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The Hematotoxic Effects of Inhaled Benzene on Peripheral Blood, Bone Marrow, and Spleen Cells Are Increased by Ingested Ethanol[1,2]

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The Hematotoxic Effects of Inhaled Benzene on Peripheral Blood, Bone Marrow, and Spleen Cells Are Increased by Ingested Ethanol. BAARSON, K. A., SNYDER, C. A., GREEN, J. D., SELLAKUMAR, A. R., GOLDSTEIN, B. D., AND ALBERT, R. E. (1982). *Toxicol. Appl. Pharmacol.* **64, 393–404.** For 13 weeks, groups of C57Bl/6J male mice were exposed to 300 ppm benzene by inhalation, 6 hr/day, 5 days/week, and to 5 or 15% ethanol in the drinking water, 4 days/week. The number and type of blood cells in the peripheral blood, marrow, and spleen were determined at regular intervals. Anemia and lymphocytopenia were observed in the peripheral blood of all benzene and benzene/ethanol-treated groups. These cytopenias, however, were more severe in the benzene/ethanol-treated mice. In addition, there was a transient increase of normoblasts in the peripheral blood of benzene/ethanol-treated mice which was not observed in mice treated with benzene alone or in control mice. Groups exposed to benzene, or benzene/ethanol displayed reduced marrow and splenic cellularities but the reductions were more severe in mice exposed to benzene/ethanol. Specifically, these reduced cellularities were characterized by decreased numbers of lymphocytes in both marrow and spleen and decreased numbers of granulocytes and normoblasts in the marrow. In benzene/ethanol-treated mice a transient increase in the numbers of splenic normoblasts was observed which coincided with the transient appearance of normoblasts in the peripheral blood of these animals. This condition was not observed in mice treated with only benzene or only ethanol. Mice exposed to benzene/ethanol presented a greater degree of atypical cellular morphology than mice treated with benzene alone. These data suggest that the ingestion of ethanol increases the hematotoxicity of inhaled benzene.

The effects of ethanol ingestion on the toxicities of certain chemicals have received much attention. It has been reported that ethanol enhances the carcinogenicity of *N*-nitrosopyrrolidine (McCoy *et al.*, 1981), the mutagenicity of dimethylnitrosamine (Garro *et al.*, 1981) and benzo (*a*)pyrene (Seitz *et al.*, 1978), and enhances the hepatotoxicity of carbon tetrachloride (Strubelt *et al.*, 1978), trichloroethane (Klaassen and Plaa,

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1966), trichloroethylene (Cornish and Adelfuin, 1966), chloroform (Kutob and Plaa, 1962), dimethylnitrosamine (Maling *et al.*, 1975), and thioacetamide (Maling *et al.*, 1975). It is believed that ethanol increases the activity of the metabolic enzymes which convert these chemicals to their ultimate toxic forms. The activities of a number of enzymes have been found to be increased by ethanol treatment, namely: hepatic cytochrome *P*-450 and epoxide hydrase (Hietanen *et al.*, 1980), hepatic and kidney 7-ethoxycoumarin-O-deethylase (Savolainen *et al.*, 1978), hepatic dimethylnitrosamine demethylase (Garro *et al.*, 1981), and intestinal cytochrome *P-450* (Seitz *et al.*, 1978).

Benzene is thought to be converted to hematotoxic metabolites via mixed function oxidase (Snyder *et al.*, 1967; Gonasun *et al.*, 1973). It has been shown that treatment of rats with ethanol will increase the rate of benzene metabolism *in vitro* (Sato *et al.*, 1980, 1981). Studies were therefore undertaken in this laboratory to examine the effects of ingested ethanol on the hematotoxicity of inhaled benzene. We recently reported that ingestion of ethanol did increase the cytopenic effects of inhaled benzene on the peripheral blood of C57Bl mice (Snyder *et* al., 1981). A second study on another group of C57Bl mice was performed to assess the effects of ingested ethanol and inhaled benzene on recognizable blood cells in the marrow and spleen.

METHODS

Exposure conditions. Male, 6-week-old C57Bl/6J mice (Jackson Laboratories, Bar Harbor, Maine) were quarantined and observed for anomalous behavior for 2 weeks before being randomly distributed into six groups of 18 mice each. Each group was assigned to one of the following combined inhalation and ingestion treatments: air + water, air + 5% ethanol, air + 15% ethanol, 300 ppm benzene + water, 300 ppm benzene + 5% ethanol, 300 ppm benzene + 15% ethanol. Solutions of ethanol in water were prepared on a weight/weight basis. Ethanol was provided for a 4-day period from Monday afternoon to Friday morning and fluid intake was monitored during this period. All animals received water *ad libitum* Friday through Sunday. When not undergoing air or benzene exposures, mice were housed three to a box over wood chip bedding in polycarbonate boxes fitted with stainless steel tops. No food or fluid was provided during the inhalation exposures.

Test and sham control animals were exposed in identical 1.0 m^3 stainless steel inhalation chambers (Drew and Laskin, 1973), 6 hr/day × 5 days/week between the hours of 8:00 AM and 4:00 PM. Sham-exposed animals were treated in all ways like benzene-exposed animals except they were exposed to filtered, conditioned air. The benzene atmosphere was generated by directing a stream of air over a reservoir of benzene. Chamber concentrations were monitored every 60 min during a daily exposure by an ultraviolet spectrophotometric technique that has been described (Snyder, *et al.*, 1978).

Blood parameters. Peripheral blood, marrow, and spleen cell counts were obtained from three mice per group after 0, 4, 32, 63, and 88 days of exposure. Free-flowing venous tail blood was obtained for peripheral blood determinations. Test and control animals were bled on the same day and within 30 min to minimize differences due to handling, circadian rhythms, etc. Red and white blood cell counts were determined on a Coulter Model ZB-I blood cell counter (Coulter Electronics, Inc., Hialeah, Fla.). Differential counts were determined manually by Wright–Giemsa-stained peripheral blood smears. Peripheral normoblasts were counted on the basis of 100 white cells. Following tail bleeding, mice were killed by cervical dislocation and the spleen and both femurs were dissected from each mouse. After the spleen was weighed, a section was removed to make a cell suspension. The remaining section was retained for histopathology. Cells were freed from the splenic capsule by gently pressing the section against a glass Petri dish with a stainless steel spatula. Cells from the broken capsule were drawn into a preweighed heparinized microcapillary tube. Marrow cells were obtained by removing the epiphyseal tip of the femur, inserting a preweighed, heparinized microcapillary tube into the hemen, and drawing a sample of cells (Fruhman and Gordon, 1955).

Microcapillary tubes containing cell samples from both organs were reweighed and washed into separate 1-ml samples of a hypotonic solution consisting of three parts heat-inactivated fetal calf serum to one part deionized water. Single cell suspensions of both samples were produced by gently impacting the cells against the bottom of the centrifugation tube with a fire-polished Pasteur pipet. A 20-μl aliquot of each sample was withdrawn to perform nucleated cell counts on the Coulter cell counter. The remaining suspensions were incubated at room temperature for 10 min and gently pelleted by centrifugation. After reducing the supernatant volume, samples were resuspended to make marrow and spleen

TABLE 1

COMPARISON OF PERCENTAGE BODY WEIGHT CHANGE ± SD

Days after first exposure	Air sham + % ethanol			300 ppm benzene + % ethanol		
	0%	5%	15%	0%	5%	15%
0	100 (21.6)[a]	100 (19.8)[a]	100 (21.6)[a]	100 (20.6)[a]	100 (19.7)[a]	100 (20.8)[a]
4	103 ± 5	109 ± 5	106 ± 5	103 ± 6	102 ± 4	99 ± 7
32	115 ± 6	120 ± 10	110 ± 6	110 ± 11	107 ± 7	104 ± 8
63	118 ± 7	134 ± 11	117 ± 7	115 ± 14	110 ± 6	91 ± 4
88	124 ± 3	136 ± 9	126 ± 6	121 ± 15	108 ± 3	87[b]

[a] Numbers in parentheses are average initial weights in grams.
[b] Only one animal remained in this group.

smears. A 200-cell differential was performed on smears which were stained with 1% dimethoxybenzidine and counter-stained with Wright/Giemsa (LoBue *et al.*, 1963).

Body weights, parhology. Body weights were recorded prior to the start of exposures and following scheduled deaths at 4, 32, 63, and 88 days. A necropsy examination was performed on each animal. Tissues sectioned from these animals included lung, liver, spleen, thymus, and bone marrow. All sections were fixed in formalin and stained with hematoxylin and eosin for histopathological examination. Lymph nodes were examined only for relative size.

Statistics. All data shown in Figs. 1–10 are presented as mean ± one standard error. Data were evaluated for differences by the Student's *t* test. Because the group size was small (3) at each data point, the Welch (1947) approximation was used to determine the numbers of degrees of freedom and the Student's *t* statistic. Except for dara concerning peripheral normoblasts, all statistical tests were two tailed with $p = 0.05$ chosen as the level of significance. No statistical treatment was a p plied to the peripheral normoblast data because of a large variance in the values for benzene/ethanol-treated mice. These data are presented as mean ± SE.

RESULTS

General

The mean daily benzene concentrations ± SD for 4, 32, 63, and 88 days of exposure were 302 ± 8.4, 301 ± 8.0, 300 ± 8.7, and 300 ± 8.2, respectively.

There were no apparent differences in the rates of body weight gain between groups exposed to ethanol alone or benzene alone (Table 1). The two groups treated with benzene/ethanol, however, showed different rates of weight change than the other groups. Mice exposed to benzene/5% ethanol showed a slower rate of weight gain than mice treated with either benzene alone or ethanol alone. Mice treated with benzene/15% ethanol showed a weight loss.

During the 4 days/week when fluid con-

TABLE 2

COMPARISON OF AVERAGE DAILY FLUID AND ETHANOL CONSUMPTION ($\bar{x}$ + SD)

Treatment	g fluid/mouse	g fluid/g body weight	g ethanol/g body weight
Air + H_2O	5.5 ± 0.7	0.24 ± 0.07	0
Air + 5% ethanol	5.1 ± 0.4	0.21 ± 0.04	0.0105 ± 0.002
Air + 15% ethanol	3.5 ± 0.3	0.14 ± 0.01	0.0210 ± 0.002
300 ppm benzene + H_2O	5.5 ± 0.7	0.25 ± 0.05	0
300 ppm benzene + 5% ethanol	4.5 ± 0.4	0.23 ± 0.03	0.0115 ± 0.002
300 ppm benzene + 15% ethanol	2.6 ± 0.4	0.13 ± 0.02	0.0195 ± 0.003

sumption was monitored, average fluid consumption was different only in the two groups ingesting 15% ethanol (Table 2). These groups ingested a little more than half the fluid of the other groups and consumed about twice as much ethanol as the mice drinking 5% ethanol.

Peripheral Blood

Peripheral erythrocyte and lymphocyte counts were depressed in all groups treated with benzene (Figs. 1 and 2). In general, peripheral erythrocyte counts were more depressed in benzene/ethanol-treated mice than in mice treated with benzene alone, with the group ingesting 15% ethanol showing the most severe decline. Peripheral lymphocyte counts were severely depressed in all groups exposed to benzene. At all times the lymphocytopenia in benzene/ethanol-treated mice was greater than or equal to that in mice treated with benzene alone.

Mice treated with ethanol alone showed peripheral erythrocyte and lymphocyte counts that were equivalent to control values. Peripheral neutrophil counts were statistically equivalent among all six groups regardless of treatment.

By the fifth week of exposure, elevated levels of normoblasts appeared in the peripheral blood of the benzene/ethanol-treated mice (Fig. 3). No peripheral normoblasts were seen in the groups exposed to benzene alone or ethanol alone. The appearance of peripheral normoblasts peaked for the benzene/15% ethanol group and the benzene/5% ethanol group at approximately 32 and 63 days, respectively.

Blood cell morphology of the benzene and benzene/ethanol-treated groups included the appearance of polychromatic and stippled mature red cells, atypical platelets, increased cytoplasmic vacuolization, neutrophilic hypersegmentation, asynchronous/atypical nuclear/cytoplasmic maturation, Howell–Jolly bodies, and atypical lymphocytes. These morphological observations were particularly noticeable in the benzene/ethanol-treated groups. Megathrombocytes were observed in the benzene/ethanol-treated mice but not in mice treated with benzene alone. Anisocytosis and poikilocytosis were more frequently observed in the benzene/ethanol-treated groups and were most severe in the benzene/15% ethanol-treated group. Macrocytosis and hypochromia were not observed in any benzene-treated groups. Blood cell morphology was normal in groups treated with ethanol alone.

Marrow and Spleen Cellularity

Marrow cellularities of all benzene-exposed mice were initially depressed after 4 days of exposure and remained depressed at relatively constant levels for the 88 days of exposure (Fig. 4). Benzene/ethanol-treated mice showed more severe depressions of marrow cellularity than mice treated with benzene alone, and the severity of the depression was related to the dose of ethanol. Spleen cellularities of all benzene-exposed mice were initially depressed after 32 days of exposure and remained depressed throughout the exposures (Fig. 5). Similar to marrow cellularities, spleen cellularities were more depressed in mice treated with benzene/ethanol than in mice treated with benzene alone. By the last exposure, the severity of the depression of spleen cellularity was related to the dose of ethanol. Marrow and spleen cellularities were not different among air sham-exposed mice, regardless of ethanol treatment.

Marrow normoblasts from all benzene-exposed groups declined in a fashion similar to total marrow cellularity (Fig. 6). Depres-

FIGS. 1–10. For all figures. points are mean values ± SE. *O*, Air + water; □, air + 5% ethanol; △, air + 15% ethanol; ●, benzene + water; ■, benzene + 5% ethanol; ▲, benzene + 15% ethanol. (a) Denotes statistically less than corresponding control; (b) denotes statistically less than benzene and water values; (c) denotes statistically less than benzene and 5% ethanol values.

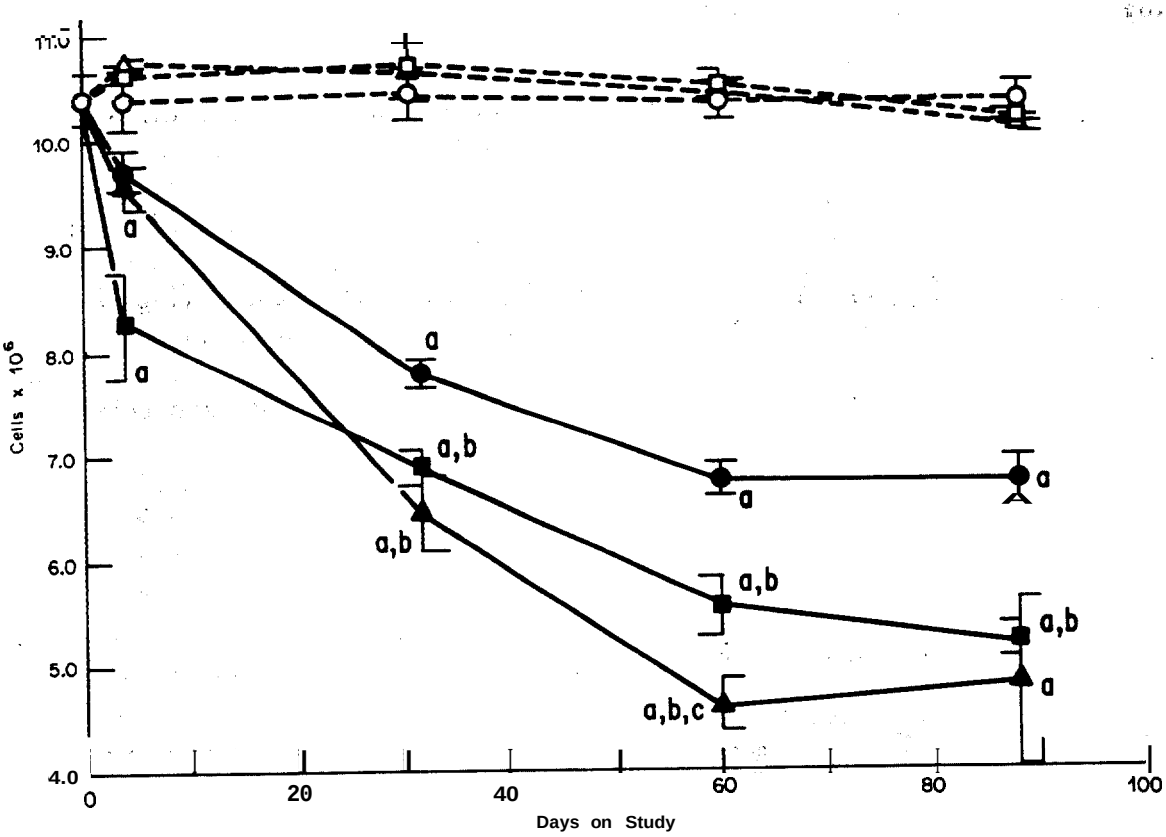

FIG. 1. Red blood cells.

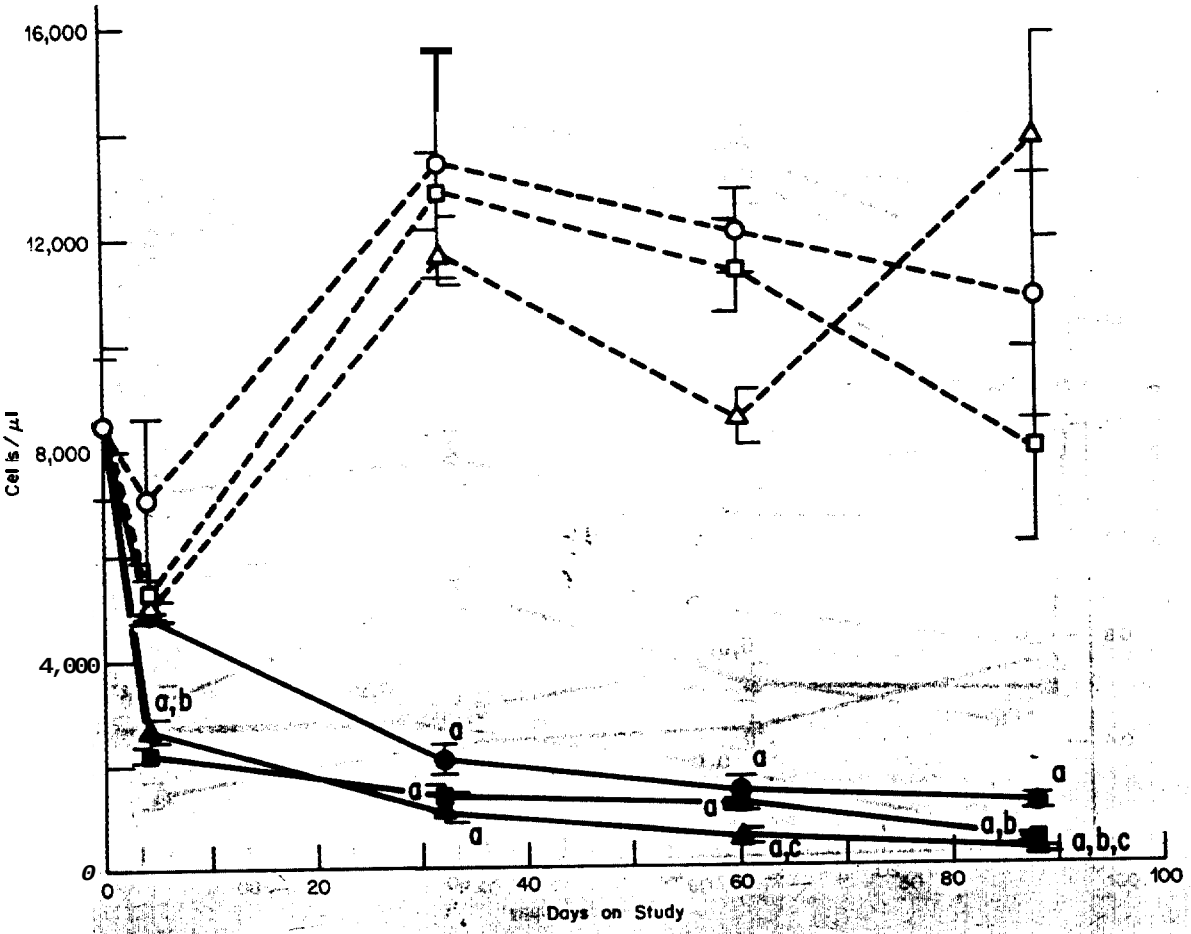

FIG. 2. Lymphocytes.

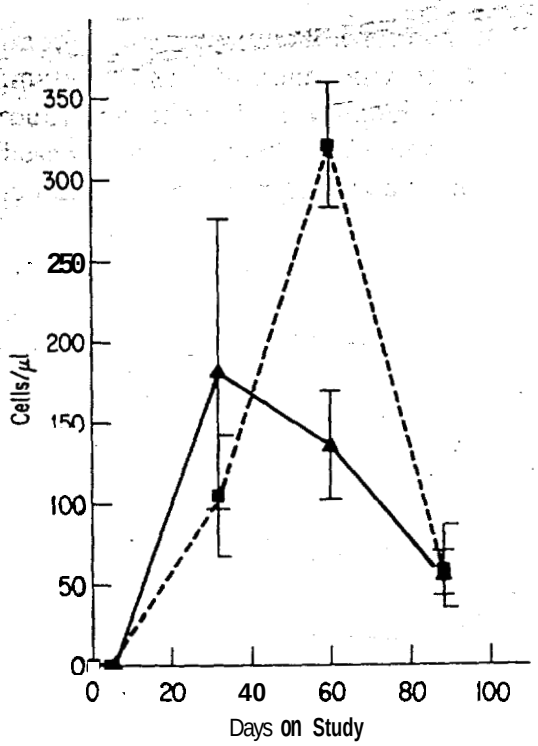

FIG. 3. **Peripheral** normoblasts.

sions were most severe in the benzene/15% ethanol-treated mice. In contrast, splenic normoblasts from benzene/ethanol-treated mice increased markedly after the first exposure week, peaked between 32 and 63 days, and then declined (Fig. 7). This increase and decline of splenic normoblasts were essentially the same for both benzene/ethanol-treated groups and coincided with the increase and decline of normoblasts in the peripheral blood of these mice. A more gradual increase in splenic normoblasts was seen in mice treated with benzene alone. This increase, however, was not associated with the appearance of any normoblasts in the peripheral blood. No differences were observed in marrow or splenic normoblast levels among any of the air sham-exposed mice regardless of ethanol treatment.

Marrow and splenic lymphocytes were significantly depressed in benzene and ben-

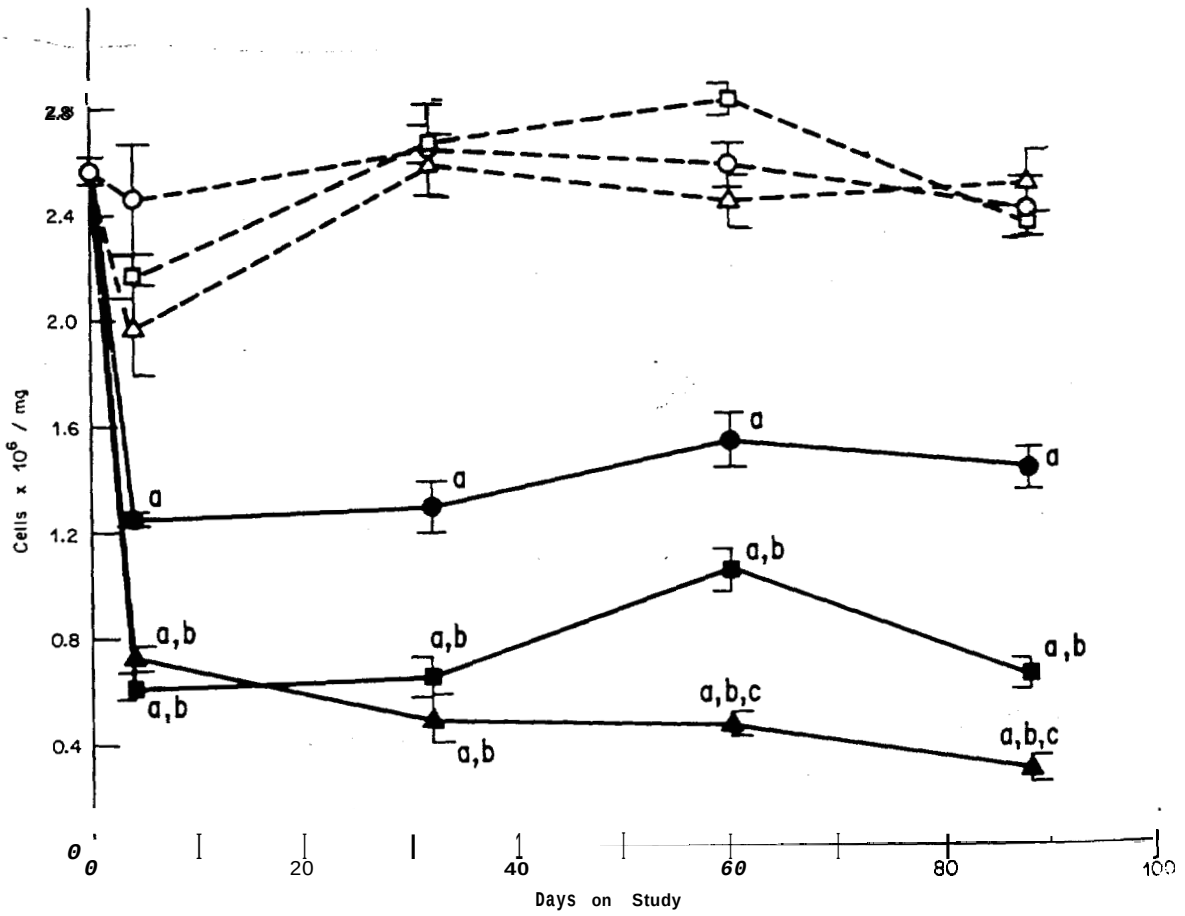

FIG. 4. Marrow cellularity.

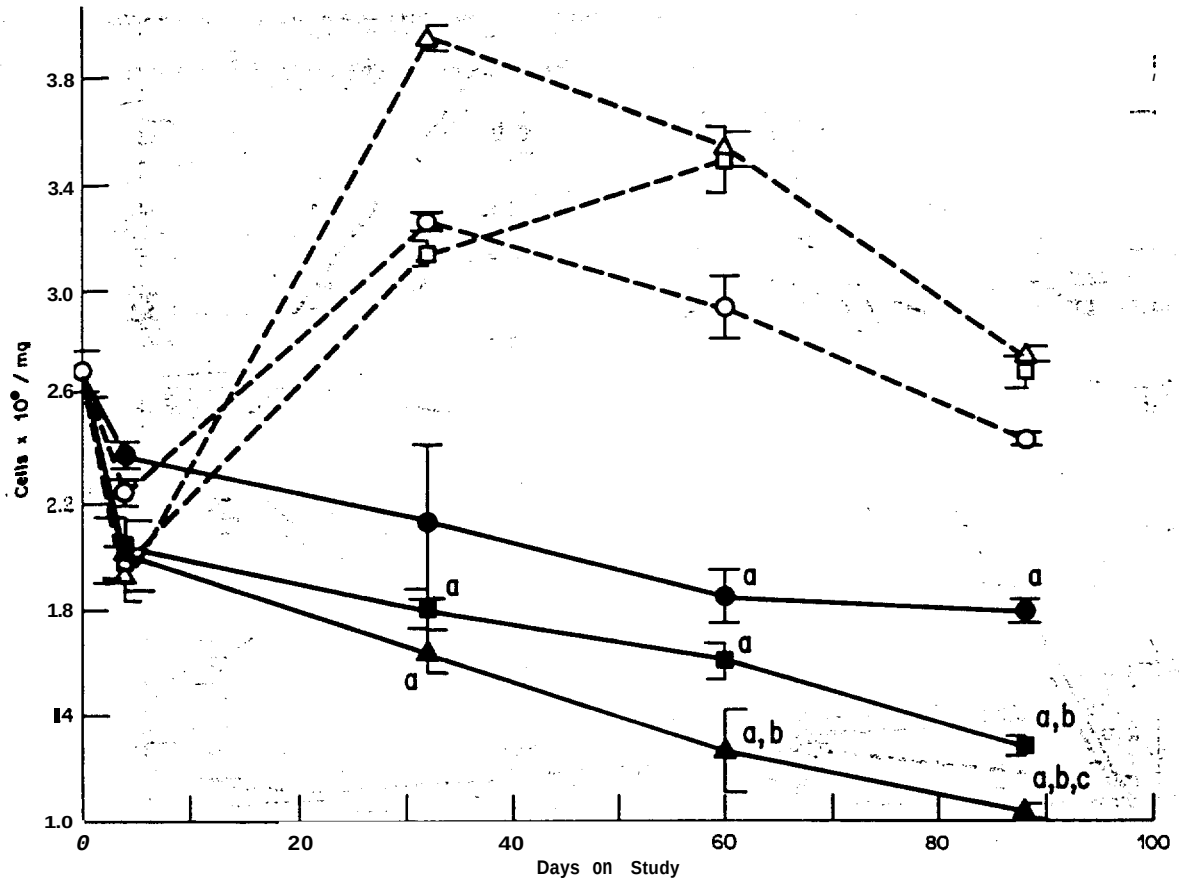

FIG. 5. Spleen cellularity per milligram.

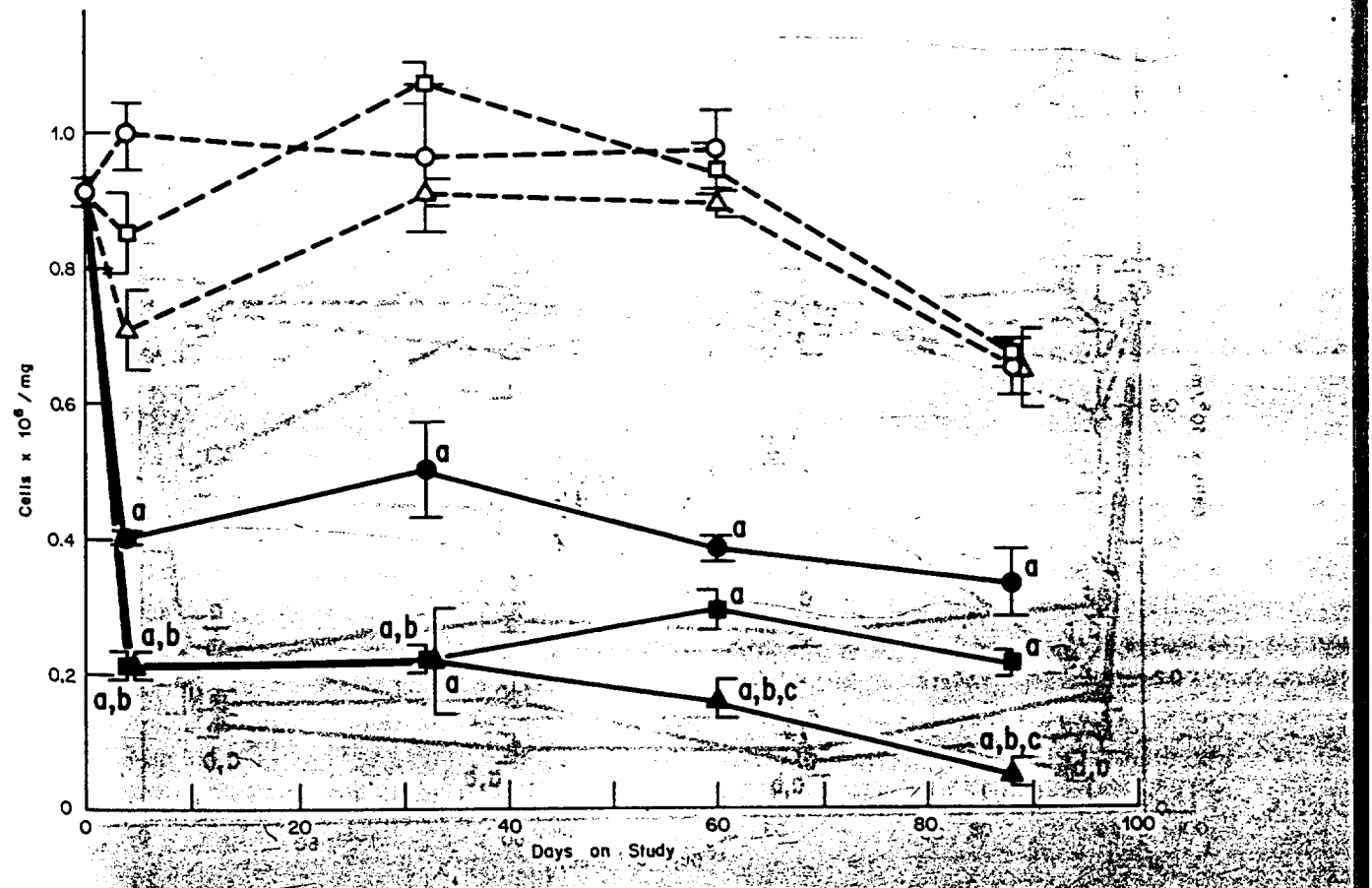

FIG. 6. Normoblasts per milligram (marrow).

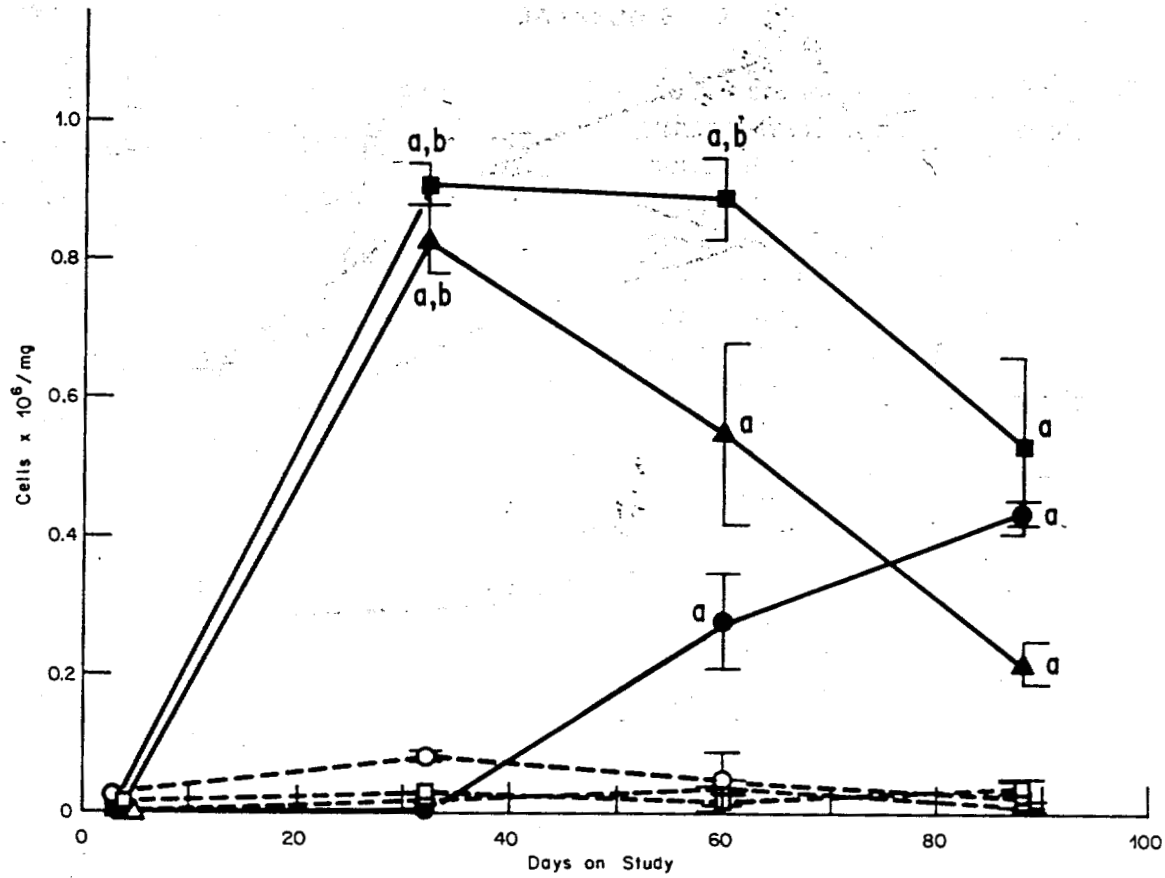

FIG. 7. Normoblasts **(spleen).**

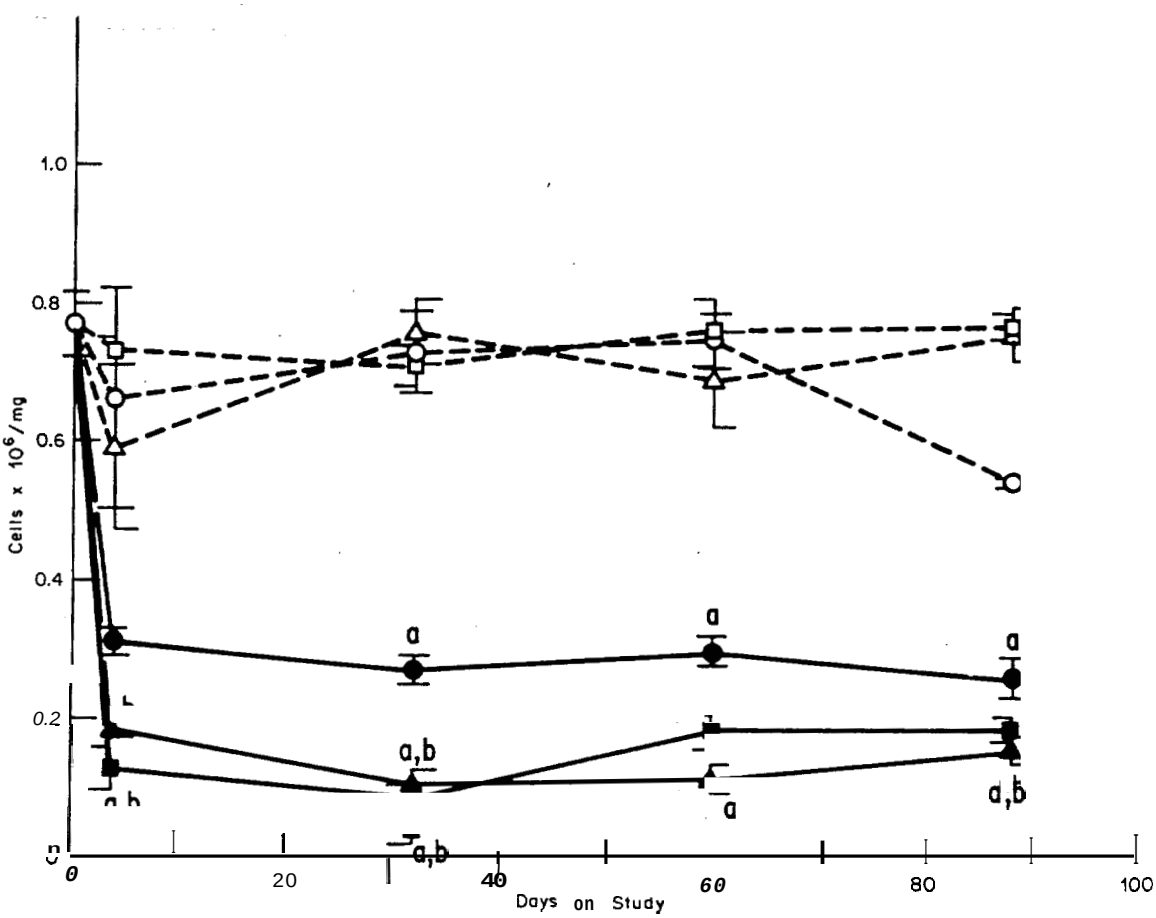

FIG. **8. Lymphocytes** (marrow).

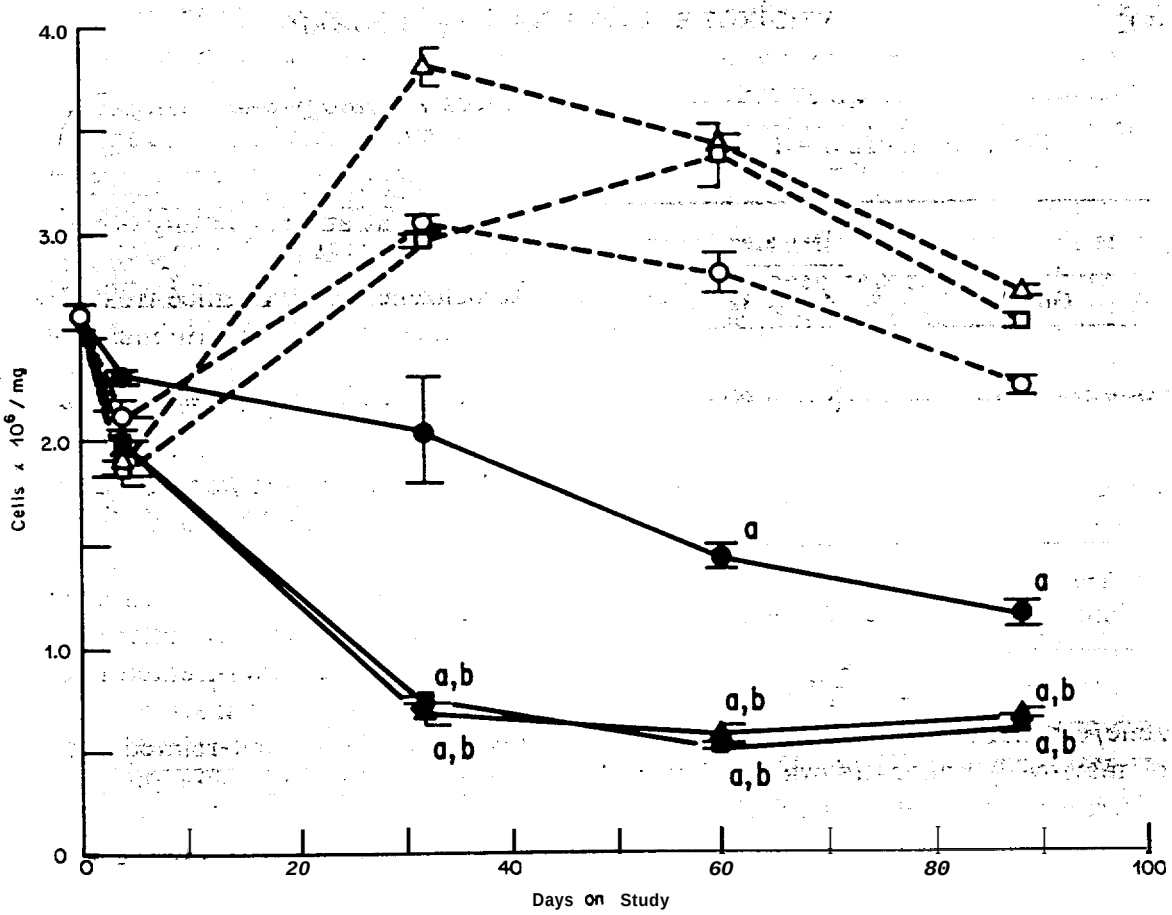

FIG. 9. Lymphocytes per milligram (spleen).

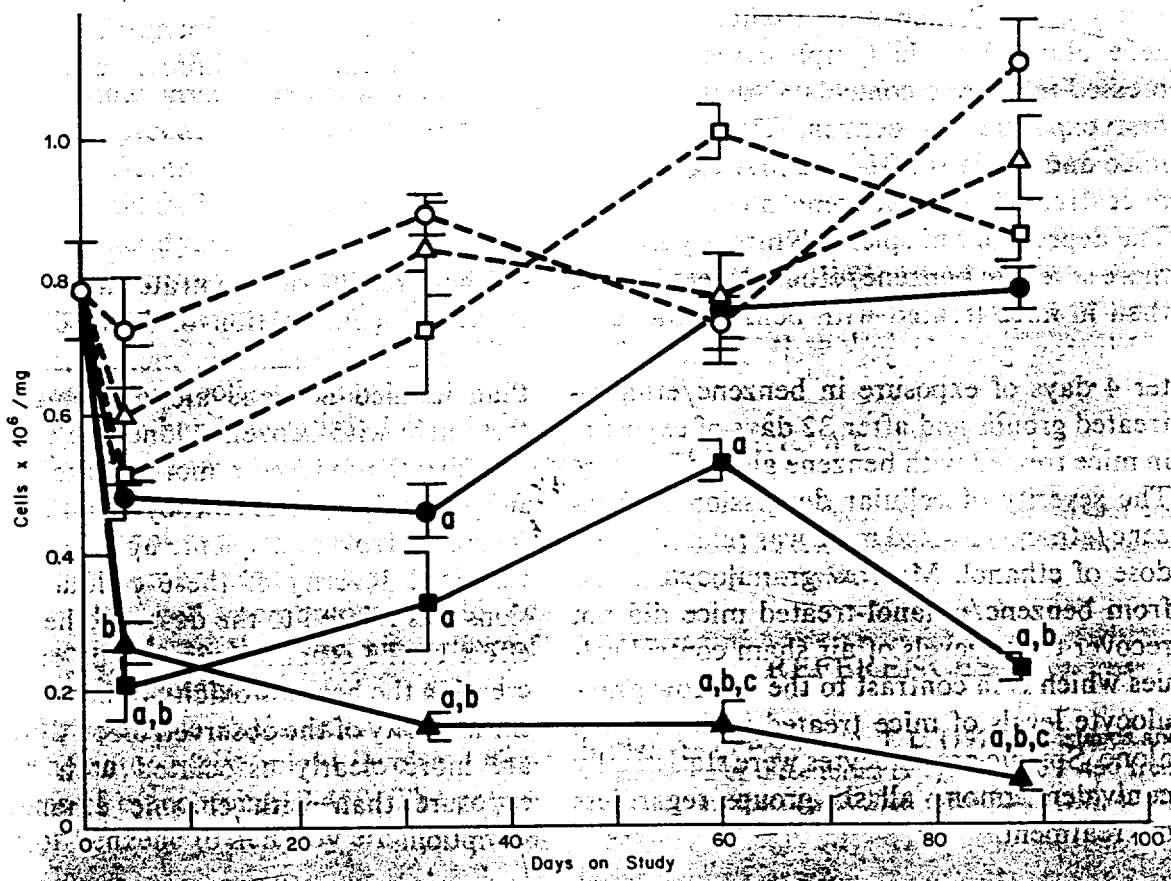

FIG. 10. Granulocytes per milligram (marrow).

TABLE 3

EFFECT OF BENZENE + ETHANOL ON SIZE OF THYMUS

Group[a]	Day after first exposure 4	32	63	88
Air + water	N[b]	N	N	N
Air + 5% ethanol	N	N	N	N
Air + 15% ethanol	N	N	N	N
Benzene + water	N	N	N	N
Benzene + 5% ethanol	N	I	I	I
Benzene + 15% ethanol	N	A	A	A

[a] Three animals *per* group.

[b] N, normal; I, greater than 50% involuted; A, absent or unidentified.

zene/ethanol-exposed mice. The depression of marrow lymphocytes was similar to the depression in total marrow cellularity. Counts were initially depressed after **4** days of exposure and remained depressed at constant levels throughout the study (Fig. 8). Benzene/ethanol-exposed mice were more severely affected than mice treated with benzene alone. Splenic lymphocytes were depressed relative to controls **32** days after the first exposures in benzene/ethanol-treated mice and 63 days after the first exposure in mice treated with benzene alone (Fig. **9).** The depressions of splenic lymphocytes were more severe in benzene/ethanol treated mice than in mice treated with benzene alone.

Marrow granulocytes were depressed after **4** days of exposure in benzene/ethanol-treated groups and after 32 days of exposure in mice treated with benzene alone (Fig. 10). The severity **of** cellular depression on benzene/ethanol-treated mice was related to the dose of ethanol. Marrow granulocyte levels from benzene/ethanol-treated mice did not recover to the levels of air sham control values which is in contrast to the marrow granulocyte levels of mice treated with benzene alone. Splenic granulocytes were statistically equivalent among all six groups regardless of treatment.

Gross Pathology and Histopathology

No significant gross abnormalities were apparent at autopsy in any organ examined from air sham-exposed mice or mice exposed to benzene alone. In mice treated with benzene and ethanol; the thymus was reduced to 50% of original size or could not be readily detected by the 32nd day of exposure. As shown in Table **3,** the incidence and extent of thymic involution were related to the dose of ethanol. By the 32nd day of exposure, the thymus was reduced to 50% of control size in benzene/5% ethanol-treated mice, but could only be detected microscopically in benzene/ 15% ethanol-treated mice.

Microscopic examination of tissues revealed few treatment-related abnormalities other than hypoplasia in the marrows and spleens of benzene-treated mice. Splenic and marrow hypoplasia was more prominent in benzene/ethanol-treated mice than in mice treated with benzene alone. No evidence of megaloblastoid marrow was observed in any animal. Mice receiving benzene and ethanol did show some very minimal periportal vacuolization in the liver.

DISCUSSION

These results demonstrate that, in C57Bl mice, the combination of inhaled benzene and ingested ethanol is more hematotoxic than inhaled benzene alone. Compared to treatment with benzene alone, the combined treatments produced a more marked depression of-peripheral erythrocytes, and a more severe marrow and splenic aplasia. In many cases the severity of these cellular depressions was related to the dose of ethanol. The effect of the ethanol seems to be one of increasing the hematotoxicity of benzene since the majority of the observed blood dyscrasias are more clearly associated with benzene exposure than with chronic ethanol consumption.

One reasonable explanation for the effects produced by the combined treatments is that ethanol, which contains seven calories per gram, may replace dailycaloric needs with nonnutritive calories. However, based on measured fluid consumption and average food consumption, the percentage of weekly caloric intake due to ethanol was calculated to be 4.3% for mice ingesting 5% ethanol 4 days/week and 7% for mice ingesting 15% ethanol 4 days/week. These values are almost identical to those found in our previous study (Snyder et *al.*, 1981). The small caloric contributions of ethanol under these conditions are unlikely to produce a nutrient deficiency.

Another explanation for enhanced benzene toxicity is that ethanol increases the levels of toxic benzene metabolites. Ethanol could increase the activities of benzene metabolizing enzymes within blood-forming organs producing, at these sites, hematotoxins in larger quantities or at faster rates than would be produced by benzene alone. Chronic treatment with ethanol has been shown to increase the rate of benzene metabolism *in vitro* without greatly increasing the content of liver cytochrome P-450 (Sato et *al.*, 1980, 1981). several investigators have suggested that ethanol may increase metabolic activity by modifying the membrane environment of the endoplasmic reticulum (Hill, 1974; Curran and Seeman, 1977). This modification may allow benzene to migrate at a faster rate to metabolizing enzymes.

On the other hand, because of its ability to modify membranes, ethanol may render cells more susceptible to the toxic effects of benzene. Ethanol has been shown to disrupt cytoplasmic membranes (Lyon *et al.*, 1981), and disrupted cell membranes would be more susceptible to the lytic properties of benzene or its metabolites.

Another possible explanation for the enhanced hematotoxicity might involve different forms of cytochrome *P*-450. The potentiation of benzene toxicity by ethanol seems to be somewhat unique. Compounds known to induce cytochrome P-450 such as phenobarbital and 3-methylcholanthrene increase the rate of metabolism of benzene but do not increase benzene toxicity (Gill *et al.*, 1979). It has been reported that there are at least three different types of rat liver cytochrome P-450 and that pretreatment with ethanol, phenobarbital, or 3-methylcholanthrene alters the relative amounts of the three forms (Comai and Gaylor, 1973). Ethanol was reported to induce form I; phenobarbital, form II; and 3-methylcholanthrene, form III. It may be that form I produces hematotoxins while forms II and III produce more benign metabolites. Perhaps form I produces lipid soluble hematotoxins which would concentrate in bone marrow whereas forms II and III produce water-soluble hematotoxins which would be eliminated before reaching appreciable concentrations in the marrow. It may also be that form I is more prevalent in the blood-forming organs than forms II and III. Investigations of these and other hypotheses would seem to be suitable for further work.

Finally, the appearance of nucleated red cells in peripheral blood is qualitatively different from any of the previous manifestations of benzene toxicity observed in this laboratory. The episode reported here represents the second that we have observed in as many studies of the interactions between benzene and ethanol.

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