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

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TITLE: Depressions in B and T Lymphocyte Mitogen-Induced Blastogenesis in Mice Exposed to Low Concentrations of Benzene

Main Point: Short-term exposure (6 days) of C57Bl mice to 10 ppm benzene (the current occupational exposure limit) reduced the mitogen-induced blastogenesis of femoral B-cells to 30% of control values. Moreover, 6 days exposure to 10 ppm was just as effective at reducing blastogenesis as 6 days exposure to higher benzene concentrations. In addition, splenic T-cell mitogen-induced blastogenesis was reduced to about 40% of control values after 6 days exposure to concentrations of benzene $\geq$ 31 ppm. These results indicate that exposures to benzene at or near the current occupational exposure limit may affect certain immune functions.

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FIELDS OF INTEREST: Toxicology
Immunology
Oncology

Scientists affiliated with regulatory agencies should be interested in this paper.

ABSTRACT

In a short-term (6 hrs/day x 6 days) benzene inhalation dose/response study, mitogen-induced blastogenesis of both B and T lymphocytes 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-induced B-colony forming ability, while total numbers of B lymphocytes at this concentration were not significantly depressed. Similarly, splenic phytohemagglutinin 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.

Exposure to benzene has been associated with hematotoxicity both in chronically exposed human populations (usually occupationally exposed groups) and in experimental animals (1-7). The most common forms of hematotoxicity associated with benzene exposure are depressions in the numbers of hematopoietic cells both in 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 (8-10).

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 (11,12). In addition, Irons and co-workers found that rat splenocytes, cultured with benzene metabolites, had depressed mitogen-induced blastogenic responses (13,14). Because of the importance of inhaled benzene as an occupational hazard, we sought to determine whether and at what levels benzene, administered by inhalations, 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 parts per million

(ppm). Mitogen-induced proliferative assays were chosen for assessing immune-associated processes. Although the assays do not give a measure of actual immune function mitototically competent lymphocytes are presumed to be a requisite for immune proficiency.

B-lymphocyte proliferative ability in response to lipopolysaccharide (LPS) was assayed by the method of Kincade et al. (15,16). T-lymphocyte proliferative ability was determined by a modification of the phytohemagglutinin (PHA) stimulation index assay as described by Stobo et al. (17). In addition, the numbers of B and T-lymphocytes were enumerated using immunofluorescent techniques (18). B-cells were enumerated using fluorescein-conjugated antibody directed against murine surface immunoglobulin M ($sIgM^+$). T-cells were enumerated using fluorescein-conjugated antibody directed against Thy1.2 antigen ($Thy1.2^+$).

Five groups of male C57Bl mice were used in these studies. Each group of 7-8 mice was exposed to one of the following mean $\pm$ SD benzene concentrations: 0 ppm (control), 10.2 $\pm$ 0.4 ppm; 31.0 $\pm$ 0.9 ppm; 100 $\pm$ 1 ppm; 301 $\pm$ 3 ppm for 6 hours/day for 6 consecutive days (19). Within 30-90 minutes after the last benzene exposure, mice were bled via the tail vein for the determination of peripheral blood cell counts (20). On the morning following the last exposure, the five 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 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.

Figure 1 shows the effects of six 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. Figure 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 $\geq$ 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 ppm and 300 ppm (data not shown). Figure 3 shows that the PHA stimulation indices of splenic T-cells were reduced at all concentrations of benzene $\geq$ 31 ppm, while splenic T-lymphocyte numbers ($Thy1.2^+$ cells) were reduced only at 100 ppm and 300 ppm. Non-stimulated spleen cultures (i.e. cultures lacking PHA) from mice exposed to all benzene concentrations had tritiated thymidine incorporations equal to that of controls (data not shown). This suggests tnat induced mitoses were

disturbed by the benzene exposures rather than the normal in vitro rates of mitoses. Likewise, non-stimulated bone marrow cultures from mice exposed to all benzene concentrations had tritiated thymidine incorporation equal to controls.

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 ppm.

Depression of peripheral blood cell counts after short-term exposures to low concentrations of benzene have not been previously reported. The depressions observed in this study are likely due to a lack of recovery between the time when the animals were exposed and the time when blood samples were taken. Animals were bled immediately after exposure rather than several hours or even days after exposure. Benzene-induced depressions in mitogen-induced lymphocyte blastogenesis have been previously reported (11-14) but this study represents the first time these depressions have been observed following exposures to inhaled benzene. Moreover these depressions occurred at concentrations equal to or near the current occupational exposure limit. Of note was that 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. These results suggest that inhaled benzene at relatively low levels of exposure may disturb immune system function.

REFERENCES

1. B.D. Goldstein, J. Toxicol. Environ. Health, Suppl. 2, 69 (1977).
2. L. Selling, Johns Hopkins Hosp. Report 17, 83 (1916).
3. T.J. Haley, Clinical Toxicology 11, 531 (1977).
4. R. Snyder, E.W. Lee, J.J. Kocsis and C.M. Witmer, Life Sciences 21, 1709 (1977).
5. B. Steinberg, Blood 4, 550 (1949).
6. W.B. Deichman, W.E. MacDonald and E. Bernal, Toxicol. Appl. Pharmacol. 5, 201 (1963).
7. C.A. Snyder, B.D. Goldstein, A.R. Sellakumar, I. Bromberg, S. Laskin and R.E. Albert, Toxicol. Appl. Pharmacol. 54, 323 (1980).
8. R.D. Irons, H. D'A. Heck, B.J. Moore and K.A. Muirhead, Toxicol. Appl. Pharmacol. 51, 399 (1979).
9. T. Matsushita and J. Nagoya, Med. Sci. 28, 204 (1966).
10. C.A. Snyder, B.D. Goldstein, A. Sellakumar, S.R. Wolman, I. Bromberg, M. Erlichman and S. Laskin, J. Toxicol. Environ. Health 4, 605 (1978).
11. D. Wierda, R.D. Irons and W.E. Greenlee, Toxicol. Appl. Pharmacol. 60, 410 (1981).
12. Ibid, Immunopharmacol. 4, 41 (1982).
13. R.D. Irons, D.a. Neptun and R.W. Pfeifer, J. Reticuloendo. Soc. 30, 359 (1981).
14. R.W. Pfeifer and R.D. Irons, Exp. Molec. Path. 35, 189 (1981).
15. P.W. Kincade, P. Ralph and M.A.S. Moore, J. Exp. Med.

143, 1265 (1976).

16. P.W. Kincade, C.J. Paige, M.E. Parkhouse and G. Lee, J. Immunol. 120, 1289 (1976).

Spleen and bone marrow cells were plated at $4x10^3$/ml and $25x10^3$/ml, respectively. On the day prior to use, RPMI 1640 medium was modified for the B-CFC assay. This modification consisted of: 0.1 g/liter streptomycin, 10^5 units/liter penicillin, 2 mM L-glutamine, 16 µg/ml L-Asparagine, 8 µg/ml L-Serine, 1 mM sodium pyruvate, 0.8% MEM essential amino acids (50x), 0.4% MEM non-essential amino acids (100x), 0.6% sodium bicarbonate solution (7.5%), $5x10^{-5}$ M 2-mercapto-ethanol, and 15% freshly thawed fetal calf serum (Gibco, Lot No. 29-N5317). The medium was then filter sterilized through a 0.45 µ Nalgene filter. on the day of use, this medium was warmed to 37°C, mixed with 1/10 volume of boiled 3% Bacto-Agar (Difco, Detroit, MI), and 25 µg/ml lipopolysaccharide (Salmonella typhosa endotoxic W0901; Difco). A small volume of cell suspension containing appropriate number of cells was added, and 1 ml proteins aliquoted to 35 mm sterile tissue culture dishes (Microbiological Associates). Cultures were prepared in triplicate. After 6 days incubation at 37°C in fully numidified atmosphere of 5-6% CO_2 in air, the cultures were examined with an inverted microscope for the presence of colonies (aggregates > 20 cells).

17. J.D. Stobo, A.S. Rosenthal and W.E. Paul, J. Immunol.

108, 1 (1972).

One million cells in 0.2 ml were cultured in triplicate, in RPMI 1640 medium which had been supplemented with 0.1 g/liter streptomycin, 10^5 units/liter penicillin and 5% heat inactivated fetal calf serum. Twelve µg of phytohemagglutinin (Difco) or medium alone was added and cultures incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 hours. Two µCi of tritiated thymidine (6.7 Ci/mmole; New England Nuclear, Boston, MA) was added for the last 24 hours of culture. Cells were subsequently harvested with an automated cell harvester (Microbiological Associates, Walkersville, MD) onto glass fiber filters. The filters containing radioactive material were placed in 7 ml glass scintillation vials containing 4 mls of Aquasol scintillation fluid (New England Nuclear, Boston, MA) and the vials shaken vigorously. The vials were then counted for tritiated thymidine content using a LKB 1215 Rackbeta liquid scintillation counter (Wallac Oy Co., Turku, Finland). The PHA-SI is the ratio of the mean tritiated thymidine incorporated in stimulated cultures (DPM) to the mean tritiated thymidine incorporated in non-stimulated cultures (DPM).

18. Becton-Dickinson, Monoclonal Antibody Source Book, section 2.1 1981.

One million cells in 50 µl of media (RPMI 1640 containing 0.1% sodium azide and 5% heat inactivated fetal calf

serum) were combined with 50 µl of fluoroscein isothiocycanate (FITC) conjugated antibody. For T-lymphocytes, the antibody used was monoclonally derived anti-Thy1.2 (Becton-Dickinson, Mountain View, CA; Lot No. C0805). For B-lymphocytes, antibody used was goat derived antimouse IgM (Tago, Inc., Burlingame, CA, Lot No. 50-5001). Both antibodies were diluted 1/50 in medium, and centrifuged at 100,000 xG for 30 minutes prior to use. Cells mixed with antibody were incubated on ice for 45 minutes and then washed 2X with medium. The washed pellet was then resuspended in 25 µl of medium. A cover smear was prepared of this suspension and the smear then enumerated for Thy1.2$^+$ or IgM$^+$ cells by immunofluorescent microscopy using a Zeiss microscope with attached UV source and appropriate filters. Between 200-400 cells per smear were counted. Preliminary experiments using FITC conjugated $F(ab')_2$ fragment of these antibodies revealed that the $F(ab')_2$ fragment or the whole antibody yielded similar enumeration results. Therefore, T and B lymphocyte enumeration of cell suspensions in these studies were performed with whole antibody.

19. Animals were exposed in whole body, dynamic inhalation chambers of 0.25 m^3, 1.0 m^3 or 1.3 m^3 volume. Benzene vapor was generated as described elsewhere (see ref. 10). Briefly, for 300 ppm or 100 ppm exposures liquid benzene was nebulized and the benzene droplets allowed

to evaporate before entry into the exposure chambers. For 30 ppm and 10 ppm exposures benzene was vaporized by gently flowing air across the surface of liquid benzene. The benzene-laden air was then metered into the exposure chambers. Benzene concentrations within the chambers were monitored at 0.5 hour intervals during the exposures by means of a MIRAN 1A infrared spectrophotometer (Foxboro Analytical, Norwalk, CT) at a wavelength of 9.8 μ. The same exposure regimes were used for benzene-treated and air control mice except that control mice were exposed to filtered, conditioned air only.

20. Blood cell counts were determined on a Coulter Model ZB-I cell counter (Coulter Electronics, Inc., Hileah, FL). Differential white cell counts were determined by counting 100 white cells from blood smears stained with Wright-Giemsa.

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.

Figure Legends

Figure 1: Six Day Dose/Response Effect of Inhaled Benzene on Peripheral Lymphocytes and Red Blood Cells (RBC)

Figure 2: Six Day Dose/Response Effect of Inhaled Benzene on Frequency of Femoral B-Lymphocyte Colony Forming Cells (B-CFCs) and on the Numbers of Femoral B-Lymphocytes ($sIgM^+$ cells)

Figure 3: Six 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)

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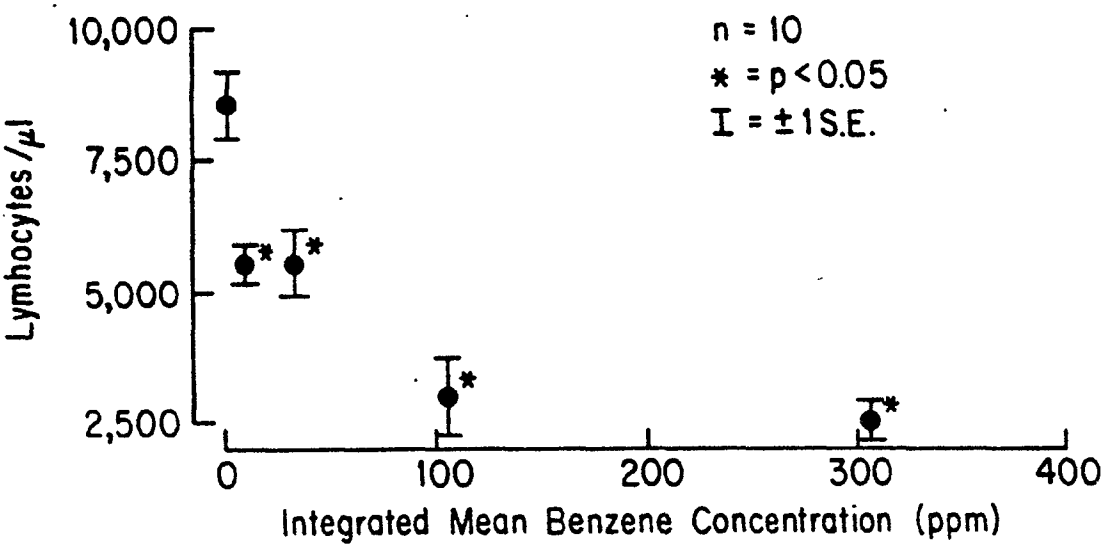
FIGURE 1


10,000
7,500
5,000
2,500
Lymhocytes /μl
n = 10
* = p<0.05
I = ±1S.E.
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)

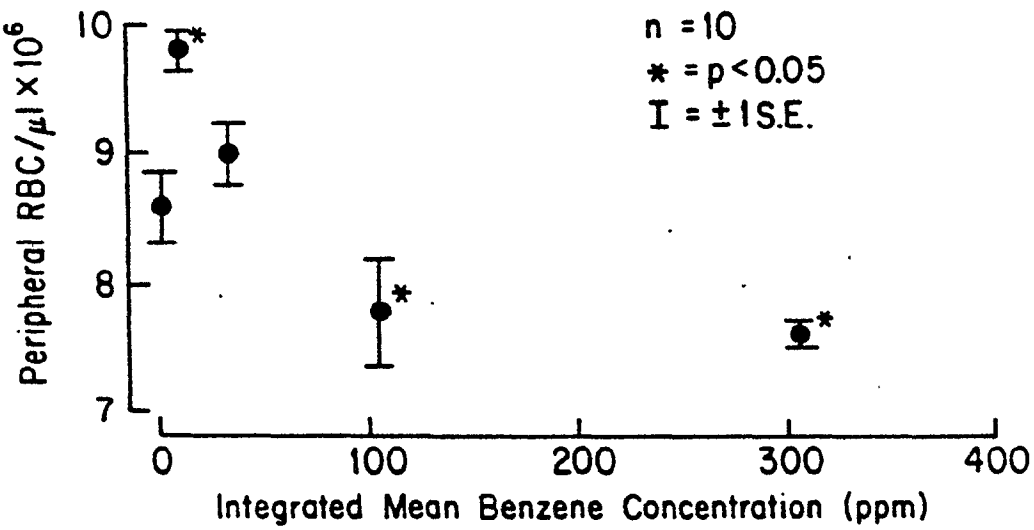
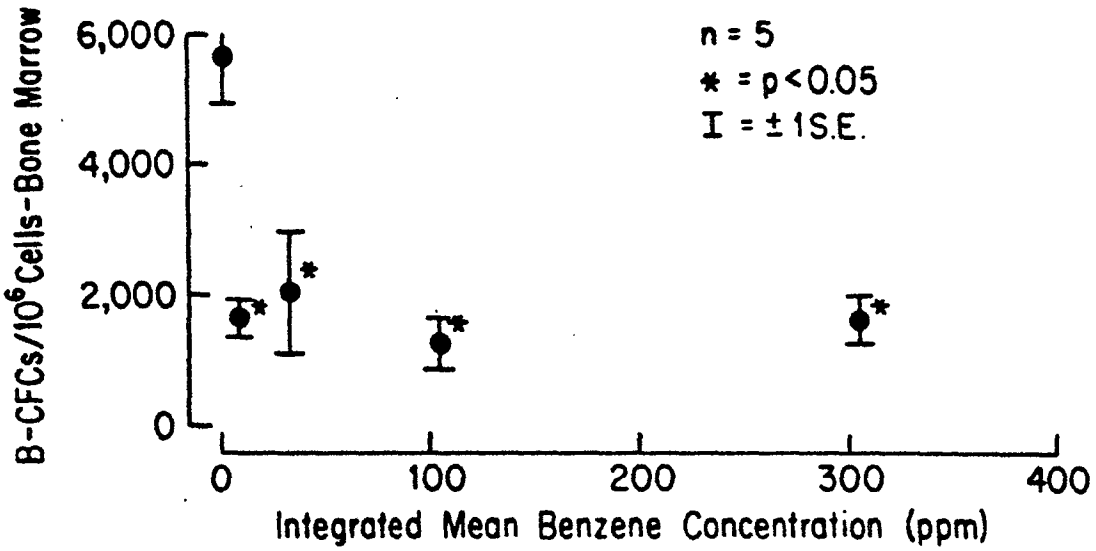

10
9
8
7
Peripheral RBC/μl x 10^6
n = 10
* = p<0.05
I = ±1S.E.
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)

FIGURE 2


B-CFCs/10^6Cells-Bone Marrow
6,000
4,000
2,000
0
n = 5
* = p<0.05
I = ±1S.E.
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)

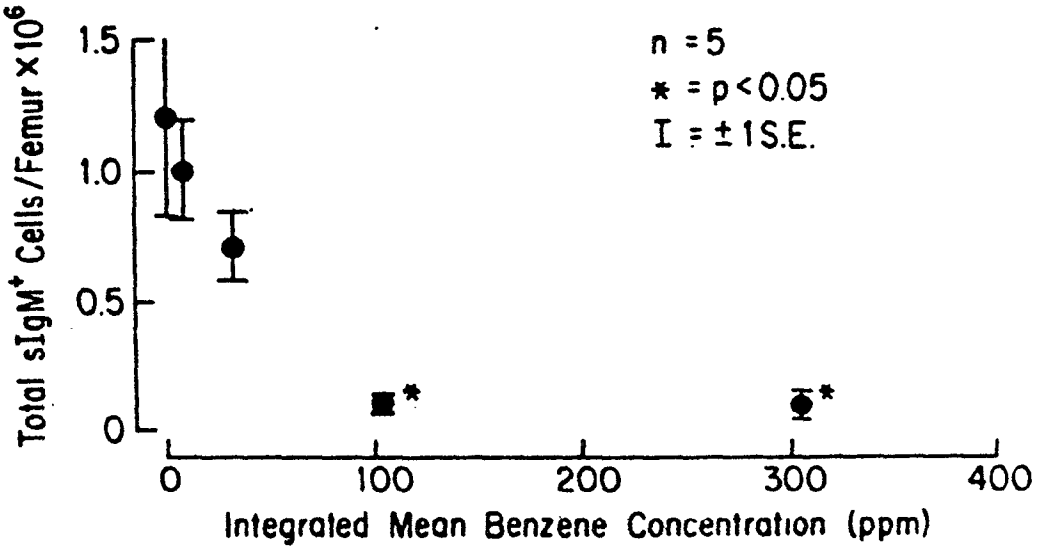
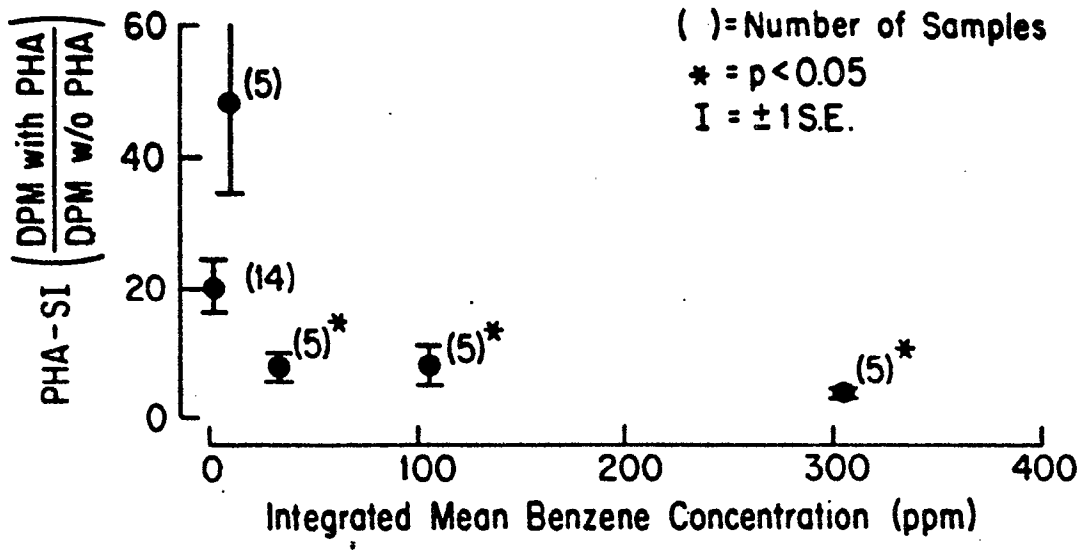
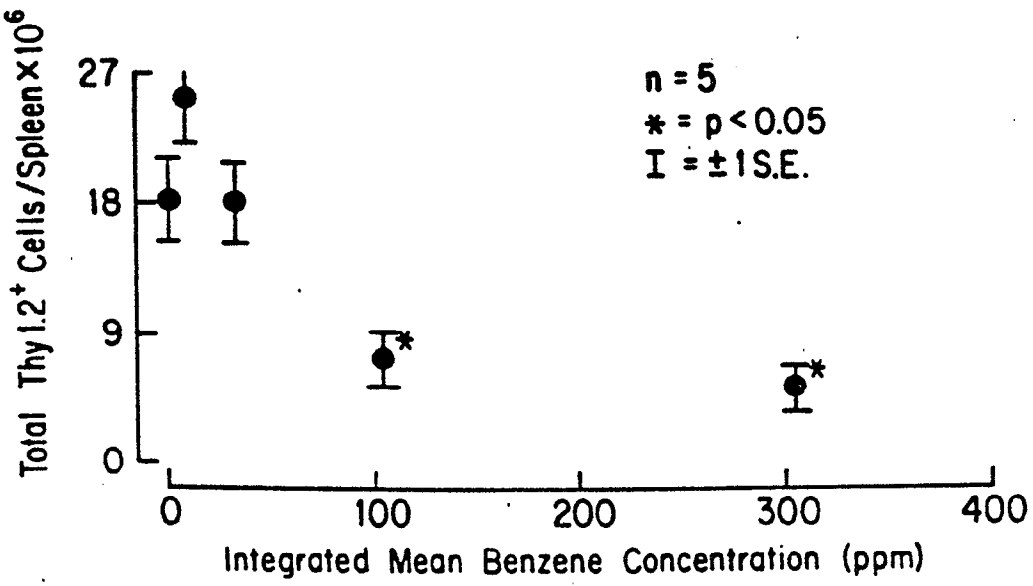

Total sIgM+ Cells/Femur X10^6
1.5
1.0
0.5
0
n = 5
* = p<0.05
I = ±1S.E.
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)

FIGURE 3


PHA-SI (DPM with PHA / DPM w/o PHA)
60
40
20
0
(5)
(14)
(5)*
(5)*
(5)*
( )=Number of Samples
* = p<0.05
I = ±1S.E.
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)


Total Thy 1.2+ Cells/Spleen X 10^6
27
18
9
0
n = 5
* = p<0.05
I = ±1S.E.
*
*
0
100
200
300
400
Integrated Mean Benzene Concentration (ppm)