group), presents the percentage of apoptosis, which increased from the control value of 1.9% ± 0.3% to 2.5% ± 0.1% for 25 mg and 13.4% ± 2.7% for 50 mg of cocaine in the respective groups. Increased apoptosis was further demonstrated by the presence of laddering (as seen in Lanes 3 and 5 of Fig. 4), after agarose gel electrophoresis of DNA extracted from thymocytes of cocaine-treated animals.

Cocaine Injections Decrease the Survival of Cultured Thymocytes. Thymocytes removed 4 hr after the last injection of cocaine or saline were suspended in 3 ml of complete medium at a concentration of 4 million cells/ml and incubated in a 5% CO_2 incubator. The number of viable cells incubated for 24, 48, or 72 hr was determined. The data in Table II demonstrates an inverse relationship between the cell viability and the amount of cocaine injected. After 24 hr of culture, a statistically significant decrease in the number of viable cells was found between the 5 and 10 mg/kg and also between the 25 and 50 mg/kg of cocaine concentrations (linear regression coefficient: $r = -0.978$). Forty-eight hours after the last injection, a significant decrease in the number of viable cells was observed between the control and 5 mg/kg groups, the 5 and 10 mg/kg groups, and the 25 and 50 mg/kg groups ($r = -0.944$). At 72 hr, about 60% of the control thymocytes were still viable with significant differences between 5, 10, 25, and 50 mg/kg of cocaine concentrations ($r = -0.973$).

Incubation of Normal Thymocytes with Cocaine Decreased the Number of Viable Cells. Thymocytes isolated from control mice were cultured with different concentrations of cocaine, and the number of viable cells determined after 24, 48, and 72 hr. The data in Figure 5 demonstrate that cocaine decreased cell numbers in both a time and concentration-dependent manner. At 24 hr, the number of viable cells decreased from 12×10^6 to 9.2×10^6/ml for the control group, compared with 7.7×10^6 and 2.6×10^6/ml for the groups incubated with 5 μg (0.016 m*M*) and 10 μg/ml (0.032 m*M*) of cocaine respectively. After the initial decrease, the number of viable thymocytes in cultures incubated with 5 μg/ml of cocaine remained constant from 48 through 72 hr, at about 4.5×10^6. Higher concentrations of cocaine decreased the number of viable thymocytes to insignificant levels.

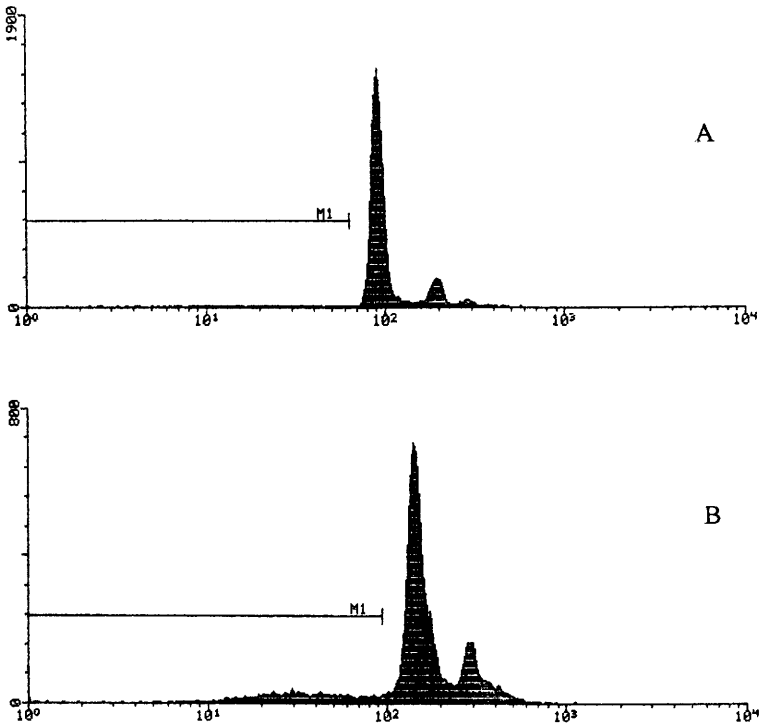

Figure 2. Mice were injected ip with saline (A) or cocaine (50 mg/kg) (B) for 5 days. Four hours after the last injection, thymocytes were removed for flow cytometric analysis of apoptosis. Abscissa: DNA content. Ordinate: cell number. (A) Reveals a typical diploid DNA peak. From cocaine injected mice. Includes a minor hypodiploid peak, which indicates apoptotic cells, located to the left of the diploid DNA. In addition, the presence of cells in hyperdiploid regions is noted. Data listed are from one of three typical experiments.

Incubation of Normal Thymocytes with Cocaine Altered the Thymocyte Cellular Composition. Normal thymocytes were incubated with different dosages of cocaine for 48 hr and the cells were stained and analyzed using flow cytometry. Table III reveals that cocaine incubation decreased the percentage of $CD4^+8^+$ subpopulation and increased the $CD4^-8^-$ subpopulation. While it appears to be a pattern of increasing $CD4^+$ and $CD8^+$ subpopula-

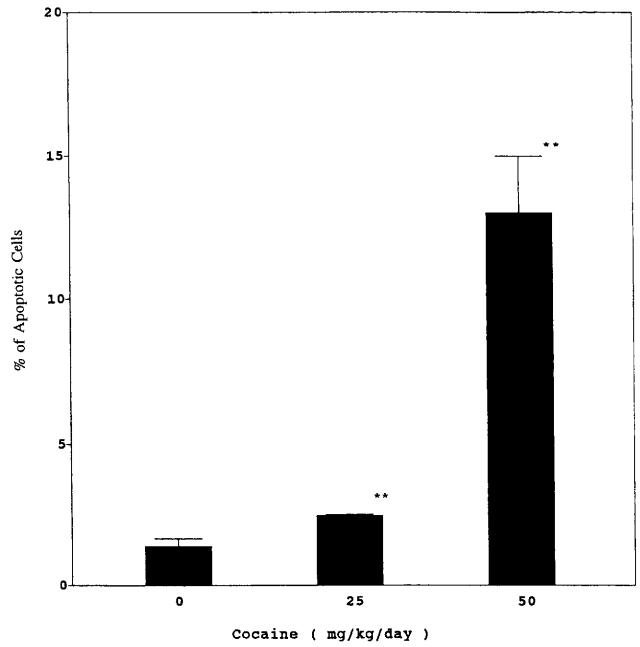

Figure 3. Mice were divided into several groups (two per group) and were injected ip with saline or different dosages of cocaine for 5 days. Four hours after the last injection, thymocytes were removed for flow cytometric analysis of apoptosis. Values are means ± SD of three experiments. $^{**}P < 0.01$.

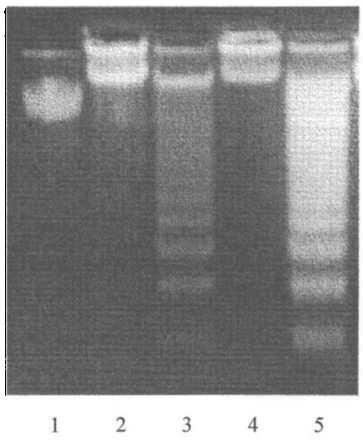

Figure 4. Mice received cocaine injections for 5 days. Four hours after the last injection, thymocytes were removed and cell suspensions prepared. Thymocytes were incubated in a complete medium for either 24 or 72 hr before extracting DNA for electrophoresis. Lane 1 includes the DNA markers. The DNA in Lanes 2 (control) and 3 (25 mg/kg of cocaine) was extracted from the cells after 24 hr, while lanes 4 (control) and 5 (50 mg/kg) includes DNA extracted after 72 hr. DNA in Lane 5 was more extensively fragmented than DNA in Lane 3, consistent with a cocaine dose-effect.

Table II. The Effect of Cocaine Injections on the Viability of the Thymocytes in Culture

Time (hr)	Thymus cells ($\times 10^6$)				
	0 mg/kg	5 mg/kg	10 mg/kg	25 mg/kg	50 mg/kg
0	12.0	12.0	12.0	12.0	12.0
24	9.5 ± 0.2^a	9.2 ± 0.6^a	8.0 ± 0.6^b	7.6 ± 0.6^b	3.1 ± 0.1
48	8.6 ± 0.4	7.2 ± 0.3	2.8 ± 0.2^c	2.4 ± 0.1^c	1.4 ± 0.3
72	6.7 ± 0.4	5.8 ± 0.4	2.8 ± 0.7	1.2 ± 0.1	0

Note. Mice randomly divided into several groups (two mice per group) were injected ip with different dosage of cocaine for 5 days. Four hours after the last cocaine injection, thymocytes were removed and cultured as described in complete medium at a concentration of 4×10^6/ml. The number of viable cells was compared after 24, 48, and 72 hr. Values are means ± SD of three typical experiments. Shared superscripts ($^{a, b, c}$) indicate no significant difference between numbers ($P < 0.01$).

tions, the changes were not significant. Multiplication of the percentages by the number of viable cells after 48 hr of incubation with cocaine shows that a dosage of 2.5 or 5.0 μg/ml significantly decreases the CD^+8^+ cells. A 5 μg/ml cocaine dose also increases the $CD4^-8^-$ cells while decreasing the $CD4^+$ cells. The alteration of $CD8^+$ subpopulation was not significant (Fig. 6).

Apoptosis Is Induced in Normal Thymocytes Incubated with Cocaine. Thymocytes from control mice were cultured with 2.5 or 5.0 μg/ml of cocaine for 24 hours. The percentage increase in apoptotic cells, as determined by flow cytometry, was as follows: control cells, 0.4% ± 0.4%, compared with 4.6% ± 1.1% and 8.0% ± 1.6% for 2.5 and 5.0 μg/ml of cocaine respectively (Fig. 7).

Discussion

In designing an experiment using animals as models, a proper dosage is always the first concern. We reviewed the literature and found that a wide range of cocaine dosages, from 5 to 60 mg/kg, had been injected into mice for various lengths of time (22–26). Our experience indicated that at 50 mg/kg, mice show some neurological symptoms immediately after cocaine injection, but recovered quickly, while no abnormalities were observed among mice injected with 25 mg/kg of cocaine. We also found that these concentrations selectively affected the thymocyte population of cocaine injected mice (18). Thus, dosages from 10 to 50 mg/kg were chosen for the continuation of this study.

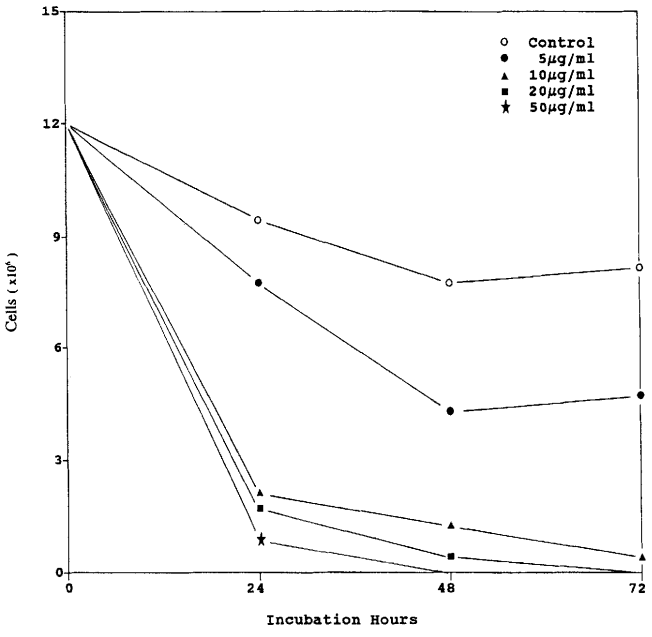

Figure 5. Thymocytes from normal mice were cultured with the indicated concentrations of cocaine for 24, 48, or 72 hr, and the viable cell number in each flask determined. Data listed are from one of three experiments.

The results presented in Table I revealed that five daily injections of cocaine in mice not only significantly reduced the number of thymocytes but also altered thymocyte subpopulations in a dose-dependent manner. The relative percentage of $CD4^+8^+$ cells decreased while the percentages of the other three subpopulations increased slightly (Table I). The absolute numbers of each subpopulation, calculated by multiplying the percentage of each subpopulation with the total cell number, revealed an extensive decline in $CD4^+8^+$, a decrease in $CD8^+$, an increase in $CD4^-8^-$ and no change in the $CD4^+$ subpopulations (Fig. 1). These results demonstrate that cocaine selectively altered survival of partially differentiated cells and suggest that cocaine may modulate T-cell maturation which could lead to an altered immune function.

The extensive decline in $CD4^+8^+$ cell number suggested that cocaine could reduce the number of thymocytes through programed cell death. Apoptosis is a common biological mechanism for removing unwanted cells without eliciting an inflammatory/immune response. The phenomenon of apoptosis has been reported in cells treated with many diverse agents (27–29). Cocaine induces apoptosis, as judged by flow cytometry and the presence of DNA laddering (Figs. 2–4). Enhanced programed cell death may be a biological response of cocaine-damaged thymocytes.

Cocaine has been detected in the thymus of cocaine-injected mice (16). In this report, we demonstrated that the ability of the cells from cocaine-injected mice to survive in culture was reduced inversely proportional to the amount of cocaine injected (Table II). This suggests that cocaine injections "prime" at least some of the thymocytes for subsequent cell death observed to be by a programed mechanism.

It has been reported that morphine treatment of C57/BL mice decreased the $CD4^+8^+$ thymic subpopulation and in-

Table III. The Effect of Cocaine on the Percentages of Cultured Thymocyte Subpopulations

Cocaine concentration (μg/ml)	% of subpopulations			
	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD8^+CD4^-$
0	6.3 ± 7.4	85.0 ± 10.3	7.4 ± 2.2	1.3 ± 1.1
1.25	9.0 ± 12.6	79.9 ± 13.4	7.2 ± 1.4	2.7 ± 2.0
2.5	37.9 ± 24.3[a]	44.2 ± 21.7[b]	11.2 ± 6.4	3.7 ± 4.9
5.0	73.0 ± 14.0[b]	13.8 ± 10.3[b]	11.5 ± 5.3	1.6 ± 2.1

Note. Three million thymocytes per milliliter from normal mice were incubated with 1.25, 2.5, or 5.0 μg/ml of cocaine. After 48 h at 37°C, thymocytes were stained with anti-Lyt2, anti-L3T4, or a combination of both antibodies, and assayed by flow cytometry. Values are means ± SD of three separated experiments.
[a] $P < 0.05$, [b] $P < 0.01$, compared with control.

creased apoptosis (27). Cocaine *in vivo* also reduces the $CD4^+8^+$ numbers and increases apoptosis, as reported in this study. To verify the possible direct cocaine effects *in vivo*, normal thymocytes were cultured with different doses of cocaine, and the effects were evaluated. Results in Figure 5 indicate that the dosages above 5 μg/ml decreased the amount of viable cells rapidly and the effects were dose and time-dependent. Flow cytometric analysis of the cell incubated with 5 μg/ml or less concentrations of cocaine revealed a substantial decrease in the percentages in $CD4^+8^+$ and an increase in $CD4^-8^-$ (Table III). When the percentage of each subpopulation was multiplied by the total cell number, it was evident that cocaine selectively altered the composition of the thymocyte subpopulation (Fig. 6) and the pattern resembles in some respects the findings described in cocaine-injected mice (Fig. 1). Lastly, the level of apoptosis in cocaine-treated thymocytes was significantly increased (Fig. 7). Thus, the findings of these *in vitro* experiments resemble those of *in vivo* experiments. This suggests that cocaine may directly affect some thymocyte functions in cocaine-injected mice. However, this finding cannot exclude the possibility that *in vivo* direct effects are also accompanied by a variety of indirect effects, resulting from drug interactions with the entire neuroimmune network (30).

In summary, we have demonstrated a selective *in vivo* cocaine-induced loss in the $CD4^+8^+$ and $CD8^+$ subpopulations and identified programed cell death with apoptosis as the likely mechanism mediating this atrophy. The consistent *in vivo* and *in vitro* results suggest that the *in vitro* thymus

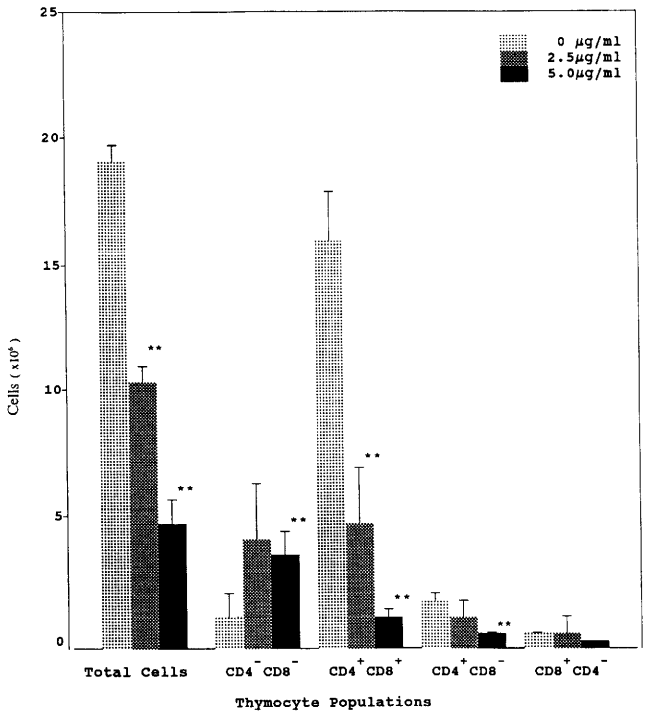

Figure 6. The effect of cocaine on cultured thymocyte subpopulation cell numbers. Three million thymocytes per milliliter from normal mice were incubated with 1.25, 2.5, or 5.0 μg/ml of cocaine. After 48 hr at 37°C, thymocytes were stained with anti-Lyt2, anti-L3T4, or a combination of both antibodies, and assayed by flow cytometry. The number of cells was calculated by multiplying the total number of thymocytes by the percentage of each subpopulation. The effect of cocaine on cells treated with 1.25 μg/ml was not shown here. Values are means ± SD of three separated experiments. $^{**}P < 0.01$, compared with control.

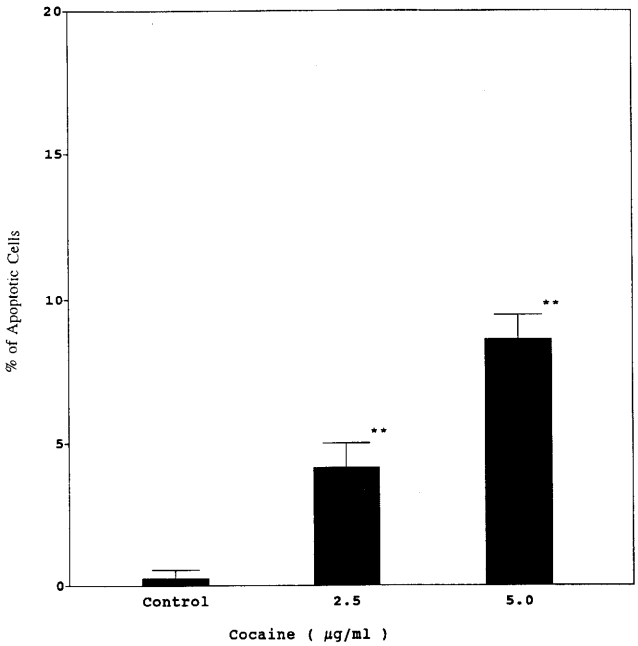

Figure 7. Three million thymocytes per milliliter from normal mice were incubated with 2.5 or 5.0 μg/ml of cocaine. After 24 hr of culturing at 37°C, the extent of apoptosis was analyzed by flow cytometer. Values are means ± SD of three separately experiments. $^{**}P < 0.01$.

model may be used to study at least some features of thymocyte response to cocaine. We plan to use the *in vitro* model further to study the mechanism of these interesting observations.

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