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SUBCHRONIC INHALATION TOXICITY OF ISOBUTYL NITRITE IN BALB/c MICE. I. SYSTEMIC TOXICITY

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The effects of subchronic inhalation exposure to isobutyl nitrite (IBN) on body weight, selected organ weights, hematology, and gross pathology and histopathology of BALB/c mice were evaluated. Mice of both sexes were exposed at 0, 20, 50, or 300 ppm IBN for 6.5 h/d, 5 d/wk for up to 18 wk. Most changes in measured indices occurred in mice exposed at 300 ppm IBN and included decreased thymus weight (females); decreased liver weight (males); decreased white blood cell counts (males); mild focal hyperplasia and vacuolization of the epithelium lining bronchi and bronchioles of the lungs (males and females). Organ weight and hematologic changes, however, were not accompanied by any observed histologic changes. In addition, elevated methemoglobin concentrations were detected in mice of both sexes exposed at 50 and 300 ppm IBN. Body weights were not adversely affected by exposure. These data suggest that mild tissue injury, restricted to the lung, and methemoglobinemia are the major toxic effects observed following exposures of mice to IBN at concentrations up to 300 ppm for 18 wk. No treatment-related effects were noted in mice exposed at 20 or 50 ppm IBN, except for slight elevations in methemoglobin concentrations in mice exposed at 50 ppm.

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INTRODUCTION

Reports of the abuse or misuse of organic nitrites by drug addicts, recreational drug users, and in particular, male homosexuals have recently appeared in the literature (Israelstram et al., 1978a,b; Dixon et al., 1981; Sigell et al., 1978). Suspicion has also been raised concerning a possible role of nitrites as immunosuppressive agents involved in the development of the acquired immunodeficiency syndrome (AIDS) (Goedert et al., 1982). Following these reports, the National Institute for Occupational Safety and Health was requested by the Centers for Disease Control to evaluate the immunotoxic potential of inhaled nitrites. Isobutyl nitrite (IBN) was selected since it was one of the more commonly abused nitrites (Dixon et al., 1981), it was commercially available, and it had relevance to occupational health with documented workplace exposures (Nickerson et al., 1979).

The intraperitoneal and oral LD50 values of IBN in adult male Swiss-Webster mice are 184 and 205 mg/kg, respectively (Maickel and McFadden, 1979; McFadden and Maickel, 1982). Quinto (1980) reported that IBN was a direct-acting mutagen in the Ames *Salmonella* microsome assay. Acute exposures of humans (Wason et al., 1980; Dixon et al., 1981; Shesser et al., 1981) and of mice (McFadden et al., 1981) have been associated with increased blood methemoglobin concentrations. Acute inhalation exposures have also been reported to produce pulmonary edema and tracheobronchitis (Shesser et al., 1981; Covalla et al., 1981). Hersh et al. (1983) reported that IBN had nonspecific cytotoxic activity for various cells in vitro. Haley (1980) pointed out the lack of toxicity data on IBN and the need for further study research.

Since there was a general lack of toxicologic data on organic nitrites and on IBN in particular, the subchronic inhalation toxicity of IBN was evaluated concurrently with the immunotoxicity assessment. Exposures were selected to mimic an occupational exposure setting in order to fill this gap in the existing literature, rather than to conduct brief, acute, high-level exposures to imitate exposure by nitrite abusers.

The purpose of this report is to present the toxicologic results of subchronic exposures of BALB/c mice to inhaled IBN. Results of the immunotoxicity evaluation of IBN are presented in the following paper (Lewis et al., 1985).

MATERIALS AND METHODS

Animals

Male and female BALB/c mice (BALB/cAnNCrlBR) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and maintained on a diet of NIH 07 pellets (Ziegler Bros., Gardner, Penn.). Four hundred mice, 200/sex, were distributed so that the control and 50-ppm groups contained 70 mice/sex and the 20- and 300-ppm groups contained 30 mice/sex. Extra mice were allocated to the control and 50

ppm groups for interim immunotoxicity evaluations following 3 and 7 wk of exposure, respectively. Mice were 10 wk of age (17-18 g) at the beginning of exposures and were selected for their usefulness in immunotoxicity testing. Mice were screened for murine viruses and *Mycoplasma pulmonis* during a 2-wk quarantine period. All findings were negative, as was a screening for cytomegalovirus at the end of the 13-wk exposure. Mice were individually identified using numbered ear tags (National Band and Tag Co., Newport, Ky.) and randomly assigned to treatment groups using a computer generated list of random numbers. Both exposed and control mice were housed two per compartment in stainless-steel wire mesh cages. Location of the cages in the chambers was systematically rotated on a weekly basis. Mice were housed in the exposure chambers continuously throughout the experiment, given food and water ad libitum except during the exposure periods, and maintained on a 12 h on/12 h off lighting schedule, with lights on from 7 a.m. to 7 p.m.

Test Chemical

IBN was obtained from Frank Enterprises, Inc. (Columbus, Ohio) as a single lot (number 188) with a stated chemical purity of 97%. Analysis of the purity of the IBN by gas chromatography/mass spectroscopy following completion of exposures suggested that some decomposition had occurred. The purity of two IBN samples was estimated to be 88 and 93%, respectively, with isobutanol concentrations of 2.4-3.1%, isobutyl isobutyrate concentrations of 1.9-3.9%, plus trace amounts of isobutyric acid and isobutyl nitrate.

Exposures

Mice were exposed to IBN vapors at airborne concentrations of 20, 50, or 300 ppm for 6.5 h/d (pump on to pump off), 5 d/wk, for up to 18 wk. Concentrations were selected based on results of 3-wk pilot studies, with the initial highest IBN exposure concentration of 200 ppm. This concentration was increased to 300 ppm after 20 d of exposure, since anticipated mortality and body weight declines were not observed in mice exposed at 200 ppm. Exposures were conducted in 4.5-m^3 stainless-steel and glass inhalation chambers (Hinners et al., 1968) fabricated by Skillcraft Sheetmetal Inc., Burlington, Ky. Chambers were operated under dynamic flow conditions at a chamber pressure of −0.254 cm of water relative to ambient. Tangential airfeed manifolds were maintained at 1.1 m^3/min (i.e., 12-15 air changes/h). Temperature and humidity were monitored hourly using a temperature/humidity indicator (Weather Measure Corp. model HT-100R, Sacramento, Calif.) and were maintained at 23 ± 3°C and 50 ± 10%, respectively. IBN exposure atmospheres were generated by pumping liquid IBN directly into the tangential filtered air intake (Fluid Metering Pump Model RPG-50, Fluid Metering, Inc., Oyster Bay, N.Y.) and diluting with filtered chamber air to the desired concentrations. IBN chamber concentrations were monitored

hourly using a Miran 1A infrared analyzer (Foxboro-Wilkes, Norwalk, Conn.), using the following instrument settings: wavelength 3.4 μm, pathlength 2.75 m, slit 1 mm. The instrument was calibrated by the closed-loop calibration method. Chamber atmospheres were monitored at least once per hour, with adjustments made to the generation systems as required to maintain the exposure concentrations at targeted levels.

Clinical Observations/Sacrifice Schedule

Individual animal body weights for test and control groups were recorded weekly. Mice were individually observed twice per day, once in the morning and once in the afternoon, for clinical signs, morbidity, and mortality. Fifteen mice/sex/group were sacrificed following 13 wk of exposure. In addition, 15 mice/sex exposed at 50 ppm were killed for immunocompetency testing after 3, 7, and 13 wk of exposure, while those exposed at 300 ppm were tested after 13 and 18 wk of exposure. These tests are discussed and results reported in the following paper (Lewis et al., 1985).

Clinical Studies

Hematologic indices including red blood cells (RBC), white blood cells (WBC), and differential counts were evaluated at each scheduled sacrifice. Hemoglobin and methemoglobin measurements were made at the 13-wk sacrifice using the method reported by Passannante and Gerarde (1966). Blood for hemoglobin/methemoglobin measurements was collected within 30 min of the end of the exposure day and analyzed within 30 min. Methemoglobin determinations were not made on mice exposed at 20 ppm IBN. In all cases blood was collected from the inferior vena cava of anesthetized mice.

Gross Pathology and Histopathology

A complete necropsy was performed on all mice which died or were killed (sodium pentobarbital, ip). Liver, spleen and thymus were weighed at each sacrifice. Ratios of organ to body weight were calculated for each animal. Complete gross examinations were performed on all mice, and the following tissues were fixed in 10% buffered formalin for histopathological evaluation: lungs, trachea, tracheobronchial lymph nodes, heart, thyroid, parathyroid, thymus, mesenteric lymph nodes, kidneys, liver, gall bladder, spleen, bone, bone marrow (femur), ileum with Peyer's patch, ovary, uterus, testes, epididymis, and adrenal gland. Tissues were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined microscopically.

Statistical Evaluation

For the body-weight data, a multivariate analysis of variance of weights versus week, using initial weight as a covariate, was used to

assess group differences. Body weights at sacrifice were compared using analysis of variance. Absolute and relative organ weights and hematology data were analyzed, across groups, using the Kruskal-Wallis test. If significant group differences were identified, pairwise differences were tested maintaining an experimentwise error rate of $\alpha = 0.05$.

RESULTS

Exposures

The actual chamber concentrations were close to the targeted concentrations with little day-to-day variation, except for the 300 ppm chamber. Chamber concentrations for the IBN exposure groups were 20.1 ± 0.5, 50.1 ± 1.0, and 296.8 ± 65.7 ppm (mean $\pm$ standard deviation), respectively.

Clinical Observations

Mice in all treatment groups tolerated the IBN exposures well. Two male mice and 1 female mouse exposed at 300 ppm IBN died during the 13-wk exposure period, but these deaths appeared to be due to dehydration and not related to IBN exposure. The mean body weight of the 50-ppm mice was significantly higher than the body weight of the controls ($p < 0.05$), although the actual differences in body weight were small (0.3 g for males, 1.6 g for females). Since no other differences in body weights between groups were observed, this finding was not considered toxicologically significant.

Clinical Studies

No differences were noted in RBC, hemoglobin, or differential counts of mice exposed to IBN. WBC counts of male mice exposed at 300 ppm were significantly reduced ($p < 0.05$) compared to controls (Table 1). Methemoglobin was detected in the blood of control and exposed mice. The highest methemoglobin level found in control mice was 2.2%, while methemoglobin levels in mice exposed at 300 ppm IBN reached 9.6%. The incidence of methemoglobinemia also increased with increasing IBN exposure.

Gross Pathology and Histopathology

No gross abnormalities were noted in any of the mice exposed to IBN nor in the controls. Organ weight data obtained from the 13 wk sacrifice are presented in Table 2. Similar data were obtained following 18 wk of exposure. Absolute and relative mean spleen weights from mice of both sexes exposed at 20 ppm IBN were statistically reduced compared to controls ($p < 0.05$); however, this effect was not observed in mice exposed at higher concentrations. Both absolute and relative thymus weights (females) and liver weights (males) were statistically reduced in mice exposed at 300 ppm IBN ($p < 0.05$) compared to controls.

TABLE 1. Hematologic Indices[a] in Groups of BALB/c Mice Intermittently Exposed to Inhaled Isobutyl Nitrite for 13 Weeks

Exposure concentration (ppm)	Male mice				Female mice			
	RBC	WBC	Hemoglobin	Methemoglobin (%)	RBC	WBC	Hemoglobin	Methemoglobin (%)
0	9.01 ± 1.12 (7.07-10.40) 11	6.43 ± 1.86 (3.80-9.10) 11	16.0 ± 0.4 (15.4-16.5) 5	0/5[b] (ND[c]-2.2) 5	9.64 ± 1.13 (7.85-11.60) 9	5.38 ± 3.01 (2.70-12.80) 9	16.3 ± 1.1 (15.4-18.1) 5	0/5 (ND-2.2) 5
20	9.86 ± 1.11 (7.48-11.80) 16	5.23 ± 1.92 (2.80-11.20) 16		[d]	11.17 ± 1.93 (8.89-15.40) 14	4.86 ± 1.28 (3.00-7.10) 14	—	—
50	10.53 ± 1.46 (9.19-14.10) 10	7.95 ± 2.25 (5.00-12.30) 10	15.9 ± 1.1 (15.0-17.5) 5	2/5 (ND-4.7) 5	9.97 ± 0.83 (8.77-11.80) 10	4.41 ± 2.91 (1.70-11.90) 10	15.8 ± 0.3 (15.4-16.0) 5	1/5 (ND-2.3) 5
300	9.49 ± 0.79 (7.71-10.30) 10	2.36 ± 0.81[e] (1.30-3.70) 9	15.5 ± 1.3 (13.5-16.5) 5	5/5 (4.8-9.6) 5	8.96 ± 0.92 (7.93-10.70) 10	5.46 ± 1.73 (3.10-8.80) 10	15.9 ± 0.4 (15.3-16.4) 5	3/5 (ND-8.3) 5

[a] Mean ± SD, with range in parentheses, and number of animals.
[b] Number of mice with detectable methemoglobin concentrations/number of mice examined.
[c] Below limits of detection.
[d] Not determined.
[e] Statistically significant difference versus controls, $p < 0.05$.

TABLE 2. Body Weights and Absolute and Relative Organ Weights[a] in Groups of BALB/c Mice Intermittently Exposed to Inhaled Isobutyl Nitrite for 13 Weeks

Exposure concentration (ppm)	Body weight (g)	Liver		Spleen		Thymus	
		g	g Body weight × 1000	mg	mg Body weight × 1000	mg	mg Body weight × 1000
				Male mice			
0	31.7 ± 2.1 (25.5-35.2)	2.14 ± 0.24 (1.85-2.67)	6.9 ± 0.6 (5.9-8.0)	199.5 ± 60.0 (99.0-340.0)	632.9 ± 183.2 (336.7-1011.9)	40.1 ± 8.5 (25.0-57.0)	128.9 ± 26.1 (86.2-180.3)
20	30.9±2.6 (24.7-34.8)	2.13 ± 0.24 (1.49-2.52)	6.7 ± 0.5 (6.1-7.5)	136.3 ± 22.0[b] (99.0-183.0)	434.2 ± 90.8[b] (284.5-627.1)	42.4 ± 11.1 (31.0-63.0)	133.4 ± 30.7 (94.8-178.0)
50	32.0 ± 2.1 (27.9-36.1)	2.23 ± 0.30 (1.73-2.76)	6.9 ± 0.4 (6.3-7.5)	176.5 ± 32.7 (114.0-240.0)	547.4 ± 67.1 (395.8-666.7)	44.1 ± 10.9 (24.0-61.0)	136.6 ± 28.0 (83.3-184.3)
300	31.4 ± 2.1 (25.3-34.7)	1.94 ± 0.20[b] (1.56-2.22)	6.3 ± 0.4[b] (5.6-6.8)	197.6 ± 42.4 (134.0-290.0)	635.4 ± 122.2 (460.6-920.6)	38.9 ± 7.7 (24.0-50.0)	126.7 ± 19.3 (92.3-158.5)
				Female mice			
0	24.9 ± 1.4 (22.5-28.6)	1.56 ± 0.15 (1.30-1.79)	6.4 ± 0.4 (5.7-6.9)	186.3 ± 38.9 (113.0-247.0)	749.8 ± 133.1 (491.3-968.6)	45.2 ± 11.4 (20.0-65.0)	184.6 ± 45.2 (87.0-261.0)
20	26.2 ± 1.5 (23.8-32.3)	1.66 ± 0.13 (1.48-1.94)	6.4 ± 0.5 (5.7-7.5)	151.2 ± 18.1[b] (114.0-184.0)	585.2 ± 69.8[b] (461.5-707.7)	41.7 ± 7.7 (32.0-53.0)	161.9 ± 31.7 (114.7-207.0)
50	26.4 ± 1.5 (23.7-29.1)	1.65 ± 0.15 (1.41-1.84)	6.5 ± 0.4 (5.5-7.2)	191.9 ± 32.8 (142.0-253.0)	763.2 ± 134.0 (563.5-984.4)	38.3 ± 10.3 (22.0-52.0)	150.8 ± 35.6 (92.4-206.4)
300	25.4 ± 1.5 (22.1-29.7)	1.53 ± 0.17 (1.20-1.76)	6.2 ± 0.5 (5.2-7.1)	231.3 ± 57.5 (146.0-390.0)	939.5 ± 244.1 (595.9-1673.8)	33.0 ± 9.0[b] (21.0-53.0)	134.5 ± 35.6[b] (89.6-210.3)

[a]Mean ± SD. There were 15 mice/sex/group. Range is given in parentheses.
[b]Statistically significant difference versus controls, $p < 0.05$.

Generally there was little or no difference in the histologic findings between control and exposed mice. A number and variety of spontaneous lesions were present in mice from each of these groups. The most commonly observed lesions, in order of decreasing frequency, included epicardial mineralization in the right ventricle, peribronchial lymphoid accumulations in the lungs, fatty change and focal accumulations of mononuclear inflammatory cells in the liver, subcapsular spindle-cell hyperplasia and deposition of ceroid in the adrenals, lymphoid and/or erythropoietic hyperplasia in the spleen, focal tubular atrophy in the testes, sinus histiocytosis in the lymph nodes, and pulmonary adenoma. Most of these lesions were mild to moderate in severity. They occurred with almost equal frequency in all groups and were not related to the administration of isobutyl nitrite.

All lymphoid and hematopoietic tissues from test and control mice were normal. No consistent differences between mice in different groups were noted. No microscopic changes were detected in either test or control animals which would suggest immunosuppression. The thymus glands from mice in each group had distinct cortical and medullary regions, and were well populated with lymphoid cells and normal-appearing epithelial elements (Hassall's corpuscles). "Starry sky" appearance in the cortex of the thymus was noted in some mice from each group, and this finding was interpreted as an early change in physiologic involution.

Alterations were observed in sections of lung from mice exposed at 300 ppm for 13 or 18 wk that were not observed in lungs from the 20-ppm and 50-ppm test groups or the controls. These changes—the only histologic findings that appeared to be agent/dose-related in this study—consisted of mild focal hyperplasia and vacuolization of the epithelium lining the bronchi and bronchioles (Fig. 1). The tracheal mucusa did not show these hyperplastic changes, and bronchopneumonia was present in only 1 mouse in the 300-ppm test groups.

DISCUSSION

In this study mice were exposed to 0, 20, 50, or 300 ppm IBN for up to 18 wk. Mice exposed at 20 ppm IBN did not show effects in any measured index of toxicity, while the only effect noted in the 50-ppm animals was a slightly elevated methemoglobin concentration. Elevated methemoglobin levels and a higher incidence of methemoglobinemia were found in mice exposed at 300 ppm IBN. Methemoglobinemia is characteristic of exposure to nitrites (Haley, 1980) and has previously been reported in animals and humans exposed to IBN following oral or inhalation exposure (Wason et al., 1980; Dixon et al., 1981; Shesser et al., 1981; McFadden et al., 1981). McFadden et al., (1981) have suggested the mechanism of organic nitrite methemoglobin formation may involve

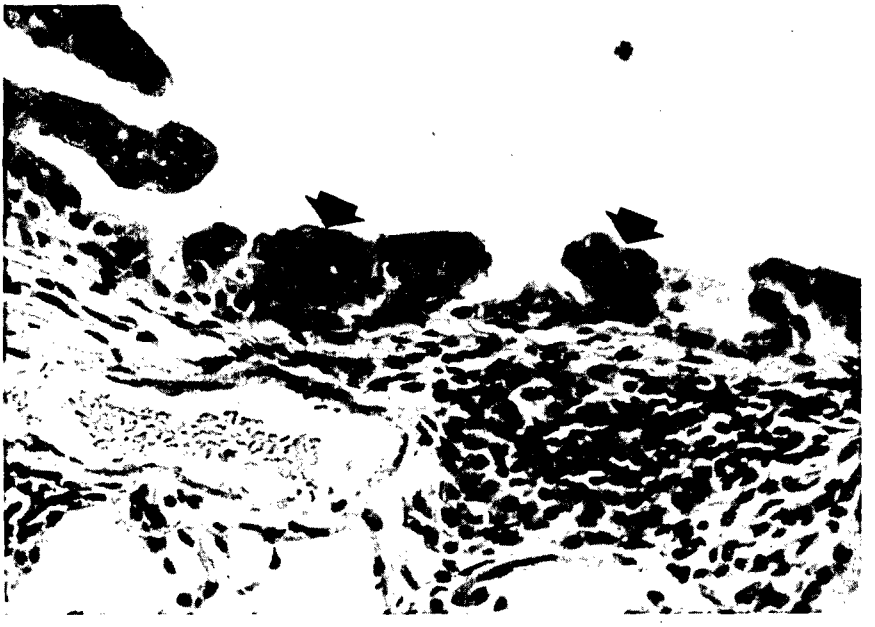

FIGURE 1. Section of lung from a mouse exposed to 300 ppm isobutyl nitrite. Note focal hyperplasia (arrows) of bronchiolar epithelium. Hematoxylin and eosin, X300.

direct oxidation of hemoglobin, hydrolysis of the nitrous acid ester to yield free nitrite ion that then oxidizes hemoglobin, or a combination of these events.

The organ weight changes that were found to be statistically significant could not be related to any gross or histopathologic damage. Similarly, changes in WBC counts in mice exposed to 300 ppm IBN were not related to any observed changes in bone marrow cytology. The remaining hematologic indices were in agreement with values reported in the literature for BALB/c mice (Frith et al., 1980).

Minor histologic changes were noted in the epithelium lining pulmonary bronchi and bronchioles. Tracheitis and edema have been reported in humans exposed acutely to apparently high concentrations of IBN (Covalla et al., 1981; Shesser et al., 1981). Although no pathologic effects of IBN exposure were observed in the tracheal epithelium of mice exposed at 300 ppm IBN for up to 18 wk, evidence of tracheitis and tracheobronchitis was seen histologically in mice exposed at 400 ppm IBN and higher concentration in pilot studies that preceded the subchronic exposures. Other microscopic lesions seen in the mice exposed to IBN were typical of spontaneous lesions reported in the literature for BALB/c mice (Frith et al., 1983).

In light of recent publicity on the nitrites and their abuse by segments of the population (Israelstram et al., 1978a,b; Dixon et al., 1981;

Sigell et al., 1978; Goedert et al., 1982), this study is significant since it provides data on the subchronic toxicity of IBN, data not currently available in the literature. The immunotoxicity of IBN was evaluated concurrently, and these data are reported in the following article. Based on the results of this study and the previously reported toxicity of IBN, it would appear that pulmonary toxicity and methemoglobin formation are the primary effects of subchronic inhalation of IBN. It is assumed that human populations, either in the workplace or by using this chemical as a recreational drug, would also be exposed by the inhalation route. Based on their increasing usage, additional research on the pulmonary toxicity of inhaled IBN and aliphatic nitrites in general may be warranted.

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