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Alterations in Splenocyte and Thymocyte Subpopulations in B6C3F1 Mice Exposed to Cocaine Plus Diazinon[1]

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ABSTRACT

Our laboratory has proposed a working model which asserts that cocaine's effects on immunity are mediated by reactive metabolites generated by the cytochrome P-450 system. This metabolic pathway is normally a minor one in humans, but takes on significance when metabolism of cocaine by the P-450 system is increased, as may occur with excessive alcohol consumption (enzyme induction) or after exposure to organophosphate pesticides (esterase inhibition). Results from our laboratory demonstrate that cocaine exerts its most dramatic effects on immunocompetence when administered to mice that have been pretreated with diazinon, an organophosphate esterase inhibitor. Most notably, we observed decreases in both the splenic T-dependent antibody response to sheep erythrocytes and the splenic T-independent antibody response to DNP-ficoll and a dramatic thymic atrophy in mice exposed to cocaine + diazinon, which were not seen in mice exposed to cocaine alone. The primary objective of the present investigation was to determine whether the exposure conditions used to produce the changes noted above are also capable of causing changes in lymphocyte cell types by use of flow cytometric analysis. Administration of cocaine after pretreatment with diazinon only modestly affected splenic lymphocyte subsets, which caused a slight decrease in the number of B cells. No effect was observed in the macrophage, T-helper or T-suppressor subpopulations in the spleen. These results suggest that changes in splenocyte subpopulations induced by cocaine + diazinon cannot account for the suppression of the antibody response. In contrast, all T-cell subsets in the thymus were decreased significantly, with immature double-positive thymocytes suffering the greatest loss in cell number. These results indicate that T cells, especially immature thymocytes located in the thymus, are sensitive to effects associated with the combined treatment of cocaine + diazinon.

Although it has been proposed that exposure to cocaine is capable of inducing marked changes in immunocompetence, very little evidence to date has been found to support this notion. Results from *in vivo* studies have been inconsistent (Holsapple and Munson, 1985; Havas *et al.*, 1987; Ou *et al.*, 1989; Starec *et al.*, 1991), and most *in vitro* studies have used concentrations of cocaine which far exceed reasonable physiological blood levels (Klein *et al.*, 1988; Martinez and Watson, 1990; Delafuente and Devane, 1991; Chao *et al.*, 1991; Luo *et al.*, 1992).

Several studies have attempted to use fluorescent monoclonal antibodies and/or flow cytometry to elucidate the cell types and subtypes which are most affected by cocaine administration. Bagasra *et al.* (1989) examined cocaine-induced alterations in splenic cell populations with use of fluorescein-labeled monoclonal antibodies and UV-light microscopy in rats. No changes were observed in the macrophage cell population or in any T-cell subsets after cocaine administration, but pan T cells exhibited a non-dose-related decrease in the relative percentage, and B cells exhibited a non-dose-related increase in the relative percentage. It is important to note, however, that absolute cell numbers were not calculated, and these results are therefore difficult to interpret.

Lopez *et al.* (1992a) similarly studied cocaine-induced modification of lymphocyte subsets by developing a mouse model with female C57BL/6 mice that were protein malnourished and infected with functional murine AIDS. After daily i.p. injections of cocaine, labeling of spleen cell subsets with monoclonal antibodies and analysis by flow cytometry revealed decreases in the percentages of pan T, T_H and T_{CTL}/T_S cells, and increased percentages and absolute numbers of B cells in non-HIV-infected saline- and cocaine-treated mice as compared with untreated controls. Non-cocaine-treated, ret-

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ABBREVIATIONS: CD, cluster designation; P-450, cytochrome P-450; DNP, dinitrophenylhydrazine; EBSS, Earle's balanced salt solution; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes; T_H, T-helper cells; T_{CTL}, T-cytotoxic cells; T_S, T-suppressor cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

rovirus-infected mice showed a decrease in percentages of pan T and T_{CTL}/T_S, and an increase in percentages and absolute numbers of IL-2R+ cells, T_H, macrophages and B cells. Treatment with cocaine had no effect on the elevated percentage of T_H cells or B cells, or the depressed numbers of T_{CTL}/T_S cells associated with virus infection. Cocaine, however, did reduce the number of activated IL-2R+ and macrophages, in addition to decreasing the total number of cells per spleen in retrovirus-infected mice only. From the data above, the author concluded that cocaine modifies cell proliferation in splenocyte subpopulations already altered by retrovirus infection (Lopez *et al.*, 1992a).

With the same murine model and dosing regimen, Lopez *et al.* (1992c) conducted additional studies to determine the effects of cocaine administration on thymocyte subpopulations. Cocaine treatment and saline treatment in non-retrovirus-infected mice resulted in absolute numbers of pan T cells, T_H cells and T_{CTL}/T_S cells that were 10% of the control values. Retrovirus infection in mice, which were not exposed to cocaine, caused decreases in the percentages and absolute numbers of these same subsets, and cocaine administration potentiated these effects. The authors postulated that cocaine may be modulating the immune system by altering the expression of T-cell differentiation markers after direct interaction with thymocytes or indirectly through the neuroendocrine-thymus axis (Lopez *et al.*, 1992b,c).

Holsapple *et al.* (1993) recently proposed a model which may account for the inconsistency of evidence. The model asserts that cocaine produces changes in immunocompetence through the generation of metabolites by P-450 metabolism in the liver. This proposal was based on the observation that hepatotoxicity, which like immunotoxicity is a minor component of cocaine's profile of toxicity, is mediated by reactive intermediates of cocaine. Although the P-450 system is normally a minor pathway for the metabolism of cocaine, it becomes an important one in situations where metabolism by esterase is inhibited, as is the case with exposure to organophosphate pesticides, such as diazinon, or when P-450 activity is induced, as occurs with excessive alcohol consumption.

Support for this model has come from two recent reports from our laboratory. First, Jeong *et al.* (1995a) demonstrated that whereas norcocaine, the primary metabolite of the P-450 pathway, was directly immunosuppressive when added to cultured splenocytes, neither cocaine nor benzoylecgonine, a primary metabolite of the esterase pathway, caused suppression. In the same paper, they showed that preincubation of splenocytes with cocaine plus liver homogenates was suppressive, whereas preincubation with cocaine alone was devoid of activity (Jeong *et al.*, 1995a). In the second study, Jeong and co-workers compared the suppression by repeated injections of cocaine alone with the suppression observed when cocaine was administered to mice pretreated with either a P-450 inducer, β-ionone, or an esterase inhibitor, diazinon (Jeong *et al.*, 1995b). Their results indicated that neither β-ionone nor diazinon had any effect when administered alone. More importantly, they showed that cocaine alone was without effect, but that cocaine was markedly immunosuppressive in mice pretreated with either β-ionone or diazinon, with the greater suppression associated with the administration of the esterase inhibitor.

These results are consistent with the metabolism model proposed above and indicate that cocaine exerts its most dramatic effects on immunocompetence after exposure to mice that have been pretreated with diazinon. To further characterize the profile of cocaine-induced immunotoxicity, the primary objective of the present study was to determine by flow cytometric analysis the changes that occur in splenic and thymic cell populations in mice treated with cocaine + diazinon. The results will be compared with mice treated with cocaine alone.

Materials and Methods

Animals. Female virus-free B6C3F1 mice, 5 to 7 weeks of age, were purchased from the Frederic Cancer Research Center (Frederic, MD). Upon arrival, the mice were randomized and housed four per cage in plastic cages containing sawdust bedding. They were quarantined for 1 week and were not used for experimentation until body weights reached 17 to 20 g. Mice were given food (Purina Certified Laboratory Chow) and water *ad libitum*. Animal holding rooms were maintained at 21–24°C and 40 to 60% relative humidity with a 12-hr light/dark schedule.

Drugs and chemicals. Cocaine hydrochloride was provided by the National Institute on Drug Abuse (Research Triangle Park, NC). Diazinon was obtained from Chem Service Co. (West Chester, PA). Dulbecco's PBS supplemented with 0.1% sodium azide and 1% bovine serum albumin, EBSS without HEPES and Roswell Park Memorial Institute (RPMI) 1640 buffer supplemented with 7.5% sodium bicarbonate and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO).

Dosing regimen. Diazinon stock solutions of 1.5 to 2 mg/ml were prepared in corn oil in advance, and cocaine stock solutions of 1 to 3 mg/ml were prepared fresh daily in saline. Mice were pretreated with 0.2 ml corn oil or diazinon (10 or 15 mg/kg) *via* i.p. injection and, 30 min later, saline or 10, 20 or 30 mg/kg cocaine was administered i.p. in a 0.2-ml volume. This regimen was administered once a day for seven consecutive days.

T-dependent antibody response to SRBC. On day 4 of the 7-day exposure, all mice were sensitized by i.p. injections of 1×10^8 SRBC in a 0.5 ml volume of EBSS. twenty-four hours after the last exposure to cocaine, mice were anesthetized with CO_2 and were sacrificed by cervical dislocation. Spleens were removed and mashed to make single-cell suspensions in 3.0 ml EBSS and the total number of cells in each suspension was counted with a Coulter counter. A 30-fold dilution of the cells was made by adding 100 μl of each cell suspension to 2.9 ml of EBSS. Aliquots of the cell suspensions were added to test tubes containing 350 μl of agar, 25 μl complement and 25 μl of SRBC in a 47°C water bath. After vortexing, the contents of the tubes were poured into 100 × 15 mm Petri dishes and covered with 45- to 50-mm microscope coverslips. After solidification of the agar, the Petri dishes were incubated at 37°C for 3 hr. At this time the antibody-forming cells (AFC), visible as clear plaques in the agar, were enumerated with a Bellco plaque viewer at a magnification of 6.5 ×.

T-independent antibody response to DNP-ficoll. The conditions for this assay were similar to the T-dependent antibody response, except that the mice were immunized with 20 ng of DNP-ficoll in 0.2 ml of EBSS. The determination of the anti-DNP antibody response required the utilization of SRBC which were densely coupled with trinitrophenyl and which were prepared according to the method of Rittenberg and Pratt (1969).

Immunofluorescent staining for flow cytometric analysis. Spleens and thymuses were isolated from mice exposed to the 7-day dosing regimen described previously. After organ weights were obtained, thymocyte and splenocyte single-cell suspensions were prepared by mashing organs and adding 3 ml PBS/0.1% sodium azide/1% bovine serum albumin. The total number of cells per suspension was enumerated on a Coulter counter, then adjusted to 1 ×

10^7 cells/ml and a 100-μl volume was added to each well of a round bottom 96-well microtiter plate (Costar). Cells were pelleted by centrifugation for 2 min at 300 × *g*. The supernatant was aspirated with a 25-gauge needle, and the plate was gently vortexed to resuspend the cell pellet. Twenty microliters of 2.4G2 was added to each well to block nonspecific Fc binding and the plates were incubated on ice for 10 min. Without washing, 3 to 4 μg of the appropriate antibody (Becton-Dickinson, San Jose, CA) was added to wells containing 10^6 cells and the plates were incubated on ice in the dark for 30 min. Phycoerythrin-conjugated anti-L3T4 and FITC-conjugated anti-Lyt2 were diluted 1:40 from the stock solution and were added to stain for the CD4+ and CD8+ T-cell subsets, respectively, in the spleen and thymus. A double-staining procedure was used to identify immature T cells (CD4+/CD8+) and prethymocytes (CD4−/CD8−) in the thymus and to identify immature thymocytes (CD4+/CD8+) and non-T cells, such as B cells and macrophages (CD4−/CD8−) in the spleen. FITC-conjugated anti-CD3, diluted 1:20, was used to stain the pan T-cell population in the spleen and thymus. Phycoerythrin-conjugated anti-CD45 (anti-B220), diluted 1:20 from a stock solution, was used to stain for the B-cell population in the spleen, and fluorescein-conjugated anti-Mac-1 was used to identify the splenic macrophage population. After incubation, the cells were pelleted and washed twice in 0.15 ml of PBS, then 0.1 ml of propidium iodide (5 μg/ml; Sigma) was added for viability analysis, allowing the positively staining population to be gated out and thus excluded from the analysis. The plate was incubated on ice for 5 min and, following two washes with PBS, cells were resuspended in 0.15 ml PBS and analyzed on a Becton-Dickinson FACScan TM Flow Cytometer equipped with a Hewlett Packard HP 9000 series computer with applications software allowing for single and multiparameter analysis of the data. 7000 viable cells were analyzed.

Statistics. The mean ± standard error was determined for each treatment group and *post hoc* analysis by Dunnett's two-tailed *t* test was performed to determine significant differences between treatment and control groups. A value of $P < .05$ was considered significant for all statistical tests.

Results

Effects of cocaine + diazinon on antibody responses. In previous studies conducted by Jeong *et al.*, a 60 to 80% suppression of the T-dependent antibody response was seen consistently in mice treated with either 10 or 30 mg/kg/day diazinon + 30 mg/kg cocaine (Jeong *et al.*, 1995b). Mice treated with cocaine and corn oil (the vehicle for diazinon) alone or with diazinon and saline (the vehicle for cocaine) exhibited minimal effects on immunocompetence. A similar type of interaction study was used to test the effects of cocaine on the T-independent antibody response to DNP-ficoll. Results indicated that cocaine (30 mg/kg) + diazinon (10 mg/kg) caused less than a 40% suppression of the T-independent antibody response (fig. 1) under the same conditions where a greater than 60% suppression of the T-dependent antibody response was observed.

In subsequent studies, the interaction between cocaine and diazinon was confirmed in the T-dependent antibody response and was further characterized by expanding the dose-response curve of cocaine. Mice were treated with either corn oil or diazinon at 15 mg/kg/day and were then treated with saline or cocaine at 5, 10, 20 or 30 mg/kg/day for 7 consectuive days. Results are presented as AFC/10^6 spleen cells (fig. 2). In agreement with previous results, cocaine alone was not suppressive and in fact caused a slight dose-related enhancement. We have previously observed an enhancement with cocaine (Holsapple *et al.*, 1993) and have speculated that this effect is mediated by cocaine-induced changes in the neuroendocrine system. Pretreatment with diazinon markedly changed the profile of activity by cocaine and results in a marked dose-related suppression with significance noted at all doses. Data were also calculated as AFC/spleen, and results were similar to those expressed as AFC/10^6 spleen cells.

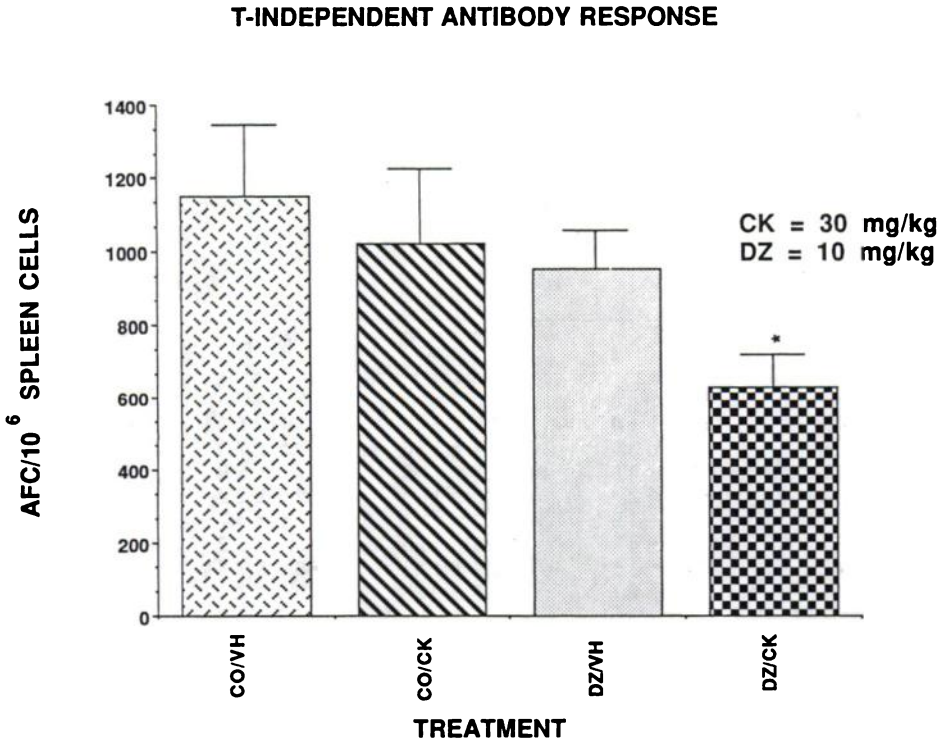

Fig. 1. Effects of cocaine on the T-independent antibody response to DNP-ficoll. Mice (n = 4) were exposed to corn oil (CO) or diazinon (DZ; 10 mg/kg) in CO, 30 min before an i.p. injection of saline (VH) or cocaine (CK; 30 mg/kg) in VH for 7 consecutive days. Mice were immunized with DNP-ficoll on day 4 and the spleens were removed 24 hr after the last exposure. The antibody responses were measured as described under "Materials and Methods." Statistical significance was determined by Dunnett's two-tailed *t* test, with * = significantly different from CO/VH control at $P < .05$, and ** = significantly different from CO/VH control at $P < .01$. Results are representative of duplicate trials.

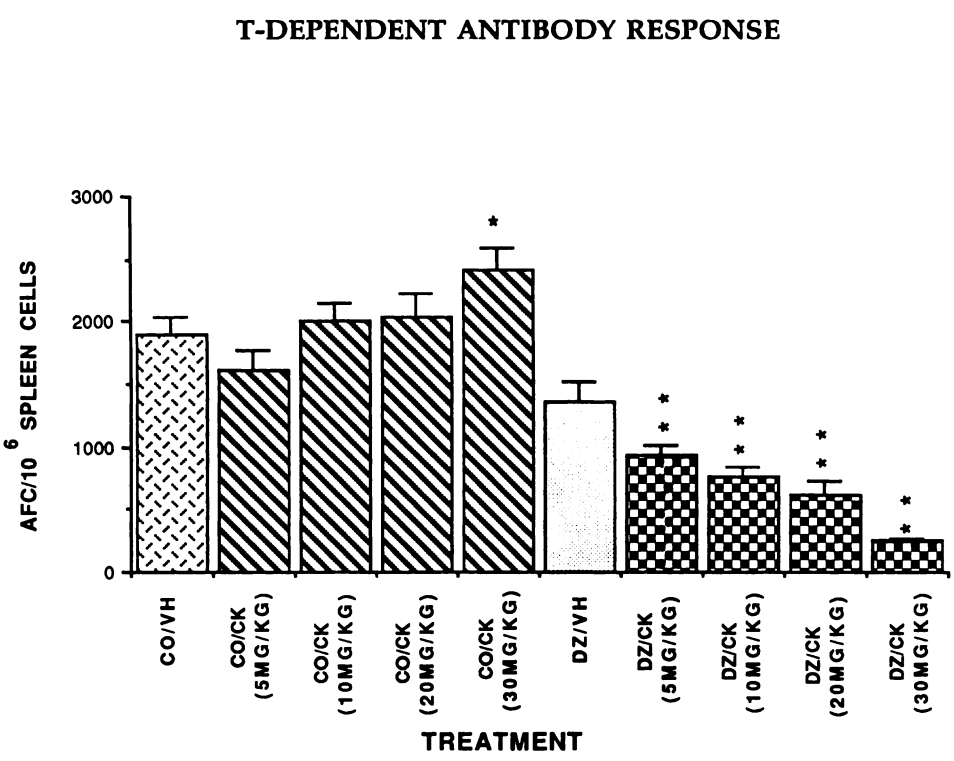

Fig. 2. Suppression of the T-dependent antibody response by cocaine + diazinon. Mice (n = 4) were exposed (i.p.) to corn oil (CO) or diazinon (DZ; 15 mg/kg) in CO, 30 min before an i.p. injection of saline (VH) or cocaine (CK; 5, 10, 20 or 30 mg/kg) in VH for 7 consecutive days. Mice were immunized with SRBC on day 4 and spleens were removed 24 hr after the last exposure. The T-dependent assay was performed as described under "Materials and Methods." Statistical significance was determined by Dunnett's two-tailed t test, with * = significantly different from CO/VH control at $P < .05$, and ** = significantly different from CO/VH control at $P < .01$. Data are expressed as AFC per 10^6 spleen cells and are representative of three trials.

Effects of cocaine + diazinon on organ weight. As indicated in table 1, thymus weight was unaffected by administration of cocaine alone. In other trials, however, effects on thymus weight have been variable, and we have seen as much as a 40% reduction in thymic weight at the highest dose of cocaine (data not shown). In contrast, dramatic decreases in thymic weight have been consistently observed after administration of cocaine + diazinon at all concentrations, with 20 and 30 mg/kg cocaine producing significant decreases in thymic weight. Similar effects on thymus cellularity were observed, with decreases of 72% and 94% at 20 mg/kg and 30 mg/kg cocaine, respectively, in diazinon-pretreated mice (data not shown).

Neither spleen weight nor spleen cellularity was affected by administration of cocaine alone or by administration of cocaine + diazinon (table 1). Other trials have indicated, however, that the highest dose of cocaine + diazinon may cause a modest but typically insignificant decrease in spleen weight and cellularity (data not shown).

Administration of cocaine alone produced no significant changes in either final body weight or liver weight. Body weight was significantly decreased only at the highest dose of cocaine + diazinon, and liver weight was significantly increased at the two highest doses of cocaine + diazinon. At these two doses, livers appeared pale and mottled.

Effects of cocaine + diazinon on spleen cell populations. Splenocyte subsets were analyzed after i.p. administration of either corn oil or diazinon at 15 mg/kg/day before i.p. exposure to saline or cocaine at 10, 20 or 30 mg/kg/day for 7 consecutive days. Immunofluorescent staining and flow cytometric analysis of CD4, CD8, B220 and MAC-1 markers were used to enumerate T_H cells, T_S cells, B cells and macrophages, respectively. Results for the individual subpopulations of spleen cells are shown in figure 3, parts A to D.

TABLE 1
Effects of cocaine (CK) + diazinon (DZ) on body and organ weights[a]

Treatment[b]	Body	Liver	Thymus	Thymus Cell No. (× 10^6)	Spleen	Spleen Cell No. (× 10^6)
	g	mg	mg		mg	
CO + VH	19.8 ± 0.5	1027.4 ± 46.8	59.3 ± 1.9	160.2 ± 14.1	67.7 ± 5.3	80.2 ± 12.3
CO + CK(5)	19.4 ± 0.5	1055.8 ± 56.9	55.8 ± 3.6	158.4 ± 10.6	78.5 ± 7.0	61.0 ± 7.1
CO + CK(10)	21.0 ± 0.9	1161.6 ± 102.1	60.0 ± 4.6	168.5 ± 11.8	77.5 ± 6.8	80.2 ± 4.3
CO + CK(20)	20.0 ± 0.6	1137.6 ± 34.9	44.5 ± 2.4	119.0 ± 11.9	82.0 ± 2.2	105.6 ± 13.6
CO + CK(30)	20.6 ± 0.3	1240.8 ± 55.2	34.5 ± 1.9**	91.0 ± 11.1**	71.0 ± 4.7	124.4 ± 5.8
DZ(15) + VH	20.0 ± 0.4	1131.4 ± 33.0	45.7 ± 4.7	114.6 ± 16.0	70.0 ± 5.2	120.8 ± 6.1
DZ(15) + CK(5)	20.1 ± 0.5	1112.6 ± 47.6	44.5 ± 2.5	113.7 ± 10.3	68.8 ± 7.8	130.4 ± 21.0
DZ(15) + CK(10)	20.7 ± 0.9	1259.0 ± 44.7	38.8 ± 2.7	111.6 ± 9.9*	79.0 ± 7.1	119.1 ± 13.6
DZ(15) + CK(20)	18.6 ± 0.3	1601.2 ± 70.1**	17.5 ± 3.6**	45.3 ± 11.2**	75.3 ± 6.8	93.8 ± 5.3
DZ(15) + CK(30)	16.3 ± 0.9**	1450.2 ± 104.9**	9.7 ± 1.8**	9.8 ± 3.6**	53.4 ± 2.0	96.7 ± 10.1

[a] Mice were sacrificed 24 hr after the last exposure and body weights were recorded. Select organs were removed and weighed. Statistical significance was determined by Dunnett's two-tailed t test. * = significantly different from CO/VH control at $P < .05$; ** = significantly different from CO/VH control at $P < .01$.

[b] Mice (n = 4) were exposed (i.p.) to corn oil (CO) or diazinon (DZ; 15 mg/kg) in CO, 30 min before an i.p. injection of saline (VH) or cocaine (CK; 5, 10, 20 or 30 mg/kg) in VH for 7 consecutive days.

Interpretation of significant alterations was facilitated by expressing results as absolute cell numbers, calculating by multiplying the percent of cells staining positively for a particular marker by the total number of cells in the spleen. The results were also analyzed as percent of control, and the trends were identical (data not shown).

No change was observed in either the T_H (CD4+/CD8−) splenic population (fig. 3A) or the T_S/T_{CTL} (CD4−/CD8+) splenic population in any of the dose groups (fig. 3B). Similarly, results from analysis of Mac-1 revealed no change in splenic macrophage cell numbers in any of the treatment groups (fig. 3D). Analysis of B-cell subsets by expression of B220 revealed a slight, dose-dependent decrease in cell numbers in treatment groups receiving cocaine alone. Administration of the highest dose of cocaine alone (30 mg/kg) resulted in a 25% decrease in cell number (fig. 3C). Administration of cocaine + diazinon similarly resulted in a dose-dependent decrease in cell number, with the highest dose of cocaine producing a 30% loss of B cells.

Effects of cocaine + diazinon on thymus cell populations. Dramatic changes were observed in the relative percentages of thymocyte subpopulations, especially at the highest dose of cocaine + diazinon (fig. 4, A–D). Again, as with results in the spleen, these data were expressed as

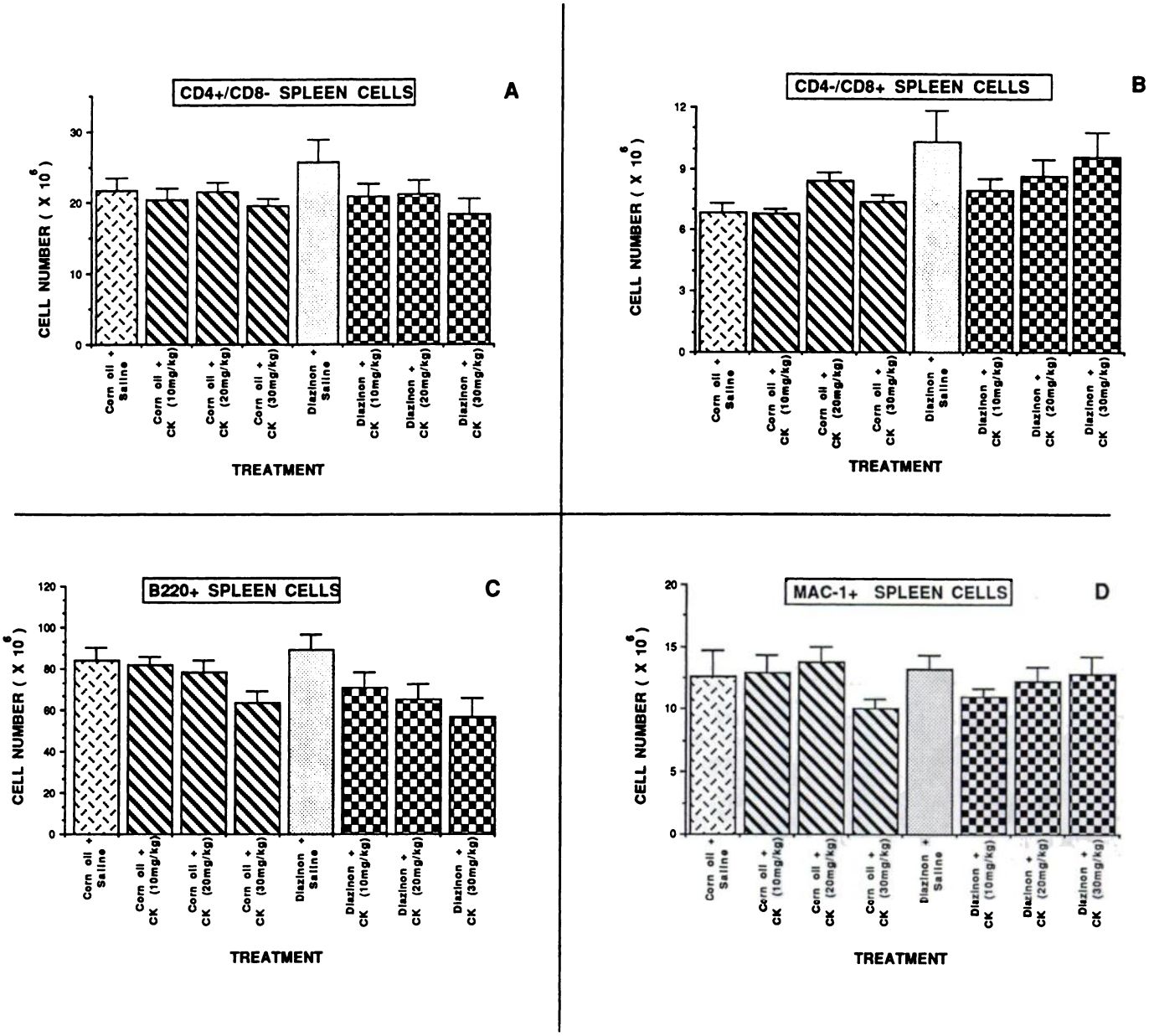

Fig. 3. Effects of cocaine + diazinon on subpopulations in the spleen. Mice (n = 4) were exposed (i.p.) to corn oil (CO) or diazinon (DZ; 15 mg/kg) in CO, 30 min before an i.p. injection of saline (VH) or cocaine (CK; 10, 20 or 30 mg/kg) in VH for 7 consecutive days. Spleens were removed 24 hr after the last injection, and cell suspensions were prepared and analyzed by flow cytometry as described under "Materials and Methods." Data are expressed as absolute cell numbers × 10^6 ± S.E. and were calculated by multiplying total number of cells in the spleen by percent of positive cells. Data are representative of two independent experiments. Statistical significance was determined by Dunnett's two-tailed t test. No significant difference was observed in any treatment groups. (A) T-helper cells (CD4+); (B) T-suppressor/T-cytotoxic cells (CD8+); (C) B cells (B220+); (D) macrophages (Mac-1+).

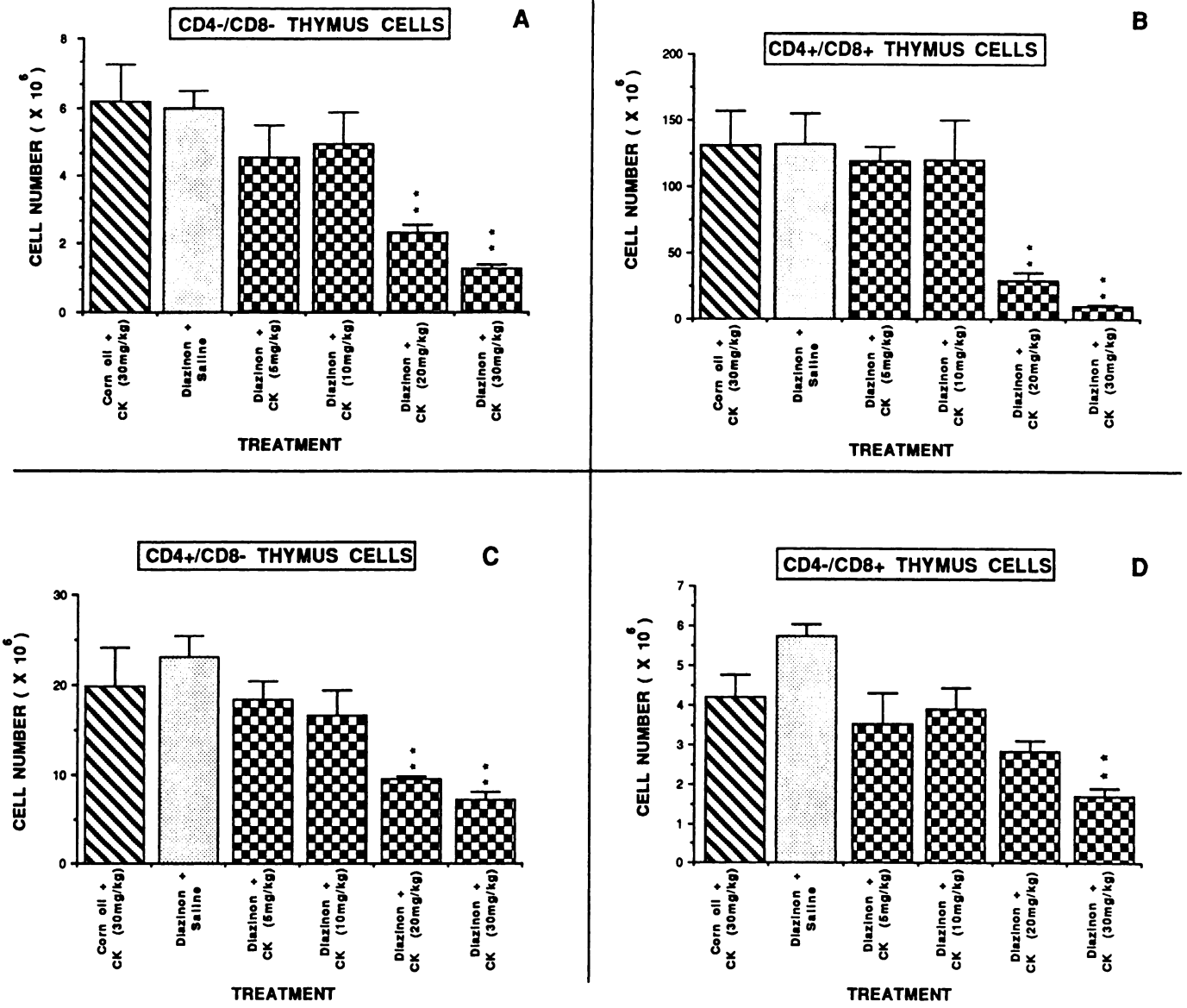

Fig. 4. Effects of cocaine + diazinon on subpopulations in the thymus. Mice (n = 4) were exposed (i.p.) to corn oil (CO) or diazinon (DZ; 15 mg/kg) in CO, 30 min before an i.p. injection of saline (VH) or cocaine (CK; 10, 20 or 30 mg/kg) in VH for 7 consecutive days. Thymuses were removed 24 hr after the last injection, and cell suspensions were prepared and analyzed by flow cytometry as described under "Materials and Methods." Data are expressed as absolute cell numbers $\times 10^6 \pm$ S.E. and were calculated by multiplying total number of cells in the spleen by percent of positive cells. Data are representative of two independent experiments. Statistical significance was determined by Dunnett's two-tailed t test. ** = significantly different from diazinon + saline control at $P < .01$. (A) Double-negative thymocytes (CD4−/CD8−); (B) double-positive thymocytes (CD4+/CD8+); (C) T-helper cells (CD4+); (D) T-suppressor/T-cytotoxic cells (CD8+).

absolute cell numbers, calculated by multiplying the percent of cells staining positively for a particular marker by the total number of cells in the thymus. No change in absolute cell number in any of the thymic subsets was observed in the treatment groups receiving cocaine alone, so the figures for the various thymic subpopulations include only the group receiving the highest dose of cocaine (30 mg/kg) for comparative purposes. As with the results from the splenocyte populations, similar trends were noted when absolute values were compared with data expressed as percent of control, so only the former is used for data presentation.

Double-negative (CD4−/CD8−) thymus cells, which represent the prethymocyte subpopulation, were suppressed only by 62% and 78% decreases at 20 mg/kg and 30 mg/kg cocaine + diazinon, respectively (fig. 4A). Analysis of the double-positive immature thymocyte population revealed a similar profile, with a decrease in cell numbers only at the two highest doses of cocaine + diazinon. Administration of 20 and 30 mg/kg cocaine to diazinon-pretreated mice resulted in immature thymocyte losses of 77% and 90%, respectively (fig. 4B). The T_H cell, or CD4+/CD8−, subpopulation was similarly affected only by exposure to 20 and 30 mg/kg cocaine in diazinon-pretreated mice. These treatment groups resulted in decreases of 58% and 67%, respectively (fig. 4C). Unlike the other thymocyte subpopulations, T_S/T_{CTL} (CD4−/CD8+) cells suffered a significant loss only at the highest dose of

cocaine + diazinon, where cell numbers were decreased by 70% (fig. 4D). Changes in pan T cells, which express the CD3 marker, were also measured. The two highest doses of cocaine resulted in losses of 67% and 93%, respectively, in the number of CD3+ cells in diazinon-pretreated mice (data not shown).

Discussion

As discussed above, evidence supporting the possibility that exposure to cocaine produces immunotoxicity has been controversial and unspectacular. Holsapple and co-workers (1993) in our laboratory proposed a model that accounted for this tenuous evidence by asserting that cocaine's immunosuppressive effects are primarily mediated through the production of reactive metabolites by the cytochrome P-450 system. Because this pathway is normally a minor one in the metabolism of cocaine, exposure to cocaine alone would not be expected to produce dramatic changes in immune function, either in terms of the magnitude of effect or in terms of the size of the population shown to be affected. This model could therefore not only account for the inconsistent results obtained thus far from animal studies, but could also explain the lack of evidence, anecdotal or otherwise, which supports a robust effect by cocaine on human immunocompetence. However, metabolism *via* the P-450 pathway takes on significance when increased, after exposure to organophosphate pesticides, which inhibit esterase activity, or after excessive alcohol consumption, which induces cytochrome P-450 activity (Boyer and Peterson, 1990). Therefore, it could be that the immunological effects of cocaine are limited to relatively small subpopulations, *i.e.*, individuals in which metabolism of cocaine by the P-450 system is increased.

Generation of reactive metabolites has also been shown to play a role in cocaine-induced hepatotoxicity, which is a minor component in cocaine's profile of toxicity, especially in man. A number of parallels can be drawn between cocaine-induced hepatotoxicity and immunotoxicity. For example, it was noted that the administration of a P-450 inducer, such as phenobarbital, or an esterase inhibitor, such as diazinon, markedly increased the hepatoxicity induced by cocaine (Thompson *et al.*, 1979; Evans and Harbison, 1978; Kloss *et al.*, 1982; Evans, 1983). Therefore, a working model was proposed which centered around a role by reactive metabolites produced by P-450-dependent metabolism of cocaine. In earlier studies, Jeong and co-workers (1995b) demonstrated, in concert with this proposed model, that cocaine exerts its most dramatic effects on immunocompetence after exposure to mice that have been pretreated with diazinon. Because exposure to organophosphates has been suggested to be associated with immunotoxicity (Newcombe and Esa, 1992), concern was raised initially over the possibility that the immunosuppression was caused, not by cocaine administration, but by the organophosphate itself. To address this concern, Holsapple and co-workers (1993) first tested the effects of diazinon administration on the T-dependent antibody response. The exposure regimen entailed administration of diazinon daily for 7 consecutive days. No change in immune function was observed, although the mice clearly demonstrated some of the signs associated with organophosphate intoxication at the highest dose tested, *i.e.*, salivation, urination and diarrhea.

In the initial studies of the present investigation, mice were pretreated with either 10 or 30 mg/kg diazinon, and then were administered either saline or 30 mg/kg of cocaine daily for 7 consecutive days. Even at the highest dose of diazinon + saline, no suppression of the antibody response was observed (fig. 1). In contrast, the antibody response by mice exposed to diazinon + cocaine was suppressed significantly, and the suppression was related to the dose of diazinon administered. The observation that both T-dependent and T-independent antibody response models were significantly suppressed suggests that cocaine treatment affects either the B cell or the macrophage; but the observation that the T-dependent response was more sensitive than the T-independent response suggests that T-helper cells may be especially sensitive to cocaine-induced changes. Overall, these results suggest that multiple cell types may be affected by cocaine exposure, but that the T cell may be the most sensitive target. The T-dependent assay was thus expanded to include multiple doses of cocaine ranging from 5 to 30 mg/kg/day, and pretreatment with diazinon was limited to a dose of 15 mg/kg. As in the earlier studies with cocaine, no effect was associated with exposure to cocaine alone, whereas exposure to diazinon + cocaine produced a dose-related suppression in which treatment with the highest dose of cocaine resulted in >80% suppression of the T-dependent antibody response.

This alteration in splenic immune function prompted the present investigation to determine whether the suppression of the spleen's ability to produce antibody was associated with changes in the numbers of any of the splenocyte subpopulations. As shown in table 1, it was also noted that, in addition to suppression of the antibody response, administration of cocaine + diazinon caused marked decreases in thymic weight. These observations led to flow cytometric investigation of thymic cell subpopulations as well. In agreement with the results from the antibody response model, it was determined that exposure to cocaine alone did not produce marked changes in either organ except for an occasional modest decrease that was noted only at the highest dose. It was also determined from these studies that administration of cocaine + diazinon did not produce a robust change in splenic cellularity and only modestly affected B-cell number by causing a slight decrease. Although the only cell type affected in the spleen was the B cell, the primary effector cell involved in an antibody response, it is difficult to imagine how a very modest reduction of ~20% in B-cell number could account for a marked reduction of >80% in the antibody response.

In contrast to results in the spleen, T-cell subpopulations in the thymus were dramatically affected, with decreases in all subsets. Immature double-positive thymocytes suffered the greatest loss in cell number and, because these cells are the immediate precursors of mature single-positive T_H and T_S cells, it follows that these mature populations would suffer a loss as well. Overall, these results indicate that a variety of cell types may be affected by administration of cocaine + diazinon, but certain types, such as immature thymocytes located in the thymus, are a more sensitive target for cocaine or cocaine metabolites.

It is tempting to speculate that the tremendous loss of functional T cells in the thymus may render the spleen unable to respond to a T-dependent antigen. However, it is

important to note that the complete cycle of T-cell maturation, from prethymocyte to migration of mature T cells out of the thymus and into the periphery, takes considerably longer than the 7 days used for the exposure regimen in these studies. Moreover, as indicated under "Materials and Methods," our protocol called for sensitization of the mice on day 4 of exposure. Therefore, the critical role of T cells in the T-dependent antibody response occurred even earlier in the exposure paradigm. As such, it is reasonable to assume that there are enough mature T cells already in the spleen at the time of sensitization to provide for an adequate T-dependent response. This speculation is also supported by the fact that no loss of mature T cells was observed in the spleen. Further evidence against a cause-and-effect association between thymocyte depletion and suppression of the antibody response is provided by previous studies examining the immune responses of thymectomized animals. When the primary and secondary antibody responses were tested 72 days after thymectomy in 8-week-old mice, it was revealed that the surgical loss of the thymus did not cause suppression of the immune response. The population of small mononuclear cells in the peripheral blood, however, had moderately decreased by this time, and was dramatically decreased when examined 100 days after the procedure (Miller *et al.*, 1963). Because cocaine + diazinon administration can be considered, in essence, a chemical thymectomy, as evidenced by decreased thymus weight and cell number, the conclusion can be drawn that thymocyte depletion is not the cause of the observed functional suppression. Although these results are not consistent with a cause-and-effect association, it is certainly possible that the two events, *i.e.*, suppression of the T-dependent antibody response and thymic atrophy, may be caused by a common, or at least similar, mechanism of action. Regardless of the mechanism, it is important to emphasize that the observed effects on immunocompetence, whether in the spleen or thymus, were produced only when animals were pretreated with diazinon.

Because of this dependence on pretreatment with diazinon, mechanisms which may account for both events involve the idea that diazinon administration causes an increase in the levels of cocaine by blocking the primary metabolic pathway, and/or an increase in the generation of cocaine metabolites, such as norcocaine, by increasing the involvement of metabolism by the P-450 pathway in the two immunocompetent organs. These elevated levels could mediate immunosuppression by directly affecting cells in the spleen or thymus. It therefore seems plausible that if a direct effect is occurring, it should be possible to measure these elevated levels in the spleen and thymus after administration of cocaine + diazinon. However, previous biodisposition studies conducted in this laboratory determined that only extremely low levels of cocaine and norcocaine were detectable in the spleen and thymus, which led to the conclusion that the cocaine-induced immunotoxicity is not being mediated by direct effects by either cocaine or norcocaine (Kump *et al.*, 1994).

There are several indirect mechanisms that may account for cocaine-induced immunosuppression. One of these mechanisms involves the release of soluble serum factors, such as transforming growth factor-β (TGF-β), from the liver. It is hypothesized that cocaine induces hepatic damage *via* reactive metabolites generated by the P-450 system, which in turn triggers the release of these soluble factors. Although their primary function is hepatic repair, it has been well documented that factors such as TGF-β also cause immunosuppression (Carlino *et al.*, 1986). Prelimary results from Matulka and co-workers (manuscript in preparation) in our laboratory indicate that administration of cocaine + diazinon results in elevated serum levels of TGF-β and that serum from cocaine + diazinon-treated mice is capable of causing suppression of the antibody response. In addition, neutralizing antibodies against TGF-β block this suppression.

Another mechanism that may account for the observed cocaine-induced immunosuppression has been proposed and is being investigated by E.D. Stanulis in our laboratory. Cocaine has been reported to cause elevations in adrenocorticotropin, which may be caused by cocaine-induced corticotropin-releasing factor release (Rivier and Vale, 1987). The increased levels of adrenocorticotropin are then able to stimulate release of corticosterone, which has been shown to suppress immune function (Watzl and Watson, 1990). Preliminary studies by Stanulis and co-workers (manuscript in preparation) has shown that administration of cocaine is associated with elevations in blood levels of corticosterone and that some of the effects by cocaine on immune function can be reversed by a glucocorticoid antagonist; effects are also reversed when cocaine is administered to adrenalectomized mice.

It is evident that further studies are necessary to elucidate the mechanism by which cocaine induces immunotoxicity. The significance of the present studies resides in the fact that the thymus has been identified as a sensitive target for the effects of cocaine. Moreover, because the effects by cocaine were only observed in mice that had been pretreated with diazinon, these results provide additional evidence in support of our model, which is centered around a role by the P-450-dependent metabolism of cocaine.

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