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FINAL REPORT

Evaluation of Genotoxic Effects From Chronic Low-Level Inhalation Exposure to Industrial Chemicals in Mice

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I. Executive Summary

The evaluation of risks from chronic exposure to toxic air pollutants is a pressing environmental health need. In 1985, we conducted studies to determine the feasability of detecting and evaluating inhalation exposures in mice to low levels of a mixture of common organic air pollutants which are known, individually, to be carcinogenic. The exposures were evaluated using a battery of tests for genetic damage in mice collectively referred to as the Combined Testing Protocol (CTP). That study produced evidence that exposure to a mixture of benzene, chloroprene, xylene, and epichlorohydrin in a ratio of 2:2:2:1 for 3 or 6 weeks caused chromosome damage at concentrations (based on benzene) of 0.1, 1.0 and 10 parts per million (ppm). In addition, exposure to 10 ppm for six weeks caused an induction of glutathione-S-transferase. The objectives of the current study were to follow-up on the previous one in three ways: 1) by evaluating each of the components (chloroprene and xylene together) individually for effects 2) by exploring the sensitivity of the assay to a lower concentration of test agent (.020 ppm of benzene and chloroprene/xylene and 0.010 of epichlorohydrin) and 3) by evaluating two tests in the CTP not used in the previous study (chromosome aberration analysis in pulmonary alveolar macrophages and unscheduled DNA systhesis in spermatids).

CD-1 mice of both sexes were exposed to each chemical at concentrations of 0, 0.02, 0.1 and 1.0 ppm (half these concentrations for epichlorohydrin) 22 hours per day for six weeks. The chemicals were delivered as a vapor by diluting commercially obtained stock vapor from cylinders with appropriate amounts of filtered and purified compressed air. The animals were housed in groups of six in small whole body inhalation chambers to which the final chemical concentration was delived at a flow

rate producing five air changes per hour. Twelve animals of each sex were exposed to each concentration of each agent. Quality control was maintained on the exposures by determining the concentrations and purity of the stock gases and of the diluted gases at the chamber inlets. The flow rates in the dilution system were also determined on a weekly basis.

Following exposure, the animals were sacrificed and the following analyses performed on tissues from the animals.

- o chromosome aberration analysis on cultured spleen lymphocytes
- o chromosome aberration analysis on pulmonary alveolar macrophages
- o enumeration of micronuclei in peripheral blood erythrocytes
- o measurement unscheduled DNA systhesis to detect DNA repair in spermatids.
- o determination of levels of glutathione-S-transferase and cytochrome P450 in liver.

In addition, specimens were collected and stored for the following analyses to be performed in the future.

- o chromosome aberration analysis on bone marrow lymphocytes
- o enumeration of micronuclei on bone marrow erythrocytes
- o assay for mutation (6-thioguanine resistance) in spleen lymphocytes
- o determination of DNA adduct formation in liver
- o chromosome aberration analysis in spermatocytes

The results of all the assays except sperm UDS analysis in the first list are presented here. The sperm UDS results are not complete at this date and will be presented later. For benzene, only exposures to 1.0 and 0.1 ppm were completed. The exposure to 0.02 ppm will be conducted at a later date. The results of benzene exposure indicated a dose responsive

increase in chromosome damage in both the spleen lymphocytes and the lung macrophages. In the animals exposed to chloroprene/xylene, a significant elevation in chromosome damage in spleen lymphocytes was observed in the males at the high dose. No dose related increase was observed in lung macrophages. In epichlorohydrin, exposed animals chromosome damage was much higher than normal at all levels including controls. Difficulties in maintaining the purity of the dilution air occurred during this exposure which probably accounted for this result. The epichlorohydrin results are considered to be uninterpretable. No elevations in liver enzymes were observed in any of the exposures.

It was concluded that among the individual components of the mixture studied in 1985 benzene probably accounted for most of the activity. Chloroprene may have accounted for some activity and the role of epichlorohydrin cannot be determined based on current data. The magnitiude of increase in chromosome damage caused by benzene alone is similar to that observed for the mixture of all the gases at the same concentrations.

These results indicate that the test system is sufficiently sensitive to be of significant value in studying the effects of long term exposure to carcinogenic chemicals in air, and that benzene may be more of a threat to health at low levels than has been previously recognized.

II. Introduction

Our laboratory has pioneered the use of a combination of short-term in vivo assasys for the detection of biological effects from exposure to mutagenic/carcinogenic chemicals. It was accomplished by incorporating a selection of highly sensitive biological assays into one testing protocol using experimental animals, the Combined Testing Protocol (CTP) (Conner et al., 1979) which has been continually updated (Legator and Harper, 1982;

Legator et al., 1986). By conducting studies with CTP, an in-depth activity profile of genotoxic and metabolic effects occurring in vivo can be obtained in the same experiment. In light of the lack of predictability from in vitro data for carcinogenicity (Ashby and Purchase, 1985), in vivo studies like ours should be a logical alternative.

In 1984, a contract was awarded by the Texas Air Control Board to conduct studies, using the CTP, to determine whether the biological endpoints in our protocol are sensitive enough to detect the effects from exposure to ambient levels of environmental mutagens and, when proven successful, whether the CTP can be adapted for use in a Mobile Animal Monitoring Unit. The study involves exposing mice by inhalation to ambient level doses of a mixture of benzene, chloroprene, epichlorohydrin and xylene for 3 and 6 weeks (for detail, see Report, 1985; Ward et al., 1986 and Au et at., 1986). The end-points used were urine mutagenesis, cytogenetic assays using bone marrow cells, lung macrophages and spleen lymphocytes and liver enzyme induction. Among them, the spleen lymphocyte chromosome aberration assay was demonstrated to be the most sensitive one by showing a dose-dependent relationship. The cytogenetic profile appears to be correlated with the induction of glutathione-S-transferase activity in the liver. On the other hand, the urine mutagenesis assay and assays using bone marrow cells (chromosome aberrations and micronuclei) were found to be insensitive under our experimental conditions. Complications were encountered in the lung macrophage assay and it was overcome eventually by applying a different mitotic arresting agent.

The complexity of conducting inhalation genetic toxicology study was appreciated during the course of the study. Modifications and improvements were accomplished.

Since exposure to the gas mixture was demonstrated to cause genomic

damage in somatic cells of mice, a second contract was awarded by TACB in 1985 to conduct studies to determine the contributions from the individual components. Mice were exposed for a duration of 6 weeks with dose ranges that reach five fold lower than those used in the mixtures. Assays shown to be effective in the initial study are used (spleen lymphocyte chromosome aberrations and liver enzyme induction) whereas those shown to be insensitive are not employed. Instead, new assays are incorporated. These include the improved lung macrophage chromosome aberration, the sperm unscheduled DNA synthesis (UDS) and the peripheral blood micronuclei assays.

In this report, results from this study are summarized with the exception of data from the sperm UDS assay. It is because slide preparations for the sperm UDS assay require a minimum of 1 month before analysis can be initiated. Results from the sperm UDS assay will be reported separately when all the data are collected and evaluated.

III. Quality Assurance of Exposures

A. Methods

1. Flow Calibration

Flow calibrations were determined using soap bubble meters as described in the 1985 fiscal year report. The flow of dilution air and test gas were determined at the inlets to the mixing chambers prior to the initiation of each exposure period. The balance of flow to the individual chambers supplied by each mixing chamber was determined at least once a week at the same time as samples were collected for chemical analysis.

The mass flow controllers were calibrated prior to use and the flow rates were checked at the chamber inlets once a week and at the flow controller outlets at the end of each exposure period. In the initial

with 2 ml of 60% acetic acid and then with Carnoy's fixative. Air-dried slides were then prepared, stained with 10% Giemsa solution for 8 min. Slides were then air-dried, mounted in Pro-Tex sealing solution and stored in slide boxes.

4. 6-Thioguanine Resistant (6TGr) Spleen Lymphocyte Assay

The lymphocytes remaining after setting up cultures for chromosome analysis were frozen for later determination of 6TG-R lymphocyte frequencies. Cells from pairs of animals from the same chamber were pooled and suspended in fetal calf serum with 10% dimethylsulfoxide at a concentration of 10(6) cells/ml. Aliquotes at 1 ml were placed in ampules and cryopreserved at -70 C.

E. Results of Performed Assays

1. Benzene Exposure

a. Spleen lymphocyte cytogenetics

Fifty metaphases were analyzed for the presence of chromosome aberrations in 24 control mice, 23 mice exposed to the medium dose (0.1 ppm) and 21 of the high dose (1 ppm) mice. Mitotic inhibition was observed in 1 medium-dose mouse and 3 high-dose mice but in none of the controls. The frequency of metaphases having chromosome damage showed a dose dependent increase from 2.25% in the control mice to 5.83 and 6.67 in the medium- and high-dose mice, respectively (Table 16). The majority of the aberrations observed were chromatid-type of deletions. There were 204 chromatid breaks observed. The other types of aberrations were 2 chromatid exchanges and 6 chromosome deletions. These two types of damage are too few to be useful for statistical analysis. A summary of the raw data is shown in Appendix II. Statistical analyses were conducted based on the frequencies of aberrant cells and of chromatid aberrations per cell. A

al (1986) demonstrated that acute treatment of male rats with benzene (20 mmol/kg, ip, for three days) increased liver glutathione content by 21% and glutathione transferase activities towards the substrates CDNB and DCNB by 70 and 20% respectively. In addition, these investigators found that this acute benzene exposure was sufficient to enhance the in vitro metabolism of benzene by 81%. The absence of benzene effects on these constituents in our study suggest that the benzene doses were too low to induce constituents involved in benzene metabolism.

2. Results of Epichlorohydrin Experiment

After the cytogenetic data were collected and the codes were broken it was realized that the background aberration frequencies in control mice were much higher than expected. It was most likely due to the presence of contaminants in the control air supply. The impact of this finding on the overall evaluation of the epichlorohydrin experiment is discussed in Section V.

3. Chloroprene and Xylene Exposure

a. Spleen lymphocyte cytogenetics

Fifty metaphases per mouse were scored from every mouse of the control and high dose groups (24 mice per group). However, only 18 and 22 mice were analyzed for the low and medium dose groups. It is because one cage of 6 female mice died of suffocation in the low dose group and 2 mice died in the medium dose group. The majority of the aberrations observed were chromatid type. The data indicate a small dose-dependent increase in the Percentage of Aberrant Cells and in the Rate of Chromatid Aberrations per 100 Cells. A detail description of the statistical analysis of the data (Table 12) is presented in Section V.

b. Lung macrophage chromosome aberrations

The same building compressed air supply was used both years. This problem does point to the need for a cleaner source of air for the system. We are currently planning to purchase a stand-alone air compressor of a type that will provide breathing quality air for future studies.

B. Interpretation of Results

1. Chromosome Aberrations

We have confirmed that the spleen lymphocyte chromosome aberration assay is a sensitive system for detection of genotoxic activities from low dose chronic exposure conditions. Among the chemicals tested, benzene is clearly the most active one in inducing highly significant frequency of chromosome damage in spleen lymphocytes. We have shown also, for the first time, that benzene exposure can induce chromosome aberrations in exposed pulmonary macrophages. Chloroprene plus xylene induced a significant increase in chromosome aberrations only in spleen lymphocytes and only in the male mice exposed to the high dose. Although the epichlorohydrin experiment is complicated by the contaminants in our control air, the variability of the results suggest that epichloro-hydrin is not likely to exhibit genotoxic activities under our experimental conditions. Our data caution that exposure to benzene, even under the current legal exposure limits, is potentially hazardous.

2. Micronucleus Studies

Tables 10, 14, 18 and 19 present the peripheral blood micronucleus results for the mixture and for the individual components. The summary table (Table 19) may be used for comparisons. The control values for each exposure over a 2-year period show some variability, and based on other studies in the literature, this strain has a lower background rate than several other strains. The rate in females was consistently lower than in

males. There is no reason to suspect that the controls for epichlorohydrin are unusually high, although all of the epichlorohydrin exposed groups are somewhat elevated. Based on the complications discussed elsewhere for this exposure and based on comparison to literature reports of positive peripheral micronuclei effects, it is concluded that this slight increase is not biologically significant, nor can the increase at the highest mixture group be considered real without confirmation. Based on the report of Erexson et al. (1986), it would be predicted that benzene might be positive at the higher doses (10 and 100 ppm if the strain (DBA12)) more sensitive to benzene were used. In designing this protocol, however, an outbred strain of intermediate sensitivity and inducibility (of cytochrome P448) was chosen as more analogous to human variability.

3. Enzyme Studies

Induction of enzymes involved in the metabolism of a xenobiotic by repeated exposure to the xenobiotic is considered an adaptive response of the organism (Reynolds and Moslen, 1985). When the induction would facilitate detoxification of the xenobiotic - as would induction of glutathione and the glutathione transferases - the effect is a beneficial adaptive response. The absense of liver enzyme induction in animals exposed to benzene or chloroprene/xylene indiciates that the doses used in the individual components studies were too low to produce this adaptive effect. The marked liver glutathione transferase induction previously observed in both male and female animals exposed to the highest dose of the mixture could have been due to additive effects of the individual components of the mixture.

C. Conclusions

Some of the assays conducted with the mixture experiment in the first

year were found to be unsuitable for use under our experimental conditions and were, therefore, not used in the current study with individual chemicals. At a result, only the spleen lymphocyte chromosome aberration and the liver enzyme induction assays were used for both experiments.

Under the similar experimental conditions of 6 week exposure to 0.1 and 1 ppm benzene, the mixture induced a 2.4 and 4.8 fold increase respectively in chromosome aberrations in spleen lymphocytes. In the current experiment, benzene alone induced a 3.2 and 3.7 fold increase whereas chloroprene plus xylene induced a 1.9 and 2.6 fold increase. It appears that benzene is solely responsible for most of the damage observed after exposure to the mixture. Furthermore, there does not appear to be a synergistic effect when the chemicals were combined together.

D. Assessment of Accomplishments of Objectives

The objectives of this study were 1) to evaluate individual components of the mixture used in the FY 1985 study 2) to test the sensitivity of our approach by evaluating the effects of exposures at 20% of the previously used concentrations (in the mixture), and 3) to incorporate new methods particularly the lung macrophage cytogenetics assay and the sperm UDS assay into the test battery. All of these objectives were met although with some limitations. The individual components of the assay were all evaluated. Unfortunately the results for epichlorohydrin were uninterpretable due to the very high background rates encountered. Useful analyses of both benzene and chloroprene/xylene were obtained. Benzene produced a dose responsive increase in chromosome damage in the spleen lymphocyte and lung macrophage down to at least 0.1 ppm. Chloroprene appears to be clastogenic in the spleen lymphocytes of male mice but not females. Additional studies will be needed to confirm or reject these findings. Levels equal to one-fifth the

lowest levels used in the mixture were tested for epichlorohydrin and chloroprene/xylene. A shortage of equipment at the beginning of the study and other difficulties (see Section VI.A., above) prevented the low dose effect for benzene from being evaluated during the study period. A careful evaluation of dose response effects with benzene at concentrations characteristic of ambient pollution levels (20-40 ppb) is the highest priority for future research. Two new methods were successfully incorporated into the CTP for this study. By optimizing conditions for mitotic arrest and cell preparation and by reducing animal numbers in chambers, we were able to routinely recover sufficient mitotic cells from the lungs to perform a chromosome aberration analysis. This resulted in positive findings for benzene but not chloroprene. The sperm UDS assay was successfully developed and integrated into the CTP. Because the autoradiographic preparations which it yields require a long exposure period, someof the slides have not yet been scored. The results for all three exposures will be reported in a separate document.

E. Future Directions

We have gained two years of experience in conducting inhalation genetic toxicology studies. Our experimental set up has proven to be economical, flexible and durable. These features are essential for adapting our system to the "Mobile Monitoring Unit." However, improvements can still be made. One is to use a more dependable source of purified dilution air. Another would be to use more conventional glass and stainless steel inhalation chambers. The gas distribution and mixing characteristics are better understood and their design makes animal care less laborious.

Among the chemicals tested, benzene has been shown to be the most

active one so that further studies should be conducted to fully evaluation the impact on human health from exposure to benzene. The major priority for future research should be to evaluate the effects of benzene in the actual range of ambient pollution levels. Levels of 40 ppb are frequently measured in polluted areas by the TACB. Final determination of the feasibility of this system for field monitoring can be made by studying levels in this range. For example, exposures for 0, 20, 40 and 100 ppb can be used.

Although the effects on somatic cells have been emphasized during the last two years, the effects on germinal cells which may lead to serious consequences for future generations should not be ignored. It should be pointed out that it is not unlikely that benzene exposure can induce genetic damage in germinal cells. It has been reported by Snyder et al (1980) that benzene is accumulated around the epidydimis after exposure. Furthermore, seruminiferous tubule degeneration has been observed after exposure to high doses of benzene (Wolff et al., 1956 and Collom and Winek, 1970). Finally, we have preliminary data to demonstrate that chromosome damage in germinal cells can occur after gavage exposure to benzene (Rithidech et al., 1986). Thus, the genotoxic effects in germinal cells after exposure to benzene under a variety of conditions (acute and subacute) should be evaluated.