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Depression of murine natural killer cell cytotoxicity by isobutyl nitrite

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Summary. *We have investigated the effect of isobutyl nitrite on murine NK-cell antitumor-directed cytotoxicity. This agent has been suggested as one of the factors underlying immunodeficiency syndrome (AIDS) in man. We demonstrated that two injections, each of 0.25 ml isobutyl nitrite, resulted in significant depression of endogenous splenic and peripheral blood natural killer (NK) cell cytotoxicity against T-cell lymphoma, YAC-1. In addition to endogenous NK cells, activity of pyrimidinol-activated NK cells was also substantially depressed by this agent. The latter observation is of the utmost importance, since it suggests that the attempt to augment NK-cell activity (to promote resistance to infections and malignancies) could fail in patients with AIDS who are isobutyl nitrite users. Isobutyl nitrite was NK-cell-suppressive not only after in vivo administration but, most importantly, also after inhalation. This indicates that isobutyl nitrite, via its NK-cell suppressive effect, could contribute to immunodeficiency in AIDS. Studies on the mechanism of NK-cell depression by isobutyl nitrite demonstrated that the NK-cell tumor-binding properties as well as NK-cell cytotoxic potential were substantially depressed. Mixing experiments failed to reveal any regulation by suppressor cell activities. The results of these studies clearly indicate that isobutyl nitrite is an immunosuppressive agent and that its use should be avoided.*

Introduction

A severe acquired immunodeficiency syndrome (AIDS) has been described recently in homosexual men [4, 10, 17, 20]. This immunodeficiency state is associated with multiple opportunistic infections [4, 17, 20] and may result in the development of Kaposi's sarcoma [10]. Immunological studies demonstrated that the subjects experiencing immunodeficiency syndrome had impairment of several parameters of immunity, as represented by distorted helper-suppressor T lymphocyte ratio, skin test anergy to recall antigens, depressed lymphocyte blastogenesis, and reduced natural killer (NK) cell-mediated cytotoxicity [4, 10, 17, 19]. Even though the etiology of the immunodeficiency syndrome is not known, various factors, such as immunosuppressive virus infection, immunosuppression mediated by exposure to allogeneic semen, and the use of 'recreational' drugs, may contribute individually or collectively to this syndrome [2, 9, 18].

NK cells have been implicated in immunosurveillance to malignancies [8, 13, 14] and in resistance to metastatic tumor growth [7]. The recreational drug isobutyl nitrite may be one of the agents contributing to the immunodeficiency syndrome, and we have previously demonstrated that it suppresses various human leukocyte functions in vitro [9]. Therefore, we have investigated the effect of this agent administered in vivo on murine NK-cell-mediated cytotoxicity against allogeneic T cell lymphoma, YAC-1.

Materials and methods

Experimental animals. Female (C57BL/6 × DBA/2) F_1 mice (abbreviated B6D2F_1) aged 8–11 weeks were used in these experiments. The mice were purchased from Cumberland View Farms (Clinton, TN) and were maintained in our animal facilities for at least 1 week after arrival to allow recovery from shipping trauma.

Preparation of target and effector cells. Murine T-cell lymphoma, YAC-1, of A strain origin was used as a target in these studies. The target cells were grown as continuous cultures in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics (500 U/ml of penicillin and 50 μg/ml of streptomycin) and HEPES buffer (supplemented RPMI 1640; S-RPMI 1640).

Spleen and peripheral blood cell suspensions were prepared as described in detail previously [12, 15, 16]. Peritoneal exudate cells were harvested by massage of the peritoneal cavity after injection of 3 ml saline and aspiration of exudate with a pasteur pipette. When large numbers of peritoneal exudate cells were required, the exudates from several animals were pooled.

Nylon wool column fractionation. Spleen cell suspensions were passed over nylon wool columns as described by Julius et al. [11]. Briefly, 150 × 10^6 splenocytes were placed on columns consisting of 0.8 g nylon wool in the barrel of a 10-ml syringe and incubated for 45 min at 37° C. After incubation, the cells not adhering to the nylon wool were eluted by washing with 20 ml warm S-RPMI 1640 and were diluted to the required concentration.

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Abbreviations used: NK, natural killer; S-RPMI 1640, supplemented tissue culture medium 1640; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TBC, tumor-binding cells; C-TBC, cytotoxic tumor-binding cells; AIPP, 2-amino-5-iodo-6-phenyl-4 pyrimidinol; LU, lytic units; T : E, target-to-effector; AIDS, acquired immunodeficiency syndrome

NK-cell cytotoxicity assay. NK-cell cytotoxicity assay has been described in detail previously [16]. Briefly, 50 µl containing 10^4 ^{51}chromium [^{51}Cr]-labeled target cells and 100 µl containing various concentrations of effector cells (see *Results*) were incubated in quadruplicate in the wells of round-bottomed microtiter plates for 4 h at 37° C in a 5% CO_2 humidified atmosphere. After incubation, the plates were centrifuged at 250 *g* for 10 min and an aliquot of supernatant was removed from each well and counted in an Autogamma scintillation spectrometer. The standard error of the mean of replicates was < 2%. Spontaneous release of ^{51}Cr was determined by incubating the target cells with medium alone, and ranged from 5% to 7%. The percentage of cytotoxicity was determined according to the formula:

$$\frac{\text{CPM experimental release} - \text{CPM spontaneous release}}{\text{Total CPM incorporated into the cells}} \times 100 .$$

In the experiments in which three different target-to-effector (T : E) cell ratios were analyzed, the data are expressed in terms of lytic units (LU). One LU represents the number of effector cells required for lysis of 10% YAC-1 target cells. LUs were determined from log linear plots of the data points and are reported as $LU_{10}/10^7$ splenocytes.

Single-cell assay. The tumor-binding and tumor-killing capacity of NK cells was measured in a single-cell assay [5]. Effector cells composed of nylon wool-filtered splenocytes were mixed with an equal number of target cells (5.0×10^5 each) in a total volume of 1.0 ml S-RPMI 1640 and incubated for 5 min. The cells were then centrifuged at 120 *g* for 5 min, suspended in a 0.5% agarose solution, and plated into 35-mm Petri dishes which had been coated with poly-L-lysine. Control dishes were composed of target cells plated alone. After plating, 1 ml S-RPMI 1640 was added to each of the petri dishes, which were incubated for 4 h at 37° C in a 5% CO_2 humidified atmosphere. Following incubation the plates were stained for 5 min with 0.1% trypan blue, washed with S-RPMI 1640, and fixed with 0.5% formaldehyde. The percentage of tumor-binding cells (TBC) was evaluated by counting 200 cells, and the percentage of cytotoxic TBC (C-TBC) by counting the number of dead target cells in 100 TBC. The background tumor death was determined by counting the percentage of dead tumor cells in the control dishes. The following formula was used for calculation of C-TBC:

(% Dead target cells in TBC) − (% Spontaneously dead target cells) × (% Dead target cells in TBC).

The frequency of C-TBC was estimated by multiplying the percentage of TBC by the percentage of C-TBC.

AIPP treatment. 2-Amino-5-iodo-6-phenyl-4-pyrimidinol (AIPP) was suspended in saline and was injected at a dose of 250 mg/kg, IP. Because of the poor solubility of AIPP, this agent was ground and vigorously mixed by Vortex to achieve a uniform suspension.

Isobutyl nitrite treatment. Isobutyl nitrite used in these studies was in the form of liquid incense, Rush (Pacific Western Distributing Corporation, San Francisco, Calif.). Rush is composed of > 90% of nitrites, with small quantities of alcohol or vegetable oil added to reduce volatilization [21]. For in vivo use, isobutyl nitrite was diluted with absolute ethanol (1 : 1 dilution) and additionally with saline (1 : 8 dilution). In most experiments 0.25 ml isobutyl nitrite solution was injected IP 48 and 24 h before the NK-cell cytotoxicity assay. This isobutyl nitrite solution contained the maximum dose tolerated by the mice. The concentration of isobutyl nitrite contained in 0.25 ml of the final isobutyl nitrite solution was more precisely estimated by high-performance liquid chromatography, as described in detail previously [9], and corresponded to 11.9 ± 1.9 µg (mean ± SE, based on four different determinations). Because of the poor solubility of isobutyl nitrite this was the most precise analysis possible. In the dose-response studies, isobutyl nitrite was injected in volumes of 0.25, 0.12, or 0.06 ml. The control mice were either untreated or received ethanol in 0.5, 0.25, and 0.12 ml IP 48 and 24 h before the NK-cell assay.

For inhalation experiments the mice were each placed twice daily, within a 5- to 7-h interval, into a 1,000-ml glass beaker which contained a 60-mm petri dish with 2-ml undiluted isobutyl nitrite. After 2–3 min the mice were removed. This treatment was repeated for 7 days. Sixteen hours after the last treatment the mice were tested for NK-cell cytotoxicity. The control mice were exposed to the same experimental conditions, but were not exposed to isobutyl nitrite.

Statistical analysis. The differences between treated and control groups were evaluated by Student's *t*-test analysis, and the *P* values were calculated.

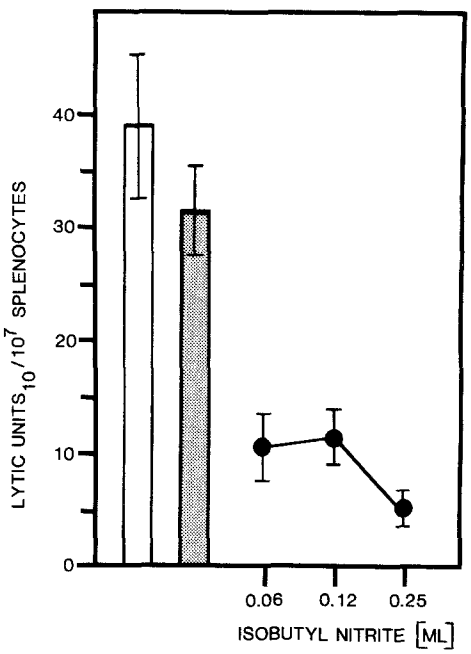

Fig. 1. Effect of various doses of isobutyl nitrite and ethanol on lytic activity of splenic NK cells of B6D2F$_1$ mice against YAC-1. □, untreated mice; ▨, ethanol-treated mice. Since none of these ethanol doses (0.5, 0.25, or 0.12 ml) affected NK-cell cytotoxicity the data were pooled. Isobutyl nitrite or ethanol was administered IP 48 and 24 h before NK-cell assay. Four to six mice were tested in each group; no difference was observed between ethanol-treated and untreated control mice; a significant difference was observed between untreated (or ethanol-treated) splenocytes and isobutyl nitrite-treated splenocytes (*P* value < 0.001 for 0.25, and < 0.01 for 0.12 and 0.06 ml isobutyl nitrite)

Results

Effect of isobutyl nitrite on splenic and peripheral blood NK-cell cytotoxicity after in vivo administration

In the first series of experiments we tested the effect of three different concentrations of isobutyl nitrite on splenic NK-cell cytotoxicity of B6D2F$_1$ mice against YAC-1 as a target. The control mice were either untreated or received ethanol, the primary diluent of isobutyl nitrite, IP (Fig. 1). It can be seen from Fig. 1 that all concentrations of isobutyl nitrite caused significant depression of NK-cell lytic activity (P value < 0.001 for 0.25 and < 0.01 for 0.12 and 0.06 ml isobutyl nitrite); the highest degree of depression of NK-cell activity was observed with the 0.25-ml volume of this agent. In contrast to isobutyl nitrite, ethanol in any of the concentrations tested did not affect NK-cell cytotoxicity levels in comparison with untreated mice; thus the data of different ethanol concentrations are expressed collectively as a single bar in Fig. 1.

Because the two injections (48 and 24 h before the NK-cell assay) of 0.25 ml isobutyl nitrite resulted in the most consistent and prominent depression of NK-cell cytotoxicity, this concentration and schedule was adhered to in all further experiments. Figure 2 illustrates the effect of isobutyl nitrite on splenic NK-cell activity of 20 B6D2F$_1$ mice. It can be clearly seen that a substantial decrease in lytic activity followed the administration of isobutyl nitrite, as evident from the mean and the range for LU values (34.4 with a range of 11.1–72 in control mice and 6.5 with a range of 1–16 in isobutyl nitrite-treated mice; $P < 0.001$). As in the spleen, peripheral blood NK-cell cytotoxicity was also significantly depressed (2-fold) after isobutyl nitrite treatment (Table 1).

Table 1. Effect of isobutyl nitrite on peripheral blood NK-cell cytotoxicity of B6D2F$_1$ mice against YAC-1

Treatment[a]	Percent cytotoxicity[b]	P value
None	10.6 ± 1.9	
Isobutyl nitrite	5.6 ± 1.2	< 0.05

[a] Mice were given two injections of 0.25 ml isobutyl nitrite at 48 and 24 h prior to NK-cell assay. This dose resulted in a slight but significant ($P < 0.05$) decrease in total WBC, which averaged from 8.8×10^6/ml to 6.4×10^6/ml before and after treatment, respectively

[b] Values represent the mean percent cytotoxicity ± SE for eight mice tested at 1 : 25 T : E cell ratio. A similar decrease in NK-cell cytotoxicity was observed when pooled peripheral blood of two mice was tested at 1 : 50 T : E cell ratio; the cytotoxicity values were 39.5 and 16.6 for control and isobutyl nitrite-treated mice, respectively

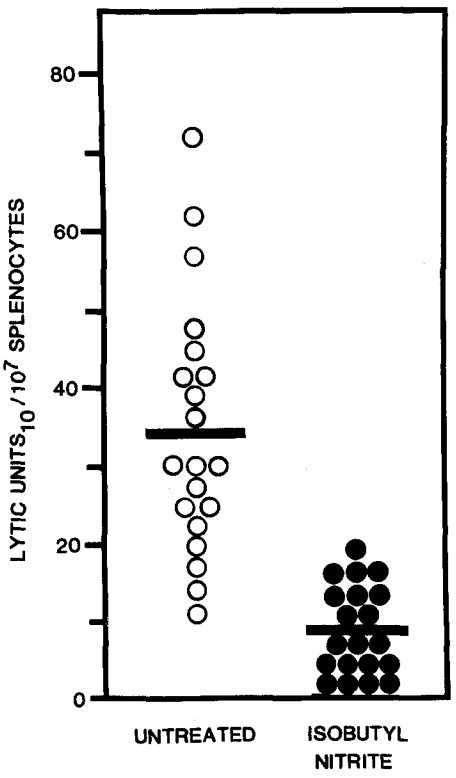

Fig. 2. Effect of isobutyl nitrite on lytic activity of splenic NK cells of B6D2F$_1$ mice against YAC-1. Isobutyl nitrite (0.25 ml) was injected IP 48 and 24 h before NK-cell assay. *Horizontal lines* represent the mean of LU. P value between NK-cell lytic potential of untreated and isobutyl nitrite-treated splenocytes was < 0.001. Isobutyl nitrite did not result in any significant changes in splenic cellularity; the total splenic cellularity averaged 168×10^6 and 155×10^6 prior to and after treatment, respectively

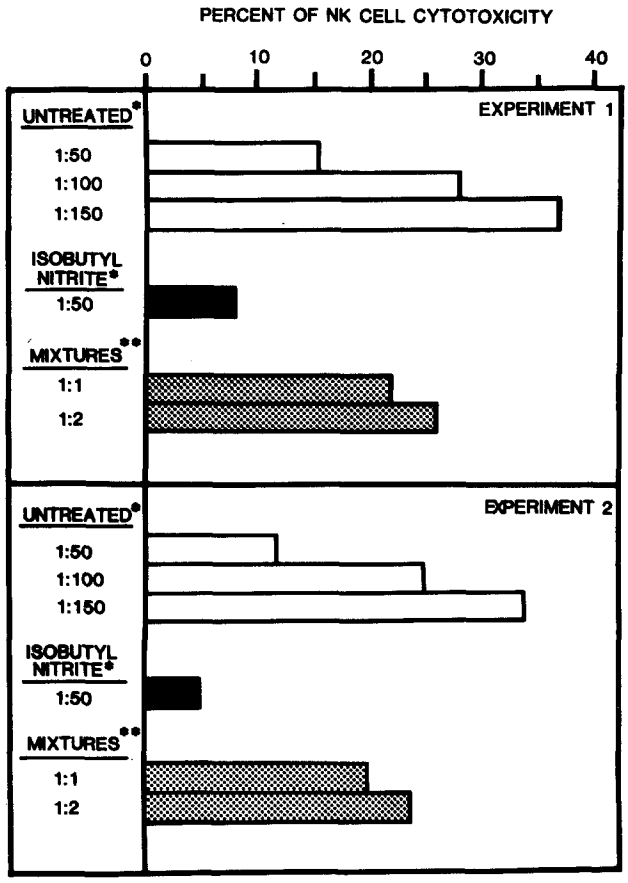

Fig. 3. Failure to detect suppressor cells in the spleens of isobutyl nitrite-treated mice. Isobutyl nitrite (0.25 ml) was injected IP 48 and 24 h before NK-cell assay. *T : E cell ratio; **ratio of control splenocytes to isobutyl nitrite-treated splenocytes

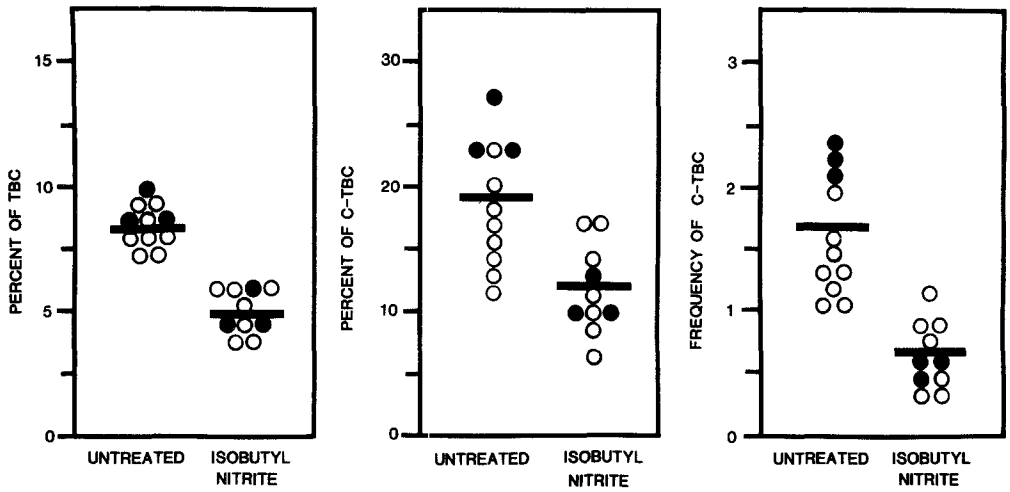

Fig. 4. Effect of isobutyl nitrite on splenic TBC and C-TBC potential and frequency of C-TBC. Mice were treated with 0.25 ml of isobutyl nitrite either 48 and 24 h (○) or 96 and 72 h (●) before single-cell assay. *P* values were < 0.001, < 0.01, and < 0.001 for TBC, C-TBC, and frequency of C-TBC, respectively. *Horizontal lines* represent the mean percent TBC and C-TBC and mean frequency of C-TBC

Table 2. Effect of isobutyl nitrite on peritoneal exudate NK-cell cytotoxicity of AIPP-treated B6D2F$_1$ mice against YAC-1

Treatment[a]	Percent cytotoxicity[b, c]		
	1 : 12	1 : 25	1 : 50
None	1.5 ± 0.6	2.2 ± 0.8	3.2 ± 1.1
Isobutyl nitrite	2.8 ± 0.8	4.8 ± 1.2	7.4 ± 2.2
AIPP	46.4 ± 2.0	59.5 ± 2.8	67.9 ± 2.2
AIPP and isobutyl nitrite	17.8 ± 3.6	26.1 ± 5.4	35.5 ± 6.3

[a] Mice received AIPP 4–5 days prior to NK-cell assay and 0.25 ml isobutyl nitrite 48 and 24 h prior to the NK-cell assay. Both agents were administered IP. The cellularity in peritoneal exudate, which in control mice averaged 4.9×10^6, was increased to 19.2×10^6 after AIPP treatment and to 10.2×10^6 after isobutyl nitrite treatment. There was no synergistic effect of both treatments on peritoneal exudate cellularity (average 18.7×10^6)

[b] Values represent the mean percent cytotoxicity ± SE for eight mice

[c] Significant reduction of NK-cell cytotoxicity was observed in mice treated with AIPP and isobutyl nitrite in comparison to AIPP-treated mice ($P < 0.001$)

Analysis of the mechanism of splenic NK-cell depression by isobutyl nitrite

To determine whether the depression of splenic NK-cell cytotoxicity by isobutyl nitrite could have been caused by the regulatory activities of suppressor cells, we performed mixing experiments. Specifically, splenocytes of normal mice were mixed at a ratio of 1 : 1 of 1 : 2 with splenocytes of isobutyl nitrite-treated mice, and tested for NK-cell cytotoxicity (Fig. 3). Cytotoxicity of normal splenocytes alone at 1 : 50, 1 : 100, and 1 : 150 T : E cell ratios (the latter two T : E cell ratios were included to correct for cell densities in the cultures) and isobutyl nitrite-treated splenocytes at a 1 : 50 T : E cell ratio were included in these experiments. It is evident from Fig. 3 that splenocytes of isobutyl nitrite-treated mice did not suppress NK-cell activity of control mice, indicating that suppressor cells were not operating in the depression of NK-cell cytotoxicity after isobutyl nitrite treatment.

Table 3. Effect of inhalation of isobutyl nitrite on splenic NK-cell activity of B6D2F$_1$ mice against YAC-1

Experiment no.	Treatment[a]	$LU_{10}/10^7$ splenocytes[b]	*P* value
1	None	15.1 ± 2.9	
	Isobutyl nitrite	5.8 ± 1.9	< 0.05
2	None	9.0 ± 1.5	
	Isobutyl nitrite	3.3 ± 1.3	< 0.02

[a] Mice were allowed to inhale isobutyl nitrite as described in *Materials and methods*

[b] Values represent the mean of LU ± SE for five to six mice

To investigate the possible mechanism of isobutyl nitrite mediated depression of NK-cell activity further, we performed the single-cell assay analysis and determined the percentage of TBC and C-TBC and the frequency of C-TBC in control and isobutyl nitrite-treated mice (Fig. 4). The latter group of mice displayed a decrease both in tumor-binding capacity (TBC) and in the lytic activity of TBC (C-TBC). Consequently, the frequency of active NK cells was significantly decreased in the spleens of treated mice compared with control mice. Since the highly purified NK-cell suspensions were not used in a single-cell assay, the involvement of effector cell populations other than NK cells in tumor binding cannot be ignored. However, it has been demonstrated that most of the nylon-wool-filtered splenocytes exhibiting tumor-binding activity are NK cells [1].

Effect of isobutyl nitrite on cytotoxicity of activated NK cells

We found previously that AIPP dramatically augmented NK-cell cytotoxicity in the peritoneal exudate of various strains of mice [16]. It was therefore of interest to determine whether the cytotoxic potential of activated NK cells would also be affected by isobutyl nitrite treatment. This may have practical relevance in patients with AIDS, in whom NK cells may be activated by the viral infections associated with this syndrome. Table 2 indicates that as in endogenous (unstimulated) NK

cells, the cytotoxic potential of NK cells activated by AIPP in the peritoneal cavity was significantly depressed (2-fold) after treatment with isobutyl nitrite.

Effect of inhalation of isobutyl nitrite on splenic NK-cell cytotoxicity

Since, in man, isobutyl nitrite is used as an inhalant, we have investigated whether the inhalation of this agent by mice affects NK-cell cytotoxic activity. Mice were allowed to inhale undiluted isobutyl nitrite twice daily for 7 days and their splenocytes were tested for NK-cell cytotoxicity 16 h after the last inhalation (Table 3). In two different experiments, inhalation of isobutyl nitrite substantially depressed (> 2-fold) the NK-cell lytic potential of mice.

Discussion

Our studies demonstrate that isobutyl nitrite, an agent frequently used as an aphrodisiac by the male homosexual population at high risk for AIDS, substantially depressed NK-cell antitumor potential in vivo. Isobutyl nitrite depressed not only cytotoxic potential of endogenous splenic and peripheral blood NK cells, but also cytotoxic activity of activated NK cells. Since in experimental animals NK cells have been implicated in the mediation of immune surveillance against tumors and resistance to various types of infections, the depression of NK-cell cytotoxicity by this agent could underlie the susceptibility of homosexual men to opportunistic infection and Kaposi's sarcoma. Furthermore, the observation that cytotoxic potential of activated NK cells was also reduced by this agent suggests that an attempt to augment NK-cell activity to promote resistance to infections and malignant disease in patients with severe immunodeficiency syndrome could fail in patients who continue to use isobutyl nitrite. Since a multifactorial depression of immunity and a certain duration of this depression probably sets the stage for successful infection of the presumed AIDS agent, the continued and prolonged use of isobutyl nitrite may play an important role in AIDS.

Studies on the mechanism of NK-cell depression by isobutyl nitrite indicate that the tumor-binding properties, cytotoxic potential, and frequency of NK cells were substantially decreased. This observation suggests that isobutyl nitrite not only affects the lytic event of NK cells, but also NK-cell activity at the target cell recognition level. Mixing experiments failed to reveal any regulation of NK-cell activity by putative suppressor cells present in isobutyl nitrite-treated mice. It is important to note not only that direct administration of isobutyl nitrite via injection decreased NK-cell cytotoxic potential, but also that inhalation of this agent was NK-cell-suppressive. The latter observation, which experimentally resembles the exposure of humans to isobutyl nitrite, indicates that this agent could contribute to the immunodeficiency in AIDS via its NK-cell-suppressive effect. Even though the in vitro suppressive effect of isobutyl nitrite on various immune functions has been demonstrated previously [9], this is the first report of which we are aware that unequivocally indicates its immunosuppressive effect in vivo. However, some indirect data suggest that the use of similar nitrites may alter T lymphocytes in human users [3]. The results of these studies indicate that immunosuppression should be added to the other reasons [6] why isobutyl nitrite should not be used by man.

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