Effect of the Recreational Agent Isobutyl Nitrite on Human Peripheral Blood Leukocytes and on in Vitro Interferon Production

Evan M. Hersh, James M. Reuben, Hal Bogerd, Michael Rosenblum, Marc Bielski, Peter W. A. Mansell, Adan Rios, Guy R. Newell, and Gerald Sonnenfeld

ABSTRACT

The effects of the isobutyl nitrite sold as incense and the chemically pure compound on various in vitro parameters of leukocyte function were studied. This was done because of the potential relationship of isobutyl nitrite use to the opportunistic infection and Kaposi’s sarcoma seen in homosexual men. Various concentrations of isobutyl nitrite dissolved in ethyl alcohol were added to various leukocyte cultures. The final added concentrations were 0.001 to 1.0%. Because of the poor solubility of the agent, the fluid concentrations were quite low after addition. Thus, the measured concentrations in the medium after preparation of 1% (v/v) solution were 0.45 µM at 1 hr, 0.04 µM at 24 hr, and 0.04 µM at 48 hr of incubation at 37° in 5% CO₂ in air. At the 1% added concentration, the agent lysed leukocytes and reduced viability from 95% to 21% in 24 hr. At an added concentration of 0.5% or below, cell count and viability were unaffected, but the agent inhibited in vitro lymphocyte blastogenic responses to phytohemagglutinin, pokeweed mitogen, and concanavalin A. It also inhibited natural killer cell activity to the K562 cell line, lymphocyte-mediated antibody-dependent cellular cytotoxicity to the CEM cell line, monocyte-mediated antibody-dependent cellular cytotoxicity to human red blood cells, and in vitro adherence and transformation of monocytes to macrophages. Inhibitory effects were greater than 90% at the 0.5% concentration and were still detectable at 0.01%. Chemically pure isobutyl nitrite and the form sold as incense had identical effects. The agent volatilized from the tissue culture medium at 37° so that its effect on cell viability was reduced about one-third after 24 hr and was gone by 48 hr. The agent inhibited leucine, uridine, and thymidine incorporation approximately equally. Within 2 hr of exposure to isobutyl nitrite, uridine and leucine incorporation were markedly inhibited. After 24 hr of exposure to the agent, the effects on various lymphocyte function parameters were not reversible. The thymidine incorporation of myeloid and solid tumor cell lines was also inhibited by the same concentrations of isobutyl nitrite which inhibited leukocyte functions. Induction of α,β-interferon by polyriboinosinic-polyribocytidylic acid in mouse embryo fibroblasts was inhibited by pretreatment of the cells with isobutyl nitrite. These data suggest that isobutyl nitrite has nonspecific cytotoxic activity for various cells in vitro and could have immunosuppressive effects on tissues exposed in vivo during its recreational use. We speculate that these immunosuppressive effects, combined with the ability of nitrates to convert amines to nitrosamines, may be related to the development of opportunistic infections and Kaposi’s sarcoma in homosexuals who use this agent.

INTRODUCTION

During the last year, there have been several reports of opportunistic infection and Kaposi’s sarcoma occurring either alone or concurrently in previously healthy young adult males, mainly homosexuals (12, 18, 22, 28). Patients in these reports, who had severe infections such as pneumocystis carinii pneumonia, were severely immunodeficient, with skin test anergy, impaired lymphocyte-proliferative responses, and low-helper or high-suppressor T-cell subsets resulting in an inverted helper:suppressor ratio (12, 18, 22, 28). The etiology of the apparent new syndrome is obscure, but it has been attributed to some component or combination of components of the homosexual life style. Possible factors include multiple sexual partners; frequent direct or indirect oral-anal contact; multiple sexually transmitted diseases, particularly viral infection such as cytomegalovirus and hepatitis; and the use of so-called recreational drugs (5). In the latter category, attention has been focused on the nitrates because they have been widely used as aphrodisiacs only since the 1970s and because it is known that nitrates convert amines and amides to carcinogenic nitrosamines in vivo (14). It is speculated that the development of the opportunistic infection and Kaposi’s sarcoma syndrome in homosexuals must involve some new element since homosexual activity itself is not new. Thus, the focus is on drug usage. In the current report, we outline the results of studies of some in vitro effect of isobutyl nitrite (both that sold for recreational use and that which is chemically pure) on various peripheral blood leukocyte parameters. At high concentrations, the drug is cytotoxic; while at lower concentrations, at which viability is preserved, it still inhibits such functions as lymphocyte blastogenesis and NK cell activity. The effect is not lymphocyte specific, however, in that the proliferation of other cell types is also inhibited in vitro. Thus, interferon production is also inhibited by agent treatment of fibroblasts. The data suggest that isobutyl nitrite does have the capacity to inhibit host defense parameters in vitro and therefore may also do so in vivo.

MATERIALS AND METHODS

Isobutyl Nitrite Preparations. Isobutyl nitrite for inhalant use is...
commercially available in a variety of brands. One of these, Rush, is sold as liquid incense by Pacific Western Distributing Corporation, San Francisco, Calif. Rush contains greater than 90% nitrates, with small quantities of alcohol or vegetable oil added to reduce volatilization (31). Chemically pure isobutyl nitrite is prepared by the action of nitrous acid on butyl alcohol and is available from Alpha Products, Danvers, Mass. Isobutyl nitrite is not soluble in water, but it is miscible with alcohol. Rush, or chemically pure isobutyl nitrite, was diluted 1:1 with 95% ethanol. The Rush-ethanol, or chemically pure isobutyl nitrite:ethanol, or 95% ethanol were then diluted with RPMI 1640 to 10, 5, 1, 0.1, and 0.01% stock solutions (v/v). The stock solutions were added in required amounts to cultures to bring about another 1:10 dilution. The alcohol-dissolved Rush, pure isobutyl nitrite, or ethanol controls had calculated final culture concentrations of 1, 0.5, 0.1, 0.05, 0.005, and 0.001% (v/v).

The true concentration of isobutyl nitrite in the cultures was determined by high-performance liquid chromatography. Cultures were set up with 1% (v/v) isobutyl nitrite in alcohol added to complete tissue culture medium. The fluid was evaluated for isobutyl nitrite content compared to a standard at 1, 2, 4, 24, and 48 hr of incubation at 37°C.

The nitrite analyses were performed with a Waters Associates liquid chromatograph consisting of a Model 710B sample processor, a Model M6000A pump, a Model 450 variable-wavelength UV detector, and a data module. A Waters analytical reverse-phase octadeylsile column (30 x 4.5 cm; 10-µm particle size) was used for all analyses. The solvent consisted of 40% methanol (Burdick and Jackson Laboratories, Muskegon, Mich.) in water delivered at a flow rate of 2 ml/min. The column eluate was monitored for UV absorbance at 356 nm. The retention time of an isobutyl nitrite standard in this system was 2.8 min.

Leukocyte Collection and Cultures. Normal human venous blood was drawn, placed in a 250-ml screw-topped Erlenmeyer flask containing glass beads, and defibrinated by swirling for 5 min. The whole defibrinated blood was separated into serum and cells by centrifugation. The cells were resuspended in HBSS to 3 time the original whole-blood volume. In 25- x 150-ml screw-topped tubes, the diluted cell suspension was fractionated by Ficoll-Hypaque density solution centrifugation for 40 min at 400 x g at room temperature. The band of mononuclear cells at the interface was removed, the cells were washed twice with HBSS, and the cell pellet was resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and 25 μg garamycin per ml (Schering Corp., Kenilworth, N. J.). The cell count was determined by Coulter counting, and a microscopic differential was performed. Cell viability was determined by trypan blue dye exclusion. In each of the experiments described below, a single individual’s lymphocytes were used as his own control.

Surface Marker Enumeration. Leukocyte cell surface markers were analyzed before and 24 hr after incubation with final added concentrations of 0.1 and 0.5% of the agent. T-cells were measured by formation of rosettes with neuraminidase-treated sheep RBC, and B-cells were measured by fluorescent microscopy after incubation with goat anti-human IgM: IgG (Cappell Laboratories, Miami, Fla.) (16). T-cells and subsets were also determined by incubation with monoclonal antibodies to the OKT3, OKT4, OKT8, and OKM1 antigens (Ortho Diagnostic Systems, Raritan, N. J.) and subsequent enumeration in a spectrum III cytofluorograph (Ortho Diagnostic Systems).

Lymphocyte Blastogenic Responses. These were measured using the standard [3H]thymidine technique (20). Each well contained 1.5 x 10⁵ lymphocytes in 0.2 ml RPMI 1640 with 10% autologous serum. Triplicate cultures were unstimulated or stimulated with PHA (Difco Laboratories, Inc., Detroit, Mich.), PWM (Grand Island Biological Co., Grand Island, N. Y.), and Con A (Difco). The mitogens were added in required amounts to cultures to bring about another 1:10 dilution. The alcohol-dissolved Rush, pure isobutyl nitrite, or ethanol controls had calculated final culture concentrations of 1, 0.5, 0.1, 0.05, and 0.005% (v/v). After 72 hr in culture at 37°C, in most, 5% CO₂ and air, 1 μCi of [³H]thymidine (specific activity, 1.9 Ci/mmol) was added for an additional 8-hr incubation. The cultures were harvested onto fiberglass filter discs with an automatic multiple-sample harvester. The incorporated radioactivity deposited on the discs was counted in a Packard liquid scintillation counter. Net cpm were calculated by subtracting the averages of the unstimulated triplicates from the average of the stimulated triplicates.

Lymphocyte Metabolism. Incorporation of [³H]thymidine (specific activity, 1.9 Ci/mmol), [³H]uridine (specific activity, 20 Ci/mmol), and [³H]leucine (specific activity, 61 Ci/mmol) was assayed in lymphocyte microcultures as described above. The cultures assaying [³H]leucine incorporation were set up using leucine-free Eagle’s minimal essential medium. The agent:ethanol mixture and ethanol were added as described above. The cultures were pulsed with 1 μCi of isotope at 24, 48, and 72 hr. After an additional 8-hr incubation, the cultures were harvested and counted as described above.

Cell Line Proliferation. The effect of the agent on tumor cell line proliferation was assayed in microcultures as described above. The cell lines used were: CEM (a T-cell lymphoma cell line); K562 (a human myeloid cell line); MB453 (a human breast cancer cell line); and H4534 (a human fibroblast cell line). Cells of each of the cell lines were plated at concentrations of 2 x 10⁵ cells/well in a 0.2-ml volume of RPMI 1640 with 20% fetal calf serum in microtiter plates. Cells were incubated for 24 hr before the addition of the agent. Agent and controls were then added, and 48 hr later 1 μCi [³H]thymidine was added. After an additional 24 hr, the cultures were harvested. CEM and K562 cultures were harvested using the method described for harvesting lymphocyte cultures. Cell lines MB453 and H4534 were harvested after washing the wells by adding 50 μl of 0.1 M NaOH to each well, which were then absorbed with 2 cotton swabs. The swabs were dried, placed in scintillation vials, and counted in a liquid scintillation counter.

Monocyte Adherence. The monocyte adherence assay was carried out by the method of Currie and Hedley (4). Mononuclear cells were adjusted to a concentration of 2 x 10⁶ cells/ml in RPMI 1640. The cell suspension (0.1 ml) and 0.1 ml autologous serum were added to microwells in microtiter plates. The agents were added to triplicate cultures for each concentration. After 7 days of incubation at 37°C in moist 5% CO₂ in air, all wells were washed with prewarmed HBSS to remove nonadherent cells and debris. Then 50 μl of WBC-lysing agent (Coulter) were added to each well and incubated for 30 sec. The number of adherent monocytes per ml of original whole blood was calculated as described previously (4) from Coulter cell counts of the washed monocytes.

ADCC and NK Cell Assays. The ADCC to HRBC and the CEM line and NK cell activity to the K562 cell line were set up as a modification of the method of Poplack et al. (27). The target cells HRBC (50 μCi) and CEM and K562 (100 μCi) were labeled with sodium [⁸¹Cr]chromate, by incubation for 40 min and washed 3 times to remove unbound ⁸¹Cr. The antiserum used for ADCC to HRBC was 1:1 dilution of Dade anti-B antiserum (American Dade, Division of American Hospital Supply Corp., Miami, Fla.). For ADCC to CEM, the antiserum was a 1:100 dilution of rabbit antiserum produced by weekly immunization of rabbits with viable CEM cells for 3 weeks. The effector:target ratios used were 1:1 and 2:1 for HRBC, 20:1 for CEM, and 20:1 and 40:1 for K562. Mononuclear cells were adjusted to 2 x 10⁵ cells/ml in RPMI 1640 with 5% fetal calf serum. For ADCC, 100 μl effectors, 100 μl targets, and 50 μl antiserum were added to each well. For NK cell cultures, 100 μl effectors and 100 μl labeled targets were added. All cultures were incubated without the agent, with the agent, and with ethanol at the above-described concentrations and were added as above; the cultures were then incubated for 4 hr at 37°C. One-half of the culture supernatant was removed and counted for ⁸¹Cr release. Specific target cell lysis was calculated as described previously (27).

Interferon Production. Mouse α,β (type I) interferon was produced in mouse embryo fibroblasts as described previously (1). Poly(I)-poly(C) was prepared by annealing polyriboinosinic acid and polyribocytidylid acid (P-L Biochemicals, Milwaukee, Wis.) by heating for 1 hr at 45°C. α,β-Interferon was induced in cells by adding 50 μg of poly(I)-poly(C)
to tissue cultures for 60 min and then adding additional fresh tissue culture medium. DEAE-dextran was included to ensure maximum interferon induction, and tissue culture supernatants were harvested at 24 hr. Interferon antiviral titers were determined by plaque reduction on murine L-929 cells using the Indiana strain of vesicular stomatitis virus (1). The interferon titer corresponded to the reciprocal of the greatest dilution of test sample that reduced virus plaques by 50%. One interferon unit in this assay was equivalent to 0.88 NIH G-002-904-511 reference units.

Statistical Considerations. The leukocyte cultures described here showed significant depression at the 5% level if counts were reduced 25%, at the 1% level if counts were reduced 50%, and at the 0.1% level if counts were reduced 75% or greater (15).

RESULTS

High-performance liquid chromatography determinations of the 1% added concentration showed 46.14 μg/ml (0.45 mm) at 1 hr of incubation in the media, 37 μg/ml (0.36 mm) at 4 hr, 3.8 μg/ml (0.036 mm) at 24 hr, and 4.1 μg/ml at 48 hr. Thus, the amount added and the amount in solution are quite different. At 0 hr and at 20°C, solubility was poor, and for this reason 1-hr incubation data are given.

At a 1% added concentration in complete media, commercial isobutyl nitrite was highly toxic to peripheral blood leukocytes. We examined the WBC, differential counts, and viabilities of Ficoll-Hypaque density solution centrifugation-separated leukocytes (originally set up at 4.5 cu mm x 10⁵) incubated with the agent for 24 hr compared to an untreated control and to ethanol controls. The 1% added concentration of the agent reduced the viability from approximately 95% to 21%, and the cell count was reduced from approximately 4000/cu mm to 1600/cu mm. The 0.5% concentration did not reduce the count or viability at all. The ethanol concentrations from 1% to 0.001% showed no effect on viability or count. This experiment was repeated 3 times with identical results. This was approached at 0 hr and at 20°C, solubility was poor, and for this reason 1-hr incubation data are given.

We next asked whether the effects of the agent were reversible by washing it out of the cultures. This was approached using cell-mediated cytotoxicity (Chart 2). Cells were incubated with commercial isobutyl nitrite for 24 hr and washed 3 times, and then target cells were added for the standard 4-hr period. The effects of the agent were generally not reversible by washing it out of the cultures. Therefore, once exposed, leukocytes can be permanently damaged by isobutyl nitrite.

It was of interest to determine whether isobutyl nitrite had similar effects on cells other than peripheral blood leukocytes. We therefore evaluated the effects of the agent on a T-lymphocyte cell line (MB453), and a fibroblast cell line (H4534). We next asked whether the effects of the agent were reversible by washing it out of the cultures. This was approached using cell-mediated cytotoxicity (Chart 2). Cells were incubated with commercial isobutyl nitrite for 24 hr and washed 3 times, and then target cells were added for the standard 4-hr period. The effects of the agent were generally not reversible by washing it out of the cultures. Therefore, once exposed, leukocytes can be permanently damaged by isobutyl nitrite.

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<tr>
<th>Agent and final culture added concentration (%, v/v)</th>
<th>Lymphocyte blastogenesis (cpm/1.5 x 10⁶ cells x 10⁵)</th>
<th>Cell-mediated cytotoxicity (% of target cell lysis)</th>
<th>Adherent monocytes/ml blood x 10⁶</th>
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<td>PHA</td>
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<td>Isobutyl nitrite</td>
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<td>Ethyl alcohol</td>
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Control                                             | 25.3  | 17.4 | 19.0  | 10.2        | 34.9      | 4.1           |

Effect of Isobutyl Nitrite on Human Leukocytes
incorporation. Thus, the inhibitory effects of these nitrites appear to be nonspecific relevant to the immune system.

Since the commercial isobutyl nitrite has not been fully characterized and may have other agents as well as various nitrites admixed, we obtained and tested chemically pure isobutyl nitrite dissolved in ethanol (Table 2). The effects on lymphocyte blastogenic responses were essentially identical to those produced by the commercial material. The minimum inhibitory concentration was between 0.05 and 0.10%. Thus, we conclude that the effects of the commercial material are due to the isobutyl nitrite itself.

Because the isobutyl nitrite is highly volatile and is reduced in concentration 90% over 24 hr in culture, we evaluated the persistence of its activity in culture. To do this, we incubated the agent at a 1% added concentration in media for up to 72 hr (Table 3). Leukocytes were added at 0, 24, or 48 hr and incubated for the subsequent 24 hr, after which viability was
incubated for the subsequent 24 hr, after which viability was evaluated. The cytotoxicity of the agent reduced at 24 hr and was completely gone by 48 hr. Thus, in longer-term cultures, such as blastogenesis and monocyte adherence, we assume that the effects of the agent would be greater if the agent did not volatilize out of the solution within a short period of time.

To further investigate this, we conducted a series of experiments in which isobutyl nitrite was added again to the culture every day. In general, the minimal inhibitory concentration was reduced one or 2 levels by this maneuver. Table 4 shows the results of readding the same concentration of the agent to cultures containing 10% fetal bovine serum and serum-free medium to more fully remove the chemicals. Interferon was then incubated for the subsequent 24 hr, after which viability was evaluated. The cytotoxicity of the agent reduced at 24 hr and was completely gone by 48 hr. Thus, in longer-term cultures, such as blastogenesis and monocyte adherence, we assume that the effects of the agent would be greater if the agent did not volatilize out of the solution within a short period of time.

To further investigate this, we conducted a series of experiments in which isobutyl nitrite was added again to the culture every day. In general, the minimal inhibitory concentration was reduced one or 2 levels by this maneuver. Table 4 shows the results of readding the same concentration of the agent to cultures at 24 and 48 hr and its effect at 24, 48, and 72 hr on monocyte adherence. Concentrations added at 0.001 % had suppressive activity.

We next evaluated the onset of the isobutyl nitrite effect on cell proliferation in culture. Chart 4 shows the effects of various concentrations of the agent on [3H]uridine incorporation by K562 cells. Isotope was added at various times during the culture period. The onset of action was rapid, and maximum inhibitory effects were already present during the first 2 hr of the culture period. Similar experiments were done with PHA-, PWM-, and Con A-stimulated lymphocyte cultures and with the CEM cell line. Leucine and thymidine incorporation were also evaluated. The results in all of the above were identical to those shown in Chart 4. All showed a very rapid onset of inhibitory activity.

Finally, we determined the effects of isobutyl nitrite on α,β-interferon production. Early-passage C3H/HeJ-derived mouse embryo fibroblasts were allowed to grow into a confluent monolayer. Different cultures were then treated for 24 hr with various concentrations of commercial or pure isobutyl nitrite. Control cultures were treated with equivalent volumes of ethanol, the solvent for isobutyl nitrite. Chemicals were then removed from the cells, and cells were washed with minimal essential medium containing 10% fetal bovine serum and serum-free medium to more fully remove the chemicals. α,β-Interferon was then induced with poly(I)-poly(C). Viability of the cell cultures was determined by trypan blue dye exclusion. Concentrations of isobutyl nitrite added of 0.1 % or greater were toxic to the cells. Concentrations of isobutyl nitrite below 0.1 % had no apparent toxic effects on the cells, and total cell viability was greater.
than 95%. Ethanol treatment had no effect on cell viability. Twenty-four hr later, the culture supernatants were harvested, and antiviral interferon titers were determined. Both commercial and purified isobutyl nitrite severely inhibited interferon production at concentrations of 0.05 and 0.01%, when cell viability was not apparently affected (Table 5). Since the interferon assay is a titration, a decrease of 50% or greater was required to have a reliable effect on interferon induction. Concentrations of isobutyl nitrite below 0.01% had no effect on interferon induction.

**DISCUSSION**

The syndrome of opportunistic infection and Kaposi's sarcoma in homosexual men is one of the most distressing and potentially serious health problems of modern American life (12, 18, 22, 28). Hundreds of cases of this syndrome are coming to medical attention each year, and the magnitude of the problem appears to be growing and spreading with time (2). The mortality of the syndrome is as high as 60%. Extensive speculations on the causes of this syndrome have been made. They include the immunosuppressive and potential carcinogenic effects of the herpesvirus group including cytomegalovirus (9), the potential immunosuppressive properties of rectally or orally ingested components of semen such as polyamines and prostaglandins (23), the extensive use of glucocorticoid-containing skin creams by these patients (24), and the use of so-called recreational drugs including marijuana, cocaine, heroin, and nitrates (11).

Volatile alkyl nitrates, such as amyl nitrite and isobutyl nitrite, have been used extensively in the homosexual community since approximately 1969 and are now being used more frequently also among heterosexuals (25). The acute transient vasodilation and tachycardia produced by these volatile nitrates apparently have aphrodisiac qualities related to the intensification of orgasm and relaxation of the anal sphincter (26). The various commercial products such as Rush, Lockeroom, Aroma of Men, and Gatorade are unregulated by the Food and Drug Administration since they are not sold as drugs but rather as incense or room odorizers. The extent of the use of these products is not known since they are also not regulated by the Consumer Products Safety Commission or the Environmental Protection Agency. However, it has been suggested that one brand has sold about 12 million 0.25-oz. bottles since 1974 (31) and that 11.1% of high school seniors report having used them as drugs (19).

Concern about these nitrates has been mainly related to their carcinogenic potential. However, they also have other acute and subacute toxicities including the production of methemoglobinemia (17), Heinz body hemolytic anemia (13), splenomegaly, skin rash (7), and death after acute overingestion causing methemoglobinemia and hypokinetic anoxia (6).

The carcinogenic potential of the nitrates and nitrates relates to their ability to nitrosate various amines and amides forming N-nitroso compounds such as dimethylcarbonitrosamine (14). The carcinogenicity of dimethylcarbonitrosamine was first described by Magee and Barnes (21) in 1956. Nitrosamines are both toxic and carcinogenic to a variety of organs including liver, lung, kidney, bladder, and the upper respiratory and gastrointestinal tracts. A variety of natural food compounds and drugs undergo nitrosation when exposed to nitrates in aqueous solution. Cancer can be induced in experimental animals by the cofeeding of natural amines or amides and nitrates (8). Some drugs can be substrates and can be nitrosated by nitrates. They include aminophenozene, disulfiram, methadone, propranolol, and phenacetin (3). It is to be emphasized that the carcinogenic potential of butyl nitrite is hypothetical at present and is extrapolated from other studies.

Furthermore, we can only speculate as to the role, if any, of these recreational nitrates in the development of opportunistic infection and Kaposi's sarcoma in homosexual men. It appears that many of the risk factors such as viral infection, sexual promiscuity, drug abuse, and the resultant or associated diseases go hand in hand. Goedert et al. (10) have demonstrated that the helper: suppressor ratio of peripheral blood lymphocytes is more inverted among nitrates users than among nonusers. This was the first indication of the potential immunological relationship to the nitrates. The concentration of nitrates in local lymphoid tissues such as in nasopharyngeal tissue could conceivably be high enough to produce both immunosuppressive and carcinogenic affects.

In the current paper, we have demonstrated that, in vitro, both "commercial" isobutyl nitrite and purified isobutyl nitrite dissolved in ethanol depress various leukocyte function parameters associated with host defense. Lymphocyte blastogenesis, cell-mediated cytotoxicity, and monocyte adherence were all suppressed by concentrations of isobutyl nitrite which were nontoxic in terms of cell viability. The data suggest but do not prove that the agents may be immunosuppressive in vivo. Preliminary data from our laboratory suggest that isobutyl nitrite does suppress the NK cell response of mice.4

These compounds are somewhat difficult to work with in that they readily and rapidly volatilize from in vitro tissue culture fluids. Therefore, cells are exposed to a progressive decrease in concentration with time. Furthermore, the effects on cellular functions were nonspecific in that the proliferation and metabolism of nonlymphoid and nonmyeloid cells were inhibited by the same concentrations which inhibit lymphocyte function. This is of some additional interest in terms of a possible relationship to the mild or moderate chronic myelosuppression which we have observed in the homosexual patients with opportunistic infection or Kaposi's sarcoma.5 The induction of α,β-interferon was also inhibited by isobutyl nitrate treatment of fibroblast cell cultures. We have shown previously that many chemicals that are carcinogenic can inhibit interferon induction (15, 29, 30). Inhibition of interferon induction might contribute to infectious disease and cancer in isobutyl nitrite users. Further characterization and clarification of the potential immunotoxic role of the nitrates may be forthcoming through studies of noncytotoxic doses in vivo in experimental systems. However, even these in vitro studies strongly suggest that the inhalant nitrates may indeed be dangerous, and their use should be condemned by those physicians who treat patients who use these drugs regularly. This is particularly supported by the evidence that 0.001% (0.09 mm) added 3 times strongly inhibited the proliferation of lymphocytes.

The added concentration of the agent used in these studies, being 0.001 to 1.0%, ranged from 0.09 to 88 mm. However, when we measured the actual concentration within the culture

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4 E. Lotzova and E. M. Hersh, unpublished data.
5 P. W. A. Mansell et al., unpublished observations.
fluid (not on the surface to avoid possible effects of phase separation), we found that the 1% added concentration yielded a fluid concentration of 46 μg/ml (0.45 mm) at 1 hr of incubation. Furthermore, this fell by 90% upon incubation for 24 hr.

It is unknown as to whether equivalent concentrations occur in the nasopharyngeal or other tissues of users.

These in vitro results are important, however, for the following reasons: (a) the potential for toxicity or carcinogenicity of a chemical upon chronic exposure can be assessed by single acute exposure at higher doses or concentrations. Since we observed suppression of leukocyte functions at noncytotoxic doses, this potential should be further explored for both in vivo and in vitro; (b) there already is some evidence that use of the agent by humans is associated with immunological abnormalities (10); (c) we have observed that the injection of nontoxic doses of the agent into mice significantly suppresses their NK cell activity. Finally, as noted above, an effect of isobutyl nitrite was evident after only 2 hr of exposure, suggesting that prolonged exposure may not be necessary. Thus, while the concentrations used in this study may or may not be achieved in a single in vivo use in humans, the observations are still relevant.

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