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Genotoxic effects of a sub-acute low-level inhalation exposure to a mixture of carcinogenic chemicals

William W. Au [1], Jonathan B. Ward Jr. [1], V.M. Sadagopa Ramanujam [1], Barbara L. Harper [1], Mary Treinen Moslen [2] and Marvin S. Legator [1]

[1] *Division of Environmental Toxicology, Department of Preventive Medicine and Community Health, and* [2] *Department of Pathology, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)*

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Summary

A study was conducted using a combined testing protocol (CTP), to determine whether short-term biological end-points, singly or in combination, are sufficiently sensitive to identify damage induced by exposure to ambient levels of industrial chemicals. A small-scale inhalation set-up which is both economical and easy to assemble was designed. Mice were exposed to 4 concentrations of a custom-blend mixture of benzene, chloroprene, epichlorohydrin and xylene in a ratio of 2 : 2 : 1 : 2, respectively. The concentrations for benzene, chloroprene and xylene were 0, 0.1, 1.0 and 10 ppm each. Concentrations for epichlorohydrin were half those for the other components. Groups of 22 male and 22 female mice were exposed to each concentration of the mixture for 3 and 6 weeks. Selected biological end-points including urine mutagenesis, bone marrow cell aberrations and micronuclei, spleen lymphocyte aberrations and liver enzyme induction were monitored. The spleen lymphocyte aberrations and liver enzyme induction were the most sensitive end-points. The lymphocytes showed a significant induction of chromosome aberrations from exposure for 3 weeks to all 3 concentrations of the mixtures. After 6 weeks of exposure, significant induction of aberrations was observed after exposure to low and medium concentrations but not to the high concentration. This lack of response at the high concentration after 6 weeks exposure, appeared to correlate with a significant induction of glutathione *S*-transferase in the liver. Since this enzyme is known to detoxify 3 of the 4 chemicals in our mixture, it may indicate a detoxification mechanism after enzyme induction.

The present study indicates that the CTP is sufficiently sensitive to identify toxicological effects after exposure to ambient levels of a gas mixture.

Correspondence: Dr. Marvin S. Legator, Division of Environmental Toxicology, Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.).

Human exposure to potentially hazardous chemicals often occurs by inhalation. A number of investigations have been conducted to study the genotoxic effects in animals after inhalation ex-

posure to environmental mutagens/carcinogens. However, most studies have been conducted with single compounds in concentrations significantly higher than those normally encountered by human populations. Although such an approach maximizes the chance of detecting induced damage and of understanding the mechanisms for damage, it is difficult to extrapolate the findings to the population as a whole for risk assessment. Furthermore, the biological consequences from such artificially high-dose acute exposures may be significantly different from the exposure conditions encountered by a human population. For example, Schmahl and Habs (1980) reported that the administered dose of diethylnitrosamine (DEN) influenced the organ specificity of its carcinogenic effect. With a single high dose, DEN induced carcinomas of the kidney in treated rats. With smaller dose levels and multiple exposures, malignant tumors of the liver were induced. Carcinomas of the esophagus were induced when even smaller doses were administered.

The determination of carcinogenic–mutagenic effects of chemicals and chemical mixtures at low levels, consistent with possibly community exposure, is a critical need in toxicology. A careful examination of the various techniques available for mutagenic studies in animal models suggested that many of these techniques can be incorporated into a single testing protocol, the combined testing protocol (Connor et al., 1979; Legator and Harper, 1982; Legator et al., 1986). In our protocol, the "CTP", an in-depth activity profile can be obtained in the same experiment. In addition, the major advantages of a multi-endpoint approach like ours are that it increases the likelihood of identifying the genotoxic activity in target tissues (e.g. lung macrophage, bone marrow, liver and germinal cells) and eliminates the variability among separately conducted experiments. In the present study, selected assays were conducted to evaluate genotoxic effects of low levels of carcinogenic mixtures in mice exposed by the inhalation route. This is one of a series of reports to determine the feasibility of the "CTP" for eventual use in in situ field monitoring.

A small-scale inhalation set-up which is both economical and easy to assemble was designed for our study. 3 industrial chemicals were selected from the priority air pollutant list of the Texas Air Control Board (TACB). All of these chemicals have been demonstrated individually to possess genotoxic and carcinogenic activities (IARC, 1982). These were benzene (BZ), epichlorhydrin (ECH), and chloroprene (CP). In addition, xylene was included since it was a stabilizer for chloroprene.

BZ is the most extensively studied chemical of the four. The toxicity of benzene has been characterized (reviewed by Snyder et al., 1982). It has been documented to be an animal and human carcinogen (e.g. Maltoni et al., 1983; Aksoy, 1985). In genetic toxicology studies, BZ has been reported to induce chromosome aberrations in bone marrow cells after acute oral exposure (Meyne and Legator, 1980; Gad-El-Karim et al., 1984) and in lymphocytes of chronically exposed workers (Tough et al., 1970; Forni et al., 1971; Watanabe et al., 1980; Sarto et al, 1984). With exposure concentrations similar to that of humans (1, 10 and 30 ppm of benzene), Cortina et al. (1984) did not observe any significant increase of chromosome aberrations in bone marrow cells of exposed mice and rats. Significant increases were observed only after exposure to high concentrations (300 ppm) of BZ (Cortina et al., 1984). However, increased micronuclei were observed in circulating erythrocytes of mice after chronic exposure (Choy et al., 1984; Barale et al., 1985) and in bone marrow cells in mice after exposure to as low as 10 ppm for 6 h (Erexson et al., 1986). BZ has also been shown to cause sister-chromatid exchanges in cells in vivo (Tice et al., 1980; Erexson et al., 1986) and in vitro (Morimoto et al., 1983; Erexson et al., 1985). In spite of its well established genotoxic effects (reviewed by Dean, 1985), BZ has not been shown to cause specific locus mutations (Dean, 1985).

Studies of ECH have shown that it can cause chromosome aberrations in exposed workers (Sram et al., 1983) and can induce cancer in animals after long-term exposure (Sram et al., 1981). Although ECH has been demonstrated to be carcinogenic in animals, its carcinogenicity in humans is still unknown (IARC, 1976; Sram et al., 1981).

CP has been demonstrated to be a weak mutagen and clastogen (IARC, 1979). There is some

evidence of CP-induced chromosome aberrations in exposed workers (Sanotskii, 1976). The carcinogenic effects of CP in exposed workers and in experimental animals have not yet been adequately demonstrated (Pell, 1978; IARC, 1979; Poncelet et al., 1984).

XY is a neurotoxic chemical. It does not induce mutation in the Ames test (Bos et al., 1981) nor chromosome aberrations in human lymphocytes in vitro (Gerner-Smidt and Friedrich, 1978; reviewed by Dean, 1985). Its carcinogenicity is unknown.

Material and methods

Overview

Mice were exposed by inhalation to 3 concentrations of a mixture of the 4 chemicals (benzene, chloroprene, xylene and epichlorohydrin) and to air only. The concentrations of each component were 10, 1.0 and 0.1 ppm except that the concentrations of epichlorohydrin were one-half these values. For exposure, the mice were housed in 3 sets of 4 inhalation chambers with 11 animals in each chamber. 2 chambers in each set housed male animals and 2 housed female animals. The durations of exposure were 3 and 6 weeks.

Inhalation apparatus

The inhalation apparatus was assembled from major components available from commercial suppliers. Animal exposure chambers were purchased from Plastic Manufacturing and Supply Co., Lansing, MI. They were constructed of ABS plastic conical top and bottom sections with a cylindrical clear acrylic center section and a floor made from plastic cube louver. Water was dispended from 100-ml glass dispensers and food from glass dishes. The chambers were sealed with circular bands of rubber. All components were connected by 1/4 in. TFE teflon tubing (Supelco, Inc., Houston, TX) and 1/4 in. Swagelok fittings made of 316 stainless steel or teflon. Mass flow controllers (Tylan Industries, carson, CA) were used for air and test gas metering. Glass mixing chambers (Tracor Inc., Austin, TX) were used to mix the gas and air streams and to distribute the final mixture to the chambers. Custom-blended gas mixtures of benzene, chloroprene in xylenes, and epichlorohydrin were supplied by Big Three Industries, LaPort, TX. All the stainless steel swagelok fittings, Teflon Unions, toggle-operated forged body shut-off valves (stainless steel), and forged body regulating valves (stainless steel) were purchased from Thomas A. Read and Company, Texas City, TX.

Animal use and care

Male and female CD-1 Swiss mice were purchased from Charles River Laboratories, Wilmington, MA. The first shipment of mice weighed 19–21 g each and they were born on the same day. The second shipment of mice weighed 22–24 g and they were born on the same day as well. All mice were inspected by the supplier and certified to be negative serologically for 16 common viruses, negative for 6 bacterial and for 2 parasite infections. Mice were acclimatized in standard animal housing conditions (tapwater, Purine Lab Chow, dried corn cob bedding and 12 h light–dark cycle) for at least 1 week before initiation of inhalation exposure. 22 mice of each sex of between 8 and 10 weeks of age were exposed per dose of mixture. Mice were exposed for a duration of 3 or 6 weeks. During the exposure period, the light cycle was reduced from 12 to 9 h/day. Exposure was continuous except for 2 h daily when cages were disassembled for cleaning, and for one 24-h period towards the end of exposure when urine was collected. Each mouse was given an ear-notch code to identify which chamber the mouse belonged in. The mice were not identified individually. Each animal was weighed weekly so that an average weight per cage was obtained each week. During the daily cleaning, food and water were replenished and the previous day's food and water consumption were recorded.

Inhalation exposure

The inhalation exposures were achieved by diluting the stock gas mixtures with compressed air. The diluted gas mixtures were delivered to the 4 exposure chambers for each dose level.

Compressed air was purified by passage through a filter and a heatless air dryer (Vacuum Technology, Austin, TX) to remove particulates, moisture and organic contaminants. The air stream was split into 3 channels and the flow rate was regulated for each channel at 6 l/min by the mass flow

controllers for delivery to the mixing chambers for dilution of the test gas.

The stock gas mixture contained 4 components, benzene, chloroprene, xylene, and epichlorohydrin in a ratio of 2:2:2:1. The concentration of epichlorohydrin was reduced because it is more acutely toxic than the other components. Two stock mixture concentrations were supplied: a high concentration in which the nominal gas concentrations were 1000/500 ppm, and a low concentration in which the nominal concentrations were 100/50 ppm. The gas mixtures were metered by the mass flow controllers at flow rates of either 6 ml/min or 60 ml/min. When air was mixed at a flow rate of 6 l/min, dilutions of 1/1000 and 1/100 were made to generate the intended final concentrations. The control chambers received air only at 6 l/min.

The mass flow controllers were calibrated prior to use against soap bubble meters. The flow rates were checked with bubble meters at the chamber inlets once a week and at the flow controller outlets at the end of each exposure period. In the initial calibration process the voltage readings from the flow controllers corresponding to the desired flow rates were determined. The voltage readings were monitored twice daily and adjusted when necessary to remain within 2–5% of the desired value. At the same time, the air and gas pressures were monitored along with the room temperature, operation of the heatless air drier, and status of the cages in use. The humidity of the final gas mixture and of chambers containing animals was determined using an electronic hygrometer (Cole Parmer, model 3309-50).

Because the apparatus could accommodate only 3 exposure conditions at one time, it was necessary to start different exposures at different times. However, arrangements were made so that overlapping concurrent controls were included with all experimental groups except the 6-week medium dose group.

Chemical analysis

The recommended methods of the Occupational Safety and Health Administration (OSHA) were followed for the diluted gas sampling at the chamber inlets and for subsequent gas chromatography (GC) analysis (NIOSH, 1977, 1984). The procedure involves: (a) sampling of organic vapors on charcoal, (b) desorption of the sample from the adsorbent using dichloromethane solvent, (c) analysis of the desorbed sample by gas chromatography, and (d) quantitation of the organic vapors. Sampling was done on the 4 chambers in each group at the same time. Charcoal tubes (ORBO-32, 20/40 mesh, 8 mm OD × 6 mm ID × 10 cm long) were connected to the gas lines at the chamber inlets. Flow through the tubes was measured using soap bubble meters. Total volumes of the gas passed through the charcoal tubes were 100–150 l and 200–300 l for the high and lower gas concentrations, respectively.

The charcoal tube samples were desorbed with dichloromethane and the samples analyzed by GC on a Varian Model 3700 (Varian Instrument Group, Palo Alto, CA) equipped with a flame ionization detector, a Varian CDS 111 Chromatography data system and a bonded methyl silicone fused silica capillary column (30 m × 0.32 mm) coated with 1.0-μm thickness SPB-1 phase (Supelco, Inc.).

The desorption efficiency of dichloromethane with the charcoal tubes was determined for each component of the mixture and the system was calibrated, using standards, for the quantitative determination of each component of the mixture.

Urine collection

Urines were analyzed for the presence of mutagenic metabolites in the Ames/Salmonella assay (Ames et al., 1973). Mouse urines were collected in metabolism cages over a 24-h period between 5 and 3 days before the termination of exposure and pooled according to exposure group. Pooled samples of 30–40 ml were analyzed for pH and specific gravity and were then centrifuged for 20 min at 250 *g* to remove sediment. Filtration through a 0.45-μm Nalgene filter effectively sterilized the urines. A 15.0-ml aliquot of the urine sample was diluted to 50.0 ml with deionized distilled water.

Urines were concentrated using a modification of the method of Durston and Ames (1974). Amberlite XAD-2 resin (20–50 mesh) was prewashed with acetone, chloroform, and deionized distilled water. The resin was packed into a 10 mm × 300 mm glass column to a height of 120 mm. The packed column was washed with 100 ml of de-

ionized distilled water. The diluted urine samples were put on the column and allowed to elute at a rate of 2–3 ml/min. The column was then washed with 1 bed vol. of deionized distilled water, eluted with 40 ml of acetone and allowed to dry. The eluate was collected in 3 fractions, the aqueous urine, the water wash, and the acetone fraction. The acetone fraction was dried to approximately 3.0 ml under a stream of nitrogen at 25 °C and resuspended in approximately 2.0 ml acetone. The above process concentrated the mouse urines approximately 5.0 times.

Urine mutagenesis

The urine concentrates were tested in the Ames/Salmonella assay using tester strains TA98 and TA100. Each concentrate was tested in 2 volumes (50 and 100 μl) in the presence and absence of post-mitochondrial liver fractions (S9). All samples were tested in triplicate. The samples were added to agar plates with Salmonella tester strains for the plate incorporation assay or allowed to interact with bacteria for the preincubation assay, and incubated further for 48 h. The resulting revertant colonies were counted on an Artek colony counter. Criterion for a positive result was an increase in colony count of 2-fold over the count in control plates.

Animal sacrifice

Mice were each injected with 0.15 ml (150 μg) of colchicine in saline intraperitoneally at 4 h before sacrifice (at the same time when exposure was terminated) in order to arrest cells at the metaphase stage of the cell cycle. They were killed by an injection of 0.2 ml of nembutal (50 mg/ml). The eleventh mouse in each cage, which did not receive colchicine or nembutal, was sacrificed by cervical dislocation and used only for histological analysis.

Each animal was weighed. Spleens were aseptically removed for isolation of lymphocytes for culture. Livers were removed next, freed of the gall bladder, rinsed with cold saline and processed immediately for enzyme analysis. Both femurs were removed. One was used for preparation of slides for micronuclei analysis and the other for chromosome aberration studies.

Bone marrow micronuclei

The procedure is the same as that described by Heddle et al. (1984). Briefly, the femur was flushed into fetal calf serum, centrifuged and the cells spread on a slide. The slides were heat-fixed and stained with Wright's and Giemsa strains. A total of 1000 polychromatic erythrocytes per mouse were analyzed for the presence of micronuclei.

Bone marrow aberrations

Marrows from femurs were flushed out using Hanks' Ca^{2+} and Mg^{2+}-free balanced salt solution. The cells were centrifuged, treated with 0.075 M KCl hypotonic solution and fixed 3 times with Carnoy's fixative. Air-dried slides were made, stained with 4% Giemsa solution and analyzed for chromosome aberrations according to standardized procedures (Savage, 1975; Preston et al., 1981). A total of 50 metaphases per mosue were analyzed.

Spleen lymphocyte aberrations

The procedure for this assay is modified from used by Kennett et al. (1978) and Wilmer et al. (1983). The removal of spleen and the isolation of spleen lymphocytes for cultures were handled aseptically. Because of the extensive amount of time required, spleens for this assay were removed from every other male mouse. Each spleen was individually rinsed in RPMI-1640 culture medium (Gibco). Approximately 20 openings were punched into each spleen with a 25-gauge needle and the cells were flushed out in 20 ml of medium by using syringes and needles. The cells were spun down and a cell count was made. Cultures were set up with $0.5 \times 10(6)$ lymphocytes/ml of complete RPMI-1640 medium. The medium contains RPMI-1640 medium, 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 2% phytohemagglutinin, 100 U/ml of penicillin, 100 μg/ml streptomycin and 0.1% heparin. Usually, 2–4 cultures were set up from each mouse in 15-ml conical tubes. The cultures were incubated at 37 °C in humidified 5% CO_2 atmosphere for 48 h. 2 h before harvest, 0.2 ml of colcemid (Gibco, 10 μg/ml) was added to each tube. Two culture tubes from each mouse were combined together, harvested and fixed as described above for bone marrow cells.

Liver enzyme assays

In order to control for circadian variations in liver enzyme activities, all male mice were sacrificed between 9:30 a.m. and 11:30 a.m. and female mice were sacrificied betwen 1:00 p.m. and 3:30 p.m. Livers were rinsed with cold saline, blotted and homogenized in 5 vol. of cold 0.25 M sucrose. The homogenate was centrifuged at 9000 $\times g$ for 8 min twice to remove cell debris and mitochondria. The 9000 $\times g$ supernatant was centrifuged at 100 000 $\times g$ for 1 h to separate microsomes from cytosol. The microsomal pellet was washed by recentrifugation at 100 000 $\times g$ for 1 h in 5 ml of 0.25 M sucrose. The cytosol supernatant was recentrifuged at 100 000 $\times g$ for 1 h to remove residual microsomes. Overlying fat was removed and the cytosol was stored at $-20\,^{\circ}$C.

On the day of sacrifice, microsomal cytochrome P-450 content was determined by the carbon monoxide spectra according to Dallner et al. (1966). Cytosolic glutathione *S*-transferase activities towards the substrates 3,4-dichloronitrobenzene (DCNB) and chloro-2,4-dinitrobenzene (CDNB) were measured at 25 °C in a Beckman DU-2 spectrophotometer according to Habig et al. (1974) and Younes et al. (1980). Protein was measured by the Lowry et al. (1951) method with bovine serum albumin as the standard.

Statistical analysis

Cytogenetic data were compiled into a data base on a microcomputer. Summary statistics from cytogenetic analysis determined for each treatment group were percentage of cells containing aberrant chromosomes and rate of aberrations/100 cells. Statistical analysis to determine the significance of differences between treatment groups were conducted using a contingency table method for percentage of aberrant cells (Whorton, 1985) and non-parametric methods for the aberration rates. The aberration rates were analyzed for significant variation among groups using the Kruskal–Wallis test (single-tailed test). Where significant variation existed a pairwise comparison between the control and combined treatment groups were made using the Mann–Whitney *U* test (two-tailed test). The *p* values were based on the tables assembled by Beyer (1966). Significance of differences in liver enzyme activities between groups was analyzed using Duncan's multiple range test. All statistical procedures were carried out using the Statistical Package for the Social Sciences (SPSS) (Hull and Nie, 1981; Nie et al., 1975) on a Cyber Computer (Control Data Corporation) at the University of Texas Health Education and Research Computer Center, Houston, TX.

Results

Exposure conditions

Both the overall flow rates to the chambers and the final concentrations of the chemical mixture were controlled by closely monitoring and adjusting the voltages on the mass flow controllers. The voltages were checked at least twice daily 7 days a week and adjusted as needed to stay within 2–5% of the values determined to provide the correct flow rate by calibration with soap bubble meters.

The delivered chemical concentrations sampled at the chamber inlets were in close agreement with the planned values with the exception of epichlorohydrin as indicated in Table 1.

Epichlorohydrin is the most unstable of these chemicals used and difficulties were encountered in preparing blends with the desired concentration. In addition, epichlorohydrin levels dropped in the stock gas over the period of use. The other chemicals were stable over the course of the experiment and were present at the intended concentrations in the stock blends. As indicated in Table 1, the correlations between the expected and observed dose values were high with slopes closely approximating the value 1.

Humidity levels measured in chambers containing 11 mice were between 85 and 90% RH. Humidity of the final gas mixtures was close to zero % RH.

Animal conditions

In general, the animals appeared to tolerate the 3- and 6-week housing in the chambers well. The consumption of food and water was qualitatively monitored and appeared to be normal. An example of the body weight of mice during the 6-week exposure is shown in Table 2. When the weights of the mice were plotted all dose groups showed

TABLE 1

CORRELATION BETWEEN EXPECTED AND OBSERVED GAS CONCENTRATIONS

Concentrations are mean values in ppm for all chamber inlet measurements at each dose level ±S.D.

	Benzene	Chloroprene	*p*-Xylene	Epichlorohydrin
High dose	9.88	11.32	9.12	4.63
($n = 6$)	(0.86)	(1.60)	(0.78)	(1.72)
Medium dose	0.718	0.853	0.685	0.062
($n = 6$)	(0.138)	(0.187)	(0.82)	(0.029)
Low dose	0.076	0.172	0.088	0.038
($n = 5$)	(0.005)	(0.072)	(0.024)	(0.014)
Correlation coefficient	0.994	0.986	0.995	0.862
Slope	0.985	1.144	0.917	0.868
y-Intercept	−0.1511	−0.1322	−0.1371	−0.0673

consistent time-dependent increase in weight. At the end of 6 weeks of exposure, the mice reached their expected mature weight and there was no dose-related reduction in average weights. The sustained and similar weight gain indicates that all the mice were in reasonably good physical conditions.

Urine mutagenesis

The mouse urine concentrates were treated in the Ames/Salmonella plate incorporation assay. Solvent (acetone and dimethyl sulfoxide) and positive chemicals (benzo[*a*]pyrene and 4-nitroquinoline oxide) were used as concurrent controls for each assay. The results of this assay showed that the two positive controls were mutagenic to both TA98 and TA100. For example, 2.5 μg/plate of BP induced 3 times the number of revertants over the solvent control whereas frequency was 10 times for 0.5 μg/ml of 4NQO in TA98. Urine concentrates from mice exposed for 3 weeks, however, did not induce mutation with or without S9. A spot test was also conducted and it demonstrated no toxicity or mutagenic activities. In order to achieve more sensitivity with the Ames

TABLE 2

EFFECTS OF 6 WEEKS INHALATION OF MIXTURES ON BODY WEIGHT OF MICE [a]

Cage No.	Starting weight (g)	Week 2	Week 3	Week 4	Week 5	Week 6
C [b] (m)	29.2±1.3 [c]	31.0±1.8	32.2±2.3	33.0±2.8	34.8±3.4	33.4±3.3
C (f)	26.4±1.8	26.2±1.9	27.1±2.0	27.8±1.6	27.1±1.7	27.6±1.7
C (m)	28.5±1.2	30.8±3.0	31.7±1.4	33.9±1.9	35.2±2.3	34.3±1.9
C (f)	26.0±1.6	30.4±3.3	26.7±2.1	27.4±2.2	27.9±2.3	27.7±2.2
L (m)	24.4±1.5	26.4±2.3	27.6±2.1	29.5±2.4	30.9±2.6	32.5±2.6
L (f)	21.9±1.2	22.1±1.3	22.9±1.7	24.0±1.9	24.9±2.0	25.3±2.1
L (m)	24.2±1.2	26.5±2.1	29.3±1.9	30.8±2.5	31.9±2.8	33.5±3.2
L (f)	21.9±0.6	22.5±1.1	23.9±1.4	24.5±1.5	25.4±1.9	25.9±2.1
M (m)	32.3±2.4	31.9±2.6	33.3±2.7	ND [d]	34.1±2.4	35.0±2.7
M (f)	24.3±1.4	24.7±1.5	25.7±1.7	ND	25.3±2.1	26.9±2.0
M (m)	32.0±3.2	31.2±3.2	33.1±3.2	ND	34.2±3.1	35.9±3.4
M (f)	25.8±2.0	25.0±2.4	25.7±2.3	ND	25.9±1.2	26.6±2.4
H (m)	28.9±1.8	28.1±2.2	29.9±2.6	32.1±2.2	33.2±2.8	32.6±2.9
H (f)	24.7±1.3	25.2±1.6	25.4±1.9	26.7±1.4	25.6±1.7	27.2±1.6
H (m)	28.3±2.2	28.0±2.3	30.5±3.0	31.6±3.5	34.4±3.9	33.2±3.6
H (f)	24.7±1.2	25.1±1.3	25.8±1.6	26.1±1.4	26.0±1.6	25.9±1.3

[a] Mice for the control and high-dose groups were purchased at the same time and have the same birth date. The same applied to mice for the low- and medium-dose group. However, the medium-dose experiment was initiated at 3 weeks after the low-dose group; therefore, the weights of mice were higher for the medium-dose animals. Each cage contained 11 mice.

[b] C, control; L, low; M, medium; H, high; (m), male; (f), female.

[c] Average weight/mouse ±S.E.

[d] ND, not determined.

test, a variation of the test was conducted with the rest of the urine samples. The samples were preincubated with the Salmonella tester strains for 20 min at 37 °C. The suspension was then plated, incubated and counted as described previously. With this modification, there was still no detectable mutagenicity in the concentrates.

Further assays were conducted with raw urine samples using the preincubation procedure. Sample concentrations were again tested and were assayed with or without the preincubation with 1000 units of β-glucuronidase for 16 h. There was again no detectable mutagenicity in samples collected from the 3- and 6-week exposure groups.

Bone marrow micronuclei

A summary of the data is shown in Table 3. The overall results indicated a trend towards more micronuclei at the highest dose, however, the increase was not statistically significant as determined by the Mann–Whitney U test. In addition, there appeared to be no difference in response between males and females.

Bone marrow aberrations

50 metaphases were analyzed from each mouse and the data are summarized in Table 4. The aberrations observed were predominantly chromatid deletion. A very limited number (less than 1%) of chromatid exchanges and chromosome types of aberrations were recorded and they were not included in our statistical analysis. We observed that the percentage of aberrant cells and the average number of chromatid aberrations/100 cells were higher in the exposed groups as compared with the control group with the exception of those mice exposed to low doses for 6 weeks. There appears to be a dose-dependent increase in aberrations. However, this slight increase in aberration frequency in the exposed groups was not found to be significantly different from that of the control group. Since there were no indications of sexual differences in responses, no attempt was made to reanalyze the data by sex.

Spleen lymphocyte aberrations

Spleen lymphocyte cultures were set up from 9–12 males of each dose–time group of mice. 3000 cells were scored to evaluate the mitotic activity in cultures of each mouse. These are expressed as a mitotic index in Table 5. Since mitotic index is a crude measurement of cell proliferation, variations do exist as shown in Table 5. For the 3-week-exposed mice, the exposed groups showed a reduction in mitotic index ($p < 0.053$) which is not significant as compared with the control group and there was no dose-dependent relationship.

TABLE 3

BONE MARROW MICRONUCLEUS FREQUENCY AFTER EXPOSURE TO GAS MIXTURE [a]

Conc.	Male		Female	
	MN/1000 PCE (S.D.)	Range	MN/1000 PCE (S.D.)	Range
3-week exposure				
Control	2.3 (2.0)	0– 7	1.4 (1.1)	0– 3
Low	1.6 (1.5)	0– 5	1.6 (1.4)	0– 5
Medium	1.4 (1.5)	0– 6	1.0 (1.3)	0– 4
High	3.1 (1.6)	0–11	3.6 (2.0)	1– 8
6-week exposure				
Control	1.8 (1.8)	0– 7	1.4 (1.4)	0–11
Low	1.3 (1.0)	0– 3	1.6 (1.5)	0– 5
Medium	2.3 (1.1)	1– 5	1.8 (1.2)	0– 4
High	3.0 (2.1)	0– 7	3.5 (1.8)	0– 8

[a] Micronucleus frequency/1000 polychromatic erythrocytes analyzed per mouse from 20 mice/group.

TABLE 4

BONE MARROW CYTOGENETIC ANALYSIS

Exposure	Number of mice	Number of cells analyzed	% Aberrant cells ± S.D.	Chromatid aberrations/ 100 cells ± S.D.
3-week exposure				
Control	32	1600	0.47 ± 0.84	0.53 ± 0.88
Low	39	1950	0.87 ± 1.44 [a]	0.92 ± 1.51
Medium	39	1950	1.18 ± 1.70 [a]	1.13 ± 1.64
High	32	1600	1.19 ± 1.33 [a]	1.25 ± 1.50
6-week exposure				
Control	30	1500	0.60 ± 1.30	0.60 ± 1.30
Low	35	1750	0.23 ± 0.65 [b]	0.23 ± 0.65
Medium	34	1700	1.12 ± 1.41 [b]	1.12 ± 1.41
High	29	1450	1.24 ± 2.36 [b]	1.38 ± 2.40

[a] Significance of difference between exposed and control mice, chi square = 1.49, $p < 0.23$.

[b] Significance of difference between exposed and control mice, chi square = 1.76, $p < 0.19$.

TABLE 5

SPLEEN LYMPHOCYTE MITOTIC INDEX

Exposure	Number of mice	Number of cells analyzed	Mean number of mitotic cells/3000 cells ± S.D.
3-week exposure			
Control	10	30000	8.4 ± 4.5
Low	9	27000	5.8 ± 2.7 [a]
Medium	10	30000	3.8 ± 2.4 [a]
High	10	30000	5.1 ± 5.1 [a]
6-week exposure			
Control	10	30000	6.3 ± 3.6
Low	10	30000	7.8 ± 3.6
Medium	10	30000	3.9 ± 1.8
High	12	36000	5.0 ± 4.4

[a] Significance of variation among groups by one-tailed Kruskal–Wallis test, $p < 0.053$.

For the 6-week-exposed mice, the mitotic indices were more variable. With the exception of the low-dose group, the exposed mice had a reduction in mitotic index compared with the control group although the difference was not significant.

TABLE 6

SPLEEN LYMPHOCYTE CYTOGENETIC ANALYSIS

Exposure	Number of male mice	Number of cells analyzed	Aberrant cells ± S.D. (%)	Chromatid breaks/100 cells ± S.D.
3-week exposure				
Control	9	450	0.67 ± 0.14	0.67 ± 0.14
Low	8	400	2.75 ± 2.38 [a]	3.50 ± 2.98 [b]
Medium	10	500	2.20 ± 2.39 [a]	2.80 ± 3.16 [b]
High	8	400	2.50 ± 2.98 [a]	3.00 ± 3.21 [b]
6-week exposure				
Control	8	400	0	0
Low	10	500	1.60 ± 2.63 [c]	1.60 ± 2.63 [d]
Medium	10	500	3.20 ± 2.35 [c]	3.20 ± 2.35 [d]
High	8	400	0.50 ± 0.93	0.75 ± 0.71

[a] Significance of difference between the exposed and control mice, chi square = 4.13, $p < 0.05$.

[b] Significance of difference between the exposed and control mice, $p < 0.028$ (Mann–Whitney U test).

[c] Significance of difference between the exposed and control mice, chi square = 6.55, $p < 0.01$.

[d] Signifiance of difference between the exposed and control mice, $p < 0.01$ (Mann–Whitney U test).

Our analysis of lymphocyte chromosome aberrations was done by analysis of 50 randomly selected metaphases from 8–10 mice per group and a summary of these data is shown in Table 6. The aberrations observed were only chromatid-type of deletions. Over 90% of them were single and isochromatid breaks. In summarizing the data for Table 6, isochromatid deletions were considered as 2 breaks each and added to the frequency of single chromatid breaks. With the exception of the high-dose 6-week-exposed mice, the exposed groups showed a significant increase in the frequencies of aberrant cells and chromatid aberrations. There was no consistent dose-dependent relationship.

Histological and liver enzyme analysis

Standard histological analysis of the lungs and livers did not indicate any observable abnormalities. Liver enzyme analyses were only conducted with mice after 6 weeks of exposure. As shown in Table 7, glutathione transferase activities of female mice exposed to the high concentration for 6 weeks were twice those of female controls, and were evident for both enzyme substrates (DCNB and CDNB) assayed ($p < 0.01$). Male mice ex-

TABLE 7

EFFECT OF 6 WEEKS INHALATION OF MIXTURE ON LIVER ENZYMES

Group	N	GSH t DCNB (nmoles/mg prot. min)	GSH t CDNB (nmoles/mg prot. min)	P450 (nmoles/mg prot. min)
Male				
Control	12	103 ± 8	6232 ± 568	1.10 ± 0.28
Low	8	102 ± 4	6277 ± 448	ND
Medium	8	130 ± 9 [b]	7334 ± 339	ND
High	8	188 ± 10 [c]	8594 ± 473 [c]	1.28 ± 0.24
Female				
Control	8	83 ± 2	1477 ± 46	1.21 ± 0.19
Low	8	79 ± 5	1270 ± 82	ND
Medium	8	91 ± 5	2035 ± 107 [b]	ND
High	8	163 ± 11 [c]	2889 ± 239 [c]	1.35 ± 0.24

[a] Values are means ± S.E.M.,; ND, not determined.

[b] $p < 0.05$ compared to controls of same sex by Duncan's multiple range test.

[c] $p < 0.01$ compared to controls of same sex by Duncan's multiple range test.

posed to the high concentration showed a lesser but consistent increase in glutathione transferase activities ($p < 0.01$). Exposure to the medium concentration was associated with increased activities from DCNB at $p < 0.05$ in males and, for the other substrate CDNB at $p < 0.01$ in females. No appreciable changes in this enzyme activity were evident in mice exposed to the low concentration. Total cytochrome P-450 levels in all mice exposed to the high concentration were similar to controls.

Discussion

The importance of developing assay systems that can be used to detect genotoxic complex mixture in our environment has been emphasized (Tice et al., 1982; Waters et al., 1983). Where such mixtures exist, a potentially hazardous environment can be created (e.g. petrochemical plants, hazardous wastes dump sites). The identification of such environments and the assessment of the hazards are not easy tasks. The use of biological systems for in situ monitoring of hazardous agents would simplify the process. The existing biological assays that are available for in situ monitoring are summarized by Ma and Harris (1985). Since human exposure to airborne chemicals are mainly by inhalation and the synergistic effects of the components of mixtures may be significant, the use of whole animals would be more appropriate than the use of lower organisms. Our study demonstrated that the combined testing protocol can be applied efficiently to large-scale chronic inhalation studies. Furthermore, our findings indicate that the protocol is sufficiently sensitive to identify damage induced after exposure to ambient levels of gas mixtures. Thus, this protocol can be useful for in situ monitoring of hazards in occupational or natural environments.

Among the end-points analyzed, the spleen lymphocyte assay was found to be the most sensitive one for detecting exposures to these particular genotoxic agents. The sensitivity of spleen lymphocytes is most likely due to their non-proliferative state in vivo which allowed the cells to accumulate DNA damage during the 3–6 weeks of exposure. The damage was converted into chromosome aberrations when the lymphocytes were stimulated to enter the cell cycle in vitro. The slight but insignificant increases in micronuclei and insignificant increase in aberration frequencies in bone marrow cells ($p < 0.23$ and < 0.19 for 3 and 6 weeks) indicate that the bone marrow cells were less appropriate cell types for the low-dose chronic exposure studies. It is possible that the damaged progeny cells were effectively eliminated during cell replication. However, that assertion needs to be confirmed which studies involving various exposure durations. Our data did not indicate the urine mutagenesis assay to be sufficiently sensitive for chronic exposures to this mixture. However, the use of improved protocol and the analysis of urine collected at multiple timepoints throughout the exposure period may be more useful.

The dose-dependent increase in hepatic glutathione transferase activities detected in this study is consistent with the known metabolism of 3 constituents of the mixture (i.e., chloroprene, epichlorohydrin and xylene) to glutathione conjugates (Summer and Greim, 1980; Gingell et al., 1985; Van Doorn et al., 1980). The glutathione transferases are generally considered to be detoxification enzymes because of their function in the transport, metabolism and storage of lipophilic xenobiotics, and their metabolites (Jakoby and Habig, 1980). Our findings indicate that exposure to the mixture produced a selective, dose-dependent alteration of the activities of liver enzyme constituents involved in the metabolism of foreign, and endogenous compounds. Thus, the increase in glutathione *S*-transferase activities, particularly after exposure to the high dose, may have protected the animals against the genotoxic effects of the mixture. This may offer an explanation for the lack of increase in chromosome aberrations in spleen lymphocytes (Table 6) exposed under the same conditions. The induction of such detoxifying activities after exposure to higher doses but not after low doses deserves further investigation.

Our combined testing protocol was developed in 1979 and improved recently for the purpose of investigating the differential effects of mutagens/carcinogens in various cell types in the same animal so that useful information on target-organ specificity, the kinetics of genotoxic activities, as well as explanations of the differential expressions of damage can be obtained. Other investigators

have also used a similar approach (e.g. Conner et al., 1979; Rao et al., 1979). The usefulness of multiple end-points for evaluating genotoxicity is well illustrated in our study. For example, the observation of liver enzyme induction suggested a possible explanation for the cytogenetic finding. A mechanistic explanation of our observation would certainly be useful in providing further understanding of the genotoxic activities of complex mixture in vivo. The application of the lymphocyte chromosome aberration assay in detecting cumulative damage is confirmed in our study. Experiments are in progress now in our laboratory to investigate the genotoxic effects of prolonged exposure to the individual components of the mixture.

In our CTP, cytogenetic end-points are emphasized because chromosome aberrations are highly correlated with serious biological consequences. In addition to the cell types and biological end-points analyzed in this report, others can be easily integrated into this protocol. Examples are cytogenetic analysis of pulmonary macrophages and germinal cells and determination of single gene mutation frequency in lymphocytes. Pulmonary macrophage assays would be of significant value for inhalation studies because they represent the cell type that is directly exposed to the hazardous agents. Cytogenetic assays involving pulmonary macrophages have been conducted (Conner et al., 1979; Scott, 1982), however, we had limited success with collecting enough mitotic macrophage cells in this experiment. Currently, experiments are being conducted in our laboratory to improve the techniques. Germinal cell assays such as the translocation test (Léonard, 1976) and the unscheduled DNA synthesis assay (Sega and Sotomayor, 1982), as well as the 6-thioguanine resistance lymphocyte assay (Albertini, 1985) and DNA adduct formation in target organs (Gupta et al., 1982) are being integrated into the protocol. As with most short-term assays, the emphasis has been on correlating positive responses with the presence of carcinogens. However, more information can be expected on short-term assays. One is the predictive value of short-term results for long-term biological consequences. In order to evaluate such relationships, sequential studies of short-term effects like our study and long-term consequences such as the development of cancer, should be conducted. Our combined testing protocol is most amenable to such a study because of the use of multiple in vivo end-points.

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