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SUBCHRONIC INHALATION TOXICITY OF ISOBUTYL NITRITE IN BALB/c MICE. II. IMMUNOTOXICITY STUDIES

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Initial epidemiologic studies of acquired immunodeficiency syndrome (AIDS) occurring in homosexual men identified the use of the inhalants amyl, butyl, and isobutyl nitrite as possible risk factors contributing to the disease. Because of the lack of immunotoxicological data on these chemicals, we studied the effects of subchronic exposure to isobutyl nitrite (IBN) on the immune system. BALB/c mice were exposed to either 50 or 300 ppm IBN for 6.5 h/d, 5 d/wk for up to 18 wk. After 7, 13, or 18 wk of exposure, mice were killed and the following assays were performed. Antibody producing cells were enumerated by a slide plaque assay on animals immunized with sheep red blood cells while still in exposure chambers. The lymphocyte proliferative response to mitogens (phytohemagglutinin, concanavalin A, pokeweed mitogen, and lipopolysaccharide) was tested using several concentrations of each mitogen. Additional mice were immunized with Freund's complete adjuvant 21 d prior to death and were tested for delayed hypersensitivity response to purified protein derivative by a radiometric skin test. Finally, the relative numbers of T cells and T-cell subsets among splenic lymphocytes from exposed and control animals were determined. At the time periods tested there were no discernable immunotoxic effects observed in the exposed animals in any of the assays performed. These results indicate that IBN, at the dosages tested, had no discernable detrimental effect on the immune system of mice.

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a recently recognized disease characterized by a profound depression of cellular immune function in previously healthy persons. This loss in immune function can lead to an increased incidence of opportunistic infections and is associated with development of rare tumors such as Kaposi's sarcoma [Centers for Disease Control (CDC), 1981a, b, c]. This syndrome was first recognized in male homosexuals (Gottlieb et al., 1981; Masur et al., 1981; Siegal et al., 1981), and initial epidemiologic studies on the outbreak of this disease among homosexual men indicated that the use of organic nitrite inhalants ("poppers") such as amyl nitrite or isobutyl nitrite (IBN) was a possible risk factor (CDC, 1982). While it has been reported that the use of nitrite inhalants is associated with altered ratios of T-helper to T-suppressor cells (Goedert, 1982), others have reported no association between the use of organic nitrites and T-helper to T-suppressor cell ratios (Kornfeld, 1982). Recently studies have been reported that show that volatile nitrites inhibit mononuclear cells' responses in vitro (Hersh et al., 1983; Jacobs et al., 1983), but the effects on the immune system of in vivo exposure has not been reported.

The pharmacologic and toxicologic properties of these compounds have been reviewed (Haley, 1980; Jackson, 1979). Volatile nitrites are known to cause hypotension by peripheral vasodilation and have been used to treat angina pectoris (Needleman and Johnson, 1980). Because of their rapid absorption and oxidant effects, these compounds have been used for emergency treatment of acute cyanide poisoning (Klaassen, 1980), but on the negative side this oxidant effect can produce methemoglobin (McFadden et al., 1981). Alykl nitrites are direct mutagens in the Ames Assay (Quinto, 1980), and all volatile nitrites are highly flammable. In addition, the side effects of nitrite use include dizziness, headache, tachycardia, syncope, hypotension, and increased intraocular pressure (Bruckner and Peterson, 1977). Thus, these chemicals are hazardous, but studies on their immunotoxic potential have not been reported.

The National Institute for Occupational Safety and Health (NIOSH), as one of the Centers for Disease Control (CDC), was requested by the CDC Task Force on AIDS Activity to perform an immunotoxicologic evaluation of aliphatic nitrites utilizing the NIOSH High Risk Exposure Chambers. We elected to use IBN as the test chemical primarily because IBN could be obtained and maintained in a more chemically pure state than amyl nitrite. IBN is used as an inhalant, and all volatile nitrites are thought to have similar physiologic effects (Haley, 1980). Mice were exposed to various concentrations of IBN and at selected time intervals removed from the chamber and tested for immunocompetency by a variety of techniques. The results of that study are the subject of this report.

MATERIALS AND METHODS

The exposure conditions and animals used are as described previously (Lynch et al., 1984). At each time interval, 20 mice (10 male and 10 female) were randomly selected from the exposure chamber along with an equal number of control animals. The animals were killed with an overdose of pentobarbital sodium, a blood sample for hematologic testing was obtained, and the spleen was aseptically removed. The spleen was weighed, a 3-4-mm slice from the center was removed and placed in formalin fixative, and the remainder of the spleen was placed in cold Hanks balanced salt solution (HBSS; GIBCO, Grand Island, N.Y.). The spleens were teased apart and filtered through an 80-mesh wire screen to remove tissue debris, and the cell suspension was collected. The cells were washed once in HBSS, counted using a Coulter Counter Model ZBI (Coulter Electronics, Hialeah, Fla.), and separated into three aliquots. One portion was used for enumerating antibody-producing cells, one for the lymphocyte blast transformation (LBT) assay, and the remainder was frozen in liquid nitrogen and used later for phenotypic typing of the T-lymphocytes. For freezing, the cells were pelleted by centrifugation at 400×g for 5 min, resuspended in freezing media (RPMI-1640 media containing 25% fetal bovine serum and 10% dimethyl sulfoxide), and slowly frozen as previously described (Hem, 1978).

Antibody-producing cells were enumerated using a plaque assay (Jerne et al., 1974) modified for slides (Dresser, 1978). Briefly, the mice were given an intraperitoneal injection of 10^9 sheep red blood cells (SRBC) and returned to the exposure chambers and 4 or 5 d later the spleen cells were assayed for antibody-producing cells. The spleen cell suspensions were washed and resuspended in HBSS to the appropriate cell density. The spleen cells were mixed with a suspension of 0.7% SRBC in HBSS containing 0.5% agarose at 42°C. A 250-μl sample of this mixture was spread over a 50 × 25-mm surface of a microscope slide, the agarose was allowed to harden, and the slide was inverted and incubated at 37°C in 5% CO_2 in air for at least 1 h. Guinea pig complement (GIBCO) diluted 1:20 with HBSS was added to the incubation chamber, and after an additional hour of incubation the slides were removed from the chamber. Slides were fixed with 1% gluteraldehyde, air-dried, stained with 0.25% eosin for 30 s, and rinsed with 80% ethanol, and the plaques were counted. The spleen-cell suspensions were prepared such that slides contained 5×10^5, 1.25×10^5, or 6×10^4 spleen cells per slide, and the mean of triplicate determinations of the number of antibody producing cells per 10^6 spleen cells was calculated.

The remaining spleen cells were used in the LBT assay (Luster et al. 1981). The cells were resuspended in RPMI-1640 containing 10% fetal bovine serum, 5m*M* glutamine, 5 m*M* HEPES buffer, 100 U penicillin/ml, and 100 μg streptomycin (GIBCO)/ml, to a concentration of 2×10^6 cells/ml. Ninety-six well microculture plates were preloaded with 100 μl

of the appropriate dilution of mitogen, and 100 μl of the cell suspension were added to each well. The mitogens and their concentrations were: phytohemagglutinin (PHA) (reagent grade, Burroughs Wellcome, Beckenham, England) at 12.5, 25, 50, 100, and 200 μg/ml; concanavalin A (Con A) (Pharmacia Fine Chemicals, Piscataway, N.J.) at 0.625, 1.25, 2.5, 5.0, and 10.0 μg/ml; pokeweed mitogen (PWM) (GIBCO) at dilution of 1/50, 1/100, and 1/200; and *Salmonella typhimurium* lipopolysaccharide (LPS) (Difco, Detroit, Mich.) at 5, 10, and 20 μg/ml. After 72 h in culture at 37°C in 5% CO_2 in air, 0.5 μCi of [^{3}H]thymidine (specific activity 2 Ci/m*M*, New England Nuclear, Boston, Mass.) were added to each well, and the cultures were harvested 18 h later using a multiple channel automatic cell harvester (BRANDLE, Rockville, Md.). The amount of [^{3}H]thymidine taken up was determined using a Beckman LS-9000 scintillation counter (Beckman Instruments, Fullerton, Calif.). Four replicate determinations were made for each dilution of mitogen for each animal. Statistical analyses were performed on the mean value counts per minute for each mitogen concentration by sex and by date of sacrifice, as well as the overall group mean values of exposed and control animals.

An in vivo test of cellular immune reactivity was performed using a radiometric skin testing procedure (Luster et al., 1981). Briefly, 10 control and 10 exposed mice were immunized by an ip injection of 1.0 ml Freund's complete adjuvant 21 d prior to sacrifice. The animals remained in the exposure chambers during this sensitization period. At 2 d prior to sacrifice, the animals were injected ip with 1.0 μCi [^{3}H]thymidine/g body weight. Skin testing was performed 24 h later by injecting 10 μl purified protein derivative (PPD, 5 μg/ml) intradermally into the right ear and 10 μl sterile saline (0.9% NaCl) into the left ear. At 24 h later the animals were killed, and the entire left and right pinnae were excised, weighed, and placed in separate scintillation vials. The tissues were digested with Protosol (New England Nuclear) and dissolved in scintillation cocktail (Econofluor, New England Nuclear). The radioactivity of each sample was determined in a Beckman LS9000 scintillation counter using an automatic quench correction program so that dpm [^{3}H]thymidine/mg tissue could be determined. The ratio of radioactivity between the right and left ears was calculated and provides an index of the degree of inflammation in the antigen-injected ear.

Spleen cells that had been frozen and stored in liquid nitrogen were used to determine the relative numbers of Thy-1, Lyt-1, and Lyt-2 positive cells present. This was accomplished by rapidly thawing the cells in a 37°C water bath, and diluting the suspension fourfold with HBSS containing 1% bovine serum albumin (BSA). Viable cells were recovered by layering the cell suspension over a density medium for isolating viable mouse mononuclear cells (Lympholyte-M; Cedarline Laboratories Limited, Hornby, Canada), centrifuging at 400×g for 20 min, and recovering the cells from the interface. The cells were washed twice with HBSS

containing 1% BSA, and total and viable cell counts performed. In general, 25-30% of the initial number of cells were recovered with greater than 95% cell viability as determined by Trypan blue exclusion. Two to five million cells were reacted with fluorescein-conjugated monoclonal antisera to Thy-1.2 (New England Nuclear), Lyt-1, or Lyt-2 (Becton-Dickinson, Mountain View, Calif.) antigen for 1 h in an ice bath. The cells were washed 3 times with cold HBSS and fixed in 100 μl of 4% neutral buffered formalin. The percent of positively stained cells for each antiserum was determined using a Ziess photomicroscope II (Morgan Instruments, Cincinnati, Ohio) equipped such that total cell counts were done by phase-contrast microscopy and fluorescent cell counting by epiillumination of the same field. At least 200 cells/sample were counted.

Statistical analysis of the data was performed using Student's *t*-test to determine the significant difference between means.

RESULTS

The results of the plaque assays for antibody producing cells are presented in Table 1. At each time interval there was no statistically significant difference in the number of antibody-producing cells found in the exposed versus control animals, with the exception of female mice exposed to 300 ppm for 18 wk. This group of exposed female mice had significantly higher counts than the control females tested the same day ($p < 0.01$).

The LBT response to mitogens was performed using spleen cells from the same animals used for the plaque assay. The dose-response curves for each mitogen at each time point were compared both for the magnitude of the response (net counts of [^{3}H]thymidine incorporated) and for the concentration of mitogen required for optimal stimulation (i.e., to see if a shift in the dose-response curve had occurred). For animals exposed to IBN at 50 ppm for 3, 7, or 13 wk and animals exposed to 300 ppm IBN for 13 wk, there were no statistically significant differences in either the magnitude of response or shape of the dose-response curves between exposed and control animals (data not shown). In addition, all data was analyzed with respect to sex, and no significant difference in the responses of male and female mice were observed. In Fig. 1 the dose-response curves are shown for animals tested after 18 wk exposure to 300 ppm IBN. As can be seen, the exposed animals had consistently higher levels of thymidine incorporation than controls, and this was statistically significant ($p < 0.01$) for all concentrations of LPS, two concentrations of PHA, and one concentration of PWM. It should be noted also that the control values—i.e., the amount of [^{3}H]thymidine incorporated by cells in media alone—were significantly higher for the IBN-exposed mice.

TABLE 1. Enumeration of Antibody-Producing Spleen Cells in Mice Exposed to Isobutyl Nitrite

IBN exposure conditions				Plaque counts[a]		
Concentration	Time[b]	Sex	N[c]	Exposed	Controls	E/C(%)[d]
50 ppm	7 wk	M	10	273 ± 82	177 ± 48	154
	(4 d)	F	10	249 ± 125	252 ± 111	99
		Total	20	261 ± 100	201 ± 101	130
50 ppm	7 wk	M	10	63 ± 11	83 ± 12	76
	(5 d)	F	10	94 ± 51	77 ± 13	122
		Total	20	78 ± 38	80 ± 12	98
50 ppm	13 wk	M	10	325 ± 56	310 ± 75	105
	(4 d)	F	10	340 ± 63	298 ± 135	114
		Total	20	332 ± 57	303 ± 103	110
50 ppm	13 wk	M	10	143 ± 49	140 ± 44	102
	(5 d)	F	10	149 ± 24	134 ± 32	111
		Total	20	146 ± 36	137 ± 37	106
300 ppm	13 wk	M	5	365 ± 77	428 ± 97	85
	(4 d)	F	5	255 ± 21	301 ± 68	85
		Total	10	310 ± 78	358 ± 102	86
300 ppm	18 wk	M	5	206 ± 63	199 ± 57	104
	(4 d)	F	5	334 ± 73	129 ± 35	259[e]
		Total	10	270 ± 93	164 ± 58	165

[a] Mean number of plaque-forming cells/10^6 spleen cells, ± SD.
[b] Duration of exposure in weeks, and in parentheses the number of days after immunization with SRBC that the plaque assay was performed.
[c] Number of animals tested.
[d] Ratio of plaque counts of exposed/control animals × 100.
[e] Significant at $p < 0.01$.

The radiometric skin testing with PPD was performed only on animals exposed to 50 ppm of IBN for 7 or 13 wk. These were a separate group of animals from those used for the plaque and LBT assay. The results of these experiments are presented in Table 2. At 7 and 13 wk, positive skin-test responses were obtained in both the exposed and control animals. While the mean values for the exposed animals were somewhat higher than the controls, the differences were not statistically significant.

Finally, we determined the percentage of spleen cells that expressed either the Thy-1.2, Lyt-1, or Lyt-2 antigens. Since we had seen statistically significant differences in the functional assays only in the animals exposed to 300 ppm IBN for 18 wk, only the data from these animals are presented. As shown in Table 3, the percentage of cells displaying these markers was not different in the exposed or control mice. Similarly, no differences were noted in the ratio of Lyt-2-positive to Thy-1.2-positive cells (i.e., ratio of suppressor/cytotoxic T cells to total T cells).

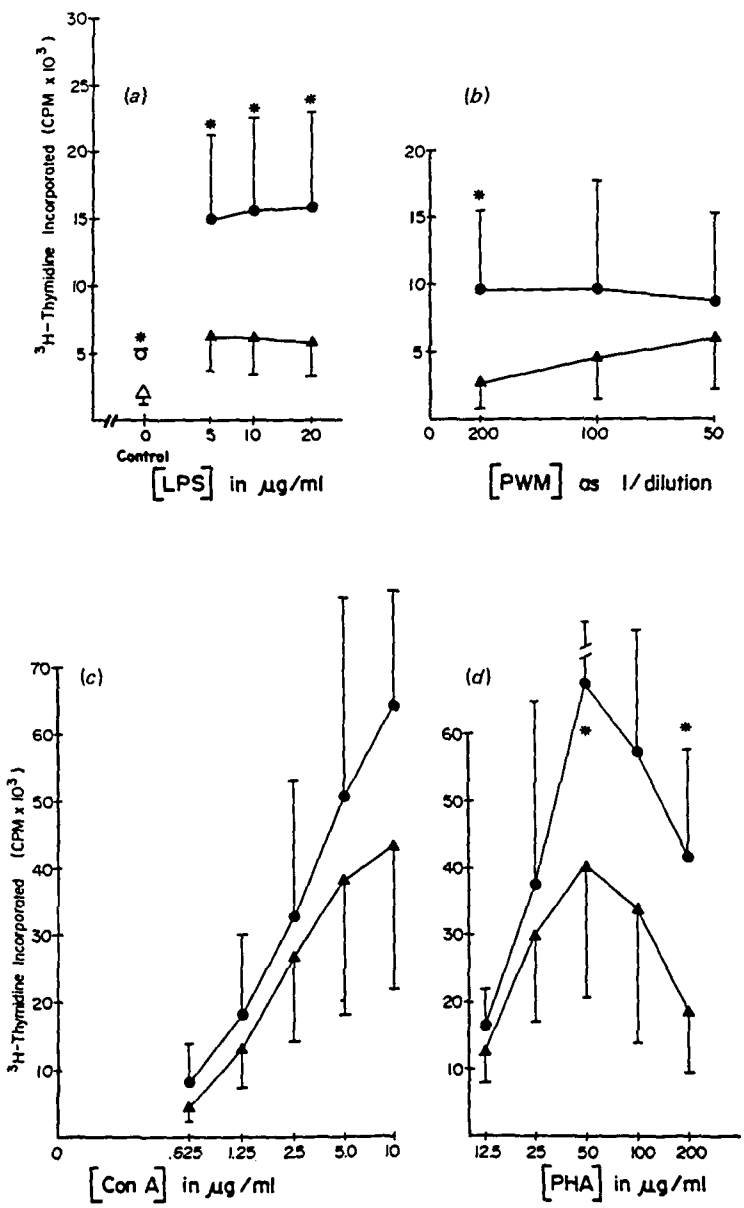

FIGURE 1. Results of the LBT assay performed on spleen cells from control mice (▲) or from mice that had been exposed to 300 ppm IBN for 18 wk (●). Mitogens used were *(a)* lipopolysaccharide (LPS), *(b)* pokeweed mitogen (PWM), *(c)* concanavalin A (Con A), and *(d)* phytohemagglutinin (PHA). Each point represents the mean ± SD of 10 animals (5 males and 5 females), with each animal's cells tested in replicates of 4 for each mitogen dilution. All animals were assayed on the same day. Control cultures (open symbols) received no mitogen and are shown in *(a)*. Points at which statistically significant differences between exposed and control animals were seen are indicated by an asterisk ($p < 0.01$).

TABLE 2. Radiometric Determination of Skin Test Sensitivity in Mice Exposed to IBN at 50 ppm

Treatment	Weeks of exposure	
	7	13
Exposed (10)[a]	2.98 ± 0.51[b]	2.49 ± 0.69
Controls (10)	1.77 ± 0.15	2.19 ± 0.39

[a]Number of animal skin tested at each time point.
[b]Ratio of [^{3}H]thymidine/mg tissue in antigen-injected ear divided by saline injected ear (mean ± SE).

DISCUSSION

The results of this study indicate that IBN was not immunotoxic at the dosages tested. The dosages used in this study were chosen based on some preliminary experiments we had conducted to determine the maximal tolerated dose (MTD) of IBN for mice. Those experiments indicated that 300 ppm was probably close to the MTD. We also exposed mice at 50 ppm IBN, since preliminary experiments indicated that this concentration would neither be stressful nor decrease body weight compared to the control groups. Mice tolerated the 300-ppm concentration, and since no treatment-related deaths were observed in this group during the first 12 wk of exposure, we decided to include this group in the immunotoxicologic study. Thus we were able to obtain data on animals that had had a wide range of exposure levels. The relevance of these dosages to human usage of these compounds is uncertain because persons who abuse aliphatic nitrites recreationally would have intermittent exposures of variable frequency at very high dosages with chemicals of unknown purity. Thus, this study did not attempt to model the recreational use of these drugs, but rather to simply evaluate the immunotoxic potential, if any, of these compounds.

TABLE 3. Relative Number of Spleen Cells Expressing Thy-1.2, Lyt-1, or Lyt-2 Antigens in Mice Exposed to IBN for 18 Weeks at 300 ppm

Treatment group	Percentage of cells expressing			Ratio of Lyt-2/Thy-1.2
	Thy 1.2	Lyt-1	Lyt-2	
Exposed (10)[a]	31.4 ± 3.6[b]	34.2 ± 5.1	19.8 ± 2.8	0.634 ± 0.085
Control (10)[a]	35.5 ± 3.5	38.6 ± 4.4	21.8 ± 3.1	0.616 ± 0.092

[a]Number of animals tested.
[b]Results expressed as mean percent of cells stained ± SD.

Although the results were uniformly negative in the sense that they showed no immunotoxic effects due to IBN exposure, some discussion of the results is needed. The plaque assay and radiometric skin testing procedures are particularly important because they measure an immune response that developed while the animals were being exposed to the chemical (Luster et al., 1981). Good delayed hypersensitivity skin test responses were obtained at the 7- and 13-wk time periods, which indicates that the animals could mount a cell-mediated immune response to tuberculin antigens while being exposed to IBN. Anergy to recall antigens is characteristic of AIDS patients (Masur, et al., 1981; Siegal et al., 1981). Thus these results indicate that IBN exposure does not produce the type of in vivo responses characteristic of AIDS.

The only statistically significant difference noted between exposed and control animals in the plaque assay occurred in female animals tested after 18 wk of exposure to 300 ppm IBN. At this time the exposed group had increased numbers of antibody-producing cells when compared to controls. However, if one compares the plaque counts of the 18-wk control females (Table 1, line 17) with the counts obtained from control females tested 4 d after immunization at the other time periods (Table 1, lines 2, 8, and 14), then it appears that the 18-wk control females had unusually low numbers of antibody-producing cells. The reasons for this are not clear. This group of five control females may have been low responders; conversely, IBN exposure may have potentiated the response of the exposed female animals. The results obtained cannot clearly distinguish between these possibilities.

The LBT assay also only revealed differences after 18 wk of exposure to the high dose of IBN. The most significant effect appeared to be in the control cultures, i.e., those cells that received no mitogen. The mean counts for the exposed group were nearly fourfold higher than those for the controls, and there was no overlap in the counts. All control animals had counts lower than all of the exposed animals. The proliferation observed in these control cultures may be due to an autologous mixed lymphocyte reaction (Kuntz et al., 1976), and the increased proliferation might reflect a change in the suppressor cell number or activity. Alternatively, organic nitrites such as IBN are known to stimulate guanylate cyclase activity and elevate cyclic guanosine monophosphate (cGMP) levels (Ignarro et al., 1981). Chronic exposure to IBN may have altered the cellular cGMP levels such that the proliferative response is more easily stimulated, because levels of cGMP have been shown to increase during the initiation of proliferative responses by mitogens (Hadden and Coffey, 1982). The biological significance of an enhanced response in the LBT assay is unclear. Chronic exposure to high concentration of IBN coupled with infectivity studies would be needed to determine if the enhanced LBT response correlates with resistance to infection, an increase incidence of autoimmune diseases, or an increase incidence of lymphoid tumors.

Monoclonal antisera were used to enumerate T cells and T-cell subsets by direct immunofluorescence, and the results indicate no differences between the exposed and control animals. The results do agree with published values for the incidence of these antigens in mouse spleen cells (Ledbetter et al., 1980) and with the manufacturer's product information sheets that came with the antisera, even though microscopic examination was used to enumerate the cells rather than flow cytometry methods. In preliminary experiment we obtained similar values using freshly isolated cells and cells that had been frozen, indicating that there was no preferencial loss of any one T-cells subset by the freeze-thaw methods used. The anti-Lyt-2 sera recognized a T-cell subset analogous to the human OKT-8$^+$ or Leu-2a$^+$ lymphocytes (i.e., the suppressor/cytotoxic subset), but Lyt-1$^+$ cells are not directly analogous to the human OKT-4$^+$ cells (i.e., helper/inducer subset) cells (Ledbetter et al., 1981). Therefore, the ratio of T-helper to T-suppressor cells could not be directly calculated. The ratio of Lyt-2$^+$ to Thy-1$^+$ cells was the same for the exposed and control groups, indicating that there was probably no reduction in the number of helper/inducer cells in the IBN-exposed animals.

Numerous reports, particularly in the popular press, have stated that aliphatic nitrites such as amyl and isobutyl nitrite are known to be immunosuppressive agents (e.g., Seligmann et al., 1983) when there are, in fact, no data to support such conclusions. To the best of our knowledge this is the first detailed investigation of the immunotoxic potential of organic nitrites in vivo, and our results do not support this popular conclusion. That volatile nitrites can inhibit cellular immune responses in vitro has been demonstrated in two recent reports. Jacobs et al. (1983) exposed human mononuclear cells to amyl nitrite vapors for 5-30 min and observed inhibition of rosette formation, mitogen- and antigen-induced proliferation, and cell-mediated cytotoxicity. In addition, they noted cell-cycle blockage in the S, G_2, and M phases and some membrane structural alterations. Hersh et al. (1983) added IBN directly to human mononuclear cell cultures and noted inhibition of the proliferative response to mitogens, and cell-mediated cytotoxicity reactions. Proliferation of tumor cell lines and interferon production by fibroblasts were also inhibited, leading the authors to conclude that IBN at subcytocidal concentrations was cytotoxic to a variety of cells. These studies clearly demonstrate the cytotoxic potential of nitrites in vitro. That we did not observe similar cytotoxic effects in the present study may be due to a number of factors. Although we exposed animals at close to the MTD, the plasma concentrations were not measured and may not have reached the cytotoxic concentrations obtained in the in vitro studies. Organic nitrite, such as IBN, are rapidly metabolized in vivo (Bruckner and Peterson, 1977), and thus the levels of IBN in the spleen may have been much lower than the levels in lungs and pulmonary vasculature.

Finally, we were not able to test mice on an exposure day; thus any acute toxic but repairable effect may have been missed. Therefore, while our results do not correlate with the in vitro studies, their findings and ours are not mutually exclusive.

While we found that IBN had no significant detrimental effects on the immune system, this does not mean that it was totally innocuous to the mice. The toxic effects of IBN observed in this study are reported separately (Lynch et al., 1985). Thus, while isobutyl nitrite may not be an immunotoxicant, it should still be considered a hazardous substance. Finally, while the inhalant nitrites are probably not responsible for the basic immune defects characteristic of AIDS, their role as a cofactor in some of the illnesses associated with this syndrome cannot be ruled out.

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