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EFFECT OF SHORT TERM BENZENE ADMINISTRATION ON CIRCULATING LYMPHOCYTE SUBPOPULATIONS IN THE RABBIT: EVIDENCE OF A SELECTIVE B-LYMPHOCYTE SENSITIVITY

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ABSTRACT

Repeated subcutaneous administration of benzene to rabbits (0.25 or 0.5 ml/kg/day) produced a progressive lymphocytopenia with a 80% reduction in total circulating lymphocytes after 10 days of treatment. This decrease in circulating lymphocytes was due primarily to a reduction in B- cells with 17-30% remaining after 10 days of treatment, depending on the dose. Immunoglobin surface receptor - negative cells, presumably T lymphocytes, were relatively unaffected until late in the experiment at the high dose. It is concluded that benzene has a relatively selective cytotoxic effect on circulating B-lymphocytes in the rabbit.

INTRODUCTION

The rabbit possesses a relatively large proportion of circulating lymphocytes exhibiting immunoglobin (Ig) surface receptors and other characteristics of B lymphocytes (Wilson, et al., 1975; Sabolović, et al., 1977; Bona, et al., 1977; Wilson, et al., 1978). These laboratories have variously reported Ig-positive lymphocytes to represent between 40% and 80% of the total circulating lymphocyte pool. The remainder of lymphocytes in the peripheral blood of the rabbit are Ig- negative cells which appear to be T cells with few or no "null" cells present (Wilson et al., 1978). Studies in our laboratory have shown that exposure of rats to high doses of benzene results in a severe and prolonged reduction in circulating lymphocytes which is accompanied by alterations in cell cycle kinetics in the proliferating cell population of the bone marrow (Irons, et al., 1979). Chronic exposure to high levels of benzene in

man similarly has been associated with both a relative and absolute lymphocytopenia (Goldwater, 1941). The purpose of this investigation was to examine the effects of benzene exposure on the major circulating lymphocyte sub-populations in the rabbit.

MATERIALS AND METHODS

Immunobead® particles, consisting of micron sized hydrophilic spheres with the $F(ab')_2$ fragment of goat IgG anti- rabbit Ig, covalently bound to the surface, were purchased from Bio-Rad Laboratories (Richmond, CA). Medium 199, fetal calf serum, and trypan blue were obtained from Grand Island Biological Co. (Grand Island, NY); Lympho-prep@ (Ficoll-sodium metrazoate solution, 1.077 gm/ml) was obtained from Accurate Chemical Co. (Hicksville, NY); FTA Hemagglutination Buffer- BBL was obtained from Becton Dickinson and Co. (Cockeysville, MD); benzene, thiophene-free, was obtained from Fisher Scientific Co. (Pittsburgh, PA) and purity was checked by gas chromatography-mass spectrometry; Female, New Zealand White, specific pathogen free rabbits, weighing between 2 and 4 kg, were obtained from Charles River (Wilmington, MA) and were maintained on Wayne Rabbit Ration (Wayne Speciality Feeds, Chicago, IL). Cell counts were made using a Coulter Counter, Model ZF® (Coulter Electronics, Hialeah, FL).

Lymphocyte separation procedure. Blood from the middle ear artery was collected in acid-citrate-dextrose solution (ACD), containing 20.5 gm dextrose, 4.2 gm NaCl, 8 gm $Na_3C_6H_5O_7$ and 1 gm NaN_3 per liter, in a ratio of 1:1 blood to ACD. Mononuclear cells were separated by density gradient centrifugation using a modification of the original method reported by Bøyum (1968). The blood-ACD mixture was diluted 1:4 with FTA hemagglutination Buffer, pH 7.2, layered over Lympho-prep® in a ratio of 8:4 ml respectively, and centrifuged at 400 X g for 30 min. at 20°C. Mononuclear cells were recovered at the buffer-Lymphoprep® interface and washed three times in Medium 199. Centrifuge tubes were coated with fetal calf serum prior to gradient centrifugation.

Determination of lymphocyte recovery and cytotoxicity. The absolute number of lymphocytes and monocytes was calculated from tha total white blood cell count and differential counts obtained from morphologic

evaluation of peripheral smears. Percent recovery of mononuclear cells was determined from cell counts made on washed cell suspensions following Lymphoprepd separation. For counting purposes, cells were washed twice in 20mM tris buffer solution, pH 7.2, containing 1% NH_4Cl to lyse red blood cells. Cytotoxicity was determined using 0.4% trypan blue.

Solid phase surface immunoglobin rosette assay (SIR). Ig-bearing lymphocytes were assayed based on their ability to form rosettes with Immunobeads®. Cells were suspended *to* give a final concentration of 1 x 10^6 - 1 x 10^7 cells/ ml Medium 199. A 0.5 ml aliquot of this suspension was incubated overnight at 5°C with 50 ul *of* reconstituted Immunobead® reagent. The preparation was then stained using 0.1% toluidine blue and a wet mount slide prepared. Between 100 and 300 cells were read per slide with rosettes comprised of three or more beads/cell scored 3s positive.

Animal treatment. Animals were kept in isolation and all procedcres were conducted under aseptic conditions. Treatment and control groups received daily subcutaneous injections of benzene (50% v/v in corn oil) or corn oil for 10 consecutive days. Each animal served as its own baseline. Treatment groups were as follows: one group of 9 animals received benzene (0.5 ml/kg/ day); another group of 8 animals received benzene (0.25 ml/kg/day). The control group consisted of 6 animals which received corn oil in voiumes equivalent to the dosing volume received by the high *dose* benzene treated animals. An additional group of 9 animals was used in characterizing the isolation and anslytical procedures. Blood was collected from the middle ear artery on the days of sampling. Animals not sacrificed on day 10 were allowed to recover for up to 30 days and were sampled at that time.

RESULTS

Cytotoxicity. Cytotoxicity was monitored twice during the experiment, immediately after density gradient centifugation and just prior to rosette assay procedures. Cell viability after separation averaged better than 97% for all preparations and losses in viability as a result of in vitro treatment averaged only an additional 5%. Cell recovery was monitcred

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throughout the study and was calculated to be 49 ± 5% for 23 samples. No significant differences in cell recovery were observed between base-, line, treatment, or control groups.

Effects of benzene on total circulating lymphocytes and monocytes. Repeated administration of benzene resulted in a rapid loss of circulating lymphocytes which appeared to be dependent on both dose and duration of exposure. Daily administration of 0.5 ml/kg benzene was accompanied by an 80% reduction in circulating lymphocytes by day 10 (Table 1). A 35% reduction was observed in animals receiving 0.25 ml/kg over the same time period. Animals allowed to recover following 10 days of repeated administration were examined between 20 and 30 days after cessation of treatment. Benzene treated rabbits demonstrated only partial recovery of circulating lymphocytes at these later points.

Circulating monocytes varied between 0.5% and 4.7% of total circulating leukocytes, depending on the treatment group. No decrease in monocytes was observed in animals receiving the low dose of benzene. Although a significant decrease (89%) in circulating monocytes was seen at day 10 of treatment in rabbits receiving 0.5 ml/kg/day benzene; monocytes, as a fraction of mononuclear cells, demonstrated no significant difference. Monocytes, expressed as a percentage of the mononuclear cell fraction, averaged 6.32 ± 4% for all groups.

Normal baseline values for the proportion of circulating lymphocytes which were 19-positive averaged 48 ± 11% (S.D., n = 32). The relative values for Ig-positive and negative lymphocytes are presented in Table 2.

Table 1. Effects of Repeated Subcutaneous Benzene Administration on Circulating Lymphocytes *

TABLE 1 Effects of Repeated Subcutaneous Benzene Administration on Circulating Lymphocytes *

Treatment Group	Corn Oil (0.5 ml/kg/day)			Benzene (0.25 ml/kg/day)			Benzene (0.5 ml/kg/day)		
Day	Total Lymphocytes	Ig Positive	Number Of Animals	Total Lymphocytes	Ig Positive	Number Of Animals	Total Lymphocytes	Ig Positive	Number Of Animals
0	3320 ± 239	1766 ± 194	23	3320 ± 239	1766 ± 194	23	3320 ± 239	1766 ± 194	23
1	3755 ± 394	1801 ± 197	6	2923 ± 326	1423 ± 241	8	3127 ± 41	1691 ± 227	9
3	—	—	—	—	—	—	2043 ± 326	774 ± 138	7
5	3703 ± 655	1395 ± 188	6	2174 ± 353	475 ± 168	8	1763 ± 193	367 ± 87	7
10	4166 ± 857	1393 ± 400	6	2173 ± 804	471 ± 85	8	701 ± 210	190 ± 68	6
28-33	3984/2948[+]	—	2	2508 ± 810[+]	—	4	1810/2753[ʃ]	451/1429[ʃ]	2
40	0731/4303	1783/1920	2	2730 ± 1056	640 ± 169	4	—	—	—

*Animals were treated daily for 10 days and allowed to recover; values are expressed as cells/mm³ of peripheral blood and represent means ± 1 S.E.

[+]Examined day 28.

ʃExamined day 33.

TABLE 2. Effects Benzene on Ig-Positive and Ig-Negative Lymphocytes*

Treatment Group	Corn Oil (0.5 ml/kg/day)		Benzene (0.25 ml/kg/day)		Benzene (0.5 ml/kg/day)	
Day	Ig Positive	Ig Negative	Ig Positive	Ig Negative	Ig Positive	Ig Negative
1	103	132	94	101	139	90
3	----	----	----	----	59+	75
5	81+	155+	29+	114	26+	45+
10	78+	163+	30+	104	17+	28+

*Values are expressed as a percent of baseline and represent the means for each treatment group, calculated from the baseline value for each individual animal (day 0 = 100%).

+Denotes values which are significantly different from baseline ($p < 0.05$) as calculated from absolute numbers (Table 2).

After 10 days of repeated administration benzene treatment resulted in a 70% and 83% loss in circulating Ig-positive lymphocytes at the low and high dose respectively. Ig-negative lymphocytes ranged from 28% to 90% of baseline values in animals receiving 0.5 ml/kg benzene; however, Ig-negative lymphocytes exhibited no depression and in fact showed a slight elevation at the low dose of benzene. In rabbits allowed to recover between 20 and 30 days after termination of treatment, recovery of circulating Ig- positive lymphocytes appeared in general to be less than 50% effective' (Table 1). Ig-positive lymphocytes remained depressed whereas Ig-negative cells were normal or slightly increased in number.

DISCUSSION

Benzene treatment results in a progressive decrease in Ig-positive cells and a commensurate loss in the total number of circulating lympho-

cytes. This is indicative of a depression of circulating E-lymphocytes. A similar decrease was not seen in the Ig-negative cell population at the low dose of benzene. Circulating Ig-negative lymphocytes were slightly elevated relative to baseline values in corn oil treated animals and in rabbits receiving the low dose of benzene. This is, perhaps, due to inflammatory response related to the route of benzene and corn oil administration; however, no neutrophilia was observed in any of the groups examined. A similar effect was noted for Ig-positive cells in corn oil treated animals and at 24 hrs in benzene treated animals. No significant dose related depression in Ig-negative lymphocytes was observed prior to 5 days of benzene exposure. Benzene appears to be a local irritant. Three animals receiving the high dose were sacrificed during the experiment because of self-inflicted wounds at the site of injection (Table 1). Animals receiving either corn oil or the low dose of benzene did not exhibit this response.

These findings, together with previously reported alterations in bone marrow cell cycle kinetics following benzene exposure, suggest the possibility that benzene acts on 6-lymphocyte precursor cells in bone marrow or directly on circulating B cells. Low doses of cyclophosphamide have been shown to induce selective B-cell depletion in guinea pigs and mice, particularly in lymph nodes and spleen (Turk and Poulter 1972; Poulter and Turk 1972; Stockman, et al., 1973); however, a selective depression in circulating 6-cells of the magnitude seen following benzene exposure has not been reported. Benzene causes an apparent block or arrest in G_2 or M phase and an increase in ^{3}H-thymidine incorporation in bone marrow, accompanied by a loss of some cells (Irons et al., 1979). As is observed with alkylating agents such as

cyclophosphamide, busulfan, chlorambucil and others, expression of benzene-induced cytoxicity appears to be dependent on DNA synthesis. Therefore, the selective cytotoxic effect of benzene on B-lymphocytes may reflect a higher rate of cell prolifer'ation in these cells, a difference in susceptibility of circulating lymphocyte subpopulations or both.

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