BENZENE–SPECIFIC INCREASE IN LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY IN RATS EXPOSED TO VAPORS OF VARIOUS ORGANIC SOLVENTS

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female Wistar rats were exposed to various solvent vapors 8 h/d for 7 d. The leukocyte suspension and serum were prepared from peripheral blood and utilized for the determination of alkaline phosphatase (AP) activity with disodium phenyl phosphate as a substrate [leukocyte AP (LAP) and serum AP (SAP) assay]. While the exposure to benzene at 20 or 50 ppm did not cause significant changes in LAP activity, the exposure at 700 and 300 pprn resulted in a dose-dependent increase of LAP activity up to more than 700% over the control. No further increase was observed at 1000 or 3000 ppm. Similar exposure at 300 ppm to either toluene, m-xylene, n-hexane, trichloroethylene, methyl ethyl ketone, ethyl acetate, or methyl alcohol did not induce any changes in LAP activity. Thus, the increase in LAP activity was considered to be specific to benzene exposure. When the animals were exposed to toluene (300 ppm) in combination with benzene (300 ppm), not only was the benzene-induced leukopenia alleviated as previously reported, but the benzene-induced increase in LAP activity was no longer observed. The parallel inhibitory effects of toluene on benzene-induced increase in LAP and leukopenia suggest that a relation may exist between increase in LAP activity and leukopenia. No changes in SAP activities were observed in the rats under the exposure conditions examined.

INTRODUCTION

The validity of the increase in activity of alkaline phosphatase (EC 3.1.3.1) in leukocytes (abbreviated as LAP) as an early indicator of benzene poisoning has been discussed by several authors (Girard et al., 1970; Moszczynski, 1980; Yin et al., 1982; Micu et al., 1985; Moszczynski and Lisiewicz, 1985). The changes in LAP activity were reported both in men occupationally exposed to benzene up to 100 ppm (e.g., Moszczynski, 1980; Yin et al., 1982) and in experimental animals, e.g.,

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in rats after exposures to benzene at 4580 ppm, 4 h/d, 6 d/wk for 3 mo (Yin et al., 1982). The possible association of such changes in LAP with myelotoxicity of benzene was also discussed (Duprat and Gradiski, 1978; Starek et al., 1978). While benzene is still in use as an unmixed solvent on rare occasions (Inoue et al., 1986), it is more often found in solvent products in combination with other organic solvents (Inoue et al., 1983; Kumai et al., 1983; Ikeda et al., 1984; Ikeda and Kasahara, 1986). Accordingly, it was considered important to investigate whether the increase in LAP activity is specific and dose-dependent to benzene or whether exposure to other solvents also results in a similar change in the enzyme activity.

The present investigation was initiated to examine the solvent specificity of changes in LAP activity by exposures of rats to the vapors of various solvents, including benzene. The effect of toluene coexposure on benzene-induced change in LAP activity was also studied.

MATERIALS AND METHODS

Exposure of Animals to Solvent Vapors

Female Wistar rats weighing 140–150 g were used. Dynamic exposure chambers with an automatic vapor concentration control system utilizing a flame ionization detector (FID) gas chromatograph as a detector (Koizumi and Ikeda, 1981) were employed for exposures to solvent vapors. Unless otherwise specified, the animals (5–7 rats per group) were exposed to various solvent vapors at 300 ppm, 8 h/d, for 7 d. Performance of the exposure system was such that the mean of the observed concentrations (measured once in every 25 min) was within the 98–101% range of the target concentration and the coefficient of variation was less than 4% (less than 10% at 20 and 50 ppm benzene) as previously reported (Kumai et al., 1984).

Preparation of Leukocyte Suspension and Enzyme Assay

Blood was sampled from the aorta on the morning of d 8. Isolation of leukocytes was conducted according to the technique of Skoog and Beck (1956), with further modifications as follows. An aliquot, 1 ml, of heparinized rat blood was gently mixed with 2 ml of 2.5% dextran in saline (i.e., 0.85% NaCl) in a test tube. The tube was placed in a horizontal shaker at an angle of 25° to the level, shaken at a rate of 132 times/min for 10 min, and then kept in a vertical position for 15 min at room temperature. The semitransparent upper layer (2 ml) was transferred to a 10-ml graded conical centrifuge tube and diluted with 4 ml saline. The tube was spun at 2500 rpm (–1200 x g) for 10 min. The upper portion (5 ml) was discarded. After the remaining 1-ml portion was diluted with additional 4 ml saline, the tube was spun at 2000 rpm (–800 x g) for 8 min. Most of the upper portion was removed and the

0.5-ml portion remaining at the bottom was gently stirred to suspend the precipitates. The suspension (containing about 1 x 10^7 leukocytes/ml) was utilized for leukocyte counting and enzyme assay.

For the assay of LAP and serum alkaline phosphatase (SAP), a commercially available kit by the phenyl phosphate method [Alkaline PhosphaK-test, Wako Pure Chemicals, Osaka, Japan; prepared after Hansen's modification (1966) of the method of Kind and King (1954)] was employed for routine studies, and the β-glycerophosphate method (Valentine and Beck, 1951) as modified by Yin et al. (1982) was used for comparison. In the former assay, 2 ml of 6 m*M* disodium phenyl phosphate in 50 m*M* carbonate buffer (pH 10) was incubated with 0.1 ml of the leukocyte suspension (or 50 μl serum) at 37°C for 15 min, and the amount of phenol formed was measured at 500 nm after a color-forming reaction with 4-aminoantipyrine and potassium ferricyanate. In the β-glycerophosphate method, a mixture of 4.5 ml of 26 m*M* β-glycerophosphate in 25 m*M* carbonate buffer (pH 9.9), 0.25 ml of 2% saponin in saline, and 0.1 ml of 50 m*M* $MgCl_2$ was incubated with 0.15 ml of the leukocyte suspension at 37°C for 60 min. The phosphate liberated was treated with aminonaphthol sulfonic acid and ammonium molybdate as originally described (Valentine and Beck, 1951), and the resulting color was measured at 660 nm. In both assays, one unit of LAP activity was defined as the activity to hydrolyze 1 nmol substrate/10^6 leukocytes (or ml serum) · min at 37°C.

Leukocyte Counting

A Coulter counter (model D) was used for counting leukocytes in whole blood samples and in suspension preparations.

Reagents

Dextran (molecular weight 17,000–20,000) and sodium β-glycerophosphate were purchased from Nakarai Chemicals, Kyoto, Japan, and Merck, Darmstadt, Federal Republic of Germany, respectively. Solvents (reagent grade, ≥99% pure) were obtained from Wako Pure Chemicals, Osaka, Japan. Trichloroethylene was stabilized by the addition of undisclosed inhibitors.

Statistical Evaluation

For the evaluation, Student's t-test was employed.

RESULTS

Selection of the Exposure Condition

In a preliminary study, rats were exposed to benzene at 0, 20, 50, 100, 300, 1000, and 3000 ppm, 8 h/d for 7 or 14 d. A nonexposed control group was set in addition to the 0-ppm (sham-exposed) group. After

the exposure for 7 d, LAP increased dose-dependently at 100 and 300 ppm, and the change was in essence inversely related to the decrease in leukocyte counts and in body weight gain (Fig. 1), while the exposure at 20 or 50 pprn was ineffective. Exposure at higher concentrations (i.e., at 1000 and 3000 ppm) for the same (i.e., 7 d) or a longer (i.e., 14 d) period did not cause any additional elevation in **LAP. SAP** had a tendency to be lower in the exposed groups than in the 0-ppm group, but the reduction was statistically insignificant ($p > 0.10$). There was no significant difference ($p > 0.10$) in both LAP and **SAP** between the nonexposed group and the sham-exposed group after 7 and 14 d of exposure. After 1 wk of exposure, about a 10% gain in body weight was observed in the 2 control groups, while there was only a 2% gain in the 300-ppm group, a 10% loss in the 1000-ppm group, and a 19% loss in the 3000-ppm group. The mean leukocyte counts in the peripheral blood of the rats in the 300-, 1000-, and 3000-ppm group were 48%, 43%, and *28%* of the sham-exposed group, respectively. Accordingly, the exposure condition was set at 300 ppm, 8 h/d for 7 d in further studies.

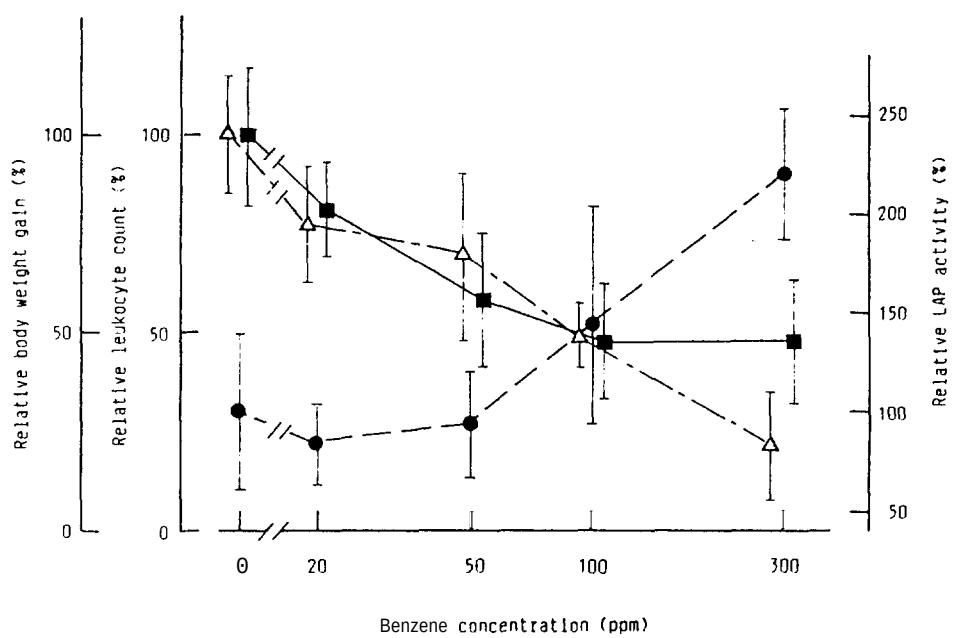

FIGURE 1. LAP activities, leukocyte counts, and body weight gain as a function of benzene concentration. Kats *(5–6* animals per group) were exposed to benzene at 20, 50, 100, and 300 ppm, 8 h/d for 7 d. On d 8, they were examined for the activity of alkaline phosphatase in leukocytes (LAP) and leukocyte counts in the peripheral blood. LAP activity, leukocyte counts, and body weight gain (during the 7-d exposure period) are expressed relative to the values in the control group, taking the latter values as 100. Symbols (circles for LAP, squares for leukocyte counts, and triangles for body weight gain) indicate means, and brackets show standard deviations.

Changes in LAP and SAP in the Rats Exposed to Various Solvents

Groups of rats were exposed to either toluene, rn-xylene, *n*-hexane, trichloroethylene, methyl ethyl ketone, ethyl acetate, or methyl alcohol at 300 ppm, 8 h/d for 7 d. The rats exposed to benzene at the same concentration served as a positive control. The results are summarized in Table I. It was evident that all of the solvents studied except benzene alone failed to induce significant changes in either LAP or SAP activities. When the rats were exposed to toluene (300 ppm) in combination with benzene (300 ppm), the LAP-inducing effect of benzene was no longer detected (experiment 3 in Table 1). Under such exposure condition, the reduction in leukocyte counts was significantly ($p < 0.01$) less in the benzene plus toluene group (77.9% of the control group) than in the benzene group (56.6%), indicating that benzene-in-

TABLE 1. LAP and SAP Levels in Rats[a] Exposed to Various Solvent Vapors

Solvents	LAP[b]	SAP[c]
Experiment 1		
Nonexposed	3.71 ± 0.87 **(6)**	362.9 ± 43.1 (6)
Benzene	5.04 ± 0.67 (7)[e]	341.4 ± 55.0 (6)
Toluene	3.49 ± 1.12 (6)	363.9 ± 39.0 (6)
rn-Xylene	3.15 ± 1.70 (6)	310.1 ■23.7 (6)[f]
n-Hexane	3.30 ± 1.23 (6)	379.7 ± 28.8 (6)
Experiment 2		
Nonexposed	3.22 ± 1.26 (7)	343.7 ± 59.4 (7)
Benzene	7.41 ± 2.20 (7)[e]	419.0 ± 38.5 (7)[f]
Trichloroethylene	3.81 ± 2.33 (7)	397.2 ± 52.3 (7)
Methyl ethyl ketone	4.06 ± 1.45 **(6)**	333.4 ± 40.2 (7)
Ethyl acetate	3.08 ± 0.65 (6)	347.6 ± 32.7 (7)
Experiment 3		
Nonexposed	3.67 ± 1.91 (6)	425.4 ± 49.7 (6)
Sham-exposed	3.06 ± 1.18 (6)	458.5 ± 37.5 (6)
Methyl alcohol	4.39 ± 2.76 (6)	482.6 ± 70.0 (6)
Benzene	'10.58 ± 6.39 (7)[f]	4'37.9 ± 99.8 (7)
Benzene plus toluene[d]	4.77 ± 2.05 (7)[g]	442.0 + 36.1 (7)

[a] Rats were exposed to each solvent vapor at 300 ppm, 8 h/d ior 7 d and subjected to the enzyme assay on d 8. **The** values in the table arc' mean ± SD, followed by the number of animals in parentheses.

[b] Leukocyte alkaline phosphatase: nmol phenol formed/10^6 leukocytes. min.

[c] Serum alkaline phosphatase: nmol phenol iormed/ml serum · min.

[d] Benzene at 300 ppm plus toluene at 300 ppm.

[e] The difference irom the control (the nonexposed) is statistically significant at $p < 0.01$.

[f] Difference trom the control statistically significant at $p < 0.05$.

[g] Difference between the benzene group and the benzene plus toluene group is statistically significant ($p < 0.05$).

duced leukopenia was alleviated by the co-exposed toluene, as previously reported (Ikeda and Hirayama, 1979). Body weight gain was also more in the benzene plus toluene group (86.3% of the control group) than in the benzene group (27.4%).

Comparison Between the Phenyl Phosphate Assay and the β-Glycerophosphate Assay

As the β-glycerophosphate method was employed in the previous study on humans and animals (Yin et al., 1982), it was considered necessary for comparison of the results to investigate the relation in the results of enzyme assays between the β-glycerophosphate method and the hand- and time-saving phenyl phosphate method. When 48 leukocyte suspensions prepared from the blood of benzene-exposed and nonexposed rats were subjected to the assays by the two methods, there was a linear correlation (Fig. 2) between the two sets of the assay results with a correlation coefficient of 0.860 ($p < 0.01$), and the regression line cut the vertical axis at the point close to the origin, indicating that the two assay results are convertible from one to the other. It is also clear from the slope of the regression line that there is a small difference in the affinity of the enzyme to the two substrates; a slightly higher activity was observed with β-glycerophosphate as the substrate than with phenyl phosphate.

DISCUSSION

The present study clearly demonstrated that the exposure of rats to benzene at 300 ppm (957 mg/m³), 8 h/d for 7 d, is associated with a statistically significant ($p < 0.01$) increase in alkaline phosphatase activity in leukocytes (Fig. 1, Table I). Although the increase after exposure for 7 d at 100 ppm was not statistically significant ($p > 0.10$; Fig. 1), it is quite possible that exposure to concentrations lower than 300 pprn for a period longer than 7 d might induce a significant increase in LAP activity.

In 1970, Girard et al. stained granulocytes in the blood smears of workers [occupationally exposed to benzene at less than 25 ppm (80 mg/m³) plus toluene at less than 200 ppm (750 mg/m³)] with fast garnet GBC salt and observed that LAP activity was lower in the workers than in the control subjects. Subsequently, Moszczynski et al. (1978) stained the neutrophils in the blood of the rats exposed to benzene at 1200 mg/m³ (376 ppm), 6 h/d, 6 d/wk for up to 32 wk and found that the alkaline phosphatase activity (as quantified by scoring) in the neutrophils was lower in the benzene-exposed animals than in the sham-exposed animals. In similar cytochemical studies in men, Moszczynski (1980) examined neutrophils of workers exposed to benzene, toluene, xylene, and n-butyl alcohol [the concentrations in the recent years being 12, 24, 130, and 119 mg/m³ (4, 6, 30, and 39 ppm) respectively, but

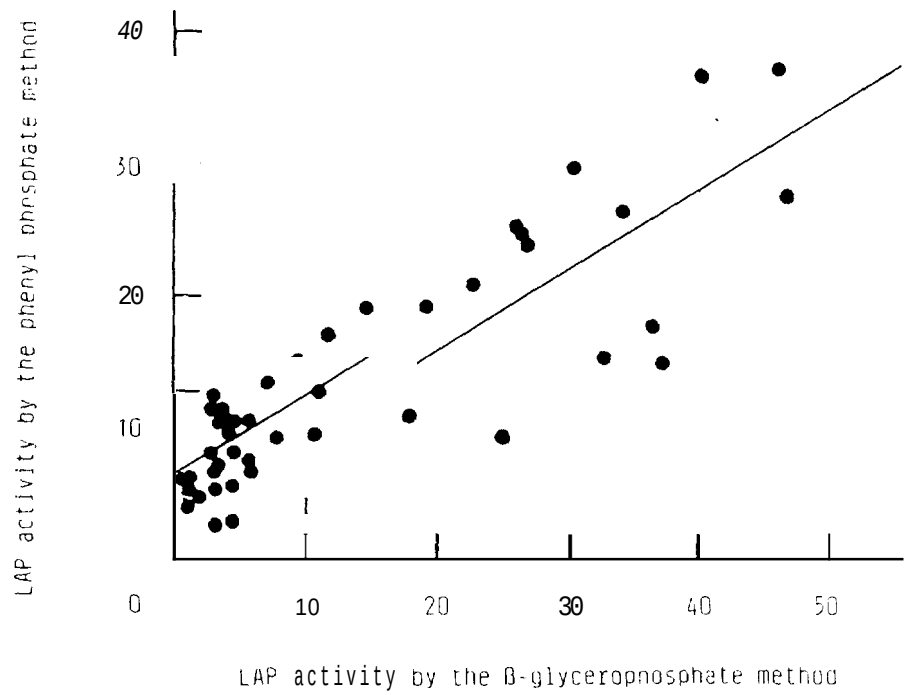

FIGURE 2. Correlation of LAP activities as measured by the phenyl phosphate method and the β-glycerophosphate method. In total, 48 leukocyte suspension samples obtained from benzene-exposed and nonexposed rats were studied. Each dot represents one pair of assay results. The line in the figure is a calculated regression line of $y = 4.03 + 0.601\,x$, where y and x are the activity (units of nanomoles substrate hydrolyzed per 10^6 leukocytes per minute) assayed by the phenyl phosphate method and the β-glycerophosphate method, respectively. The correlation coefficient r is 0.860 ($p < 0.01$; $n = 48$).

higher in the past] and observed that LAP was significantly lower in the workers than in controls. Moszczynski and Lisiewicz (1985) conducted a cytochemical study of the activity of nonspecific esterase (NE) in lymphocytes of workers exposed to a mixture of benzene [up to 370 mg/m^3 (116 ppm)], toluene [up to 580 mg/m^3 (155 ppm)], and xylene [up to 560 mg/m^3 (129 ppm)]. It was found that counts of lymphocytes with intact NE-positive lysosomal granules decreased and counts of lymphocytes with damaged NE-positive lysosomes increased in workers with service history of 55–122 mo.

In a study utilizing a modification of the β-glycerophosphate method (Valentine and Beck, 1951), however, Yin et al. (1982) observed an increase in LAP in rats exposed to benzene at 14,600 mg/m^3 (4580 ppm), 4 h/d for up to 20 wk. The increase was detectable as early as wk 3 of exposure and was more prominent in females than in males. In men (Yin et al., 1982), LAP was three times higher in workers with occupational exposure to benzene at about 100 mg/m^3 (~31 ppm) as com-

pared to control subjects. As the results by the phenyl phosphate method and the β-glycerophosphate method are interconvertible, as shown in Fig. 2, it is reasonable to conclude that the findings in the present study with the phenyl phosphate method were in agreement with the observation by Yin et al. (1982). Thus, it may be deduced that the benzene-induced increase in LAP is detectable when the enzymological method is employed. Cytochemical study gives reverse results, with the one exception of Starek et al. (1978), who reported in a cytochemical study an increase in LAP in rats exposed to benzene at 27,000 mg/m^3 (−8460 ppm), 6 h/d for 10 d.

The exposure-related elevation in LAP activity is specific to benzene and not induced by any other aromatics (e.g., toluene and rn-xylene), a six-carbon aliphatic (n-hexane), or other popular solvents such as methyl ethyl ketone, ethyl acetate, trichloroethylene, or methyl alcohol. Thus, it is possible that the increase in LAP observed among the subjects in solvent workplaces is solely attributable to benzene exposure. In contrast, it was demonstrated in the current study that co-exposure to toluene may prevent the benzene-induced LAP increase and leukopenia. According to Yin et al. (1980), alkaline phosphatase activity increased (by 20 and 42% on cell number basis) in the bone marrow of rats exposed to benzene at 11,400 mg/m^3 (3570 ppm), 4 h/d, 6 d/wk for either 1 or 6 mo, respectively, which suggests that changes of AP in leukocytes are probably initiated while the cells are in the bone marrow. Some authors have discussed the possibility that the benzene-induced leukopenia may be associated with changes in enzyme activity in leukocytes (e.g., Duprat and Gradiski, 1978; Starek et al., 1978). There is, however, no definitive evidence yet to show direct casual relationship between increased LAP activity and leukopenia.

No significant changes were observed in the current study in serum alkaline phosphatase after exposure to benzene and other solvents (Table 1). Such negative findings are in accordance with the observations *of* Rao and Pandya (1978) and Kala et al. (1978), in which serum alkaline phosphatase activity did not show remarkable changes in rats given either a single oral dose of 1.5 ml (1.32 g) benzene/kg body weight or 7 ip doses of 2 ml (1.78 g) benzene/kg · d, despite a significant ($p < 0.05$) increase of AP activity in the liver in both studies.

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