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3-Methylindole-induced Splenotoxicity: Biochemical Mechanisms of Cytotoxicity[1]

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3-Methylindole-induced Splenotoxicity: Biochemical Mechanisms of Cytotoxicity. UPDYKE, L. W., YOON, H. L., KIORPES, A. L., ROBINSON, J. P., PFEIFER, R. W., AND MARCUS, C. B. (1991). *Toxicol. Appl. Pharmacol.* **109**, 375–390. 3-Methylindole (3-MI) is a pneumotoxic metabolite of L-tryptophan that can form in the digestive tracts of humans and ruminants as a result of microbial protein metabolism. Alternatively, human lungs can be directly exposed to 3-MI formed during protein pyrolysis and inhalation of tobacco smoke. 3-MI has been shown to cause acute lung injury in both ruminants and rodents. The present studies demonstrate that the spleen is also a target for 3-MI-induced toxicity. A dose-dependent decrease in splenic weight (24–75%) and nucleated splenic cell number (22–68%) was observed 24 hr after intraperitoneal injection of 3-MI (50–300 mg/kg) to intact and adrenalectomized rats. These findings were associated with significant alterations in splenic histopathology. Mice appeared less affected by 3-MI than rats as no splenotoxicity was observed at doses less than 200 mg/kg. Other mono- and dimethyl-substituted indoles did not decrease mouse spleen cell numbers when administered *in vivo*. Phenobarbital pretreatment *in vivo* protected against 3-MI-induced splenotoxicity, suggesting a role for cytochrome P450-mediated metabolism of 3-MI in the splenotoxicity of this compound. Exposure of rat or mouse splenic cells to 3-MI (1 mM) *in vitro* resulted in toxic changes over 24 hr. However, equimolar concentrations of the structurally related mono- and dimethylindoles were also toxic *in vitro*, and preincubation with a variety of inhibitors of cytochrome P450 or prostaglandin synthase *in vitro* failed to protect against 3-MI-mediated toxicity to splenic cells in culture. These results suggest mechanisms of 3-MI splenotoxicity also exist that do not require bioactivation, and indicate a possible role for alkylindoles in suppression of immune function.

3-Methylindole (3-MI) is a pneumotoxic product of L-tryptophan metabolism formed in the digestive tract of ruminants and, less commonly, humans (Fordtran *et al.*, 1964; Huang *et al.*, 1977; Yokoyama and Carlson, 1979). Absorption of 3-MI from the gut causes acute lung injury characterized by bronchiolar alveolar epithelial necrosis and pulmonary edema in cattle (Carlson *et al.*, 1972), sheep (Bradley *et al.*, 1978), and goats (Huang *et al.*, 1977; Bray and Carlson, 1979; Mesina *et al.*, 1984). Human exposure to 3-MI may occur following microbial metabolism of trypto-

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phan-containing foods with subsequent colonic absorption of 3-MI (Fordtran *et al.*, 1964). Patients suffering from liver disease may be at increased risk for 3-MI-induced toxicity (Suyama and Hirayama, 1988). Of potentially greater significance, human lungs are directly exposed to 3-MI and other alkylindoles by inhalation of these compounds (which are formed as pyrolysis products of proteinacious material) during tobacco smoking. A single cigarette can produce from 4 to 50 μg of these compounds (Wynder and Hoffman, 1967; Hoffman and Rathkamp, 1970). Similarities between some of the lung lesions observed in animals following acute 3-MI exposure and emphysematous changes seen in persons chronically exposed to tobacco smoke have led to speculation that 3-MI might be a contributing factor in this disease (Huang *et al.*, 1977).

As demonstrated for other pneumotoxins, target organ metabolism of 3-MI by cytochrome P450 mixed-function oxidases to toxic intermediates is believed to be a critical event leading to disease (Hammond *et al.*, 1979; Huijzer *et al.*, 1987, 1989; Leung *et al.*, 1983; Nocerini *et al.*, 1983, 1985; Turk *et al.*, 1986). Glutathione conjugation at the *methyl* position of 3-MI suggests the formation of a highly reactive electrophilic imine methide intermediate (Nocerini *et al.*, 1985). Other studies have suggested bioactivation of 3-MI by cooxidation by prostaglandin synthase (Formosa and Bray, 1988; Formosa *et al.*, 1988). Nonsteroidal anti-inflammatory cyclooxygenase inhibitors have reportedly reduced 3-MI-induced lung lesions in cattle (Gibbs, 1984). This alternative pathway could be an important bioactivation route in organs containing low levels of cytochrome P450 (e.g., kidney and spleen). Mechanisms of 3-MI-mediated pneumotoxicity have recently been reviewed (Yost, 1989; Yost *et al.*, 1989; Bray and Kirkland, 1990).

Cattle are most susceptible to 3-MI pneumotoxicity (due, in part, to high levels of ruminal 3-MI production following sudden dietary changes; Carlson *et al.*, 1972). Mice and rats administered 3-MI develop lung lesions similar to those seen in ruminants, although higher doses are required (Turk *et al.*, 1984; Kiorpes *et al.*, 1988). In previous reports, histologic examination of mice following exposure to 3-MI confirmed the lung as the primary target organ; no evidence of histopathological changes were observed in the brain, heart, kidney, liver, and spleen (Turk *et al.*, 1984).

This report presents evidence that the spleen may be a second target organ for 3-MI in rats and mice, and describes the effects of 3-MI on splenic cells. The specific objectives of the studies reported here were to determine whether the observed 3-MI splenotoxic changes following 3-MI administration: (1) resulted from endogenous release of adrenal glucocorticoids, (2) were species-specific, (3) occurred both *in vitro* and *in vivo*, (4) were also caused by other indole congeners, and (5) required metabolic activation of 3-MI.

MATERIALS AND METHODS

Chemicals. 1-, 2-, 4-, 5-, 6-, and 7-monomethyl substituted indoles, and 1,2-, 2,3-, and 2,5-dimethylindole (DMI) were purchased from Aldrich Chemicals (Milwaukee, WI). 3-MI, indole, L-tryptophan, L-glutamine, indomethacin, 3-4,5-dimethylthiazo(-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and α- and β-naphthoflavone (ANF) were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT); RPMI-1640 medium from GIBCO (Grand Island, NY), and penicillin and streptomycin were purchased from Eli Lilly and Co. (Indianapolis, IN). SKF-525A was a generous gift from Smith, Kline and French, 1-phenylimidazole (PI) was from Fairfield Chemical Co. (Blythewood, SC), and phenobarbital sodium (PB) from Baker Chemicals (Phillipsburg, NJ). 1-Aminobenzotriazole (ABT) and deuterated 3-MI were generous gifts of Dr. Garold Yost (University of Utah).

Animals and animal treatments. Male Sprague–Dawley naive, sham-operated, and adrenalectomized rats, 125–150 g, were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Rats were housed in metal cages on wire screens over Bed-O'Cobs bedding (The Andersons-Industrial Products Division, Delphi, IN). All rats were provided 1% NaCl solution for 10 days postsurgery, thereafter receiving tap water. Food (Rodent Laboratory Chow No. 5001, Purina Mills Inc., St. Louis, MO) and water were provided *ad libitum*. A 12 hr light:12 hr dark photoperiod was maintained. Animals were allowed to accli-

matize to their new housing for at least 1 week prior to use. Rooms were maintained at 22–24°C and 40–60% relative humidity.

Rats were administered 50–400 mg/kg of test compound in 0.5 ml corn oil or corn oil alone intraperitoneally (ip). In some experiments, rats were pretreated with ip injections of 100 mg/kg phenobarbital in 0.5 ml phosphate-buffered saline (PBS) or PBS alone once daily for 3 consecutive days prior to 3-MI exposure.

In parallel experiments, male non-Swiss albino (NSA) mice, 6–8 weeks of age (Harlan Sprague–Dawley, Indianapolis, IN), were administered 25–400 mg/kg of selected mono- and dimethyl-substituted indole congeners ip in 0.2 ml corn oil. Other groups were administered 60 mg/kg PB (0.2 ml saline, ip) or 40 mg/kg β-naphthoflavone once daily for 3 consecutive days (0.2 ml corn oil, ip), or a single dose of 50 mg/kg SKF 525A, 2 mg/kg indomethacin (0.2 ml corn oil, ip), or 600 μl/kg diethylmaleate 1 hr prior to 3-MI treatment.

Preparation of splenic cell suspensions. Rodents were lightly anesthetized with ether then euthanized by cervical dislocation. The spleens were removed aseptically and weighed. Single-cell splenic suspensions were prepared as previously described (Updyke *et al.*, 1988). Briefly, spleens were dispersed by gentle pressure through a stainless-steel mesh screen. Erythrocytes were lysed with 0.15 M Tris-buffered ammonium chloride (pH 7.2) and cells were washed three times with fresh medium. Following the final wash, cell pellets were resuspended in RPMI-1640 medium supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (complete medium). Viability and cell counts were assessed by trypan blue exclusion on a hemocytometer. In experiments requiring macrophage (MP)-depleted splenic cell suspensions, MPs were removed by adherence during 2-hr incubations in plastic tissue culture flasks as previously described by Updyke *et al.* (1989).

In vitro treatments with alkylindoles. 3-MI and congeners were dissolved in vehicle (2-, 3-, deuterated-3-, 5-, and 7-MI; 1,2-; 2,3-, 2,5-DMI, L-tryptophan, and indole in 95% ethanol; or 1-, 4-, and 6-MI in dimethyl sulfoxide) and diluted in complete medium prior to addition to splenic cell cultures to achieve a final concentration of 1 mM. The cytochrome P450 inhibitors were dissolved in dimethyl sulfoxide, and indomethacin was dissolved in 95% ethanol prior to dilution. Each were incubated with splenic cells at final concentrations of 100 μM PI, 10 μM ANF, 100 μM SKF 525A, 5 mM ABT, and 100 μM indomethacin for 30 min prior to exposure to 3-MI. Solvents and inhibitors alone at these concentrations were not cytotoxic in control incubations (except for SKF 525A which resulted in a 30% reduction in viability at the 100 μM concentration utilized in these studies). Higher concentrations of these inhibitors were either cytotoxic or insoluble in the incubation media. Splenic cell suspensions with or without test compounds were incubated at 37°C in humidified, 5% CO_2–air for up to 24 hr. Aliquots of cells were removed at various time points to assess cell viability.

MTT assay for splenocyte viability. An assay of cell viability was utilized to assess the toxicity of alkylindoles to spleen cells. This assay, a modification of that used by Leeder *et al.* (1988), is based on the ability of mitochondrial dehydrogenases in viable cells to reduce MTT to a water-insoluble purple formazan. At various time points, 0.9 ml (1.8×10^6 cells) was removed from incubations and centrifuged at 200*g* for 5 min and resuspended in 0.9 ml fresh medium. Twenty-five microliters of MTT (final concentration 2.4 mM) was added to 100 μl of cell suspension in each well of a 96-well U-bottom microtiter plate. The plates were incubated for 3 hr at 37°C in humidified, 5% CO_2–air and then centrifuged at 1000*g* for 10 min. The supernatant was removed by gently inverting the plate and the insoluble pellets were dissolved in 100 μl DMSO. Plates were left at room temperature protected from light for 30 min to allow complete crystal dissolution. The absorbance of each well was determined at 540 nm using a Model E-307 microELISA reader (Biotek Instruments, Inc.). Toxicity was evaluated based on the reduction in dye intensity compared to vehicle-exposed controls.

Histopathology. Cellular damage by alkylindoles to both rat and mouse spleens was evaluated at the light microscopic level. Spleens were removed 24 or 48 hr after treatment with corn oil vehicle or 400 mg/kg 3-MI in corn oil for rats, 300 mg/kg for mice. Tissues were gently washed with phosphate-buffered saline (PBS) pH 7.4, and placed in 10% Bouins fixative for at least 24 hr. After fixation, tissues were cleared in 70% ethanol, embedded in paraffin, cut into 5-μm transverse (mouse) or parasagittal (rat) sections, and stained with hematoxylin and eosin. Tissue samples were evaluated by light microscopy at magnifications shown of 100× (for rat sections) and 150× (for mouse).

Statistical analysis. For all *in vivo* experiments at least four animals were used per group. Triplicate samples of pooled cells (from at least two animals) were used for all *in vitro* assays. Mean differences between control and treatment groups were evaluated for statistical significance by Student's two-tailed *t* test or by one-way analysis of variance (ANOVA) followed by application of Newman–Keuls' range test to assess intergroup variance. The acceptable level of significance was established at $p < 0.05$. All experiments presented were repeated at least twice with similar results.

RESULTS

Splenotoxicity following in Vivo Exposure to 3-MI in the Rat and Mouse

In vivo splenotoxicity was observed following ip administration of 3-MI to rats. A dose-dependent decrease in viable nucleated splenic cell numbers and organ weights, 22–68% and

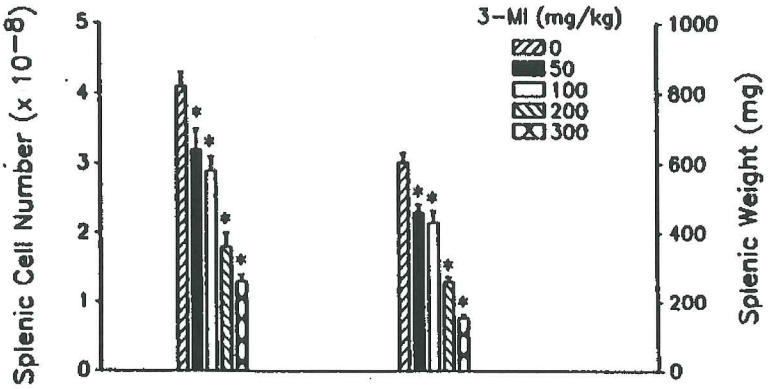

FIG. 1. Nucleated splenic cell number and splenic weight in rats 24 hr after ip administration of 50–300 mg/kg 3-MI. Bars represent mean of four animals ($n = 4$) ± SE. An asterisk indicates a significant difference ($p < 0.05$) from vehicle-treated controls.

24–75%, respectively, relative to vehicle controls (Fig. 1), was observed 24 hr after ip injection of rats with 50–300 mg/kg 3-MI in corn oil. An identical pattern of splenotoxicity was noted when Cremophore EL was used as the vehicle for 3-MI (data not shown), a carrier used in initial studies of 3-MI pneumotoxicity in the rat (Kiorpes *et al.*, 1988). Thymic weights and cell numbers in rats administered 300 mg/kg 3-MI were not significantly decreased compared to vehicle-treated control rats (data not shown).

A similar dose-dependent reduction in splenic cell numbers was observed in mice treated with 3-MI (Fig. 2), although this species appears to be less sensitive to splenotoxicity than rats since significant decreases were not observed until a dose of at least 200 mg/kg was administered. A time course of splenotoxicity suggested that significant reductions in numbers of viable mouse spleen cells occurred as early as 8 hr after administration of 300 mg/kg 3-MI, and significant reductions in cell numbers persisted at least 48 hr following treatment (Fig. 3).

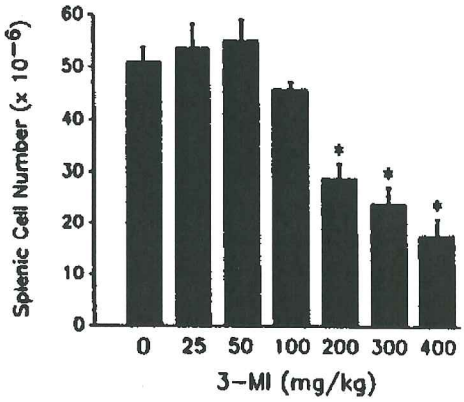

FIG. 2. Nucleated splenic cell number in mice 24 hr after ip administration of 25–400 mg/kg 3-MI. Bars represent the mean of three animals ($n = 3$) ± SE. An asterisk indicates a significant difference ($p < 0.05$) from vehicle-treated controls.

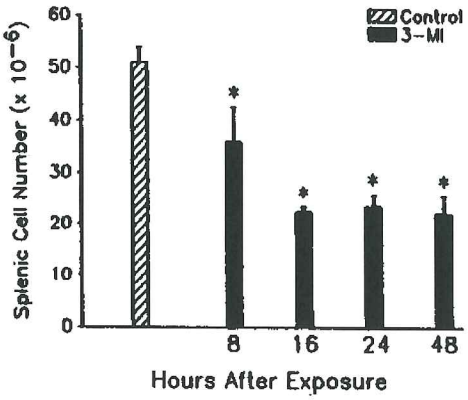

FIG. 3. Nucleated splenic cell number in mice 8–48 hr after ip administration of 300 mg/kg 3-MI. Bars represent the mean of three animals ($n = 3$) ± SE. An asterisk indicates a significant difference ($p < 0.05$) from vehicle-treated controls.

Splenotoxicity following in Vivo Exposure to 3-MI in the Adrenalectomized Rat

In order to determine the importance of indirect, stress-related glucocorticoid release in 3-MI-induced splenotoxicity, effects of 3-MI on adrenalectomized rat spleens were examined. Adrenalectomized (adx) and sham-operated (sham) rats were allowed to stabilize for 10 days after surgery prior to treatment with 3-MI. Doses of 200 and 300 mg/kg 3-MI ip caused a decrease in nucleated splenic cell numbers in control (nonoperated) and sham as well as adx rats (Fig. 4).

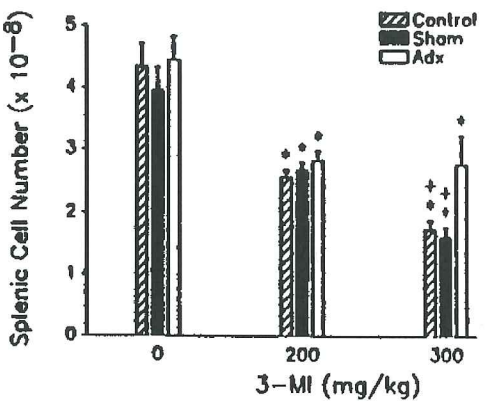

FIG. 4. Nucleated splenic cell numbers in control, sham-operated (sham), and adrenalectomized (adx) rats 24 hr after ip administration of 200 or 300 mg/kg 3-MI. Bars represent mean of four animals ($n = 4$) ± SE. An asterisk indicates a significant difference ($p < 0.05$) from vehicle-treated controls. A plus sign indicates a significant difference ($p < 0.05$) from animals receiving 200 mg/kg 3-MI.

Splenic Histopathology Following 3-MI Exposure in the Rat and Mouse

Forty-eight hours after ip administration of 400 mg/kg 3-MI to rats, dramatic changes in splenic tissue organization were observed. Compared to controls, rats treated with 3-MI had markedly less distinct periarteriolar lymphatic sheaths (PALS) and marginal sinuses, with expanded marginal zones containing an increased number of red blood cells. The red pulp appeared more disorganized, and the distinctive islands of prorubricytes (sites of extramedullary hematopoesis) were not as apparent as in untreated controls (Figs. 5A and 5B). Splenic sections viewed at higher magnifications (not shown) confirmed these observations.

In contrast, the effect of 3-MI (300 mg/kg) on mouse spleens 48 hr after treatment was less dramatic (Fig. 6C) compared to that of the rat (Fig. 5B), although the dose administered was somewhat less. Changes in mouse spleens were more subtle when viewed at magnifications used to examine rat spleens (100×, micrographs not shown) but were more clearly visible at greater magnification (150×) as shown in Figs. 6B and 6C. The architecture was preserved and the major change was a decrease in the density of lymphocytes within the PALS. Other changes included increased presence of vacuoles and pycnotic nuclei. The marginal zone and sinus were both present. Similar changes in mouse spleens were visible by 24 hr following administration of 400 mg/kg 3-MI (Fig. 6B). Spleens from mice treated with 300 mg/kg of other mono- and dimethylindole congeners of 3-MI (Table 1) were not different in appearance from vehicle controls 48 hr after treatment even at the higher magnification (data not shown).

Role of Metabolism in 3-MI-induced Splenic Toxicity: Cytochrome P450 and Prostaglandin Synthase Inducers and Inhibitors

Since cytochrome P450-mediated bioactivation of 3-MI to a reactive imine methide by lung microsomal cytochrome P450 appears to be a critical step in 3-MI pneumotoxicity (Huijzer *et al.*, 1987, 1989), the potential role of P450-mediated bioactivation in splenic 3-MI toxicity was investigated. Cytochrome P450 inhibitors and inducers were tested *in vitro* and *in vivo* for their ability to modulate 3-MI-induced splenotoxicity, and various analogs of 3-MI were also tested *in vitro* to examine the structure–activity relationship for alkylindole cytotoxicity.

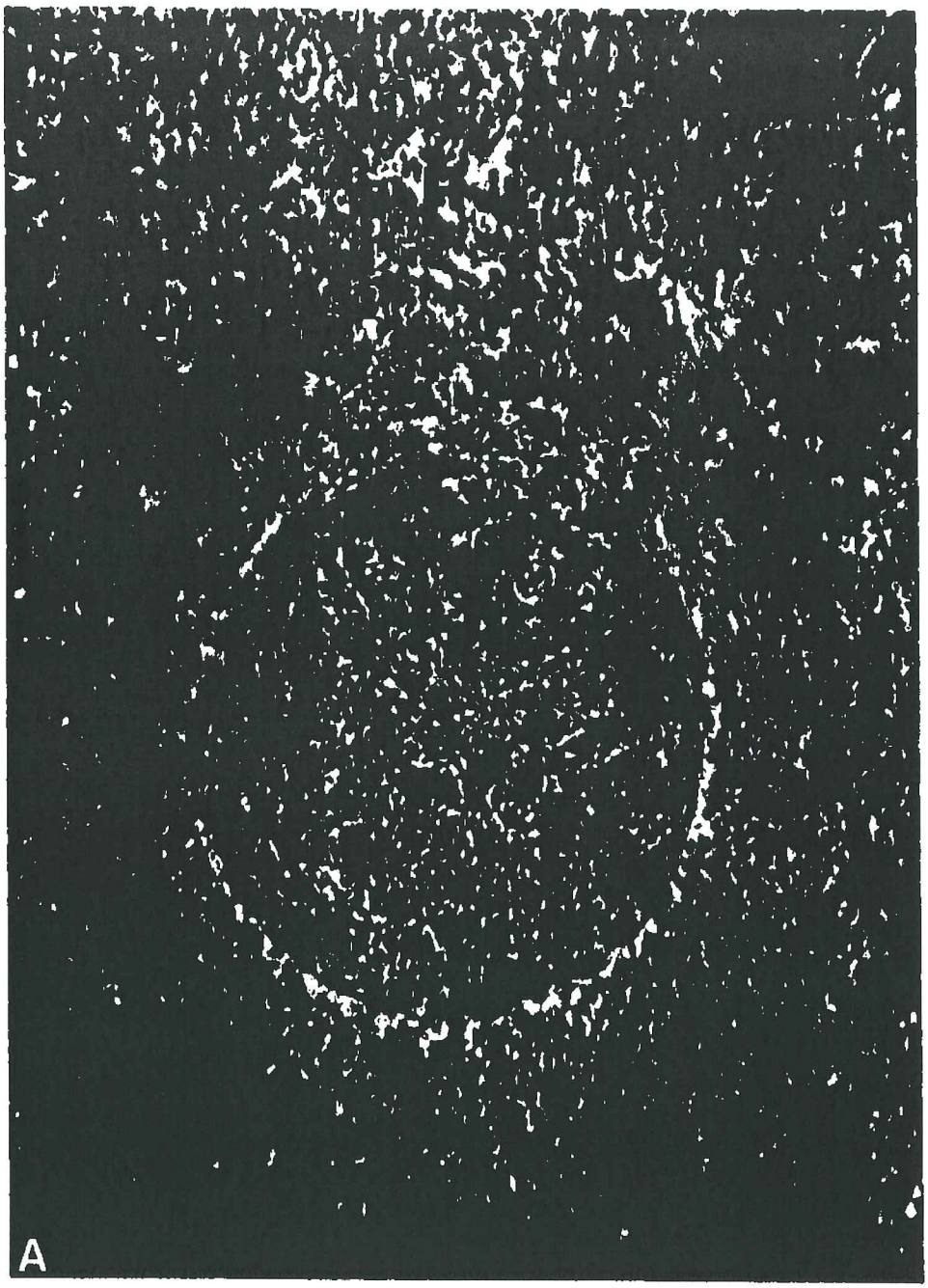

FIG. 5. Parasagittal Sprague–Dawley rat spleen sections (5 μm) prepared 48 hr after treatments, stained with hematoxylin and eosin. (A) Control rat administered corn oil only (0.5 ml ip). Note that marginal zone (Z) and central artery (A) are quite distinct. Marginal sinuses (indicated by arrows) are visible between periarteriolar lymphatic sheaths (PALS) (P) and marginal zone (Z), and the cells within both regions are of uniform density and staining. (100×) (B) Rat administered 3-MI (400 mg/kg in 0.5 ml corn oil, ip). Note the loss of marginal zone (Z) compared with corn oil-treated controls (see Fig. 5A). The central artery (A) is also less distinct than in controls. The remnants of the marginal sinuses (indicated by arrows) outline the PALS (P) containing many more pycnotic cells compared to controls. The red pulp (R) surrounds the PALS. (100×).

In Vivo Studies

Phenobarbital-inducible P450 isozymes have been implicated as contributing significantly to 3-MI bioactivation (Huijzer *et al.*, 1989). In order to test for a potential role of bioactivation by these isozymes in 3-MI-induced splenotoxicity, rats were treated *in vivo*

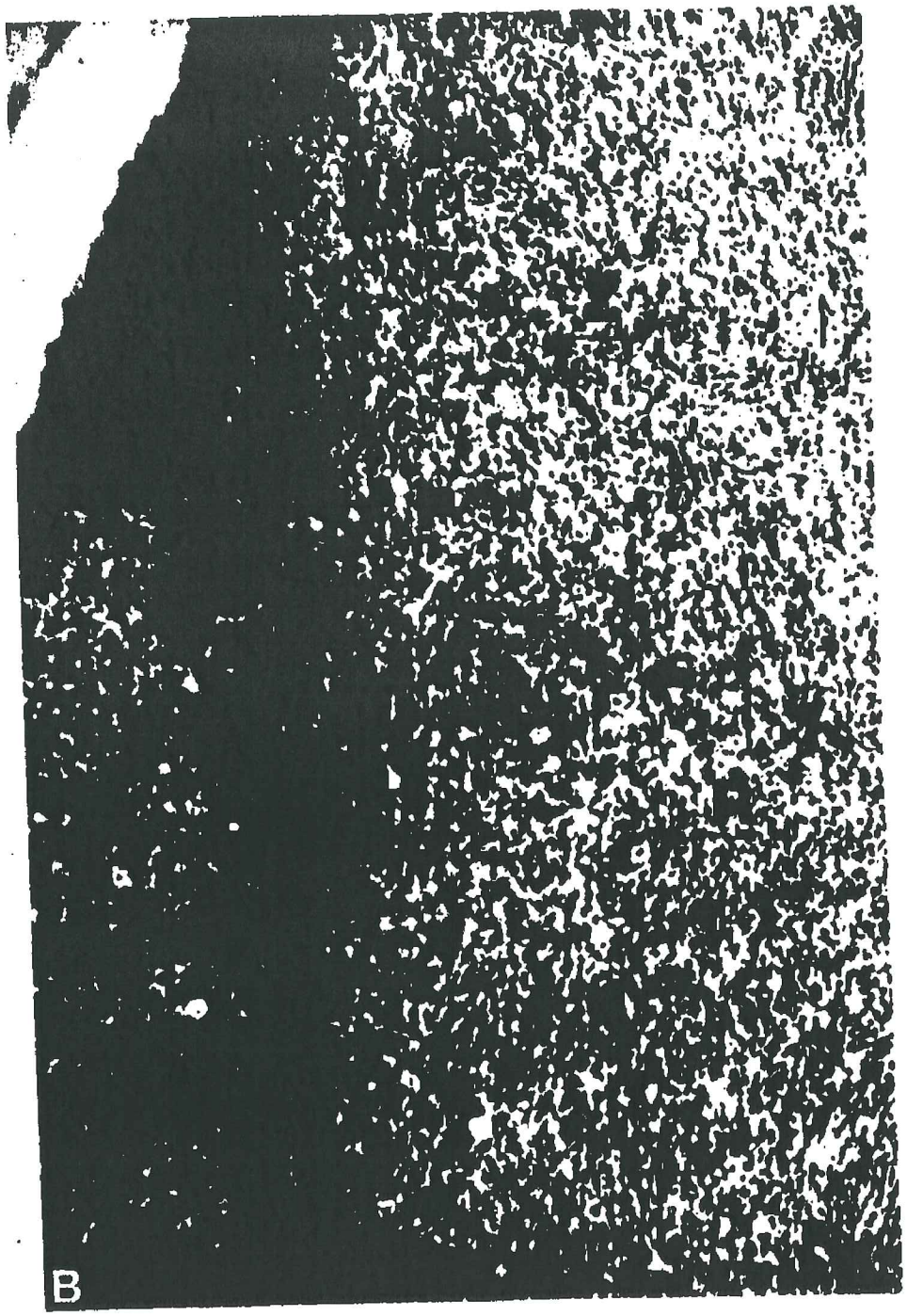

FIG. 5—*Continued*

with PB, a potent inducer of the cytochrome P450IIB family of isozymes in rat liver. Rats receiving daily ip injections of 100 mg/kg PB for three consecutive days prior to exposure to 300 mg/kg 3-MI still demonstrated a significant decrease in splenic weight and cell number. However, 3-MI splenotoxicity in PB-treated animals was significantly *less* than control animals which received vehicle only (saline) pretreatment for 3 days (Fig. 7).

As shown in Table 1, a similar trend was obtained for PB-induced mice when subsequently treated with 3-MI. However, with mice the decrease in toxicity was not statistically significant. Treatment of mice with 50 mg/kg SKF 525A, 40 mg/kg β-naphthoflavone, 600 μl/kg diethylmaleate or 2 mg/kg indomethacin prior to administration of 300 mg/kg 3-MI did not significantly alter 3-MI splenotoxicity compared to the results shown in Table 1 (data not shown). Studies with additional mono- and dimethylindole congeners of 3-MI administered *in vivo* indicated that only treatment with 3-MI resulted in reduc-

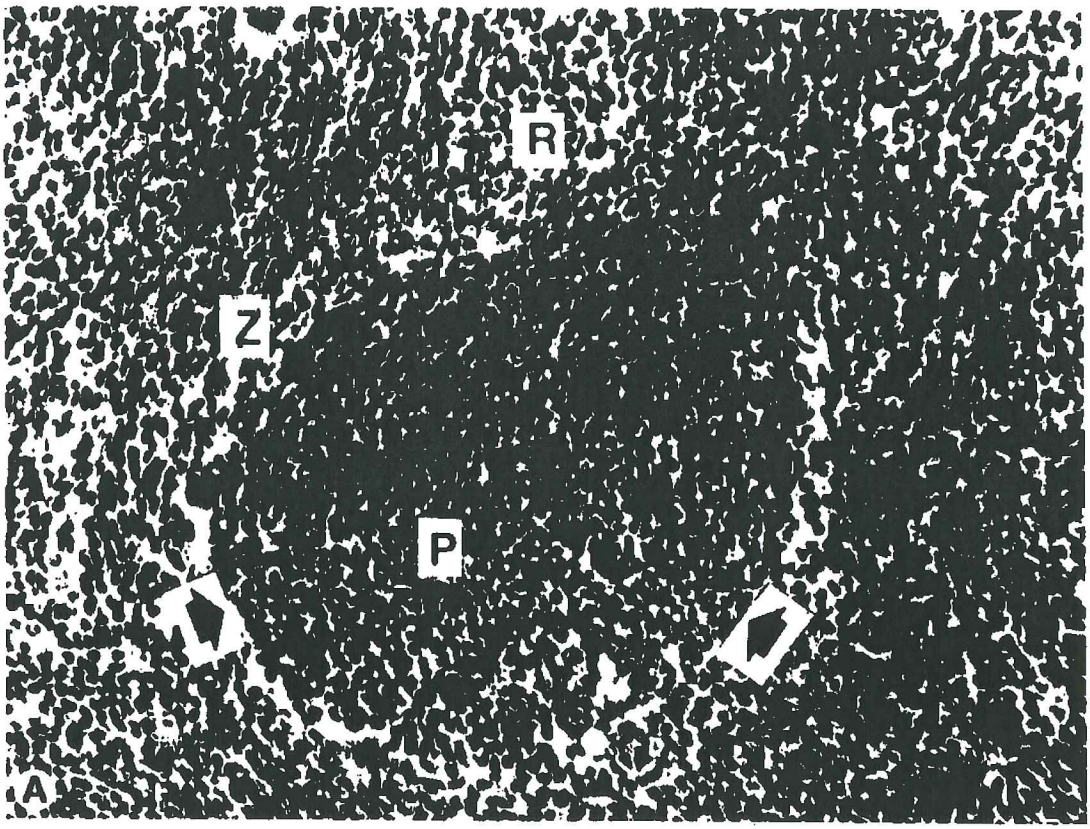

FIG. 6. Transverse non-Swiss albino (NSA) mouse spleen sections (5 μm), stained with hematoxylin and eosin. (A) Control NSA mouse receiving corn oil (0.1 ml, ip), 48 hr following treatment. Note that the marginal zone (Z) and sinuses (indicated by arrows) are distinct, and cells within the periarteriolar lymphatic sheaths (PALS) (P) are of uniform density and staining. (150×). (B) NSA mouse administered 3-MI (400 mg/kg in 0.1 ml corn oil, ip), 24 hr prior to tissue sectioning. Note that marginal zone (Z) and sinus (indicated by arrow) are less distinct than in controls (see Fig. 6A). There is decreased density of cells and uniformity of staining in the PALS (P), and the number of pycnotic cells is increased. (150×). (C) NSA mouse administered 3-MI (300 mg/kg in 0.1 ml corn oil, ip), 48 hr prior to tissue sectioning. Note that the marginal zone (Z) is distinguishable but only remnants of sinuses (indicated by arrow) remain. PALS (P) cells are nonuniform in density and staining and many are pycnotic. The red pulp (R) surrounds the PALS and marginal zones. Also note similarity to Fig. 6B. (150×).

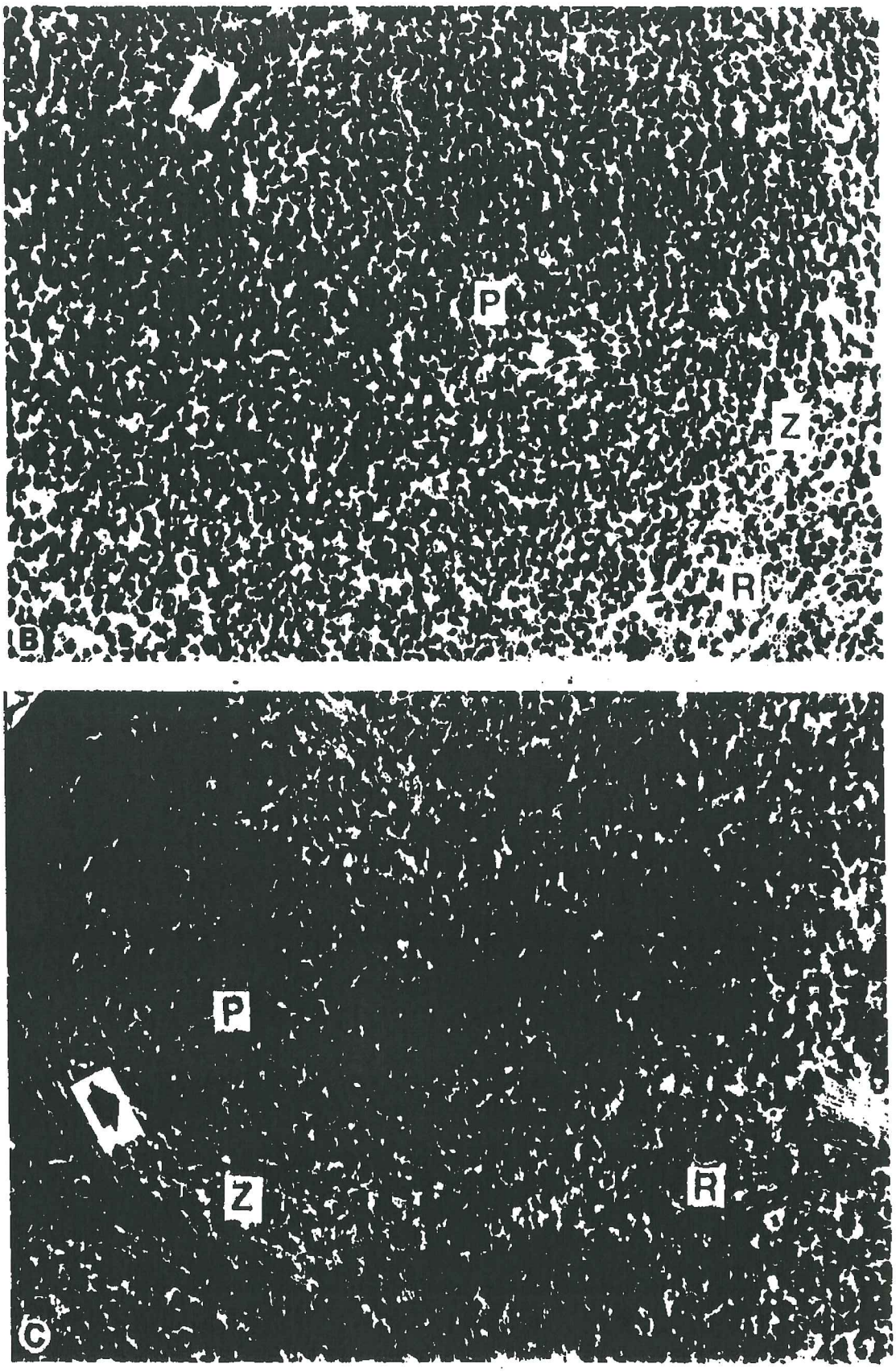

FIG. 6—*Continued*

TABLE 1

In Vivo EFFECTS OF ALKYLINDOLES AND CYTOCHROME P450 INDUCERS ON MOUSE SPLENIC CELL NUMBERS

Treatment	Pretreatment	Splenic cell number[a]
Corn oil	—	5.10 ± 0.48
2-MI	—	5.06 ± 0.62
7-MI	—	4.12 ± 1.19
1,2-DMI	—	4.84 ± 1.51
2,3-DMI	—	3.82 ± 0.61
Tryptophan	—	4.97 ± 0.25
3-MI	—	2.40 ± 0.57*
Indole	—	2.94 ± 0.33*
Corn oil	PB	5.95 ± 0.47
3-MI	PB	3.51 ± 0.67*

[a] Numbers represent the mean of at least three animals ($\times 10^7$) ± SD. Alkylindoles were administered ip at 300 mg/kg in 0.5 ml corn oil 24 hr prior to euthenization. PB was administered ip at 60 mg/kg in 0.3 ml normal saline for three consecutive days prior to administration of 3-MI. An asterisk indicates a significant decrease ($p < 0.05$) relative to vehicle-treated control animals.

tions in mouse splenic cell numbers (Table 1), also suggesting a role for selective bioactivation of alkylindoles for 3-MI splenotoxicity. These results parallel our histopathology observations for these compounds with mice, which also indicate a lack of toxicity by substituted alkylindoles other than 3-MI (micrographs not shown) and splenotoxicity for unsubstituted indole (Table 1).

In Vitro Studies

Additional *in vitro* experiments were conducted to more closely examine the potential role of *splenic* metabolism in 3-MI-induced splenotoxicity, particularly whether alkylindoles might be bioactivated *in situ* in rat splenocytes, or whether reactive metabolites might be formed in other organs and then transported to the spleen (Yost *et al.*, 1990). Splenocytes from naive (untreated) rats were incubated with 0.1 to 1 mM 3-MI (Yost, 1989; Yost *et al.*, 1990) *in vitro* for 24 hr. 3-MI caused a time- and concentration-dependent increase in the number of nonviable cells as assessed by MTT reduction (data not shown).

Further *in vitro* experiments were conducted to assess the structure–activity relationships for alkylindole splenotoxicity. Isolated rat splenic cells were incubated *in vitro* with 1 mM concentrations of all other possible monomethylindole congeners (1-, 2-, 4-, 5-, 6-, or 7-methylindole), and 1,2-, 2,3-, and 2,5-

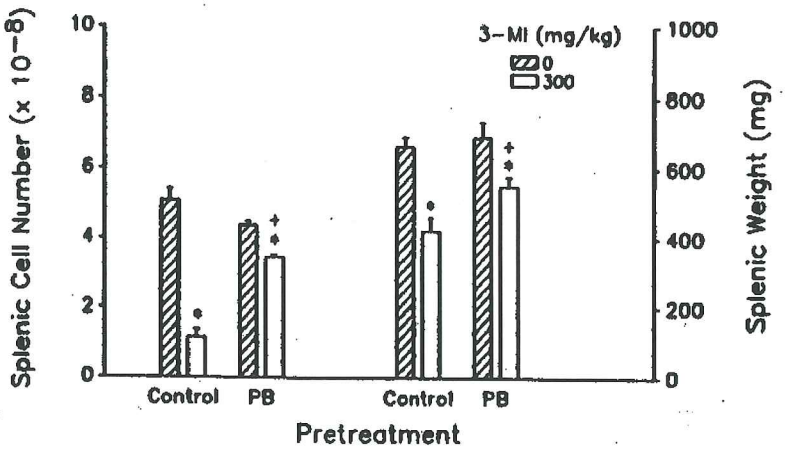

FIG. 7. Effect of 3-day *in vivo* pretreatment of rats with phenobarbital (PB) or saline (control) on nucleated splenic cell number and splenic weight following 24 hr ip injection of corn oil or 300 mg/kg 3-MI. Bars represent the mean of four animals ($n = 4$) ± SE. An asterisk indicates a significant difference ($p < 0.05$) from controls. A plus sign indicates a significant difference between control and PB-pretreated animals.

dimethylindole. All of these substituted alkylindoles caused increases in cell death equivalent to those of 3-MI as measured by the MTT assay of cell viability (Table 2). Although the 3-MI precursor L-tryptophan, at equimolar concentrations, did not demonstrate time- or concentration-dependent toxicity, the structurally related compound indole also demonstrated toxicity to the splenic cells (Table 2). Similar findings were obtained for mouse spleen cells incubated with the same series of alkylindole congeners under the same experimental conditions (data not shown).

To test for isozyme-specific bioactivation of alkylindoles by P450, isolated rat splenocytes were coincubated for 24 hr with 1 mM 3-MI *plus* 100 μM PI, a selective inhibitor of cytochrome P450IIB; or 10 μM ANF, a selective inhibitor of cytochrome P450IA; or 100 μM SKF 525A, a nonselective inhibitor of cytochrome P450 isozymes. None of these compounds protected against 3-MI-induced toxicity under these conditions (data not shown). ABT, (5 mM), a selective suicide substrate inhibitor of cytochrome P450IIB-mediated 3-MI bioactivation (Huijzer *et al.*, 1989), was also coincubated with splenocytes and 1 mM 3-MI for 24 hr, but also did not protect against *in vitro* 3-MI splenotoxicity (Table 2). Similar findings were obtained for mouse splenocytes incubated with 3-MI and these metabolic inhibitors under the same experimental conditions (data not shown).

In a final experiment to test for potential cytochrome P450-mediated bioactivation of 3-MI by splenocytes, 1 mM deuterated 3-MI was incubated with rat splenocytes for 24 hr *in vitro*. Deuteration of the 3-methyl group of 3-MI has been previously demonstrated to retard 3-MI bioactivation and reduce 3-MI pneumotoxicity (Huijzer *et al.*, 1987). However, deuterated 3-MI was found to be equally as toxic to cultured splenic cells as was 3-MI (Table 2). Again, similar results were obtained for mouse spleen cells incubated with deuterated 3-MI in parallel experiments (data not shown).

TABLE 2

In Vitro EFFECT OF ALKYLINDOLES ON RAT SPLENOCYTE VIABILITY AS ASSESSED BY REDUCTION OF MTT

Indole congeners	% Reduction in cell viability[a]
3-MI	69.9 ± 0.3
Deuterated 3-MI	66.3 ± 3.5
1-MI	53.7 ± 1.3
2-MI	70.5 ± 0.3
4-MI	56.4 ± 1.1
5-MI	69.0 ± 0.8
6-MI	54.3 ± 1.4
7-MI	70.9 ± 0.5
1,2-DMI	65.6 ± 1.6
2,3-DMI	70.9 ± 0.5
2,5-DMI	64.7 ± 0.3
Indole	73.2 ± 1.0
ABT (5 mM)	3.6 ± 2.8
ABT + 3-MI	62.9 ± 0.5

[a] Reduction in viability of rat splenic cells, compared to vehicle-treated controls, following 24 hr *in vitro* exposure to 1 mM mono- and dimethylindoles. 1-Aminobenzotriazole (ABT), a cytochrome P450 suicide substrate inhibitor, was incubated in some cultures for 30 min prior to exposure to 3-MI and remained in the cultures throughout the duration of the experiment. Values represent the mean reduction in viability of three separate cultures of pooled cells ($n = 3$) ± SE that were sampled in quadruplicate. All values except ABT alone were significantly different ($p < 0.05$) from controls.

Cooxidation of 3-MI by prostaglandin synthase (PGS) has been suggested to also contribute to 3-MI bioactivation (Formosa *et al.*, 1988; Bray and Kirkland, 1990). To test whether this metabolic pathway might play a significant role in the splenic toxicity of 3-MI, experiments were conducted with the specific PGS inhibitor indomethacin. Indomethacin (100 μM) was incubated with isolated rat splenic cells for 30 min, and then 1 mM 3-MI was added to the cultures and coincubation continued for an additional 24 hr. Under these conditions, indomethacin also failed to protect against 3-MI-mediated toxicity (Table 3). Similar results were again obtained with mouse splenic cells (data not shown). Finally, removal of splenic macrophages (with poten-

TABLE 3

EFFECT OF REMOVAL OF ADHERENT CELLS (MPS) OR INDOMETHACIN PREINCUBATION *In Vitro* ON 3-MI-INDUCED REDUCTION IN SPLENIC CELL VIABILITY

Preincubation	% Reduction in cell viability[a]
No preincubation	76.4 ± 0.2
Adherent cell (MP) removal	77.2 ± 1.4
Indomethacin (100 μM)	74.2 ± 1.0

[a] Reduction in spleen cell viability, compared to vehicle-treated controls, of rat splenic cells following *in vitro* incubation with 1 mM 3-MI. Macrophages (MPs) were removed by plastic adherence for 2 hr on tissue culture flasks followed by collection of nonadherent populations for subsequent incubation. Indomethacin preincubation involved continuous exposure of cells to indomethacin from 30 min prior to 3-MI exposure and throughout the 24-hr treatment period. Values represent the mean reduction in viability of three separate cultures of pooled cells ($n = 3$) ± SE that were sampled in quadruplicate. All values were significantly different ($p < 0.05$) from vehicle-treated controls, but not from each other.

tially significant levels of PGS) from these mixed cultures of isolated spleen cells by plastic adherence prior to 8–24 hr exposure to 3-MI was also ineffective in protecting against 3-MI splenotoxicity *in vitro* (Table 3).

DISCUSSION

The studies reported here demonstrate that the spleen is also a target organ for 3-MI. In previous studies by Turk *et al.* (1984), no significant toxicity was noted during histopathologic examination of spleens from mice treated with 400–600 mg/kg 3-MI *in vivo*. Failure to observe toxicity may be due to a lack of examination of splenic weights and nucleated cell numbers, which are more sensitive indicators of toxicity. Indeed, splenic histopathologic changes observed in the present studies (Figs. 6B and 6C) may not have been detectable. Spleen sections from mice examined 24 hr after administration of 400 mg/kg of 3-MI showed very subtle changes that could easily have been interpreted as normal variations. Furthermore, although both rats and mice demonstrated dose-dependent decreases in splenic cell numbers and weights, the mouse appears less susceptible to splenotoxicity, as no significant changes were noted at doses of less than 200 mg/kg of 3-MI. Even 48 hr following administration of 300 mg/kg of 3-MI, mouse spleens evidenced less histopathological changes than would be expected compared to rat.

Recent studies by Yost *et al.* (1990) indicate that, under certain circumstances, other organs besides the lung may become susceptible to 3-MI toxicity. Yost *et al.* (1990) have reported that significant renal damage occurs in mice with a relatively low dose (75 mg/kg) of 3-MI, but only after glutathione has been significantly depleted in hepatic tissues. The splenotoxicity induced by 3-MI reported herein occurs in the absence of any such prior experimental manipulation.

The decrease in splenic cell numbers observed in the present studies occurs as a result of *in vivo* molar doses of 3-MI in the same range as those required for the immunotoxin benzo[*a*])pyrene (B[a]P) (Kawabata and White, 1987; Ginsberg *et al.*, 1989) and equal to or less than that for 3-MI pneumotoxicity in rodents (Yost *et al.*, 1989; Yost *et al.*, 1990). 3-MI splenotoxicity does not appear to be mediated by stress-induced release of glucocorticoids secondary to 3-MI pulmonary insufficiency, since adrenalectomized rats demonstrate decreases in splenic cell number similar to those observed in naive and sham-operated control animals following 3-MI challenge (Fig. 4). While these decreases in control and sham-operated rats were dose-dependent, both 200 and 300 mg/kg 3-MI caused similar decreases in adx rats (Fig. 4), the reason for which is unclear at this time.

Three adult female goats were administered: (a) a single dose of 3-MI by iv infusion (150 mg/kg 24 hr prior to tissue removal), (b) five repeated doses by iv infusion (40 mg/kg once per day for 5 days, prior to tissue removal), or (c) vehicle only (Cremophore EL) as a control. Acute treatment led to a 36 and 42% decrease

in splenic weight and nucleated cell number, respectively, compared to control. Chronic treatment caused a 45 and 62% decrease in splenic weight and cell number, respectively. Histopathologic changes similar to those observed in rats were also observed in the goats administered both single and repeated doses of 3-MI. Therefore, species-dependent differences in 3-MI splenotoxicity between rats, mice, and goats appear to be much smaller than those observed for 3-MI pneumotoxicity. The dramatic changes in rat splenic architecture observed by histopathologic studies, combined with the reduction of splenic cell numbers, suggests a generalized toxicity to all splenic leukocyte subpopulations.

Based on previous studies indicating the apparent requirement of bioactivation for 3-MI pneumotoxicity, 3-MI-induced splenotoxicity might be hypothesized to result from local bioactivation within the target organ (i.e., spleen) as occurs for 3-MI pneumotoxicity. Alternatively, as suggested by Ginsberg *et al.* (1989) for the *in vivo* immunotoxicity of B[a]P, 3-MI may be bioactivated in organs that contain significant amounts of cytochrome P450 (e.g., liver or lungs) and the toxic metabolites systemically transported to the spleen. Such transport of toxic metabolites to extrahepatic target sites has been suggested by Yost *et al.* (1990) in studies demonstrating that a shift in organ selectivity of 3-MI from lung to kidney occurs as a result of glutathione depletion of hepatic tissues.

Support for the hypothesis that metabolism may mediate or modulate 3-MI splenotoxicity is derived from the finding that 3-day pretreatment with PB led to a significant (although incomplete) reduction in nucleated splenic cell cytotoxicity arising from *in vivo* exposure to 3-MI. Such protection may be due to induction of enzymes responsible for alternative metabolic pathways that either directly detoxify 3-MI, enhance excretion, or otherwise lower the effective circulating concentration of 3-MI. Lower levels of circulating 3-MI could decrease any direct cytotoxicity mediated by the compound, or reduce the amount of 3-MI available for bioactivation. Specifically, this might include the extensively documented increases in selected isozymes of cytochrome P450 (Gonzalez, 1989), or increases in certain phase II conjugation enzymes (e.g., glucuronyl transferase) that occur following treatment with metabolic inducers (Greenlee and Irons, 1981).

The time-dependent increase in toxicity to splenic cells exposed to 3-MI *in vitro* suggests a requirement for metabolism to toxic intermediates. Concentrations of 3-MI in the millimolar range are required to demonstrate any significant *in vitro* cellular toxicity both in these studies and in previous studies on 3-MI pneumotoxicity *in vivo* and toward isolated rabbit Clara cells *in vitro* (Yost *et al.*, 1989), suggesting that the parent 3-MI is either a relatively nontoxic compound and/or that bioactivation is required to form reactive intermediates.

Despite the above considerations, aromatic compounds may themselves be directly cytotoxic. For example, the rank order of potency for rabbit erythrocyte hemolysis (Rogers, 1969) and arthritogenic potential in the rabbit knee (Rogers *et al.*, 1969) was directly proportional to the lipophilicity of a series of aromatic compounds. Aromatic compounds have been speculated to cause inflammation via disruption of lysosomal membranes leading to release of lysosomal contents and cytotoxicity (Nakoneczna *et al.*, 1969). Consistent with this model of cytotoxicity, in the present studies all the mono- and dimethylindole congeners tested were equally cytotoxic *in vitro* to spleen cell suspensions (Table 2). However, unsubstituted indole was the only other compound tested besides 3-MI to demonstrate splenotoxicity *in vivo*, the significance of which is unknown at the present time, although this compound has been reported to produce inflammatory responses *in vivo* (Nakoneczna *et al.*, 1969).

Previous reports describing the ability of cytochrome P450 inhibitors to protect against 3-MI pneumotoxicity (Huijzer *et al.*, 1987, 1989; Yost *et al.*, 1989) and B[a]P-induced

toxicity (Kawabata and White, 1987), and reports of reduced pneumotoxicity of deuterated 3-MI (Huijzer *et al.*, 1987), all suggest a role for P450-mediated bioactivation in 3-MI toxicity. In contrast to these findings, the present studies do not indicate these inhibitors significantly reduce 3-MI splenotoxic effects *in vitro*. Therefore, 3-MI-induced splenotoxicity may be mechanistically similar to that suggested by Ginsberg *et al.* (1989), who have demonstrated that loss of splenic cell viability during B[a]P-induced immunosuppression *in vitro* is due to *both* direct cytotoxic effects and DNA adduct formation with reactive metabolites.

Formosa and Bray (1988) and Formosa *et al.* (1988) have suggested cooxidation of 3-MI by prostaglandin synthase as an alternative bioactivation pathway for 3-MI pneumotoxicity, and others have also postulated this as an alternate route for bioactivation of B[a]P (Sivarajah *et al.*, 1981). Since macrophages synthesize copious amounts of prostaglandins (Adams and Hamilton, 1984), significant amounts of prostaglandin synthase in these cells could potentially metabolize 3-MI to toxic intermediates within the spleen. Removal of macrophages, however, and/or incubation with indomethacin (at concentrations known to inhibit synthesis and release of prostaglandins), did not protect splenic cells against 3-MI toxicity *in vitro* (Table 3) or *in vivo*, suggesting this pathway is not significant in 3-MI splenotoxicity.

3-MI administered *in vivo* clearly alters splenocyte viability, as evidenced by changes in both splenic histopathology and cell numbers. The time- and dose-dependence of the *in vivo* splenotoxicity of 3-MI in combination with the selectivity of 3-MI toxicity *in vivo* compared with other alkylindoles and the partial protection against 3-MI splenotoxicity *in vivo* by PB all suggest a role for metabolism in the cytotoxic effects of 3-MI toward spleen cells. However, the results obtained *in vitro* with cytochrome-P450 and prostaglandin synthase inhibitors also suggest that if metabolic transformation plays a key role in the splenotoxicity of 3-MI, these reactions may not all occur in the splenocytes themselves. Furthermore, the *in vitro* studies of alkylindoles with isolated spleen cells suggest the possibility that 3-MI splenotoxicity may be mediated, at least in part, by a direct cytotoxicity, although this mechanism would not explain the apparent selectivity of 3-MI for lung and spleen cells.

In conclusion, these studies demonstrate that 3-MI, a known systemic pneumotoxin, induces a previously unreported splenotoxicity. Unlike 3-MI pneumotoxicity, 3-MI splenotoxicity is markedly less species-specific. Furthermore, 3-MI splenotoxicity appears unrelated to a release of endogenous adrenal glucocorticoids. This splenotoxicity occurs following both *in vitro* and *in vivo* exposure to 3-MI, although possibly via different mechanisms. Additional studies are in progress to investigate the mechanism(s) of splenotoxicity following *in vitro* and *in vivo* exposure to 3-MI.

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