PENGAD-Bayonne, N.J.
PLAINTIFF'S EXHIBIT
898

Toxicology Letters, 20 (1984) 343–349
Elsevier

DEPRESSIONS IN B- AND T-LYMPHOCYTE MITOGEN-INDUCED BLASTOGENESIS IN MICE EXPOSED TO LOW CONCENTRATIONS OF BENZENE

(Benzene; immunotoxicity; dose-response)

MICHAEL G. ROZEN. CARROL A. SNYDER. and ROY E. ALBERT

New York University Medical Center, Institute of Environmental Medicine, 550 First Avenue, New York, NY 10016 (U.S.A.)

(Received November 2nd. 1983)
(Accepted November 4th, 1983)

SUMMARY

In a short-term (6 h/day × 6 days) benzene inhalation dose-response study. mitogen-induced blastogenesis of both B- and T-lymphocytes in male, C57Bl mice was observed to be significantly depressed at relatively low levels of benzene. Exposure to 10 ppm benzene resulted in a significant depression in femoral lipopolysaccharide (LPS)-induced B-colony-forming ability, while total numbers of B-lymphocytes at this concentration were not significantly depressed. Similarly. splenic phytohemagglutinin (PHA)-induced blastogenesis was significantly depressed at 31 ppm. without a concomitant significant depression in numbers of T-lymphocytes. These data indicate that concentrations of benzene at or near the current standard for occupational exposure (10 ppm) can affect certain immune-associated processes

INTRODUCTION

Exposure to benzene has been associated with hematotoxicity both in chronically exposed human populations (usually occupationally exposed groups) and in experimental animals [1–5]. The most common forms of hematotoxicity associated with benzene exposure are depressions in the number of hematopoietic cells both in

*To whom all correspondence should be sent

Abbreviations: LPS. lipopolysaccharide; PHA. phytohemagglutinin: PHA-SI. PHA stimulation index: sIgM, surface immunoglobulin M .

0378-4274/84/$ 03.00 © Elsevier Science Publishers B.V.

the peripheral blood and in the hematopoietic organs. Even though all hematopoietic lineages (erythroid, myeloid and lymphoid) exhibit susceptibility to the toxic effects of benzene exposure, the lymphoid line appears to be the most sensitive [6–8].

Benzene-induced reductions in lymphocyte numbers raise the possibility that benzene can impair immune response since lymphocytes play a fundamental role in immunological processes. In several studies, Irons and co-workers demonstrated depressions in both mitogen-induced blastogenesis and plaque-forming cells when mice were administered benzene or some of its metabolites by parenteral injection [9, 10]. They also found that rat splenocytes, cultured with benzene metabolites, had depressed mitogen-induced blastogenic responses [11, 12]. Because of the importance of inhaled benzene as an occupational hazard, we sought to determine at what levels benzene, administered by inhalation, interferes with certain immune associated responses. A dose–response exposure regime was employed in which the lowest exposure concentration was the current occupational exposure limit, 10 ppm. Mitogen-induced proliferative assays were chosen for assessing immune-associated processes. Although the assays do not give a measure of actual immune function, mitotically competent lymphocytes are presumed to be a requisite for immune proficiency.

METHODS

Inhalation exposures

Male, 6-week-old, C57Bl/6J mice were received from Jackson Laboratories, Bar Harbor, ME. Animals were quarantined for 2 weeks during which time they were acclimatized to a 12-h light/dark cycle and observed for anomalous behaviour and disease. Animals were then randomly distributed into five groups of 7–8 mice per group.

Animals were exposed in whole–body, dynamic inhalation chambers of 0.125 m^3, 1.0 m^3 or 1.3 m^3 volume. Benzene vapor was generated as described elsewhere [8]. Benzene concentrations within the chambers were monitored at 0.5 h intervals during the exposures by means of a MIRAN 1A infrared spectrophotometer (Foxboro Analytical, Norwalk, CT) at a wavelength of 9.8 μm. Animals were exposed for 6 h/day for 6 consecutive days. The same exposure regime was used for benzene-treated and air control mice except that control mice were exposed to filtered, conditioned air only.

Lymphocyte assays

Within 30–90 min after the last benzene exposure, mice were bled via the tail vein for the determination of peripheral blood cell counts. These were performed using standard hematological methods [8]. On the morning following the last exposure, the 5 animals in each group which had blood counts closest to their respective

group's mean blood count were selected for the lymphocyte assays. Single-cell suspensions [13] were made of the marrow and spleen cells from these animals. Aliquots of these cell suspensions were used for T- and B-cell enumeration and for the mitogen-induced proliferative assays.

B-lymphocyte proliferative ability in response to LPS was assayed by the method of Kincade et al. [14, 15]. Spleen and bone marrow cells were plated at $4 \cdot 10^3$/ml and $25 \cdot 10^3$/ml, respectively. Cell cultures from each animal were prepared in triplicate. After 6 days incubation at 37°C in fully humidified atmosphere of 5-6% CO_2 in air, the cultures were examined with an inverted microscope for the presence of colonies (aggregates > 20 cells). T-lymphocyte proliferative ability was determined by a modification of the PHA stimulation index assay as described by Stobo et al. [16]. One million spleen cells per animal were cultured in triplicate. The PHA-SI was taken as the ratio of the mean levels of tritiated thymidine incorporated into PHA-stimulated cultures to the mean levels of tritiated thymidine incorporated into non-stimulated cultures. The numbers of B- en T-lymphocytes were enumerated using immunofluorescent techniques [17]. B-cells were enumerated using fluorescein-conjugated antibody directed against murine $sIgM^+$. T-cells were enumerated using fluorescein-conjugated antibody directed against Thyl.2 antigen ($Thyl.2^+$). Cells were counted using an immunofluorescent microscope. Between 200–400 cells per smear were counted. Preliminary experiments using an FITC-conjugated $F(ab')_2$ fragment of these antibodies revealed that the $F(ab')_2$ fragment or the whole antibody yielded similar results. Therefore, T- and B-lymphocyte enumeration of cell suspensions in these studies were performed with whole antibody. Statistical significance for differences between control and treatment groups was determined by a one-way ANOVA test. Statistical analysis was performed with a Minitab Data Analysis Package on a Control Data Corp. CYBER 170/730.

RESULTS

The mean daily benzene concentrations ± SD for the five exposures were: 301 ± 3 ppm; 100 ± 1 ppm; 31.0 ± 0.9 ppm; 10.2 ± 0.4 ppm; and 0 ppm (control). Fig. 1 shows the effects of 6 consecutive days of exposure on peripheral lymphocyte and erythrocyte (RBC) counts. Lymphocyte counts were depressed at all exposure levels while erythrocyte counts were elevated at 10 ppm, equal to controls at 30 ppm and depressed at 100 and 300 ppm. Fig. 2 shows the effects of the benzene exposures on femoral B-lymphocyte numbers ($sIgM^+$ cells) and on femoral B-lymphocyte colony-forming ability (B-CFCs). All benzene concentrations reduced the marrow B-lymphocyte colony forming ability to about 30% of the control values. Femoral B-lymphocyte numbers were reduced only after exposures to concentrations ≥ 100 ppm. Splenic B-lymphocyte colony forming abilities were reduced only at 300 ppm and the numbers of splenic B-lymphocytes were depressed only at 100 pprn and 300 ppm (not shown). Fig. 3 shows that the PHA stimulation indices of splenic T-cells

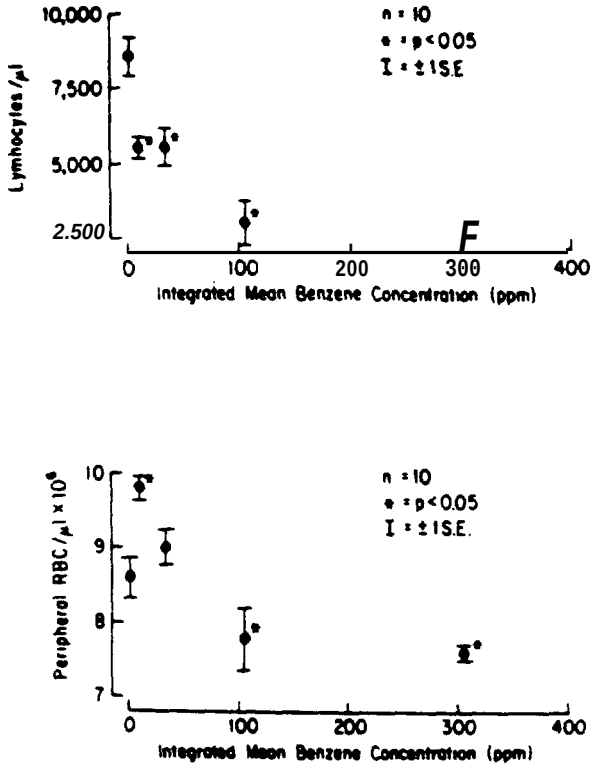

Fig. 1. 6-day dose-response effect of inhaled benzene on peripheral lymphocytes and red blood cells (RBC).

were reduced at all concentrations of benzene ≥ 31 ppm, while splenic T-lymphocyte numbers (Thyl.2$^+$ cells) were reduced only at 100 ppm and 300 ppm. Non-stimulated spleen and femoral cultures (i.e., cultures lacking PHA) from mice exposed to all benzene concentrations had tritiated thymidine incorporations equal to that of controls (not shown).

DISCUSSION

These results demonstrate that short-term inhaled benzene even at relatively low exposure concentrations can cause reductions in certain immune-associated processes. Levels of circulating lymphocytes and mitogen-induced blastogenesis of femoral B-lymphocytes were depressed after 6 days exposure to 10 ppm benzene. In addition, mitogen-induced blastogeneses of splenic T-lymphocytes were depressed after 6 days exposure to 31 pprn.

Benzene-induced depressions in rnitogen-induced lymphocyte blastogenesis have been previously reponed [10–12] but this study represents the first time these depres-

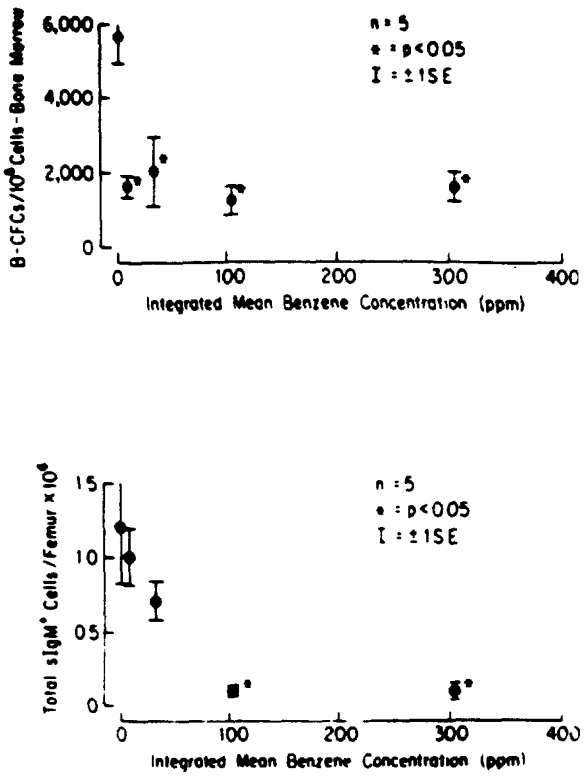

Fig. 2. 6-day dose-response effect of inhaled benzene on frequency of femoral B-lymphocyte colony forming cells (B-CFCs) and on the number of femoral B-lymphocytes (sIgM⁺ cells).

sions have been observed following exposures to inhaled benzene. Moreover the depressions occurred at concentrations equal to or near the current occupational exposure limit. 6 days exposure to 10 ppm benzene was just as effective at reducing femoral B-cell blastogenesis as 6 days exposure to higher benzene concentrations.

Normal T-lymphocyte S-phase was apparently undisturbed by the exposures since levels of tritiated thymidine incorporated into unstimulated cells were unaffected by the exposures. This, coupled with the observed reductions in thymidine incorporation following mitogen-induced blastogenesis, suggests that exposures to low levels of benzene may disturb those immune responses requiring heightened lymphocyte proliferation. Perhaps future work should concern the effect of low level benzene exposure on various immune functions requiring heightened lymphocyte proliferation.

ACKNOWLEDGEMENTS

Work completed in partial fulfillment of requirements for degree of Doctor of

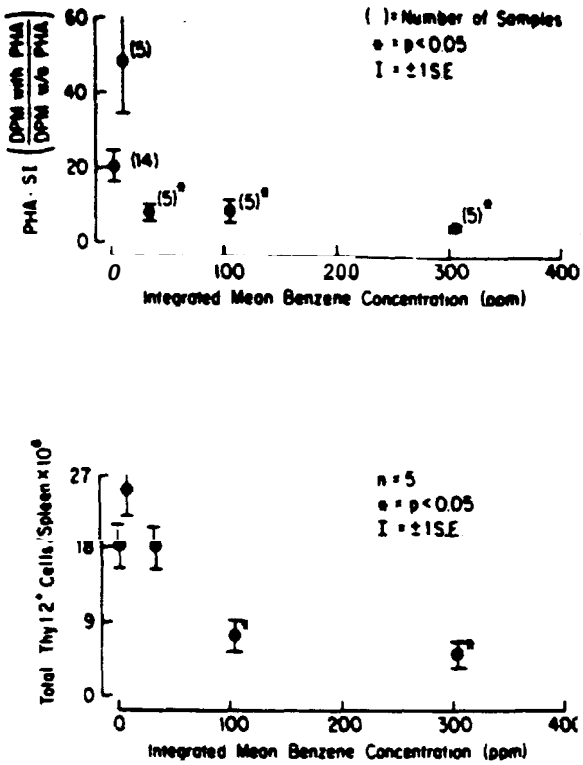

Fig. 3. 6-day dose-response effect of inhaled benzene on splenic cells phytohemagglutinin stimulation indices (PHA-SI) and on the numbers of splenic T-lymphocytes (Thy1.2+ cells).

Philosophy (senior author) under the sponsorship of Dr. Snyder. This research was supported in part by Center grant No. ES00260 from the National Institutes of Environmental Health Sciences. and by center grant No. CA13343 and grant No. CA26724 both from the National Cancer Institute. The authors wish to acknowledge the efforts of Kenneth Magar and Paul Priest Jr. for operating the inhalation chambers and Maureen Freitag for typing the manuscript.

REFERENCES

1 B.D. Goldstein. Hematotoxicity in humans. in Benzene Toxicity, A Critical Evaluation. J. Toxicol. Environ. Health, Suppl. 2. Nov. (1977) pp. 69–106.

2 L. Selling. Benzol as a leucotoxin. Studies on the degeneration and regeneration of the blood and hematopoietic organs, Johns Hopkins Hosp. Rept.. 17 (1916) 83–142.

3 R. Snyder and J.J. Kocsis, Current concepts of chronic benzene toxicity. CRC Crit. Rev. Toxicol.. June (1975) 265–288.

4 W.B. Deichman, W.E. MacDonald and E. Bernal, The hematopoietic tissue toxicity of benzene vapors, Toxicol. Appl. Pharmacol., 5 (1963) 201–224.

5 C.A. Snyder, B.D. Goldstein, A.R. Sellakumar, 1. Bromberg, S. Laskin md R.E. *Albert.* The inhalation toxicology of benzene: Incidence of hematopoietic neoplasms urd hematotoxicity of AKR/J and C57Bl/6J mice. Toxicol. Appl. Pharmacol., 54 (1980) 323-331.

6 R.D. Irons. H. D'A. Heck. B.J. Moore and K.A. Muirhead, Effects of short-term benzene administration on bone marrow cell cycle kinetics in the rat, Toxicol. Appl. Pharmacol., 51 (1979) 399-409.

7 T. Matsushita. Experimental studies on the disturbance of hemopoietic organs due to benzene intoxication, Nagoya J. Med. Sci., 28 (1966) 204–234.

8 C.A. Snyder, B.D. Goldstein. A. Sellakumar, S.R. Wolman, 1. Bromberg, M. Erlichman and S. Laskin, Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm, J. Toxicol. Environ. Health, 4 (1978) 605–618.

9 D. Wierda, R.D. Irons and W.F. Greenlee, Immunotoxicity in C57Bl/5 mice exposed to benzene and Aroclor 1254. Toxicol. Appl. Pharmacol., 60 (1981) 41—47.

10 D. Wierda and R.D. Irons. Hydroquinone and catechol reduce the frequency of progenitor B-lymphocytes in mouse spleen and bone marrow, Immunopharmacology, 4 (1982) 41-54.

11 R.D. Irons. D.A. Neptun and R.W. Pfeifer, Inhibition of lymphocyte transformation and microtubule assembly by quinone metabolites of benzene: Evidence for a common mechanism, J. Reticuloendo. Soc., 30 (1981) 359–372.

12 R.W. Pfeifer urd R.D. Irons. Inhibition of lectin stimulated lymphocyte agglutination and mitogenesis by hydroquinone: Reactivity with intracellular sulfhydryl groups. Exp. Mol. Pathol.. 35 (1981) 189-198.

13 J.D. Green, C.A. Snyder. J. LoBue, et al. Acute and chronic dose/response effects of inhaled benzene on multipotential hematopoietic stem (CFU-S) and granulocyte/macrophage progenitor (GM-CFU-C) cells in CD-I mice. Toxicol. Appl. Pharmacol., 58 (1981) 492–503.

14 P.W. Kincade, P. Ralph and M.A.S. Moore. Growth of B-lymphocyte clones in semisolid culture is mitogen-dependent, J. Exp. Med., 143 (1976) 1265-1269.

15 P.W. Kincade, C.J. Paige, M.E. Parkhouse and G. Lee, Characterization of murine colony forming B-cells, I. Distribution resistance to anti-immunoglobulin antibodies, and expression of Ia antigens. J. Immunol.. 120 (1976) 1289-1296.

16 J.D. Stobo, A.S. Rosenthal and W.E. Paul, Functional heterogeneity of murine lymphoid cells, I. Responsiveness to md surface binding of concanavalin A and phytohemagglutinin, J. Immunol., 108 (1972) 1–17.

17 Becton-Dickinson, Monoclonal Antibody Source Book. Section 2.1 (1981).

timulation

urch was
tutes of
ant No.
wish to
g the in-

. Toxicol.

blood and

Toxicol..

f benzene