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Carcinogenicity of Inhaled Butadiene Diepoxide in Female B6C3F1 Mice and Sprague-Dawley Rats

Rogene F. Henderson,[1] Fletcher F. Hahn, Edward B. Barr, Steven A. Belinsky, Margaret G. Ménache, and Janet M. Benson

Lovelace Respiratory Research Institute, P.O. Box 5890, Albuquerque, New Mexico 87185–5890

Previous studies suggest that the greater sensitivity of mice, compared to rats, to the carcinogenicity of 1,3-butadiene (BD) is linked to higher rates of BD metabolism to butadiene diepoxide (BDO_2) by mice than rats. The purpose of this study was to determine the tumorigenicity of BDO_2 in mice and rats exposed by inhalation to the same concentrations of the agent. Female B6C3F1 mice and Sprague-Dawley rats, 10–11 weeks old, 56/group, were exposed to 0, 2.5, or 5.0 ppm BDO_2, 6 h/day, 5 days/week for 6 weeks. At the end of the BDO_2 exposure, 8 animals/group were evaluated for toxicity. The remainder of the exposed rats and mice were held for up to 18 months for observation of tumor development. At the end of the exposure, rats had no biologically significant alteration in standard hematological parameters, but mice had a dose-dependent increase in neutrophils and decrease in lymphocytes. Most of the significant lesions in both species were in the nose, concentrated around the main airflow pathway. Necrosis, inflammation, and squamous metaplasia of the nasal mucosa, as well as atrophy of the turbinates, were all present in animals exposed to 5.0 ppm. In mice, necrosis and inflammation subsided within 6 months, but squamous metaplasia remained. In rats that died after exposure, squamous metaplasia was seen in areas of earlier inflammation and extended beyond those areas with time. The metaplasia was severe enough to restrict and occlude the nasopharyngeal duct. Later, keratinizing squamous-cell carcinomas developed from metaplastic foci in rats, but these were not seen in mice. At the end of 18 months, the only significant increase in neoplasia in the exposed rats was a dose-dependent increase in neoplasms of the nasal mucosa (0/47, 12/48, and 21/48 for the control, 2.5 ppm, and 5.0 ppm exposures, respectively). Neoplasia of the nasal mucosa did not increase significantly in the mice. Neoplastic lesions in the mice were observed in reproductive organs, lymph nodes, bone, liver, Harderian gland, pancreas, and lung, but the only significant increase in neoplasms in a single organ in the mice was in the Harderian gland (0/40, 2/42, and 5/36 for the control, 2.5 ppm, and 5.0 ppm exposures, respectively). This tumor accounts for the apparent trend toward an increase in total neoplastic lesions in mice as a function of dose (10/40, 7/42, and 16/36 for control, 2.5 ppm, and 5.0 ppm, respectively). These findings indicate that the metabolite of BD, BDO_2, is carcinogenic in the upper respiratory tract of rats. An increase in upper respiratory tract tumors was not observed in similarly exposed mice, despite the fact that preliminary studies indicated mice should have received twice the dose to tissue than did the rats. Higher cytosolic activity of detoxication enzymes has been reported in the liver and lung cells of the mouse compared to the rat, and this may account, in part, for the differences in response. The transport of externally administered BDO_2, into the cell and through the cytoplasm, might allow detoxication of the molecule before it reaches critical sites on the DNA. The results indicate that the site of formation of the BDO_2 is important for tumor induction.

Butadiene (BD), a widely used industrial chemical, is carcinogenic in animal bioassays. However, there is a large species difference in the response of B6C3F1 mice and Sprague-Dawley rats to inhaled BD (Huff *et al.*, 1985; Melnick *et al.*, 1990; Owen *et al.*, 1987). This may be due to differences in the metabolism of BD between these two species. BD is biotransformed into a reactive intermediate, butadiene diepoxide (BDO_2), a suspected potent carcinogen. The observation that the sensitive animal species produces more BDO_2 than the insensitive species suggests that BDO_2, rather than BD, may be the primary carcinogen and should be of more concern. Because risk assessment of BD in humans is based in part on animal data, an investigation of BDO_2 carcinogenicity and possible differences among species is warranted.

Mice are more sensitive to the tumorigenic effects of inhaled BD than rats. In chronic studies, B6C3F1 mice exposed to high levels of BD (200 ppm or higher) had increased lethal lymphocytic lymphomas (Huff *et al.*, 1985). At lower exposure concentrations, the mice had increased lung neoplasia (at 6.25 ppm BD in females and 62.5 ppm BD in males) and hemangiosarcomas of the heart (62.5 ppm BD in males) (Melnick *et al.*, 1990). Neoplasias in the liver, mammary gland, and ovary were also increased. Besides neoplasia, the exposed mice had macrocytic anemia. When chronically exposed to BD over a 2-year period, Sprague-Dawley rats had increased tumors in mammary glands at the 1000-ppm dose level, and they had increased neoplasia in the thyroid, testes, uterus, pancreas, Zymbal's gland, and mammary glands after exposures to 8000 ppm (Owen and Glaister, 1990). Exposed rats did not develop macrocytic anemia or lymphomas. Thus, responses of the two species differed in the level of BD required to induce neoplasia and in the site of tumor induction.

Recent evidence suggests large species differences in the

[1] To whom correspondence should be addressed. Fax: (505) 845-1198. E-mail: rhenders@lrri.org.

formation of BDO_2. Csanády *et al.* (1992) found that mouse livers, but not rat or human livers, could oxidize butadiene monoepoxide (BDO) to BDO_2. In studies conducted *in vivo*, BDO_2 was detected in the blood of mice, but not in rats exposed to low levels of BD (100 ppm or less for 4 to 6 h) (Bechtold *et al.*, 1995; Himmelstein *et al.*, 1994, 1995). However, more recently, Thornton-Manning *et al.* (1995a,b) used a more sensitive method and found a small amount of BDO_2 in BD-exposed rats. Vangala *et al.* (1993) observed DNA cross-linking, an effect that could be caused by BDO_2 but not BDO, in the livers of BD-exposed mice, but not in rats. Therefore, BDO_2 may be the potent genotoxic metabolite of BD. This could provide at least one explanation for the large species differences in response to inhaled BD.

Studies by Boogaard and Bond (1996) and Boogaard *et al.* (1996) indicate species differences in the detoxication routes for the BDO_2. Human liver microsomes hydrolyze the diepoxide faster than rat liver microsomes, which is faster than is observed in mouse liver microsomes (Boogaard and Bond, 1996). The detoxication via cytosolic conjugation with glutathione follows the reverse order, with mouse liver cytosol catalyzing the reaction faster than the rat liver cytosol, which is faster than the human liver cytosol.

The current study was designed to determine tumorigenic responses of rats and mice following direct exposure to BDO_2.

METHODS AND STUDY DESIGN

Experimental design. Female B6C3F1 mice and Sprague-Dawley rats were used because large differences in response to BD occur between these species in BD chronic-inhalation bioassays. In addition, females had been the more sensitive gender for induction of lung tumors in the 2-year bioassay studies (Huff *et al.*, 1985; Melnick *et al.*, 1990; Owen *et al.*, 1987).

Animals were exposed by inhalation for the following reasons. BDO_2 should be a direct-acting carcinogen that is most likely to cause tumors near the site of administration. The lung and heart were the two most sensitive sites for induction of tumors in the mice exposed to BD, and exposure by inhalation should deliver high doses to those sites (Henderson *et al.*, 1999). Finally, inhalation exposures would avoid the repeated bolus dosing involved in intratracheal depositions. Based on preliminary studies (Henderson *et al.*, in press), concentrations of 0, 2.5, and 5 ppm BDO_2 were chosen for the 6-week exposures.

The experimental design of this study is presented in Table 1. Groups of 56 animals per species were exposed to 0, 2.5, and 5.0 ppm BDO_2. Animals were exposed 6 h/day, 5 days/week for 6 weeks. Eight animals from each group were killed at the end of the exposures, for observation of histopathology. A second interim sacrifice of mice was made at 6 months after the end of the exposure, to determine if tumors or pre-neoplastic lesions were present. There was no second interim sacrifice of rats due to high mortality in this species. All remaining animals, except those that had died or were sacrificed as moribund, were held for 18 months for the final sacrifice.

Animals. Four-week old female B6C3F1 mice and Sprague-Dawley rats were purchased from Charles River Laboratories (Pontane, MI) and quarantined for 2 weeks. At initiation of exposures, the animals were 6 weeks old. Viral antibody screens for common rodent viruses, which were performed at the end of the quarantine, were negative. Animals were randomly assigned to each exposure group by species and weight within 6 days before the start of exposures. Randomization was accomplished using the Path/Tox Computer System (Xybion Corporation). Rats and mice were individually identified by tail tattoo and individually housed in stainless steel wire-mesh cages within Hazleton 2000 chambers during quarantine and exposures.

TABLE 1
Fate of Animals in Exposure Groups

	0 ppm	2.5 ppm	5.0 ppm
Rats			
Died in exposure	0	0	0
Scheduled interim sacrifices[a]	8	8	8
Moribund sacrifices/deaths	31	37	46[b]
Final sacrifices[c]	17	11	2
Deaths from end of exposure to final sacrifice	65%	77%	94%
Mice			
Died in exposure	0	0	4
Scheduled interim sacrifices end of exposure	8	8	8
6 months after end of exposure	4	4	3
Moribund sacrifices/deaths	4	2	5
Final sacrifices[c]	40	42	36
Deaths from end of exposure to final sacrifice	9.1%	4.5%	11%

Note. For all treatment groups, $n = 56$.
[a] At end of exposure.
[b] Includes one accidental death.
[c] 18 months after end of exposure

The rats and mice were fed Teklad Certified Rodent Diet (Harlan Teklad, Madison, WI). Once exposures began, food was withheld during exposure hours, and water was supplied *ad libitum*. The light/dark cycle was 12-h light/12-h dark with lights on at 0600. The chamber temperature was maintained at 75 ± 3°F and the relative humidity at 40–70%. At the end of the 6-week exposure, animals were housed in polycarbonate cages (3 mice per cage and 2 rats per cage) containing hardwood-chip bedding and filter caps; food and water were available *ad libitum*.

Chemical. Butadiene diepoxide (BDO_2 CAS #30031–64–2) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The compound was a racemic mixture of the stereoisomers of BDO_2 with none of the meso form present. BDO_2 is a liquid with a molecular weight of 86.1 and density of 1.113. Purity of the compound was checked before the exposure began; the commercial product (>97% pure) was further purified to >99% by redistillation. Purity was determined by nuclear magnetic resonance analysis.

Exposure system. Exposures were conducted in stainless steel inhalation chambers (H2000, Lab Products, Inc., Aberdeen, MD). The rate of airflow through these chambers was 15 ± 2 cubic feet per minute, which provided 15 chamber air changes/hr. All chamber supply air was HEPA-filtered before being introduced into the chamber supply system.

BDO_2 vapors were generated using a heated J tube (120°–140°C) located within a lexan enclosure connected to the facility exhaust. The liquid BDO_2 was supplied to a heated J tube using a syringe pump at a controlled-feed rate. Nitrogen was used to carry the vapors from the tube to the chamber. The temperature of the J tube, the BDO_2 feed rate to the J tube, and the flow rate of the carrier gas (N_2) were optimized to provide stable vapor concentrations within the chambers throughout the 6-h daily exposure period.

Vapor concentration in each exposure chamber was monitored periodically by infrared (IR) absorbance, using a MIRAN-IA IR spectrometer (Foxboro, MA) to ensure that the real-time concentration was stable throughout the exposure day. Vapor concentration was determined by extracting chamber air through a bubbler system, which consisted of two 100-ml volume glass impingers connected in series. Each bubbler was filled with 50 ml of ethyl

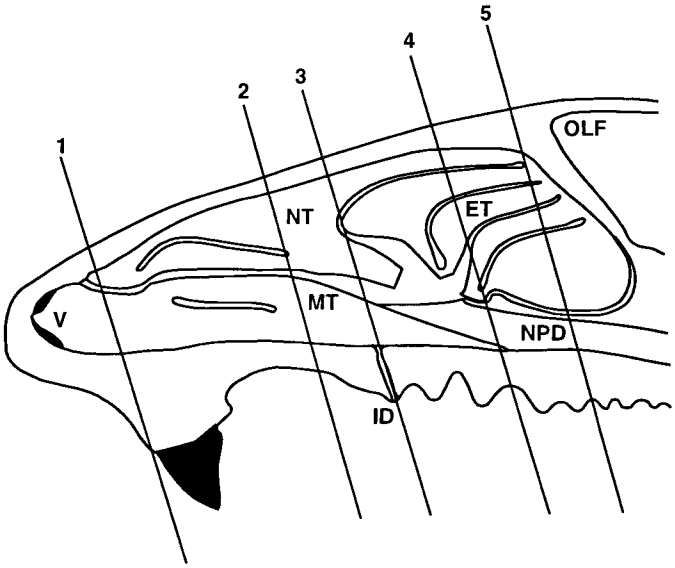

FIG. 1. Diagram indicating the cross-sections of the nose taken for histologic evaluation. V, vestibule; NT, nasoturbinate; MT, maxilloturbinate; ID, incisive duct; ET, ethmoturbinate; NPD, nasopharyngeal duct; OLF, olfactory bulb.

acetate. Three 1-h bubbler samples were taken each exposure day. Ten-milliliter samples of each impinger were taken and analyzed by gas chromatography (GC). The generation system was adjusted as needed based on the previous day's GC data.

Clinical observations. Rats and mice were observed once in the early morning and once in the afternoon, for dead or moribund animals. Detailed clinical observations were made prior to exposures, 1 week after the BDO_2 exposures began, at the end of the exposures, and at monthly intervals thereafter.

Sacrifices and necropsies. Animal sacrifice schedules are shown in Table 1. At the time of necropsy, the animals were weighed and sacrificed using an overdose of pentobarbital (250–300 mg/kg) administered by IP injection. The chest cavity was opened immediately, and the animals exsanguinated by cardiac puncture using a syringe and needle. All animals, including those that had died or were euthanized, were given a complete necropsy of all organ systems. Only target organs noted in the rat or mouse bioassays were routinely trimmed and sectioned for histologic examination. These included heart, lung, stomach, liver, Harderian gland, ovary, pancreas, uterus, thyroid, Zymbal's gland, mammary gland, and kidney. In addition, any organs suspected of having an increased incidence of neoplasia based on gross necropsy results were trimmed and sectioned. Target tissues were fixed in 4% buffered paraformaldehyde overnight and transferred to 70% ethanol. Tissues for histopathological evaluation (including nose and left femur after decalcification) were trimmed, embedded in paraffin, sectioned at about 5 μm intervals, and stained with hematoxylin and eosin for histopathological evaluation.

The nose was decalcified in 13% formic acid solution. Four cross-sections of the nose were taken initially (Levels 2–5, Fig. 1). Because extensive lesions were noted in the nasal mucosa of the rats, a fifth section was added (Level 1). Lesions noted histologically in the nasal mucosa were recorded on nose maps (Mery *et al.*, 1994) to document the distribution of the lesions throughout the nasal cavity.

Hematological parameters, evaluated in blood obtained at sacrifice, included complete blood count, differential cell counting, hematocrit, and hemoglobin content.

Statistical Methods

The statistical methods implemented to analyze three types of data (body weight measurements, hematology, and neoplastic lesions) are described below. A general survival analysis was also performed. All analyses were performed separately for mice and rats. SAS statistical software was used for the analyses (SAS, 1989).

Survival analysis. The survival distribution function (SDF) was estimated for each stratum (BDO_2 exposure level) using the nonparametric Kaplan-Meier method. The log rank test and the Wilcoxon test were used to evaluate homogeneity across all strata (0, 2.5, and 5.0 ppm BDO_2). If homogeneity across all strata was rejected ($p \leq 0.05$) by either test, pair-wise comparisons were made, and a Bonferroni-type adjustment was applied.

Body weight analysis. Body weight measurements were made prior to initiation of exposure, 1 week after the exposure began, at the end of the exposure, and at monthly intervals thereafter for 18 months. For the mice, a multivariate, repeated-measures procedure was performed to compare body weights among the 3 exposure groups at 6 identified time points: immediately prior to exposure (to test that all animals had similar body weights at the beginning of the experiment); at the end of the exposure (to test acute effects of the 6-week exposure); at 1, 6, and 12 months after the end of the exposure (to test any developing effects due to the exposure); and at sacrifice. Statistical significance ($p \leq 0.05$) was assessed by the Hotelling-Lawley trace. The multivariate tests determined the next level of analysis. If there were statistically significant effects, then univariate analyses of variance (ANOVA) were examined. A statistically significant factor effect ($p \leq 0.05$; F-test) permitted comparison of the body weights in the different exposure groups at the single point in time. These comparisons were made with t-tests. Due to the step-down nature of the analysis, no corrections for multiple comparison were made.

Because of the large number of early deaths in the rats, a repeated-measures analysis was used to look at only 4 time points: immediately prior to exposure; at the end of exposure; and at 1- and 6-months postexposure. These corresponded to relatively early time points in the study.

Hematology data analysis. Data were grouped for statistical treatment and analyzed in a step-down fashion. First, a vector of primary variables, considered important in identifying potential effects from the exposure, was identified. The 4 variables in this vector (white blood cells, red blood cells, platelets, and mean cell volume) formed the dependent variable vector of the primary variable multivariate analysis of variance (MANOVA).

If statistically significant effects ($p \leq 0.05$, Hotelling-Lawley trace) were observed, then the ANOVAs for each primary variable were examined. Statistical significance was evaluated using the factor F tests ($p \leq 0.05$). Subtesting of statistically significant effects was performed using uncorrected t-tests. If there was an ANOVA concentration effect but no concentration by time interaction, then the t-tests were performed on the concentration factor means (i.e., averaged over the 2 time periods).

Neoplastic lesion analysis. Due to the extensive deaths in the rats, the statistical approach for analyzing the lesion data was different from that used for the mice. In the rats, a survival analysis was performed using only those animals found dead or sacrificed moribund. The analysis follows the techniques described earlier for the general survival analysis. The results reported, however, are only the median survival time and its standard error.

The neoplastic lesion data for the mice were analyzed using contingency tables. Only those animals that survived until final sacrifice were included. Lesions were observed in several tissues although generally there was only one lesion in any mouse. As a result, the contingency tables were constructed 4 ways. First, the mice were classified according to whether they had any lesion in any tissue or no lesions at all. Second, based on findings in historical studies, the mice were classified on whether they had a lesion in the reproductive organs. Third, based on both historical information and the observations from the rats, the mice were classified on whether they had a lesion in the respiratory tract. Finally, a single contingency table was constructed to test for the effects of BDO_2 exposure on the Harderian gland, because this gland is in the eye and would be directly exposed to BDO_2 in the nose-only exposure system.

Because the contingency tables had 3 rows, corresponding to the 3 dose levels of BDO_2 (0, 2.5, or 5.0 ppm), a significant chi-square test ($p \leq 0.05$) was sub tested by constructing the 2×2 contingency tables. The chi-squares from these tests were adjusted for multiple comparisons.

RESULTS

Exposures. The mean ± SD of the BDO_2 concentrations in the 3 chambers were 0 (control), 2.48 ± 0.42 (low level), and 5.07 ± 0.65 (high level) ppm BDO_2. The mean concentrations were within 1.5% of the target values. The coefficients of variation were <20%.

Clinical Observations. The predominant clinical findings attributable to BDO_2 exposure in rats and mice were due to lesions in the upper respiratory tract. Rats inhaling 2.5 and 5.0 ppm BDO_2 developed a broad spectrum of clinical signs associated with obstruction of the upper airways; however, unlike mice, these signs occurred only after the BDO_2 exposures had ended. The major clinical sign was dyspnea, which was first observed in rats approximately 4 weeks after termination of exposures. The incidence peaked in 2.5 ppm- and 5.0 ppm-exposed rats approximately 12 and 8 weeks after exposure, respectively, and remained elevated in both groups until approximately 24 weeks after exposure. The time course of development of this clinical symptom in rats is illustrated in Figure 2.

Abdomens of rats inhaling 2.5 and 5.0 ppm BDO_2 were periodically swollen due to their swallowing air. The time course for development of swollen abdomens in each dose group paralleled that of dyspnea. The obstruction of the upper airways prevented eating, leading to weight loss prior to death, or moribund sacrifice.

No control mice or mice exposed to 2.5 ppm BDO_2 displayed clinical signs of toxicity. All mice exposed to 5.0 ppm BDO_2 had dyspnea by the fifth week of the exposure, but the prevalence dropped to less than 10% following termination of exposures. The time course for occurrence of dyspnea in mice is illustrated in Figure 2.

Body Weights. The mean monthly body weights of the rats and mice are shown in Figure 3. There was ~20% reduction in body weight in the rats exposed to 5.0 ppm BDO_2 compared to control rats (Fig. 3). This difference was maintained throughout the recovery period. Similarly, ~20% reduction in body weight was observed in mice exposed to the high level of BDO_2 (Fig. 3).

Survival. The fates of rats and mice in each exposure group are shown in Table 1, respectively. Survival curves for all groups are shown in Figure 4. The control rats did not survive as long as the mean survival of female Sprague-Dawley rats in 15 2-year studies as published by the supplier (Charles River Laboratories, 1992). However, the survival of both rats (log-rank and Wilcoxon, $p < 0.01$) and mice (log-rank, $p = 0.05$; Wilcoxon, $p = 0.03$) differed by

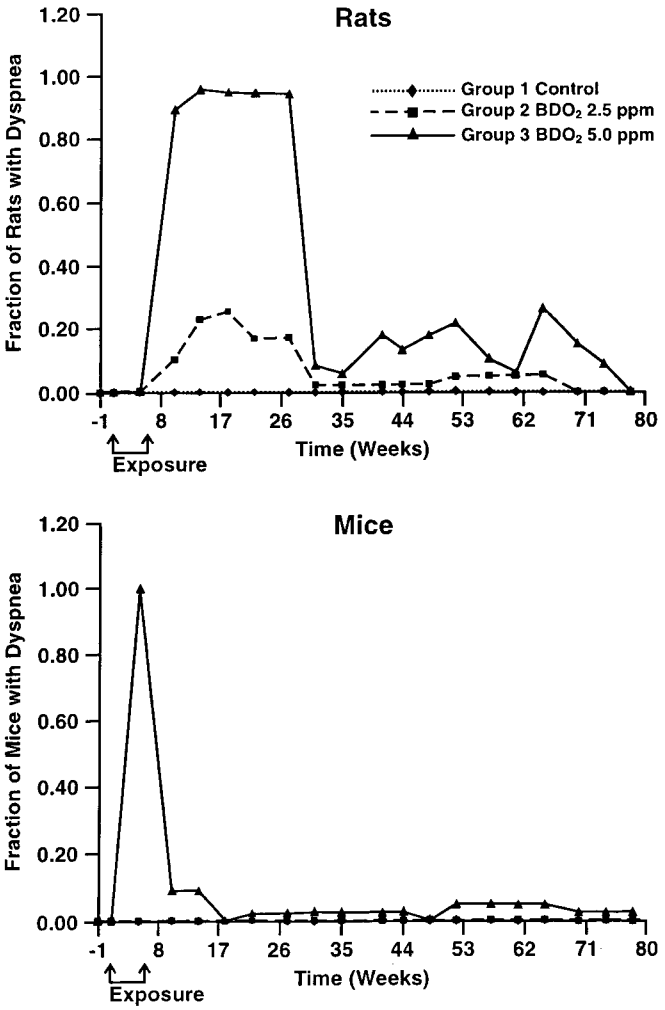

FIG. 2. Time course for observation of dyspnea in BDO_2-exposed rats and mice.

exposure group, with the control animals outliving the treated animals.

No rats died during the exposure, but 13 rats (12 exposed to 5.0 ppm, 1 exposed to 2.5 ppm) died within 3 months of the exposure. Deaths were due to blocked nasal passages caused by necrosis, inflammation, and squamous metaplasia of the nasal mucosa. As illustrated in Figure 4, exposure to 5.0 ppm BDO_2 was associated with significantly reduced survival of the rats, relative to either exposure to control air or 2.5 ppm BDO_2. The decrease in survival in rats exposed to 2.5 ppm BDO_2 was not significantly different from control when the Bonferroni adjustment was made.

Four of the mice exposed to the high level of BDO_2 died during the last week of exposure due to blocked nasal passages. After the end of the exposure, survival of mice was good in all groups. As indicated by the pair-wise comparisons with a Bonferroni correction (Fig. 4), there were no statistically significant differences in the survival of mice in the three exposure groups.

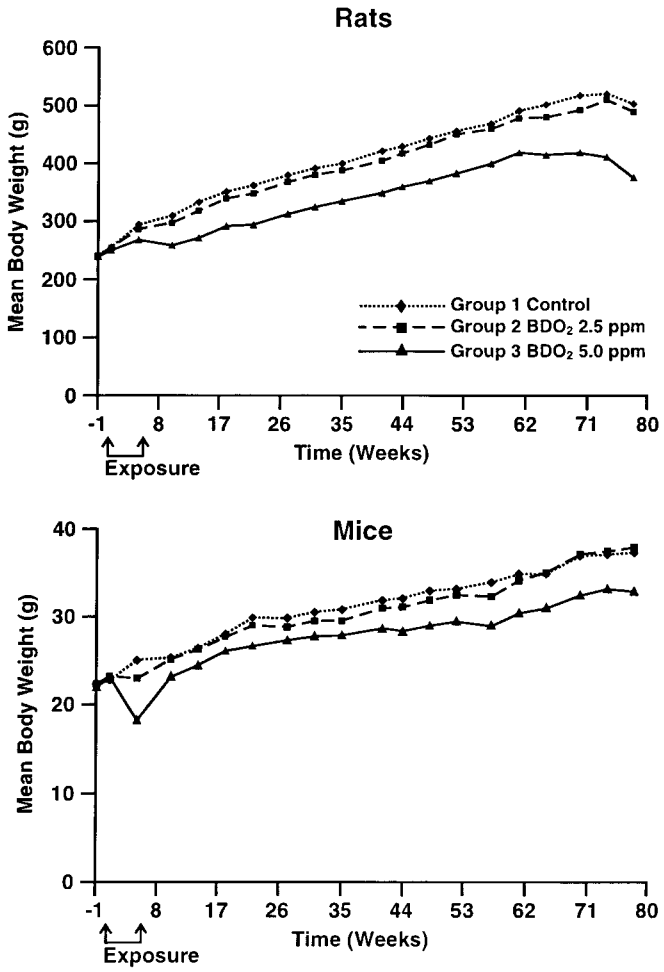

FIG. 3. Mean body weights of rats (upper graph) exposed for 6 weeks to BDO_2. By the end of the exposure, the body weights of rats exposed to 5.0 ppm BDO_2 were significantly depressed ($p < 0.01$) relative to the other two exposure groups and remained depressed at 10 and 30 weeks post exposure. Mean body weights of mice (lower graph) exposed for 6 weeks to BDO_2. By the end of the exposure, the body weights of mice exposed to 5.0 ppm BDO_2 were significantly depressed ($p < 0.01$) relative to the other two exposure groups and remained depressed at all subsequent time points.

Observations at Sacrifices

Hematology. Hematological parameters were measured at the end of the exposure and at the final sacrifices. There were no biologically significant alterations in standard hematological parameters in the exposed rats. Differences in total white blood cell counts were not statistically significant among the different exposure groups, but statistically significant concentration effects in mice were observed in the cell differential variables. Segmented neutrophils increased significantly in a dose-dependent fashion, whereas lymphocytes decreased in a dose-dependent fashion (Table 2). The increase in neutrophils may have been associated with the dose-dependent increase in nasal lesions in the mice (see section on nonneoplastic lesions in the mice).

Histopathology

Rats

Nonneoplastic lesions. The only exposure-related, nonneoplastic lesions in the rat were in the nasal mucosa. These lesions were centered around the main airflow pathway through the nose. The distributions of the lesions are noted (Fig. 5, end of exposure; and Fig. 6, >60 days after end of exposure). The

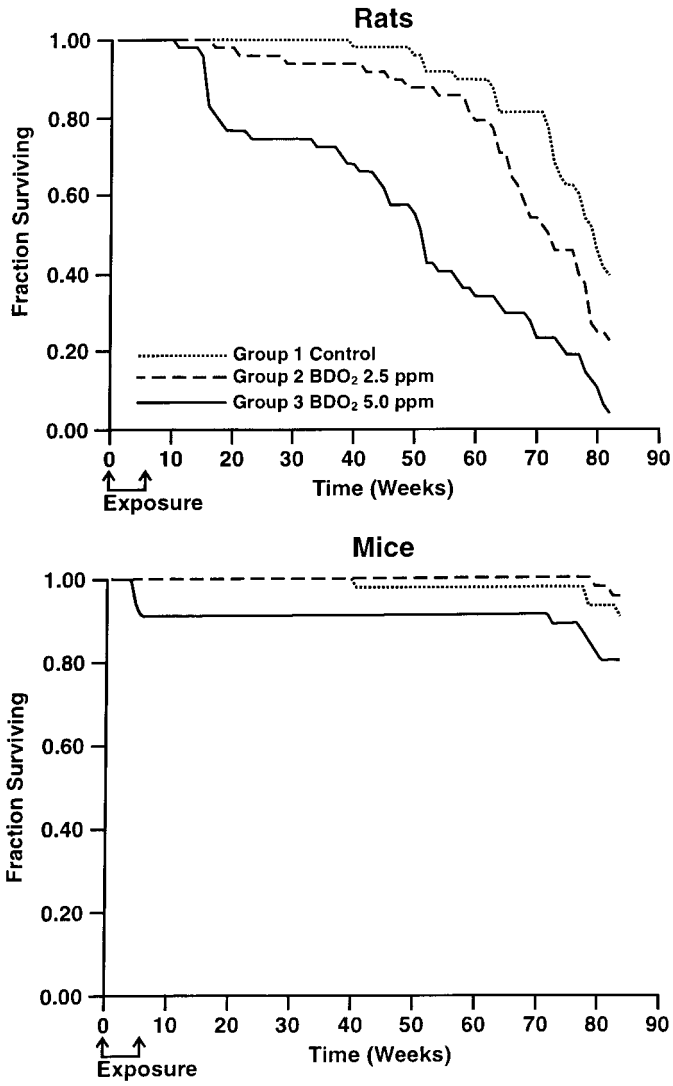

FIG. 4. Survival curves for rats (upper graph) held for long-term observation following a 6-week exposure to BDO_2. Mean survival times ± SE were 520 ± 10 days for controls, 479 ± 16 days for rats exposed to 2.5 ppm BDO_2, and 349 ± 24 days for rats exposed to 5.0 ppm BDO_2. There was no statistically significant difference in overall survival of rats exposed to clean air or 2.5 ppm BDO_2. Survival was decreased in rats exposed to 5.0 ppm relative to the other two groups ($p < 0.05$ with multiple comparison adjustment). Survival curves for mice (lower graph) held for long-term observation following a 6-week exposure to BDO_2. Mean survival times ± SE were 580 ± 8 days for control mice, 581 ± 1 days for mice exposed to 2.5 ppm BDO_2, and 516 ± 24 days for mice exposed to 5.0 ppm BDO_2. There was no statistically significant difference for any pairwise comparisons of the 3 groups.

TABLE 2
Mouse Hematology Data

Variable:	BDO_2 (ppm)	*n*	% White blood cells (least squares $\bar{x} \pm$ SE)[a]	Cell counts, $10^3/mm^3$ ($\bar{x} \pm$ SE)
Segmented neutrophils	0	46[b]	34.1 ± 1.9	59.9 ± 10.1
	2.5	50	43.3 ± 1.9*	77.6 ± 4.7
	5.0	44	52.5 ± 2.0*	103.1 ± 6.5*
Lymphocytes	0	46	61.1 ± 1.9	101.0 ± 17.3
	2.5	50	52.3 ± 1.9*	78.4 ± 5.3
	5.0	44	44.0 ± 1.9*	83.4 ± 5.5

Note. Data represent average of the 6-week and 18-month values.

[a] Statistical significance ($p < 0.05$) from uncorrected *t* tests is indicated by an asterisk. Comparisons are to controls (0 ppm BDO_2). Differences in total white blood cell counts were not statistically significant among the exposure groups.

[b] Two samples from this group were lost to follow-up.

extent and severity of the lesions varied with dose and time after exposure.

In the rats sacrificed at the end of exposure, lesions were found only in the nose. In the rats exposed to 5.0 ppm, the lesions were characterized by necrosis, inflammation, squamous metaplasia of the mucosa lining the anterior nasal cavity, and atrophy of the underlying maxilloturbinate and nasoturbinate bones (Table 3). In rats exposed to 2.5 ppm BDO_2, the lesions were similar, but much less severe and frequent.

In rats that were sacrificed moribund or died after the end of the 6-week exposure, marked squamous metaplasia and chronic inflammation characterized the nasal lesions and involved primarily the transitional and respiratory epithelia (Table 3). The lesions were more extensive and severe in the rats exposed to 5.0 ppm than in rats exposed to 2.5 ppm BDO_2, and more severe in rats sacrificed moribund than in rats sacrificed at the end of exposure.

A dose response was apparent for the inflammatory/meta-

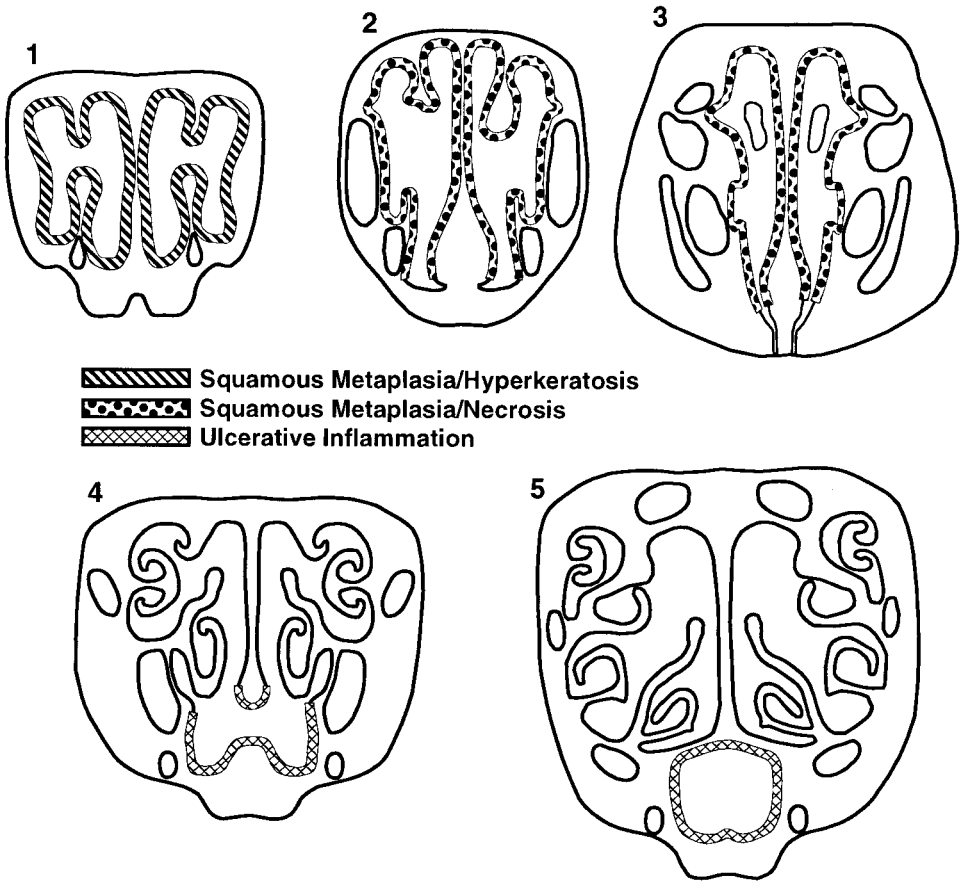

FIG. 5. Distribution of nasal lesions in rats at the end of exposure to BDO_2. The cross sections are those shown in Figure 1.

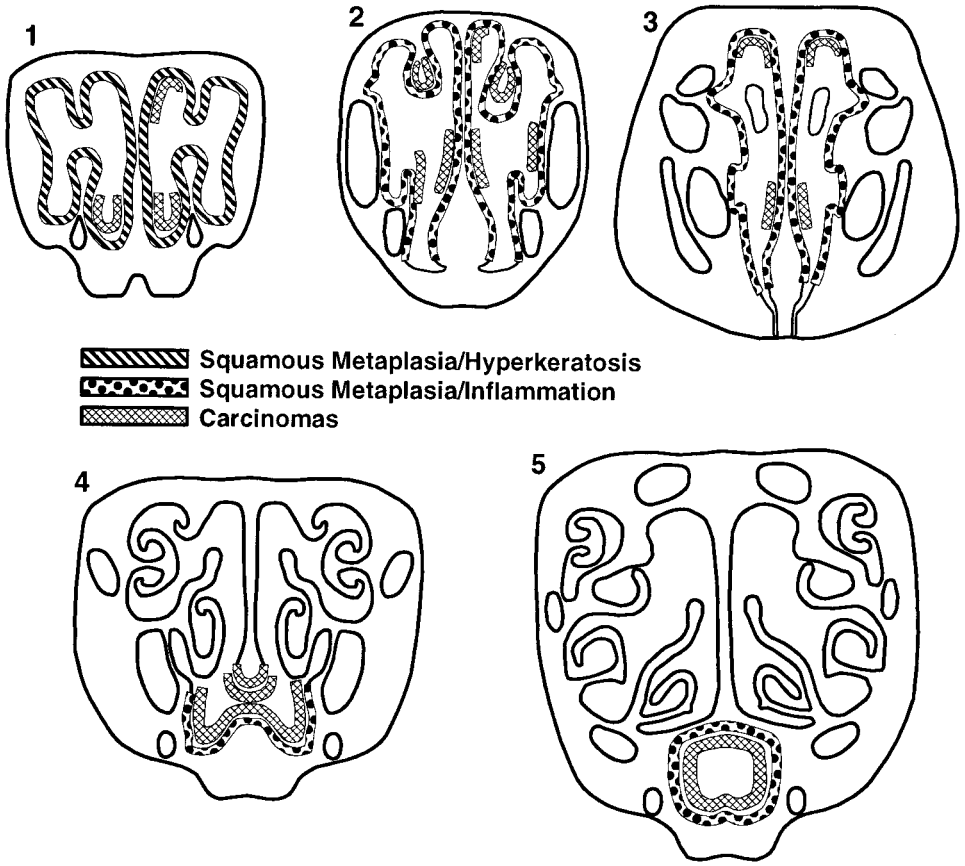

FIG. 6. Distribution of nasal lesions in rats more than 60 days after the end of exposure to BDO_2. The cross sections are those shown in Figure 1.

plastic lesions. They were more severe and extensive at the high concentration (Table 3). Moreover, after the end of the exposure period, these lesions persisted for the length of the study with a nearly consistent intensity. It was not easy to differentiate the morphologic appearance of lesions of those animals that died 300 days after exposure from those that died 582 days after exposure.

Neoplasia. The only significant increase in neoplasia in exposed rats was in the nasal mucosa. Of the 48 rats held for long-term study in the 5.0 ppm group, 21 had neoplasms of the nasal mucosa. Two rats in the high-exposure group had 2 malignant neoplasms each, and one rat had both a benign and a malignant neoplasm. In the 2.5-ppm group, 12 of 48 rats had similar neoplasms (Table 4).

Of the 12 rats with neoplasms following exposure to 2.5 ppm BDO_2, eight deaths were attributed to the lesions (22% of the total deaths in the group). The mean survival time of these eight rats was 403 ± 43 days (Table 4). In contrast, the mean survival time of all 48 rats exposed to 2.5 ppm BDO_2 was 479 ± 16 days. The percentage of rats with neoplastic lesions following exposure to 5.0 ppm BDO_2 (44%) was nearly double that of rats exposed to half the concentration (25%). The percent of fatal tumors, however, was similar in the 2 groups (22% and 26%).

The vast majority of the neoplasms arose in the respiratory epithelium of the nasal mucosa (Fig. 6). Of the 27 sites that could be identified, 23 were in the respiratory epithelium. Several sites of origin within the nasal cavity were identified: the epithelium lining the vestibule, the septum, the lateral wall, the nasoturbinate, and the nasopharyngeal duct. In 14 cases, the site of origin was the nasopharyngeal duct; in 7 cases, it was the septum. Three tumors arose in the vestibule and 3 in the nasoturbinates or lateral wall. In 9 cases, such a site could not be identified with certainty, but they did not involve the nasopharyngeal duct. Thus, the tumors were distributed throughout the nasal mucosa where there was respiratory epithelium along the main airflow pathway (Fig. 7).

The first death with a neoplasm of the nasal mucosa occurred 144 days after initiation of exposure and 102 days after cessation of exposure. The neoplasm was a squamous cell carcinoma of the epithelium surrounding the nasopharyngeal duct. The neoplasm had occluded the duct, resulting in the death. This site was the most frequent in rats that died within 9 months after exposure. Sites anterior in the nasal cavity (epithelium lining the septum, lateral wall, and nasoturbinates) dominated at longer times after exposure.

The most common neoplastic lesions were squamous cell carcinomas. These were associated with prominent squamous metaplasia and prominent chronic inflammation of the mucosa, especially in the mucosa lining the main airflow pathway

TABLE 3
Prevalence (%) of Nonneoplastic Lesions in Nasal Mucosa of Rats Exposed to BDO_2

	Exposure group		
	0 ppm	2.5 ppm	5.0 ppm
Sacrifice at end of exposure			
Number examined	8	8	8
Necrosis/nasal mucosa	0	38%	100%
Inflammation/nasal mucosa	0	63%	63%
Squamous metaplasia/nasal mucosa	0	25%	88%
Atrophy/turbinates	0	75%	100%
Unscheduled deaths			
Number examined	30[a]	37	46
Necrosis/nasal mucosa	0	0	11%
Inflammation/nasal mucosa	6.6%	84%	85%
Squamous metaplasia/nasal mucosa	0	92%	100%
Atrophy/turbinates	0	89%	93%
Final sacrifice			
Number examined	17	11	2
Necrosis/nasal mucosa	0	0	0
Inflammation/nasal mucosa	24%	100%	100%
Squamous metaplasia/nasal mucosa	0	100%	100%
Atrophy/turbinates	0	100%	100%

[a] Tissues from one rat listed in this group in Table 1 were lost to follow-up.

through the nose. One neoplasm, arising from the nasopharyngeal duct, was classified as an adenocarcinoma of the respiratory epithelium, and two were classified as sarcomas.

Mice

Nonneoplastic lesions. Few morphologic lesions were found in the 4 mice exposed to 5.0 ppm BDO_2 that died during the last week of exposure. A mild inflammation of the nasal mucosa was present in only 1 animal. Two mice had a reduced number of erythropoietic cells in the bone marrow. No conclusions could be made on the cause of death in any of these mice.

As in rats, the only exposure-related, nonneoplastic lesions found in mice at the end of exposure or later were in the nasal mucosa. These lesions were centered in the anterior portions of the nasal mucosa around the main airflow pathway through the nose (Fig. 8). However, the nasopharyngeal duct was not affected. The extent and severity of the lesions varied with dose and time after exposure. Lesions in mice sacrificed at the end of exposure to 5.0 ppm were characterized by necrosis and inflammation of the mucosa lining the anterior nasal cavity and by minimal atrophy of the underlying turbinate bones.

Neoplasia. Neoplastic lesions in mice are listed in Table 5. In general, the mice had only one neoplasm per animal; however, some animals did have multiple neoplasms in the same or in different organs. Based on individual organs or tissues, the number of observed lesions was generally too small to perform a meaningful statistical analysis. The exception to this was the Harderian gland, for which a contingency table was constructed. The chi-square test indicated a statistically significant effect associated with the high dose of BDO_2 ($p < 0.05$).

The one tumor in the nasal mucosa was a squamous cell carcinoma *in situ*. The neoplasm arose from a field of squa-

TABLE 4
Prevalence of Metaplastic and Neoplastic Lesions in Nasal Mucosa of Rats Exposed to BDO_2[a]

Exposure group	0 ppm	2.5 ppm	5.0 ppm
Number examined	47	48	48
Total primary neoplasms	0	12	24
Rats with one or more	0	12	21
Percent with one or more	0	25%	44%
Total benign neoplasms	0	0	1
Squamous papilloma (%)	0	0	4.2%
Total malignant neoplasms	0	12	23
Squamous cell carcinoma (%)	0	23%	44%
Adenocarcinoma (%)	0	0	4.2%
Sarcoma (%)	0	4.2%	4.2%
Total squamous metaplasia	0	45	48
Percent with squamous metaplasia	0	94%	100%
Total squamous cyst	0	1	0
Percent with squamous cyst	0	4.2%	0
Animals with fatal tumors	0	8	12
Percent fatal tumors	0	22%	26%
Mean survival of rats with fatal tumors (days)	—	403 ± 43	420 ± 31
Mean survival of all rats	520 ± 10	479 ± 16	349 ± 24

[a] Based on unscheduled deaths and final sacrifice.

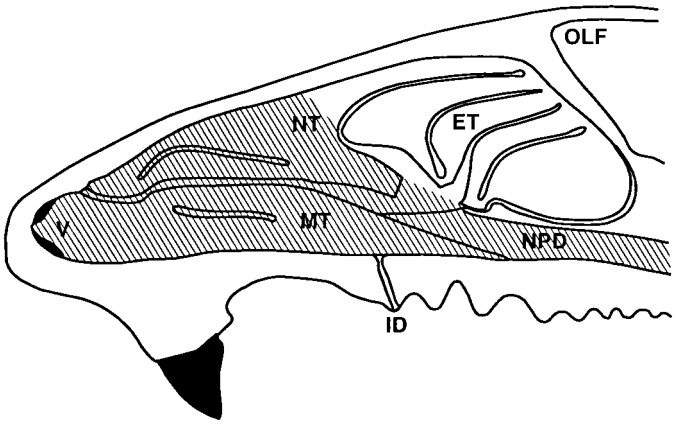

FIG. 7. Distribution of lesions of the nasal mucosa in rats exposed to BDO_2. V, vestibule; NT, nasoturbinate; MT, maxilloturbinate; ID, incisive duct; ET, ethmoturbinate; NPD, nasopharyngeal duct; OLF, olfactory bulb. Cross-hatching indicates area of lesions.

mous metaplasia on the lateral wall of the nasal cavity normally lined with transitional nasal epithelium.

The Harderian gland tumors were all adenomas. They appeared to arise *de novo*, not from areas of pre-existing lesions as was the case for the tumors in the nasal epithelium.

DISCUSSION

General

The results of the study were contrary to what was predicted. The mice were expected to have at least as many tumors induced by inhalation exposure to BDO_2 as the rats, but that was not the case. Despite indications from preliminary studies that mice received approximately twice the dose of BDO_2 to blood and lungs, as did rats (Henderson *et al.*, in press), the mice did not develop the number of nasal tumors observed in rats.

The daily dose of BDO_2 in the lungs of the exposed rats and mice can be estimated from earlier studies. The original intent was to expose rodents to enough BDO_2 to induce lung tumors, but not lymphomas, which cause premature deaths in mice. The highest exposure to BD that caused tumors in mice but not lymphomas was the repeated exposure to 62.5 ppm BD (Melnick *et al.*, 1990). According to Thornton-Manning *et al.* (1995a,b), a 4-h exposure of mice to 62.5 ppm BD results in lung concentrations of BDO_2 equal to 114 ± 37 pmol/g tissue. The data indicated that the level of BDO_2 in lung increased linearly during the 4-h exposure. If one extrapolates linearly, one can estimate that the lung levels of BDO_2 would be approximately 180 pmol/g lung after a 6-h exposure to 62.5 ppm BD.

Preliminary dosimetry studies indicated that a 6-h exposure to 12-ppm BDO_2 would yield a level of BDO_2 in the lung of 2900 pmol/g tissue (Henderson *et al.*, 1999). If the total pmol/day/g tissue of BDO_2 dose to lung in the chronic exposure ($\sim$520 days) is calculated, the estimated integrated dose is 93,600 pmol/day/g of BDO_2. This can be compared to the total dose of BDO_2 to lung in the current study of 36,250 pmol/day in mice exposed for 6 weeks to BDO_2. Thus, the BDO_2-exposed mice received approximately 40% (5.0 ppm) or 20% (2.5 ppm) of the BDO_2 dose received by the lungs of mice in chronic exposures to 62.5 ppm BD. Considering also that lung tumors were observed in mice inhaling only 6.25 ppm BD in the chronic studies (Melnick *et al.*, 1990), the dose to the lung in the BDO_2-exposed mice was predicted to be within the range of BDO_2 doses received by BD-exposed mice that developed lung tumors.

It should be noted that the above estimates are based on single exposures, and the extrapolation to repeated exposures may not be linear. Repeated exposures might induce enzymes that lead either to increases or decreases in the lung BDO_2 levels. Note, too, that the time course of the rise in lung levels of BDO_2 in mice exposed directly to BDO_2 may be more rapid than the rise in lung levels of BDO_2 in mice exposed to BD (due to time required to metabolize BD to BDO_2). If this is so, our calculations would underestimate the relative lung dose of BDO_2 received by the rodents exposed directly to BDO_2 compared to rodents exposed to BD. Thus, one must consider the estimates of dose as "ballpark" figures, rather than being precise.

Even if the estimate of the absolute dose received by the rodents had a good deal of uncertainty associated with it, it is clear from the preliminary studies that the respiratory tract and blood of the mice had higher concentrations of BDO_2 than did those in the rats (approximately 2-fold). The dose received by the mice was sufficient to cause a decrease in blood lymphocytes. Yet, upper respiratory tract tumors occurred to a much higher degree in the rats than the mice. One may conclude that the epithelial tissues of the upper respiratory tract of the rat are sensitive enough to the tumorigenic activity of BDO_2 that, if

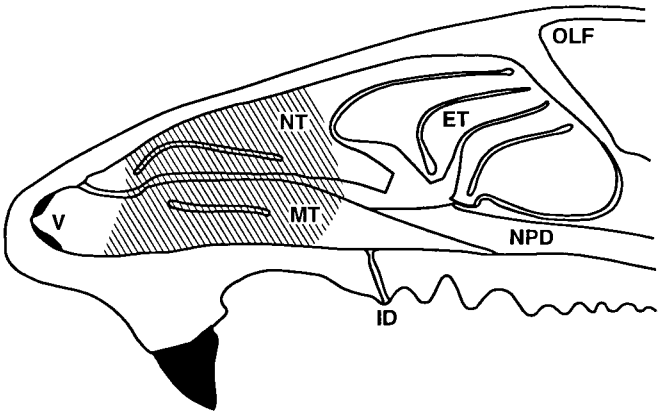

FIG. 8. Distribution of lesions of the nasal mucosa in mice exposed to BDO_2. V, vestibule; NT, nasoturbinate; MT, maxilloturbinate; ID, incisive duct; ET, ethmoturbinate; NPD, nasopharyngeal duct; OLF, olfactory bulb. Cross hatching indicates area of lesions.

TABLE 5
Neoplasms in Mice Exposed to BDO_2 (Number Observed/Number Examined)

Organ	Exposure Groups		
	0 ppm	2.5 ppm	5.0 ppm
Final sacrifice			
Nasal mucosa, malignant	0/40	0/42	1/36
Harderian gland, benign	0/40	2/42	5/36
Ovary, benign	0/40	0/41	2/36
Lung, benign	1/40	1/42	4/36
Lung, malignant	2/40	1/42	0/36
Uterus, benign	0/40	0/42	1/36
Femur, malignant	0/40	1/42	0/36
Lymphoma, malignant	2/40	1/42	2/36
Liver, benign	2/40	1/42	1/36
Liver, malignant	1/40	1/42	0/36
Mammary gland, malignant	0/40	0/42	0/36
Pancreas, benign	0/40	0/42	1/36
Pancreas, malignant	1/40	0/42	0/36
Unscheduled deaths			
Nasal mucosa, malignant	0/4	0/2	0/3
Harderian gland, benign	0/4	0/2	0/8
Ovary, benign	0/3	0/2	0/6
Lung, benign	0/4	0/2	0/8
Uterus, benign	0/4	0/2	0/6
Femur, malignant	1/4	0/2	0/7
Lymphoma, malignant	0/4	1/2	1/8
Liver, benign	0/4	0/2	0/7
Mammary gland, malignant	1/3	0/2	0/8
Pancreas, benign	0/4	0/2	0/7

Note. Neoplasms in protocol tissues are not listed. Sometimes tissues were lost to follow-up.

the metabolite were present *in vivo* following BD exposures, one would expect to see tumors. Thus, the minimal tumorigenic response of rats to BD inhalation is not due to lack of sensitivity to BDO_2.

The question remains as to why the mice, which are exquisitely sensitive to inhaled BD, did not develop respiratory tract tumors after exposure to BDO_2. One explanation might be that the dose was not high enough. The calculated estimates of dose suggest that it should have been sufficient, and the exposure was sufficient to cause portal-of-entry tumors in rats. A more likely explanation is that the site of formation of BDO_2 within the cell may be important for its tumorigenic action. The transport of externally administered BDO_2 into the cell, then on to the critical sites on the DNA, may allow detoxification of the molecule before it reaches the target site. Thus, the biologically effective dose may be greatly diminished by administering the compound externally to the cell compared with the metabolic formation of the compound within the cell.

In support of this hypothesis is the observation that glutathione transferase (GST) activities, which lead to detoxication of the diepoxide, are much higher in the cytosol of mouse tissues than in rat tissues (Boogaard *et al.*, 1996). The externally administered diepoxide would have to pass through the cytosol containing the GST before reaching the target DNA molecules in the nucleus. The GST activity in the cytosol would be expected to detoxicate the diepoxide to a greater extent in mice than in rats, which would lead to a greater tumor response in rats.

One aspect of the exposure that must be considered is the stereochemical form of the diepoxide used in the exposures versus the form of the diepoxide produced by metabolism of BD *in vivo*. Studies by Nieusma *et al.* (1997) using hepatic microsomes indicate that mouse microsomes produce slightly more S-BDO than R-BDO and produce significantly more BDO_2 from S-BDO than from R-BDO. R-BDO is more toxic toward rat hepatocytes than S-BDO, and meso-BDO_2 is the most toxic BDO_2 enantiomer. The same investigators (Nieusma *et al.*, 1998) reported that R-BDO depletes cytosolic GSH in isolated rat hepatocytes faster than does S-BDO and that SS- and meso-BDO_2 deplete cytosolic GSH faster than the RR-BDO_2. Krause and Elfarra (1997) reported that human cytochrome P4502E1 preferentially forms meso-BDO_2 from BDO, and that the meso-BDO_2 is preferentially hydrolyzed by human liver microsomes. However, in BD inhalation studies (exposures to 0, 20, 62.5, or 625 ppm BD, 6 h/day, 5 days/week for 4 weeks) reported by Koc *et al.* (in press), mice and rats formed the same amount of the racemic trihydroxybutane adduct on N-7-guanine as did the meso form of the adduct. The source of those adducts would be either the butadiene diepoxide or its hydrolysis product, the 1,2-dihydroxy-3,4-epoxybutane; the data indicate that most of the adduct comes from the epoxy diol. In similar studies conducted by Oe *et al.* (in press) on mice and rats exposed to BD at a higher dose rate (1250 ppm, 6 h/day, 5 days/week for 2 weeks), the amount of the racemic form of the adduct was higher than the meso form in both species. Thus, the data from two studies indicate that there is not a preferential formation of meso-BDO_2 *in vivo* in mice and rats.

Histopathology

The neoplastic and nonneoplastic lesions induced in rats by BDO_2 were distributed throughout the nasal mucosa, from the vestibule in the anterior portion of the nasal cavity to the nasopharyngeal duct in the posterior portion. Only the olfactory epithelium was spared. This was not unexpected, because metabolism may not be required for BDO_2 to be toxic or carcinogenic, and the high enzymatic activity of the olfactory tissue may have hydrolyzed the BDO_2.

The distribution of lesions covers much more area than other nasal toxicants or carcinogens and may be related to the high-exposure concentration. A lower concentration may give a more restricted distribution pattern of lesions, as was observed for formaldehyde (Morgan, 1997). On the other hand, the distribution of BDO_2 lesions may be the result of characteristics related to its chemical nature. For example, exposure of

rats to formaldehyde results in inflammatory lesions and carcinomas of the respiratory epithelium lining the lateral meatus and nasal septum in the anterior nasal passages (Morgan *et al.*, 1986). Exposure of rats to ozone results in inflammatory lesions of the transitional epithelium lining the lateral wall of the anterior nasal passages (Johnson *et al.*, 1990). Formaldehyde and ozone probably act as direct genotoxic agents or nasal irritants. The location of the lesions may relate to the relatively soluble nature of these agents, with most of the dose being delivered to the anterior portion of the nasal cavity.

The site specificity of nasal lesions has been noted (Morgan and Monticello, 1990; Morgan, 1997). The wide distribution of lesions in the nasal mucosa after inhalation of BDO_2 is somewhat similar to that of nasal lesions in rats after inhaling alkylating agents (Sellakumar *et al.*, 1987). Like BDO_2, alkylating agents are direct acting carcinogens. A study of five alkylating agents with differing hydrolysis rates showed two agents, beta propriolactone and methlymethane sulfonate, produced high rates of nasal neoplasms. The lesions and neoplasms were site-specific, involving primarily the respiratory and transitional epithelium of the anterior portion of the nasal cavity. As with BDO_2-exposed rats, no direct compound-related lesions were seen in the olfactory epithelium of the posterior region of the nasal cavity. The involved epithelium showed necrosis, ulceration, and acute inflammation. In some cases, squamous metaplasia and dysplastic lesions led to carcinogenesis. The carcinomas were primarily squamous, involving the nasomaxillary turbinates, septum, and lateral walls. Even with these potent carcinogens, however, lesions and neoplasms were not seen in the nasopharyngeal duct.

The fact that BDO_2 induced a much higher prevalence of nasal tumors in rats than in mice is consistent with reports that rats are more susceptible to the induction of epithelial tumors in the nasal cavity than are mice (Brown *et al.*, 1991). The induction of squamous cell carcinomas in the nasal cavity of rats by another irritant, formaldehyde, has been well-described (Swenberg *et al.*, 1980). As in the current study, squamous metaplasia developed in the nasal cavities of both rats and mice when both were exposed to formaldehyde for 24 months, but squamous-cell carcinomas developed almost exclusively in the rats (Kerns *et al.*, 1983).

In contrast to the current study, nonneoplastic lesions in the nasal cavity of both the rats and the mice regressed after the exposures to formaldehyde ended. Regression of nasal lesions after stopping exposure to an irritant was also observed in rats exposed to tobacco smoke (Maples *et al.*, 1993). However, in the current study, the BDO_2-induced nasal lesions regressed in the mouse but not in the rat. In fact, the nasal lesions in the rat worsened with time after exposure. The basis for this difference in progression of nasal lesions from metaplasia to neoplasia in the two species could be the subject of an important follow-up study.

The squamous tumors arose from squamous metaplasia in the nasal mucosa of rats. Stages leading from well-differentiated squamous metaplasia to dysplasia to anaplasia were seen in tissue sections. Chronic inflammation was invariably associated with the metaplastic lesions. It is tempting to speculate that the initial exposure to BDO_2 resulted in genetic changes in the exposed epithelium and the subsequent inflammation and that the associated increased cell proliferation promoted the initiated cells. This scenario is similar to that proposed for the induction of nasal tumors by formaldehyde (Monticello and Morgan, 1994).

In summary, the results of this study indicate that BDO_2, a metabolite of BD, is carcinogenic in the upper respiratory tract of exposed rats. An increase in respiratory tract tumors was not observed in similarly exposed mice, despite the fact that preliminary studies indicated mice should have received twice the dose to tissue compared to rats. Detoxication of BDO_2 by the GST activity in cytosol, which is higher in mouse cells than rat cells, may explain, in part, the species differences in response to inhaled BDO_2. The results indicate the importance of the BDO_2 formation site in the expression of its carcinogenicity.

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