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Immunologic, Hematologic, and Endocrine Responses to Subacute and Subchronic Exposures to Graded, Subanesthetic Levels of Nitrous Oxide in CD-1 Mice

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IMMUNOLOGIC, HEMATOLOGIC, AND ENDOCRINE RESPONSES TO SUBACUTE AND SUBCHRONIC EXPOSURES TO GRADED, SUBANESTHETIC LEVELS OF NITROUS OXIDE IN CD-1 MICE

by

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Charles E. Healy

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Immunologic, Hematologic, and Endocrine Responses to Subacute and Subchronic Exposures to Graded, Subanesthetic Levels of Nitrous Oxide in CD-1 Mice

by

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Nitrous oxide (N_2O) oxidizes vitamin B_{12}, disrupting deoxyribonucleic acid (DNA) synthesis. Occupational exposures to subanesthetic levels of the gas have been documented that may result in suppressed proliferative cell activities. Male CD-1 mice were exposed to 0, 50, 500, and 5000 parts of N_2O per million parts of air (ppm) for 6 hr/day, 5 days/week for 2 and 13 weeks. Splenic lymphocytes were assayed for responsiveness to mitogens and for the ability to produce interleukin-2 (IL-2). Tritiated-thymidine ([^{3}H]-TdR) uptake was measured in CD-1 splenic lymphocytes cultured in a mixed-lymphocyte culture (MLC). Cytolytic cell activity was measured by a ^{51}chromium release assay. Antibody-mediated immunocompetency was determined for sheep red blood cell (SRBC)-sensitized animals by plaque-forming cell (PFC) assay and sera anti-SRBC antibody titer. Deoxyuridine suppression tests (dUdRST) were performed on bone marrow cells. Serum adrenocorticotropic hormone and corticosterone levels were determined.

There was significantly decreased splenic lymphocyte uptake of [^{3}H]-TdR by cells cultured with mitogenic substances and in MLC following 2-week animal exposures to 5000 ppm. After 13-week exposures, the animals' splenic lymphocytes showed decreased [^{3}H]-TdR uptake following low N_2O dosing and nonsignificantly increased responsiveness at the higher gas exposures in both the blastogenic and MLC assays. Compared to control animals, the 5000-ppm-exposure group had significantly depressed PFC activity and circulating anti-SRBC immunoglobulin M levels following 13-week gas exposures, and all three subchronic exposure groups demonstrated both decreased liver weights and leukopenia. Bone marrow activity at these dosing levels was dose-responsively depressed following subchronic gas exposures. No hormonal effect appears to be attributable to N_2O exposure.

(97 pages)

CHAPTER I

INTRODUCTION

Since it was observed to have narcotic properties in the mid-nineteenth century, nitrous oxide (N_2O) has been used as an anesthetic and an analgesic gas in surgical, dental, and veterinary operatories. Its principle use today is as an adjuvant to other anesthetic drugs such as barbiturates (Gilman *et al.*, 1980). Lesser uses of N_2O include its incorporation in rocket fuels and as a propellant in whipped-cream dispensers (Clayton and Clayton, 1978). Historically, N_2O has been considered to be a relatively innocuous gas with few toxic properties unless administered in hypoxic concentrations. While the exact mechanism of its anesthetic effect has not been resolved to date, it is believed that N_2O is generally not metabolized in the body and leaves by simple expiration following administration (Trudell, 1985).

Within the last 30 years, a number of studies have implicated N_2O as a mediator of a variety of physiologic disorders in humans, including embryopathies, neuropathies, hematopathologies, and cancer, as well as some immunomodulations. These have been reviewed by several authors (Brodsky, 1983, 1985; Brodsky and Cohen, 1987; Chanarin, 1982; Eger, 1985; Nunn, 1987). However, the precise mechanism(s) by which these disorders are produced, and the duration and concentration of exposure required to bring them about, are still unknown. In fact, there is no current United States statute which defines a "safe" or acceptable exposure limit for N_2O, although a recommended standard of 25 parts of N_2O per million parts of air (ppm) based primarily upon behavioral performance studies by Bruce and Bach (1975,

1976) and Bruce *et al.* (1974) has been proposed by the National Institute for Occupational Safety and Health (NIOSH) (Archer *et al.*, 1977).

Due to the interactive nature of the various systems of the body, a substance that causes a disorder in either the immune, hemopoietic, endocrine, or nervous system could very easily impact upon one or any of the others. For example, patients acutely exposed to anesthetic concentrations of N_2O have sometimes presented with megaloblastic hemopoiesis (megaloblastic anemia) and bone marrow depression (Amess *et al.*, 1978; Amos *et al.*, 1982; Lassen *et al.*, 1956; Nunn *et al.*, 1986a; O'Sullivan *et al.*, 1981; Skacel *et al.*, 1983; Sourial *et al.*, 1985). Since immune system cells ultimately arise from pluripotent stem cells in the blood-forming system (Kimball, 1986), N_2O-induced hemopoietic disorders such as these may be indicative of alterations within the immune system also.

Additionally, N_2O exposure, like other environmental exposures (Monjan and Collector, 1977), might cause or be associated with a generalized stress of the central nervous system (CNS), which could lead to some form of immunomodulation. This immunomodulation might be mediated by neuroendocrine alteration of biogenic amine levels (Locke and Colligan, 1986). Recent reviews of the interactions between the immune, endocrine, and nervous systems are provided by Cavagnaro *et al.* (1988), Locke and Colligan (1986), and Solomon (1987).

There appears to be a growing volume of literature describing the immunotoxicology of various compounds to which people may be exposed (although this is not so for N_2O). Nevertheless, immunologic endpoints are not presently used as measures of toxicity in an occupational standards setting. The American Conference of Governmental Industrial Hygienists

(ACGIH), for instance, has recently proposed an occupational health standard of 50 ppm for N_2O (Anonymous, 1987), but immunotoxic endpoints were not used in defining the chosen value.

Because of recent advances in our understanding of the immune system and its relevance to the entire being, there are now those who believe (Exon *et al.*, 1986) that the time is soon coming when immunotoxicologic assessment, in addition to current safety assessment procedures, may be required by governmental regulatory agencies prior to approval of many chemicals and drugs. The importance of determining whether or not chemicals or drugs affect the immune system can be appreciated in light of their potential to mediate either autoimmune disorders or simply as an immunosuppression that might increase susceptibility to infectious or other diseases (Dean and Vos, 1986; Luster and Blank, 1987; Sharma, 1984). The present study, therefore, was undertaken to assess whether immunomodulation occurs in a murine model following subacute and subchronic inhalation exposures to low concentrations of N_2O.

CHAPTER II

LITERATURE REVIEW

In 1956, after administering 50% N_2O for between 5 and 6 days to 6 patients as a tetanus treatment, Lassen *et al.* observed that the subjects developed megaloblastic anemia with decreased circulating neutrophil and platelet counts. The toxic potential of N_2O was not appreciated, however, until the publication of several epidemiologic studies in the 1970s. These studies indicated that chronic exposure of medical and dental personnel to trace concentrations of anesthetic gases result in increased incidences of teratogenicity and spontaneous abortion both for women directly exposed to the gases and for the wives of men who are exposed (Askrog and Harvald, 1979; Cohen *et al.*, 1971, 1974, 1980; Knill-Jones *et al.*, 1972, 1975; Rosenberg and Kirves, 1973; Tomlin, 1979).

A few of these studies also suggested the presence of kidney and liver disease and the possibility of higher cancer rates among exposed personnel. Some of the studies reported partially negative results, and others, while reporting a statistically significant positive correlation between anesthetic gas exposure and the above-mentioned disorders, attributed their findings to factors such as occupational stress (Rosenberg and Kallio, 1977; Rosenberg and Kirves, 1973).

Despite these differences of opinion and the technical flaws in the studies themselves (which disallowed drawing any firm conclusions regarding N_2O toxicity), the scientific and medical communities were nevertheless alerted to the need to further study N_2O's capacity to be involved in various pathologies. In fact, the U. S. Food and Drug Administration (FDA) issued two bulletins

(1980, 1981) warning health practitioners of the potential of the anesthetic gas to produce teratogenic and various other effects. The FDA has apparently taken no action since then, however, to regulate N_2O exposures, although a number of animal studies (Corbett *et al.*, 1973; Fink *et al.*, 1967; Lane *et al.*, 1980; Mazze *et al.*, 1982, 1984, 1986; Ramazzotto *et al.*, 1979; Shah *et al.*, 1979; Vieira, 1979, Vieira *et al.*, 1977, 1980, 1983) have generally confirmed the findings of the human epidemiologic studies. Occupational health standards for N_2O will be discussed later.

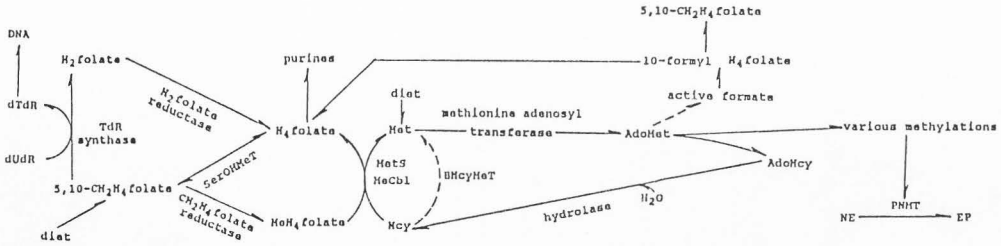
BIOCHEMICAL CONSEQUENCES OF N_2O/VITAMIN B_{12} INTERACTIONS

No experiments were performed in this research to specifically define either vitamin B_{12} levels or oxidation states. However, a brief description of previous work describing the effect N_2O has on the B_{12} molecule is important to lay the groundwork for assessing the potential hematologic and immune system effects of the gas. Two separate studies (the first apparently unnoticed for several years) were performed that demonstrated N_2O's ability to oxidize vitamin B_{12} *in vitro* from an active (cob(I)alamin) to an inactive (cob(III)alamin) form (Banks *et al.*, 1968; Blackburn *et al.*, 1977). Once formed, cob(III)alamin may then combine with other cob(I)alamins to produce the equally inactive cob(II)alamin. These reactions are both nonenzymatic and irreversible (Nunn, 1984, 1987).

Kolhouse and Allen (1977) identified two forms of physiologically active vitamin B_{12}. The first, adenosylcobalamin, is required by the mitochondrial enzyme methylmalonyl coenzyme A mutase in the conversion of

nethylmalonyl coenzyme A to succinyl coenzyme A. Methylmalonyl coenzyme A mutase does not appear to require the reduced form of vitamin B_{12} for its activity, however.

The second form of vitamin B_{12} has a methyl group substituted for the adenosyl moiety. This form of the B_{12} molecule is required by the cytosolic enzyme methionine synthase (synonym: 5-methyltetrahydrofolate-homocysteinemethyltransferase) (MetS). A lack of active methylcobalamin (MeCbl), brought about by both acute and chronic N_2O exposures, has been shown to result in marked decreases in MetS activity both in animals (Black and Tephly, 1983; Brodsky *et al.*, 1984; Deacon *et al.*, 1978, 1980b, 1985; Frasca *et al.*, 1986; Hansen and Billings, 1985; Koblin *et al.*, 1981; Kondo *et al.*, 1981; Lumb *et al.*, 1983; Nunn and Chanarin, 1985; Nunn and Sharer, 1981; O'Sullivan *et al.*, 1981; Sharer *et al.*, 1983; van Tonder *et al.*, 1986; Viña *et al.*, 1986; Wilson and Horne, 1986; Xue *et al.*, 1986) and in humans (Kano *et al.*, 1981; Koblin *et al.*, 1982). Methionine synthase catalyzes the conversion of methyltetrahydrofolate to tetrahydrofolate with the concomitant conversion of homocysteine to methionine (Zubay, 1983). There are a number of possible consequences resulting from hinderance of this reaction, and these can be readily appreciated by referring to figure II-1. The principle consequence is that there may be cessation of mitotic replication due to suppressed deoxyribonucleic acid (DNA) synthesis. This, of course, would be detrimental to physiologic systems such as the hemopoietic and immune systems which undergo frequent or continuous cellular replication.



Legend:

dTdR deoxythymidylate	DNA deoxyribonucleic acid
dUdR deoxyuridylate	Met methionine
H_2folate dihydrofolate	Hcy homocysteine
H_4folate tetrahydrofolate	AdoMet S-adenosylmethionine
CH_2 methylene group	AdoHcy S-adenosylhomocysteine
Me methyl group	H_2O water
TdR synthase thymidylate synthase	NE norepinephrine
MetS methionine synthase	EP epinephrine
PNMT phenynthanolamine N-methyl-transferase	MeCbl methylcobalamin
BHcyMeT betaine homocysteine-methyltransferase	SerOHMeT serine hydroxy-methyltransferase

FIG. II-1. Various interrelated biochemical pathways that may be affected by nitrous oxide inactivation of methylcobalamin.

HEMOPOIETIC RESPONSES TO N_2O EXPOSURE

Because it oxidizes MeCbl (Banks *et al.*, 1968; Blackburn *et al.*, 1977), N_2O is able to induce megaloblastic hemopoiesis (MH) in human bone marrow (Amos *et al.*, 1982; Lassen *et al.*, 1956; Nunn *et al.*, 1986a; O'Sullivan *et al.*, 1981; Skacel *et al.*, 1983; Sourial *et al.*, 1985). In their studies on N_2O mediated MH, Amess *et al.* (1978) and Sweeney *et al.* (1985) measured total plasma cobalamin (Cbl) levels and found no difference between N_2O exposure and control values, yet deoxyuridine (dUdR) suppression tests indicated decreased bone marrow DNA synthesis similar to that seen in megaloblastic anemia patients who had dietary deficiency of either vitamin B_{12} or folinic acid (Herbert, 1985). These studies confirmed those of Banks *et al.* (1968) and Blackburn *et al.* (1977) in that they indicated that N_2O produces a functional change in the vitamin B_{12} molecule which then results in the biochemical alterations discussed previously.

Nitrous oxide also appears to have deleterious synergistic hematologic effects when administered to rats (Lumb *et al.*, 1981b; O'Leary *et al.*, 1985; van de List *et al.*, 1986) and humans (Nunn *et al.*, 1986b) already dietarily deficient in vitamin B_{12} or folinic acid. Several studies have also indicated that B_{12} or folinic acid administration either prior to or following N_2O exposure assists in overcoming the hematologic effects of the gas (Amos *et al.*, 1984; Deacon *et al.*, 1980a; Kano *et al.*, 1984; O'Sullivan *et al.*, 1981).

In MH, DNA synthesis is inhibited, but ribonucleic acid and protein syntheses are not; this results in enlargement (macrocytosis) of both erythrocytic and leukocytic precursor cells (Wintrobe, 1981). While experimental animals exposed to N_2O remain normoblastic (Deacon *et al.*,

1980a), there are biochemical manifestations, such as decreased MetS activity and abnormal dUdR suppression tests, and other physiologic indications, such as leukopenia, that both humans and animals suffer similar hematologic insult from N_2O exposure (Deacon *et al.*, 1978, 1979, 1980a; Kripke *et al.*, 1977; McKenna *et al.*, 1980; Nunn *et al.*, 1976). Variability was observed when comparing the response rates to N_2O of different animal species as well as of man, with man responding somewhat more slowly than animals (Deacon *et al.*, 1980a; Green, 1968; Green and Eastwood, 1963; Koblin *et al.*, 1981, 1982).

The studies mentioned above involved acute or subacute N_2O exposures at concentrations ranging from 50% to 80% (500,000 ppm to 800,000 ppm of N_2O). A few articles have been published that describe the hematologic effects of the longer-term, lower-level concentrations of N_2O to which medical, dental, and veterinary personnel are routinely exposed, but these are inconclusive and contradictory.

One of these (Sweeney *et al.*, 1985) describes the bone marrow responses of 20 dentists exposed to N_2O concentrations ranging from 159 ppm to 4600 ppm. Seven of the 20 dentists were exposed to N_2O for 10 or more hr per week; 3 of these had abnormal dUdR suppression tests, and 2 of the 3 also displayed abnormal leukocyte morphologies. In a study of hospital operatory personnel exposed to between 155 ppm and 860 ppm of N_2O for unspecified periods of time, Salo *et al.* (1984) reported a slight incidence of macrocytosis with some hypersegmentation of neutrophils. Unlike Sweeney *et al.* (1985), these researchers felt that their findings were not indicative of significant hematologic insult by N_2O. Finally, Blanco and Peters (1983) reported the case of a dentist exposed to N_2O for 4 months who presented with macrocytosis

and hypersegmented neutrophils as well as manifestations of subacute combined degeneration of the spinal cord. His exposure parameters were not given, however. In the one subchronic animal study performed to date, Rice *et al.* (1985) exposed Swiss Webster mice to between 5000 ppm and 500,000 ppm of N_2O for 4 hr per day, 5 days per week for up to 14 weeks with no lowering of erythrocyte or leukocyte totals or differential cell counts.

IMMUNOTOXICOLOGIC EVALUATION OF XENOBIOTICS

Over the last 10 to 15 years it has become increasingly apparent that the immune system may act as a "target organ" for a number of different xenobiotics (Dean and Vos, 1986; Luster *et al.*, 1988). As such, it acts as most other body tissues and organs, sometimes being stimulated to overactivity and sometimes being depressed to lessened activity. The consequences of these immunomodulations may present various problems for the person thus affected, such as increased risk of autoimmune disorder in a case of immunostimulation or a greater likelihood of susceptibility to contracting infectious disease in a case of immunosuppression. Therefore, it is important to determine "safe" levels of xenobiotic exposures to which people may be routinely exposed without incurring immunotoxicologic insult.

While a number of studies have been published that describe the effects of chemicals and drugs on the immune systems of both humans and various laboratory animal models, much work yet remains to be done both in defining whether various xenobiotics may be immunotoxic and in relation to extrapolating animal research data to the human experience. At present, the most widely accepted method for determining the immunotoxicologic potential of chemicals or drugs involves a tiered system of assays (Dean and Thurmond, 1987; Luster *et al.*, 1988). This system is based on the assumption

that xenobiotics that are toxic to the immune system will manifest such toxicity following a battery of tests conducted at the tier I level, weeding out those compounds which are probably not toxic and, at the same time, pinpointing those compounds for which further research needs to be performed (tier II level).

The tier I level of research (Dean and Thurmond, 1987; Luster *et al.*, 1988) includes histopathologic examination of various immune system organs, enumeration of body and organ weights and splenic cellularity, routine hematology, and the following immunoassays: mixed lymphocyte culture (MLC); plaque-forming cell (PFC) assay; immunoglobulin quantitation, usually assessed by enzyme-linked immunosorbent assay (ELISA); mitogen assays; and either cell-mediated cytolytic (CMC) or natural killer-cell (NK) assay.

The MLC is an assay in which lymphocytes from the experimental animals are co-cultured with an allogeneic cell line. Differences noted in the stimulation indeces between the cultures from xenobiotic-exposed animals and those from non-exposed animals following pulsing with tritiated-thymidine ([^{3}H]-TdR) are indicative of altered competency of cell-mediated immunity (CMI). A PFC assay evaluates the animals' ability to provide antibody-mediated immunity (AMI). Experimental animals are challenged with sheep red blood cells (SRBCs) ip 4 days prior to sacrifice, and their splenic lymphocytes are thereafter cultured with SRBCs and guinea pig complement. The ability of the cells to produce immunoglobulin M (IgM) is measured by observing zones of hemolysis surrounding individual PFCs. The total number of PFCs are counted in a given volume of culture solution, and the results are presented as both PFCs/10^6 splenic cells and PFCs/spleen (both values are usually presented in case of variation in splenic cellularity caused

by xenobiotic exposure). Further assessment of AMI is performed by quantitating circulating plasma IgM levels by ELISA.

Mitogen assays are performed to assess the proliferative capabilities of both B and T lymphocytes following incubation with polyclonal activators such as lipopolysaccharide (LPS), a B-cell mitogen; concanavalin A (Con A) and phytohemagglutinin (PHA), T-cell mitogens; and pokeweed mitogen (PWM), which stimulates both lymphocyte populations. The proliferative capabilities of the cells is measured following the uptake of [^{3}H]-TdR. As pointed out by Luster *et al.* (1988), mitogen assays may not always correlate with the assays described above and are not necessarily indicative of altered CMI or AMI. Non-specific immune responsiveness is measured by either CMC or NK assays, in which cells from the experimental animals are co-cultured with ^{51}Cr-labeled tumor cells. Tumor cell killing by experimental animal cells is quantitated by measuring radioisotope release into the culturing media. A CMC assay involves both cytotoxic T lymphocytes and NK cells, whereas the NK assay is performed following separation of these two cell types from one another.

Tier II immunotoxicologic testing is performed as a follow-up to positive tier I assessment and includes the following assays: Quantitation of both B and T lymphocytes from the spleen; quantitation of immunoglobulin G levels; determination of cytolytic T-cell functionality; delayed hypersensitivity response; macrophage enumeration and a determination of their phagocytic abilities; and assessment of host resistance to antigenic challenge from syngeneic tumor cells, bacteria, viruses, or parasites (Luster *et al.*, 1988). The latter series of assays, i. e., assessment of host resistance to various antigenic challenge models, are of particular importance. This is because they represent the determination of an indirect or secondary effect of toxic

xenobiotic exposure, the increased potential of incurring infectious or opportunistic disease following the direct or primary effect of the chemical in question. The direct effect in this case involves a suppression of the immunocompetency of the exposed person or animal.

Immunotoxicologic assessment is not currently required as part of the risk assessment process for environmental or occupational exposures to chemicals. There are some researchers, however, who feel that the time is soon coming when such screening or assessment procedures will be legislatively required by various governmental regulatory agencies (Exon *et al.*, 1986). The applicability and significance of positive tier-I results, demonstrating an immunotoxicologic chemical effect, are still points of dispute in some circles (Loose, 1984, 1986, 1988). In fact, Luster *et al.* (1988, p. 4) readily admit that ". . . Tier I [testing] provides little information on the specificity of immune defect or its relevance to the host." They go on to say, however, that such testing ". . . can readily discern an immune alteration resulting from chemical exposure."

IMMUNOTOXICOLOGIC EFFECTS OF N_2O EXPOSURE

Beyond the studies discussed above, which demonstrate that N_2O adversely affects the hemopoietic system and may therefore have an impact upon the immune system, there are other studies which more directly describe N_2O's effect upon various immunological parameters. Linnell *et al.* (1978) and Quadros *et al.* (1979) have shown that N_2O depresses the conversion of cyanocobalamin (CnCbl, the normal dietary form of vitamin B_{12}) into MeCbl in human lymphocytes *in vitro* while having no apparent effect on the conversion of CnCbl to AdoCbl. However, Bruce (1976) showed that *in vitro*

exposure to 70% N_2O for between 3 and 72 hr failed to inhibit the proliferation of lymphocytes treated with phytohemagglutinin (PHA), as did Cullen and van Belle (1975) with cells from patients with minor or no surgery, although they did observe suppressed blastogenesis in the cells of patients who underwent what they termed stress-induced surgeries.

Cobalamin oxidation by unreported amounts of N_2O following 0.5 hr and 4 hr *in vitro* exposures has resulted in decreased purine nucleotide and Met syntheses in human lymphoblasts (Boss, 1985). Rats exposed to 20% and 40% N_2O were lymphocytopenic after 21 days, although there were no abnormalities observed upon histologic examination of the spleen, submaxillary lymph nodes, and intestinal tract (Kripke *et al.*, 1977). The greatest effect, however, occurred among the myeloid cells, which decreased both in total cell number and in mature forms (Kripke *et al.*, 1977).

Nitrous oxide exposure has been related to effects upon both leukocytic chemotaxis and phagocytosis, although there is some controversy as to whether the effects are the direct result of exposure to the gas or are due to some surgically induced stress mechanism(s) involving either biogenic amines or hormones or both. The chemotactic studies have been contradictory. Nunn and O'Morain (1982) exposed human neutrophils for 15 min to 80% N_2O *in vitro*, after which cell cultures displayed decreased mobility. Another *in vitro* study involving both neutrophils and monocytes and 70% N_2O for 30 min produced similar results (Moudgil *et al.*, 1984). On the other hand, Hill *et al.* (1978), in an *in vivo* study, exposed 7 human volunteers to 60% N_2O for 1 hr. Total and differential leukocyte counts were normal, but neutrophil chemotaxis was increased rather than decreased as in the *in vitro* studies. These researchers suggested that the *in vivo* response to N_2O may

have been due to the gas somehow altering circulating blood catecholamine levels. Finally, Welch and Zaccari (1982) demonstrated no abnormal chemotaxis by neutrophils, as measured by luminol dependent chemiluminescence, following exposure to 80% N_2O *in vitro*.

Only one study on phagocytosis has been found in the literature in conjunction with N_2O administration. In this study (Cullen, 1974), 80% N_2O exposure of human leukocytes *in vitro* for 15 min resulted in a statistically non-significant decrease in the ability of the phagocytes to engulf latex particles or to demonstrate nitroblue tetrazolium reduction. (NBT reduction involves a spectrophotometrically observable color change, which is indicative of active phagocytic processes and the cell's ability to kill ingested bacteria (Green, 1970)).

Another area of interest is the influence N_2O exposure may have upon immune system cells in their performance of cytotoxic functions, especially in relation to immunologic cancer surveillance (Kimball, 1986). Cullen *et al.* (1976) described the *in vitro* exposure of mouse peritoneal exudate cells to 80% N_2O for 4 hr. These cells were placed in culture with allogeneic tumor cells, and a 12% reduction in cytotoxicity was observed. Griffith and Kamath (1986) collected whole blood samples from female patients with either benign or malignant breast carcinomas. They then exposed these cells to 66% N_2O and 34% O_2 for 5 min *in vitro*, following which the cells were co-cultured with K562 leukemia cells in a whole blood, natural cytotoxicity assay (Rees and Platts, 1983). Control assays were run following whole blood exposures to air only. No differences were noted between the cytotoxic abilities of the cells exposed to N_2O and those exposed to air only from either the benign- or the malignant-tumor patients.

Vose and Moudgil (1975) collected and isolated peripheral blood leukocytes from 26 women with mammary carcinomas prior to and after the patients underwent their individual surgeries. The cytotoxic abilities of the cells taken from the patients following *in vivo* administration of N_2O (along with several other anesthetic drugs) was determined after culturing the isolated leukocytes *in vitro* with tumor cells taken from other mammary carcinoma patients. The results indicate that there was a decrease in the cytotoxic abilities of the leukocyte preparations postoperatively, compared to preoperative observations. Kumar and Taylor (1974) demonstrated a transient decrease in the *in vitro* cytotoxicity of lymphocytes from surgical patients exposed to N_2O in combination with most of the same anesthetic drugs as above. Whether the decreased cytotoxicities were due to N_2O exposures or to the presence of the other drugs is unknown, and the possibility of surgically induced stress could not be ruled out (see also Pollock *et al.*, 1987).

EFFECT OF N_2O UPON BIOGENIC AMINES

A brief discussion of the effect of N_2O upon biogenic amines (BA) is necessary to facilitate the discussion of the interactions between the immune, nervous, and endocrine systems. Few studies have been performed that assess the effects of N_2O exposure upon BA concentrations in either the blood, the CNS, or the peripheral nervous system. Those studies that have been conducted have involved exposures to high doses of the gas. Additionally, N_2O is generally administered in combination with other drugs, often at the time the person or animal undergoes surgery. These two factors may complicate the assessment of N_2O effects.

Eisele (1985) reviewed a number of studies and case reports of the cardiovascular effects of N_2O exposure and concluded that the results can be variable depending upon the combination of drugs and gases used with N_2O. In a study on plasma BA levels during surgical procedures, Halter *et al.* (1977) determined that surgical stress, and not anesthetic exposure, plays a more important role in observed neurochemical increase. In one particular study, in which the researchers attempted to control these factors (Eisele and Smith, 1972), human volunteers were exposed to 40% N_2O for 30 to 45 min per day for 3 to 4 weeks. The volunteers' urinary epinephrine (E) and norepinephrine (NE) levels increased slightly, their plasma E levels were variable, and in all cases their plasma NE concentrations rose slightly. However, none of these changes were statistically significant.

Several researchers have described physiologic responses to N_2O that are indicative of biogenic amine fluctuations, but the results are not consistent (Eisele, 1985; Rorie *et al.*, 1986). While the evidence is still hypothetical that N_2O and morphine may produce similar biochemical consequences, studies have been conducted that relate the analgesic mechanism of N_2O to that of morphine with its concomitant impact upon BA metabolism. For instance, Hynes and Berkowitz (1979, 1981, 1983) have shown that N_2O and morphine both appear to induce concentration- and dose-dependent mouse locomotor stimulation, that this stimulation can be inhibited by certain narcotic antagonists such as naloxone (although moreso in the case of morphine), and that both the gas and the drug have similar effects upon the same strains of mice. Morphine administration has been shown to be associated with the release of brain biogenic amines when stimulating mouse locomotor activity (Rethy *et al.*, 1971; Villarreal *et al.*, 1973). In connection with the

morphine/N_2O link discussed above, one might anticipate a similar result for N_2O administration (Hynes and Berkowitz, 1983), but no studies were found on this point.

RELATIONSHIPS OF THE IMMUNE, ENDOCRINE, AND NERVOUS SYSTEMS

It was formerly thought that regulation of the immune system beyond genetic controls (the major histocompatibility complex, for example) is completely autonomous (Locke and Colligan, 1986). Evidence has been presented in the last few years, however, which strongly indicates that the immune and nervous systems act in concert, even to the point of sharing some reciprocal regulatory mechanisms in conjunction with the endocrine system (Besedovsky *et al.*, 1983). Studies have been performed that are indicative of the following dynamic interactions among the three systems: 1) there is adrenergic innervation of lymphoid organs such as the spleen, thymus, lymph nodes, bone marrow, and gut-associated lymphoid tissue (Felten *et al.*, 1985; Reilly *et al.*, 1976, 1979); 2) there are adrenergic and neurohormone receptor sites located on some lymphocytes (Hadden *et al.*, 1970; Singh *et al.*, 1979); 3) alterations of brain biogenic amine levels may have either an enhancing or a suppressive effect upon lymphocyte functions (Besedovsky *et al.*, 1981; del Rey *et al.*, 1981; Sanders and Munson, 1985); and 4) immunocytes produce a number of chemical substances that can alter biochemical pathways within the CNS (Besedovsky *et al.*, 1983, 1985; Blalock, 1984).

Others have described some of the interactions in further detail, in particular the relationships between the endocrine and immune systems and

between the endocrine and nervous systems. For instance, Claman (1972) discussed the effects of adrenal glucocorticosteroids with both humoral and cell-mediated immunities, concluding that these hormones do indeed interact with lymphoid cells, although the cells' responses are both varied and species-dependent. In an editorial, Talal and Ahmed (1987) also briefly described these interrelationships and stated that, in addition to adrenal steroid hormones, other hormones, such as pituitary, growth, adrenocorticotropin (ACTH), and sex hormones, also regulate the immune system at least to some extent.

Further evidence of immunoendocrine communication has recently been presented by Besedovsky *et al.* (1986), whose work suggests that an immunoregulatory feedback mechanism exists between the T-cell lymphokine interleukin-1 and the endocrine hormones ACTH and glucocorticosteroids. Finally, McEwen (1987) reviewed the literature regarding the interactions between the endocrine and the nervous systems in relation to their modulatory effects on mood and behavior. He concluded that glucocorticosteroids: 1) raise serotonin (5-HT) and norepinephrine (NE) and possibly dopamine (DA) levels in the adrenal medulla and brainstem, 2) maintain phenylethanolamine N-methyl transferase (PNMT) activity in the adrenal medulla and brainstem and DA beta hydroxylase activity in the hypothalamus, and 3) increase tyrosine hydroxylase (TyrH) activity in the autonomic nervous system on a short-term basis; but after several days decrease NE and 5-HT effects on the cerebral cortex and hippocampus.

OCCUPATIONAL EXPOSURES TO N_2O

It can be readily appreciated that N_2O exposure has the potential to

produce, either directly or indirectly, varied effects within the body. These effects include bone marrow depression with concomitant decreases in leukocyte (myeloid and lymphoid) numbers; altered immune responses to exogenous or endogenous insult due to decreased Ig production, cell chemotaxis, or cytotoxicity; and either positive or negative fluctuations in BA levels that may evolve from and result in a number of outcomes. As mentioned previously, there is no current United States' health standard which defines a "safe" level for occupational N_2O exposure. Nevertheless, NIOSH (Archer *et al.*, 1977) has recommended that N_2O exposures for health professionals be maintained at or below 25 ppm time-weighted average (TWA). The TWA is the average concentration to which most people may be exposed for up to 8 hr per day for 40 hr per week without any adverse effects. A TLV-TWA of 50 ppm has recently been recommended by the ACGIH (Anonymous, 1987). Several European countries have established legal standards for N_2O exposures (5 ppm in Yugoslavia and 100 ppm in Denmark, Norway, and Sweden) (Venables *et al.*, 1983).

Whether these health standards (both NIOSH's and the Europeans') are too stringent remains a point of dispute. The NIOSH recommended standard, for instance, is based primarily upon studies by Bruce and Bach (1975, 1976) and Bruce *et al.* (1974), which purportedly demonstrate human behavioral effects following several hr exposures to 50 ppm of N_2O rather than 25 ppm. However, studies by Allison *et al.* (1979), Smith and Shirley (1977, 1978), and Venables *et al.* (1983) have failed to reproduce the results of Bruce and Bach (1975, 1976) and Bruce *et al.* (1974), even with N_2O concentrations as high as 500 ppm. Based upon teratogenic studies, Vieira *et al.* (1980) suggested that a standard of 30 ppm is too low, but they made no recommendations of their

own. Several studies have reported N_2O concentrations in various occupational settings to be between 10 ppm and 3000 ppm in hospital operatories (Davenport *et al.*, 1980; Sonander *et al.*, 1985), 78 ppm and 4600 ppm TWA in dental operatories with peak values as high as 24,000 ppm (Hannifan *et al.*, 1978; Sweeney *et al.*, 1985), and approximately 25 ppm TWA in veterinary operatories (Johnson *et al.*, 1987).

As has been pointed out, most of the studies performed to date have assessed the toxic potential of N_2O during acute, high-level exposures. It is felt that further study of occupational exposures that are representative of subchronic or chronic time periods is needed. This study was performed to assess some of the effects discussed above at levels that more realistically reflect exposures of personnel to N_2O in occupational settings.

CHAPTER III

METHODOLOGY

One-hundred forty-four male CD-1 mice (Charles River, Wilmington, MA) were numbered and randomly assigned to 24 groups. One-half of the animals were exposed to either N_2O or control air for a 2-week period (subacute study) and the other half for a 13-week period (subchronic study). When dosing was initiated, the mice were 4 to 8 weeks old and weighed between 26 and 38 g. Food and water consumption was measured weekly per group, and each animal was weighed initially and at the end of each week throughout the experiment. The day following the second week of exposure for the subacute study and the thirteenth week for the subchronic study, the mice were sacrificed by decapitation and spleens, livers, kidneys, thymuses, and adrenal glands were removed and weighed. Blood and bone marrow were also removed.

ANIMAL ACCLIMATION

One week prior to their being exposed to N_2O, the mice were acclimated to a daily routine of a 12-hr light period. Ambient room temperature was maintained at 75°F±5°F with a relative humidity of between 34% and 62%. Each day during the acclimation period, excluding the weekend, the animals were placed in 750-l, whole-body, dynamic air-flow inhalation chambers (Wahmann Manufacturing Co., Timonium, MD) for 6 hr and exposed to approximately 10 chamber air changes per hr (ACH) of filtered room air. Chamber environmental conditions were the same as those in the ambient

room environment. The mice were housed by group outside of the chambers during non-exposure periods and were maintained on commercial lab chow and water *ad libitum*.

ANIMAL EXPOSURES TO N_2O

Following the acclimation period, N_2O exposures were initiated (the N_2O used in these studies was > 99% pure and was obtained from Ogden Welders Supply, Logan, UT). After placing the mice in the inhalation chambers, N_2O was introduced through flowmeters (Cole-Parmer Inc., Chicago, IL) into three of the chambers such that concentrations of 50.6 ppm ± 0.3 ppm N_2O, 502 ppm ± 1.4 ppm N_2O, and 4990 ppm ± 19 ppm N_2O were achieved (Appendix A). The fourth chamber received filtered room air only and housed the mice used as non-exposed controls. A schematic diagram of the exposure set-up is provided as figure III-1. As during the acclimation period, 10 ACH were maintained in each chamber, and environmental and all other conditions (temperature, humidity, food, time, etc.) were controlled as previously described. The chambers were maintained under a slightly negative pressure, and no N_2O was detected in the room air. Nitrous oxide concentrations were measured using a MIRAN I infrared gas analyzer (Foxboro Company, Foxboro, MA). It was calibrated prior to use by injecting a known volume of N_2O into the machine and noting the absorbance reading. Appendix B shows the calibration curve obtained for the infrared analyzer.

Prior to initiating animal exposure to N_2O, sampling was performed to determine gas concentrations at different points throughout the chambers, especially at the level where the animal cages were to be placed during the

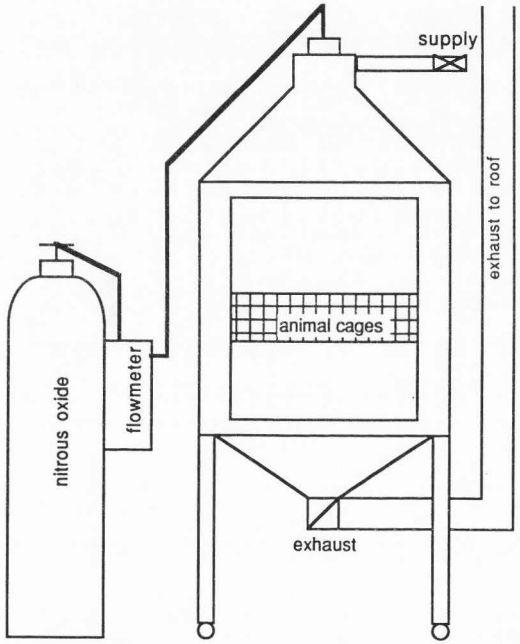


FIG. III-1. Schematic diagram of the inhalation chamber set-up used to administer nitrous oxide to the CD-1 mice.

dosing regimen. There was an even distribution of N_2O throughout each chamber, with < 4% variation from sample point to sample point. Even so, the animals were randomly placed in different locations within the chambers on a daily basis to control for the possibility of any variances in air flows and N_2O concentrations that might occur.

Immediately after the mice were placed in the chambers, N_2O and mixing air were introduced by opening regulating valves to pre-determined settings. The time needed to achieve 99% of the maximum test concentration in each chamber was determined to be 15 min, after which time the 6 hr exposure period was observed. At the end of the exposure period, N_2O flow into the chambers was cut off, and the chambers were allowed to exhaust any remaining N_2O for 15 to 20 min, after which the mice were removed from the chambers until the next day's exposure was initiated. All of the N_2O introduced to the chambers was exhausted through outlets on the roof of the animal test facility. Nitrous oxide concentrations in the inhalation chambers were routinely measured every 2 hr throughout the day, beginning immediately after achievement of the 99% concentration.

IMMUNOLOGIC EXPERIMENTS

The methods used were similar to those described by Hsieh *et al.* (1988) and Srischuart *et al.* (1987), with minor modifications. Spleens were removed aseptically and maintained in ice-cold, low-endotoxin RPMI 1640 media (Hyclone Laboratories, Inc., Logan, UT) supplemented 5% with fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT), 100 units of penicillin and 100 μg of streptomycin (Fisher Scientific Co., Pittsburg, PA) per ml of

media. The spleens were mashed in a model STOM 80 Stomacher® Lab Blender (Tekmar® Company, Cincinnati, OH), producing mononuclear splenic cell suspensions. These were transferred into 15-ml sterile conical centrifuge tubes, and the non-cellular debris was allowed to settle in the tubes for 10 min, after which the contents were decanted into new sterile tubes. Cell numbers were determined by counting with a model D-2 Automatic Blood Cell Counter (Coulter Electronics, Inc., Hialeah, FL). The cell suspension volumes were adjusted to between 1 to 2 X 10^7 cells/ml of media. The following assays were performed with the splenic cell cultures.

Mitogen Assays

Fifty μl of splenic cells (approximately 0.5 to 1 X 10^6 cells/well) were cultured in triplicate in 96-well, flat-bottomed microtissue culture plates (Becton Dickinson & Company, Oxnard, CA) either with or without 50 μl of media containing concanavalin A (Con A) (Difco Laboratories, Inc., Detroit, MI), lipopolysaccharide (LPS) from *E. coli* (Sigma Chemical Company, St. Louis, MO), purified phytohemagglutinin (PHA) (Wellcome Reagents Division, Greensboro, NC), or pokeweed mitogen (PWM) (Sigma Chemical Company, St. Louis, MO). The optimal concentration for splenic cell growth was determined by titration to be 0.39 μg/ml for Con A, 50 μg/ml for LPS, 1.56 μg/ml (subacute study) and 3.13 μg/ml (subchronic study) for PHA, and 12.5 μg/ml for PWM. These concentrations were used in the assays. Control wells (those without mitogen) received 50 μl of supplemented RPMI.

An additional 50 μl of supplemented RPMI was added to each well, after which the cultures were incubated for 42 hr at 37°C in humidified air with 4% CO_2. Each culture was then pulsed with 0.5 μCi (18.5 kilobequerels (kBq))

of tritiated-thymidine ([^{3}H]-TdR) (specific activity 2 Ci/mmol (74 gigabequerels (gBq)/mmol) (New England Nuclear, Boston, MA) in 50 μl of media. Incubation was continued for 6 hr, after which the splenic cells were harvested with a Brandel model M-12 cell harvester (Biomedical Research and Development Laboratories Inc., Gaithersberg, MD) onto 2.1 cm glass microfiber filters (Whatman Ltd., Maidenstone, Eng.). The filters were then placed into 7 ml scintillation counting vials and dried overnight in the dark at room temperature. The following day 5 ml of ScintiVerse™ liquid scintillation cocktail (Fisher Scientific Co., Pittsburg, PA) was added to each vial, after which the amount of tritium activity was determined in each well in a Packard model Tri-Carb 2660 liquid scintillation counter (Packard Instrument Company, Inc., Downers Grove, IL).

Mixed Lymphocyte Culture

A one-way, mixed lymphocyte culture assay (MLC) was performed using mitomycin C-treated stimulator YAC-1 mouse splenic cells (Moloney virus induced T-lymphoma cells from A/Sn [H-2] mice, American Tissue Culture Collection (ATCC), Rockville, MD). Two sets of splenic responder cells were utilized, one treated with (control) and one without (experimental) mitomycin C. For the control cultures, 50 μl (0.25 to 0.5 X 10^6 cells/well) of treated responder cells were co-cultured in triplicate in 96-well, flat-bottomed microtissue culture plates with 50 μl (0.125 to 0.25 X 10^6 cells/well) of non-mitomycin C-treated CD-1 mouse splenic cells. These cultures were then pulsed with 0.5 μCi (18.5 kBq) of [^{3}H]-TdR (6 hr pulsing for the subacute study, 24 hr for the subchronic study, both performed during the latter portion of the culture incubation period) in 50 μl of supplemented RPMI media. Finally, 5 X 10^{-5} M mercaptoethanol was added to each well. The

procedure for the experimental cultures was the same as for the control cultures, except that 25 μl of the YAC-1 splenic cells (0.125 to 0.25 X 10^6 cells) was mixed with 100 μl of the untreated responder cells (0.25 to 0.5 X 10^6 cells) in each well. The cultures were incubated for 72 hr at 37°C in humidified air with 4% CO_2. Cell-harvesting and liquid-scintillation procedures were the same as described for the mitogen assays.

Interleukin-2 Assay

An interleukin-2 (IL-2)-dependent mouse cell line (HT-2) (from BALB/c mice) (Watson, 1979) was cultured in triplicate with test supernatants of varying concentrations produced from the T lymphocytes of the N_2O exposed CD-1 mice. The HT-2 cell line was maintained in medium consisting of 60% (by volume) RPMI supplemented 10% with FBS, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 40% (by volume) of RPMI containing 5 units of rat spleen cell IL-2 (Sigma Chemical Company, St. Louis, MO) per ml of media, and 5 X 10^{-5} M mercaptoethanol. Prior to being mixed with the CD-1 test supernatant, the HT-2 cells were washed and resuspended in fresh RPMI-FBS-antibiotic medium at a concentration of 1 X 10^5 cells/ml. Fifty μl of the HT-2 cell culture (5 X 10^3 cells) was then added to each well of 96-well, flat-bottomed microtissue culture plates along with varying concentrations of test supernatants (undiluted, 50%, 25%, and 12.5%). The cultures were incubated for 24 hr at 37°C in humidified air with 4% CO_2. One-half of 1 μCi (37 kBq) of [^{3}H]-TdR in 50 μl of supplemented RPMI was then added to each well and incubation was continued for another 4 hr, after which the cells were harvested and counted by liquid scintillation as previously described.

Cell-Mediated Cytolysis

The cytolytic activity of cytotoxic T lymphocytes and nonspecific accessory cells, such as natural killer (NK) cells, was measured as described by Grabstein (1980), with modifications (Hsieh *et al.*, 1988). YAC-1 cells were utilized as the antigenic target (stimulator). Prior to their use in the assay, the YAC-1 cells were checked both to ensure that they were in a log-growth phase and at least 90% viable. They were then suspended in RPMI media supplemented 10% with FBS and 50 μg/ml streptomycin and 50 units/ml penicillin at a concentration of 1 X 10^6 cells/ml. For every 1 ml of cell suspension 50 μg of mitomycin C was added, after which the cells were incubated at 37°C in humidified air with 4% CO_2.

At the end of 1 hr, 1 X 10^7 cells were transferred to a 50-ml conical centrifuge tube and 250 μCi (9.25 MBq) of ^{51}Cr (specific activity 200-900 Ci/g (7.4-33.3 teraBq/g) New England Nuclear, Boston, MA), given as sodium chromate, was added. This mixture was incubated for 1 hr with gentle agitation. Subsequently, the cells were pelleted by centrifugation for 10 min at 200 X g and 4°C. The supernatant was then decanted, and the cells were resuspended by the addition of approximately 5 ml of FBS. The cells were cultured for 30 min, re-centrifuged, then re-suspended in supplemented RPMI at a concentration of 1 X 10^5 cells/ml.

Varied concentrations (1 X 10^7, 5 X 10^6, and 2.5 X 10^6 cells/ml) of the CD-1 mouse splenocytes (effector or responder cells) were co-cultured in triplicate in 100 μl volumes with 100 μl of the YAC-1 cell cultures in 96-well, flat-bottomed microtissue culture plates for 4 hr at 37°C in humidified air with 4% CO_2. Control cultures measuring either the spontaneous or the maximal release of ^{51}Cr were run concurrently with the experimental cultures. The

control cultures had 100 μl of cell growth media or 100 μl of 1% (w/v) saponin (NP40) added, respectively, rather than the CD-1 splenocyte suspension. Also, 10μl (150 units) of mouse fibroblast interferon (Sigma Chemical Company, St. Louis, MO) was added to all except the control wells. At the end of the incubation period, the cultures were centrifuged at 250 X g for 10 min at 4°C. Being careful not to disturb the cell pellet, 100 μl of the supernatant was removed from each well, placed into scintillation vials, and measured for radioactivity with a model 578 gamma scintillation counter (Packard Instrument Company, Inc., Downers Grove, IL).

Plaque-Forming Cell Assay

A plaque-forming cell (PFC) assay was performed to determine the ability of splenic lymphocytes from the test mice to respond to an antigenic challenge by sheep red blood cells (SRBCs) following N_2O exposures. The methods employed were basically as described by Jerne and Nordin (1963). Ninety-six hr prior to their being sacrificed, the mice were given, ip, 0.25 ml of a solution consisting of 20% (by volume) SRBCs in sterile physiologic saline solution. Following the injections, the animals continued to be exposed to N_2O as before until they were sacrificed. Spleens were aseptically removed and weighed, and a monocellular splenic cell culture was made as previously described. In order, 450 μl of freshly prepared SRBC solution (10% SRBCs, by volume), 100 μl of guinea pig complement (Gibco Laboratories, Grand Island, NY), and 450 μl of the splenic cell suspension (1 X 10^7 cells/ml) were added to sterile 12 X 75 mm test tubes and mixed thoroughly. Aliquots of the mixtures (1 per test animal) were then added to the preweighed microchambers described previously (Cunningham and Szenberg, 1968). The chambers were then sealed with a paraffin/Vaseline mixture. The chamber cultures were

incubated for 1 hr at 37°C, after which the number of plaques per chamber were counted.

Immunoglobulin Quantitation

An antibody (Ab) titer for immunoglobulin M (IgM) was conducted by an indirect, enzyme-linked immunosorbent assay (ELISA) (Voller and Bidwell, 1986). The blood samples used in the assay were obtained from the same mice used in the PFC assay. Ninety-six-well, flat-bottomed, polystyrene microtissue culture plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 μl of SRBC ghosts (prepared as described by Hanahan and Ekholm (1974)) diluted to a protein concentration of 3.6 μg/ml in pH 9.6 carbonate-bicarbonate buffer. After the plate wells were filled with the SRBC ghost solution, the plates were covered with parafilm and stored at 4°C for 24 hr. Following the storage period, the plate wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween).

Serum samples from the test animals were collected and diluted 1:50, 1:100, and 1:200 in PBS-Tween, after which 100 μl of each serum sample dilution was added in triplicate to the ghost-coated wells. The samples were incubated for 1 hr at room temperature. The wells were then re-washed three times with PBS-Tween and 100 μl of peroxidase-conjugated goat anti-mouse IgM, diluted 1:1000, was added to each well for 1 hr. The wells were washed as before, then 100 μl of substrate (0.4 mg/ml o-phenylenediamine in pH 5 phosphate citrate buffer and 0.4 μl/ml of 30% H_2O_2) was added to each well. After 20 min, the reaction was stopped by adding 50 μl of 1 N HCl to each well. The optical density was determined for each well on a model EL309 microplate autoreader (Bio-Tek Instruments, Inc., Burlington, VT) using an absorbance of 490 nm. Non-SRBC-sensitized mouse serum served as a negative control.

BLOOD SYSTEM ASSESSMENTS

Routine Hematology

Total red and white blood cell counts were performed using a model D-2 Attomatic Blood Cell Counter (Coulter Electronics, Inc., Hialeah, FL). Differential leukocyte counts were conducted following standard white blood cel staining procedures. Additionally, hematocrit determinations were made fo each animal using microhematocrit equipment, and hemoglobin coicentrations were defined using a model Hemo-W® hemoglobinometer (Coulter Electronics, Inc., Hialeah, FL).

Deoxyuridine Suppression Test

A deoxyuridine suppression test was performed as described by Town *et al.* (1986), with slight modifications. Cell samples were obtained from the long bones of the hind limbs of the mice as described by Mishell and Shiigi (1980). After producing monocellular bone marrow suspensions, 100 μl (5 X 10^5 cells) of the cell cultures was added in two triplicate sets per animal to 96-well flat-botomed microtissue culture plates along with 10 μl of autologous serum. Next, either 20 μl of thymine- and thymidine-free 2'-deoxyuridine (dUdR) (Sgma Chemical Company, St. Louis, MO) at a concentration of 0.1 μmol/ml or 20 μl of Hank's Balanced Salt Solution (HBSS) was added to each well. The well voumes were adjusted to 190 μl with the addition of HBSS, and the cultures were then incubated for 1 hr at 37°C in humidified air with 4% CO_2.

At the conclusion of the incubation period, each well was pulsed with 5 μCi (185 MBq) of [^{3}H]-TdR (specific activity 2 Ci/mmol (74 MBq/mmol)) in 9.5 μl of HBSS. The cell cultures were then re-incubated for an additional hr. Cell havesting and liquid scintillation procedures were as previously described

for the mitogen assays.

ENDOCRINE HORMONE MEASUREMENTS

Radioimmunoassay (RIA) procedures were utilized to measure serum levels of both adrenocorticotropic hormone (ACTH) and corticosterone. To control for the variability often seen in endocrine hormone serum concentrations throughout the day, samples were obtained and assays run at the same time of day for all of the animals in both the subacute and subchronic N_2O-dosing groups. Kits for the RIAs were obtained from Radioassay Systems Laboratories, Inc., Carson, CA, and were used as directed with slight modifications. Plasma samples were obtained from each animal by collecting blood from the carotid artery into chilled, NaEDTA-coated tubes at the time of animal sacrifice. The cellular constituents of the samples were pelleted by centrifugation at 200 X g for 10 min at 4°C, after which the plasma was carefully suctioned out of the tubes by pipet, placed into 1.5 ml tubes, and frozen at -20°C until the hormone analyses were performed later during the week.

Adrenocorticotropic Hormone

Just prior to their use, lyophilized reagents were reconstituted and mixed with kit-supplied water and allowed to sit at 4°C for 15 min, after which 0.1 ml of diluent water, varied standard ACTH concentrations, or plasma was pipetted into duplicate kit vials and kept on ice throughout the procedure. Next, 0.1 ml of anti-ACTH was added to all tubes except those with diluent water, followed by the addition of 0.1 ml of ^{125}I-ACTH. Tube contents were mixed thoroughly and then incubated at 4°C for 16 hr. At the end of the incubation period, 0.5 ml of precipitant solution was added to each tube and mixed in well. The

tubes' contents were centrifuged at 1000 X g for 15 min at 6°C, and the supernatants were decanted into a liquid-waste container. Radioactivity in each pellet was measured with a model 578 gamma scintillation counter (Packard Instrument Company, Inc., Downers Grove, IL).

Corticosterone

Plasma samples were obtained from the mice as described above for the ACTH assay but were diluted 1:200 with the steroid diluent provided with the kit (10 μl of plasma in 2 ml of diluent). All reagents were brought to room temperature prior to use, then 0.1 ml of diluent buffer, varied standard corticosterone concentrations, or diluted plasma samples were added to duplicate 10 X 75 mm polystyrene centrifuge tubes. Next, 0.2 ml of ^{125}I-corticosterone was added to each tube, followed by 0.2 ml of anti-corticosterone. The tube contents were mixed followed by incubation for 2 hr at room temperature. At the conclusion of the incubation period, 0.5 ml of precipitant solution was added to each tube and mixed for 20 sec. The tubes' contents were centrifuged for 15 min at 1000 X g, and the supernatants were then decanted into a liquid-waste container. The radioactivity in each pellet was measured by a gamma counter as described above.

STATISTICAL ANALYSES

The data from each of the above experimental procedures were tested for honoscedasticity by Bartlett's method (Snedecor and Cochran, 1967). Data for which homogeneity of variance could be assumed were tested for statistical significance by one-way analysis of variance (ANOVA) using the F statistic. Intergroup comparisons were made by Student-Newman-Keuls' procedure (Dowdy and Wearden, 1983). Values $\leq$ 0.05 were used to determine statistical

significance. Data for which heterogeneity of variance was assumed were tesed for statistical significance ($p \leq 0.05$) by the non-parametric Mann-Whitney U-test (Sokal and Rohlf, 1973).

CHAPTER IV

RESULTS

The results obtained from monitoring food and water consumption are unremarkable, insofar as the observation of any specific trends are concerned. It should be noted, however, that the use of these items by the different animal groups fluctuated greatly throughout the study period, and that the control animals in the 13-week study appeared to consume more food and water on a weekly basis than did the animals exposed to N_2O. The results of these measurements are presented as Table IV-1 (food and water consumption for the 2-week study animals) and Figures IV-1 and IV-2 (food and water consumption for the 13-week study animals).

SUBACUTE EXPERIMENTS

Body and Organ Weights

By the end of the first week of exposure, mice from the 500-ppm dosing level demonstrated depressed body weight gains ($p < 0.05$), compared to both the control animals and those at the 5000-ppm level (Table IV-2). This trend was observed to continue through the second week as compared to the high-dosing level (5000 ppm) animals only. Relative liver weights of animals exposed to 500 ppm were decreased ($p < 0.05$) compared to controls and 5000 ppm animals (Table IV-3).

Immunologic Assessments

Both B- and T-splenic lymphocyte responsiveness to various mitogens were significantly depressed ($p < 0.02$ for LPS and $p < 0.01$ for Con A, PHA, and

TABLE IV-1

FOOD (G) AND WATER (ML) CONSUMPTION BY CD-1 MICE DURING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Food consumed (g)		Water utilized (ml)	
	Week 1	Week 2	Week 1	Week 2
0	224.1	231.6	493.2	559.3
50	202.1	204.7	552.8	447.8
500	220.8	231.1	480.2	480.6
5000	215.7	224.6	552.3	527.2

PWM) at 5000 ppm, compared with 0 ppm mouse cells when measured as total [^{3}H]TdR cellular incorporation (Table IV-4). Interestingly, however, Con A and PHA, immature and total T-cell mitogens, respectively, and LPS, a B-cell stimulator, produced noticeable but statistically non-significant decreased mitogenic activity at 50 ppm compared with 0 ppm cells. The activity observed in the median-dosing group was comparable to the activity noted in the cells from animals not exposed to N_2O. Pokeweed mitogen (PWM) stimulation dose-responsively decreased with increasing N_2O exposure.

As shown in Table IV-5, both the 500 ppm animals' cells ($p < 0.05$) and the 5000 ppm animals' cells ($p < 0.01$) took up less [^{3}H]-TdR than did the 0 ppm animals' cells, whether they were co-cultured with YAC-1 cells or not. T-lymphocyte interleukin-2 (IL-2) production, shown in Table IV-6, was

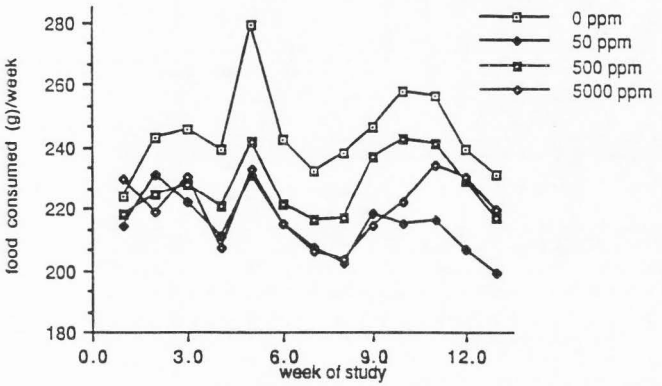


FIG IV-1. Food consumption (g) by CD-1 mice during 13-week exposures to nitrous oxide.

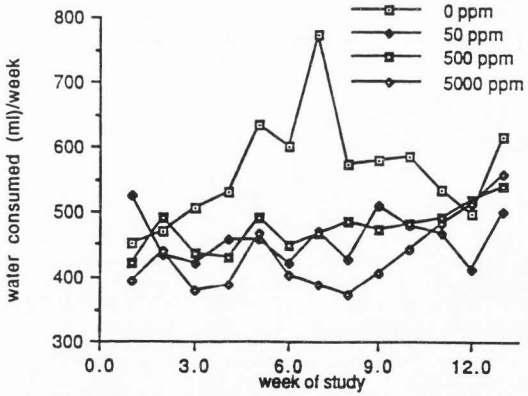


FIG. IV-2. Water utilization (ml) by CD-1 mice during 13-week exposures to nitrous oxide.

TABLE IV-2

BODY WEIGHTS[a] OF CD-1 MICE DURING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Body weight	
	Day 7	Day 14
0	103.33±0.77	107.75±1.66
50	101.95±0.81	104.58±1.03
500	100.93±0.61[b,c]	103.75±0.61[b]
5000	103.53±0.66	107.25±1.41

a - Data presented as the means±SE (n = 6). Expressed as a percentage of the body weights (g) measured prior to exposure regimen initiation.

b - Significantly different from 0 ppm at $p < 0.05$.

c - Significantly different from 5000 ppm at $p < 0.05$.

TABLE IV-3

ORGAN WEIGHTS[a] OF CD-1 MICE FOLLOWING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen	Thymus	Liver	Kidneys	Adrenals ($X 10^2$)
0	0.34±0.02	0.10±0.01	5.81±0.11	1.65±0.06	1.25±0.14
50	0.28±0.02	0.10±0.01	5.78±0.16	1.61±0.12	1.15±0.17
500	0.35±0.05	0.10±0.01	5.35±0.06[b]	1.61±0.09	1.32±0.26
5000	0.32±0.02	0.10±0.01	5.85±0.14	1.73±0.05	1.15±0.25

a - Data presented as the means±SE (n = 6). Expressed as the organ weights (g)/100 g body weight.

b - Significantly different from both 0 ppm and 5000 ppm at $p < 0.05$.

virtually unaltered following N_2O exposures. No differences were noted in cell-mediated cytolytic capabilities (Table IV-7).

Humoral responses, as measured by a plaque-forming cell (PFC) assay and by enzyme-linked immunosorbent assay (ELISA), quantitating sera anti-SRBC antibody titers are presented in Table IV-8 and Figure IV-3, respectively. The number of PFCs observed at each exposure level was approximately equal whether calculated as PFCs/10^6 or PFCs/total spleen cells. Circulating antibody levels for SRBCs were similar for all exposure groups with only one significantly different IgM concentration noted at one serum dilution.

TABLE IV-4

CD-1 MOUSE SPLENIC LYMPHOCYTE PROLIFERATIVE RESPONSES TO MITOGENIC SUBSTANCES FOLLOWING 2-WEEK ANIMAL EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen cellularity[a]	Mitogenic responses[b]				
		No Mitogen	Con A	PHA	LPS	PWM
0	1.12±0.03	4.68±0.35	94.81±25.64	79.26±15.14	78.32±21.34	24.24±6.42
50	1.19±0.09	4.52±0.33	41.39± 7.48	32.48± 4.81	52.72±14.07	18.99±5.58
500	1.22±0.12	3.90±0.24	81.84±30.90	62.07±21.33	61.52±18.45	12.21±3.72
5000	1.02±0.04	4.23±0.24	13.98± 2.97[d]	14.67± 3.28[d]	16.74± 3.66[d]	6.55±1.00[d]

a - Number of lymphocytes recovered/spleen X 10^{-8}.

b - dpm/10^6 splenic lymphocytes X 10^{-3}, response evaluated by ^3H incorporated after 6 hr pulsing. Data presented as the means±SE (n = 6 except 0 ppm where n = 5).

c - Significantly different from 0 ppm at $p < 0.01$.

d - Significantly different from 0 ppm at $p < 0.02$.

TABLE IV-5

TRITIATED-THYMIDINE UPTAKE BY CD-1 MOUSE SPLENIC LYMPHOCYTES IN MIXED CULTURE WITH MITOMYCIN-C TREATED YAC-1 CELLS FOLLOWING 2-WEEK ANIMAL EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Responders dpm/10^6 cells (X 10^{-3})	Responders/ stimulators dpm/10^6 cells (X 10^{-3})
0	6.6±1.6	8.7±2.0
50	4.8±0.6	4.6±0.6
500	3.2±0.3[b]	4.1±0.4[b]
5000	2.0±0.1[c]	2.1±0.2[c]

a - Data presented as the means±SE (n = 6 except for 0 ppm where n = 5).

b - Significantly different from 0 ppm at $p < 0.01$.

c - Significantly different from 0 ppm, 50 ppm, and 500 ppm at $p < 0.01$.

TABLE IV-6

HT-2[a] CELL RESPONSE TO INTERLEUKIN-2 PRODUCED BY SPLENIC LYMPHOCYTES FROM CD-1 MICE EXPOSED TO NITROUS OXIDE FOR 2 WEEKS

Nirous oxide concentration (ppm)	Stimulation indices[b,c] IL-2 concentration[d]			
	100	50	25	12.5
0	16.0±1.2	13.8±1.2	10.6±0.4	9.4±0.7
50	16.2±1.8	14.2±1.4	11.9±2.3	9.9±0.9
500	12.1±1.3	8.1±0.5[e]	9.3±0.9	6.8±0.4
5000	13.1±1.8	12.1±0.8	9.4±0.9	7.2±1.0

a - HT-2 cells are dependent upon the presence of IL-2 for proliferative re:ponse.

b - Data presented as the means±SE (n = 6 except for 0 ppm where n = 5).

c - Ratio of dpm from ^3H incorporation into HT-2 cells cultured in supernatant containing IL-2 from CD-1 mouse splenic lymphocytes cutured with Con A to dpm from ^3H incorporation into HT-2 cels cultured in supernatant containing IL-2 from CD-1 mouse splenic lynphocytes cultured without Con A.

d - % of original splenic lymphocyte supernatant containing IL-2.

e - Significantly different from 50 ppm at $p < 0.05$.

TABLE IV-7

CELL-MEDIATED CYTOLOYSIS OF YAC-1 TARGET CELLS BY SPLENIC LYMPHOCYTES AND NON-SPECIFIC KILLER CELLS FROM CD-1 MICE EXPOSED FOR 2 WEEKS TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Percent ^{51}Cr release[a,b]		
	100:1[c]	50:1[c]	25:1[c]
0	797±185	462±261	710±205
50	478±237	101±125	-[d]
500	924±125	821±136	529±107
5000	563±143	-[d]	363±143

a - Data presented as the means±SE (n = 6 except for 0 ppm where n = 5).

b - Expressed as: (dpm/10^6 target cells minus spontaneous dpm/10^6 target cells) X 10^{-2}.

c - Effector cell:target cell ratio.

d - Not reported due to technical error.

Note: Maximal ^{51}Cr release was measured as 3753 dpm/10^6 target cells and spontaneous as 945 dpm/10^6 target cells, both X 10^{-3}.

TABLE IV-8

HUMORAL IMMUNE RESPONSES[a] OF CD-1 MICE FOLLOWING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen cellularity[b]	PFC[c]/10^6 spleen cells	PFC/total spleen cells (X 10^{-3})
0	1.72±0.21	1323±146	235.2±43.9
50	2.02±0.23	1167±183	233.9±39.3
500	1.86±0.09	1448±146	266.0±25.6
5000	1.64±0.23	1405±484	244.0±79.3

a - Data presented as the means±SE (n = 6).

b - Number of lymphocytes recovered/spleen X 10^{-8}.

c - Plaque forming cells.

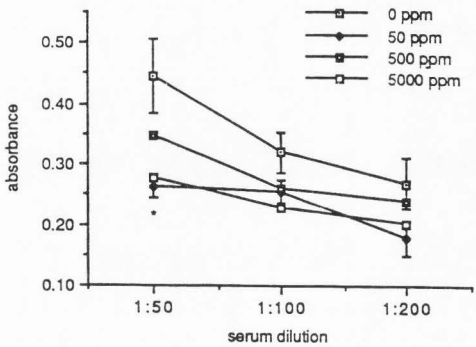


FIG IV-3. Enzyme-linked immunosorbent assay quantitation of anti-sheep red blood cell immunoglobulin M levels from the sera of CD-1 mice exposed to nitrous oxide for 2 weeks. Titers measured in absorbance units. Presented as the means±SE (n = 6). *50 ppm significantly different from 0 ppm at 1:50 dilution, $p < 0.05$.

Hematologic Parameters

The results of the routine hematologic assessments are presented in Table IV-9 While statistical significance was not observed, the total number of leukocytes in the high-dose group was between 13% to 17% less than that measured in any of the other three groups.

There were no obvious trends noted in the cellularity of the animals' bone marrows (Table IV-10) and, although there were no statistical differences seen among the various dosing groups in the deoxyuridine suppression test (dUdRST), all three groups receiving N_2O had dUdR suppression values 10% higher than those seen in the control group (Figure IV-4).

Endocrine Hormones

No intergroup differences were noted in either the serum corticosterone or ACTH level (data not presented).

TABLE IV-9

HEMATOLOGICAL PARAMETERS[a] FROM CD-1 MICE FOLLOWING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Erythrocytes (10^6/mm^3)	Hematocrit (%)	Hemoglobin (g/dL)	Leukocytes (10^3/mm^3)	Leukocyte differentials (%)		
					Lymphocytes	Neutrophils	Others[b]
0	10.01±0.40	48.9±1.3	17.7±0.6	6.37±0.29	70±4	24±4	6±1
50	10.44±0.14	47.6±0.5	16.8±0.4	6.71±0.37	74±3	21±3	5±1
500	9.64±0.07	49.2±0.7	17.3±0.2	6.59±0.68	67±6	26±5	7±1[c]
5000	10.25±0.23	49.1±1.2	17.8±0.5	5.55±0.14	69±5	26±4	5±1

a - Data presented as the means±SE (n = 6).

b - Includes monocytes, eosinophils, and basophils.

c - Significantly different from 50 ppm at $p < 0.05$.

TABLE IV-10

BONE MARROW CELL RESPONSES[a] OF CD-1 MICE FOLLOWING 2-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Bone marrow cellularity[b]	dUdR suppression value (%)[c]	Normalized[d] dUdR suppression value (%)[c]
0	6.42±0.38	87.4±2.9	100.0±3.3
50	5.73±0.31	97.3±3.9	111.3±4.5
500	5.75±0.81	97.3±5.2	110.4±5.9
5000	6.40±0.21	96.5±7.7	111.3±8.8

a - Data presented as the means±SE (n = 6).

b - Expressed as the total number of bone marrow cells collected from both femurs X 10^{-7}.

c - Expressed as the amount of [^{3}H]-TdR uptake after preincubation with deoxyuridine (dUdR) as a percentage of uptake without preincubation with dUdR.

d - Normalized to 100% for 0 ppm values.

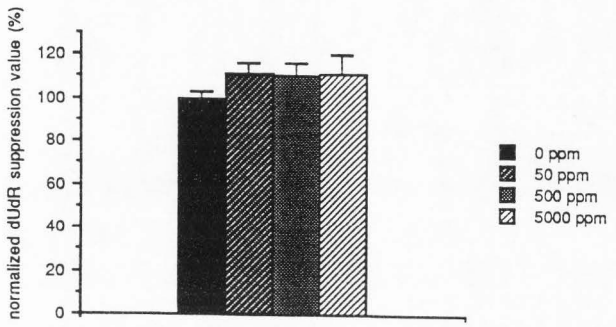


FIG. IV-4. Deoxyuridine suppression test performed on CD-1 mouse bone marrow cells following 2-week animal exposures to nitrous oxide. Presented as the means±SE (n = 6).

SUBCHRONIC EXPERIMENTS

Body and Organ Weights

While no statistically significant differences were observed between the dosing groups, Figure IV-5 shows that the body weights of the animals in all three groups receiving N_2O appeared to be slightly less than those receiving no N_2O, especially during the second half of the exposure regimen. These same animals' relative liver weights (Table IV-11) were substantially ($p < 0.01$) reduced compared with control animal liver weights, as were the adrenal weights in the animals from the 500-ppm dosing group ($p < 0.05$).

Immunologic Assessments

As seen with the subacute mice, the splenic lymphocytes from the subchronic animals dosed with 50 ppm of N_2O manifested decreased [^{3}H]-TdR uptake after culturing with either Con A, LPS, or PHA (Table IV-12), but, as at 2 weeks, the lower numbers observed were not statistically different from the values associated with the 0-ppm cells. However, there was increased

TABLE IV-11

ORGAN WEIGHTS[a] OF CD-1 MICE FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen	Thymus	Liver	Kidneys	Adrenals ($X 10^2$)
0	0.31±0.02	0.04±0.01	5.78±0.14	1.79±0.09	1.00±0.05
50	0.22±0.01	0.07±0.01	5.04±0.07[b]	1.58±0.06	0.86±0.06
500	0.29±0.03	0.04±0.01	5.13±0.11[b]	1.70±0.04	0.77±0.09[c]
5000	0.29±0.03	0.06±0.01	5.01±0.14[b]	1.58±0.07	1.01±0.04

a - Data presented as the means±SE (n = 6). Expressed as the organ weights (g)/100 g body weight.

b - Significantly different from 0 ppm at $p < 0.01$.

c - Significantly different from 0 ppm and 5000 ppm at $p < 0.05$.

TABLE IV-12

CD-1 MOUSE SPLENIC LYMPHOCYTE PROLIFERATIVE RESPONSES TO MITOGENIC SUBSTANCES FOLLOWING 13-WEEK ANIMAL EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen cellularity[a]	Mitogenic responses[b]				
		No mitogen	Con A	PHA	LPS	PWM
0	1.70±0.14	11.52±1.22	184.96±47.91	301.46±77.22	177.11±30.35	32.96±10.53
50	1.57±0.04	10.06±2.33	101.45±44.01	106.06±38.72	74.59±26.40	42.96±17.01
500	1.18±0.06[c]	27.21±4.56	331.29±78.49	415.60±29.67[d]	241.47±33.07[d]	140.99±37.52
5000	1.64±0.14	33.65±9.52[e]	354.00±80.24	397.72±67.26[d]	304.94±26.36[d]	146.51±43.31

a - Number of lymphocytes recovered/spleen X 10^{-8}.

b - dpm/10^6 splenic lymphocytes X 10^{-3}, response evaluated by ^3H incorporated after 6 hr pulsing. Data presented as the means±SE (n = 6).

c - Significantly different from both 0 ppm and 5000 ppm at $p < 0.05$.

d - Significantly different from 50 ppm at $p < 0.01$.

e - Significantly different from both 0 ppm and 50 ppm at $p < 0.05$.

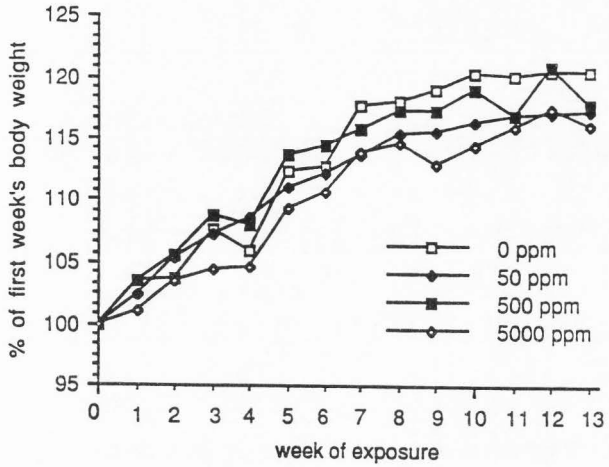


FIG. IV-5. Body weights of CD-1 mice during 13-week exposures to nitrous oxide. Presented as the means±SE (n = 6) and expressed as a percentage of the body weights measured prior to exposure regimen initiation.

radioisotopic uptake at the two higher N_2O concentrations, significantly so ($p < 0.01$) for PHA- and LPS-cultured cells compared to 50 ppm cells. Control (no mitogen) cell-culture [^{3}H]-TdR uptake at 0 ppm and 50 ppm was similar, but there was significantly increased tracer uptake ($p < 0.05$) at 5000 ppm compared to both lower-exposure groups. There was also increased uptake of [^{3}H]-TdR by 500 ppm cells, which approached levels of significance ($0.05 < p < 0.10$) compared to 0 ppm and 50 ppm cells.

Unlike their 2-week counterparts (Table IV-5), in which decreased [^{3}H]-TdR uptake was noted at higher N_2O exposure levels, the CD-1 splenic lymphocytes in MLC following 13-week gas exposures manifested increased radioisotopic uptake ($p < 0.05$) compared with both 0-ppm and 50-ppm cells

(Table IV-13).

The IL-2 assay, while performed under indentical conditions as those in the subacute study, resulted in no HT-2 cell response (data not presented) to Con A-stimulated CD-1 mouse lymphocyte supernatant versus non-stimulated cell supernatant. The cell-mediated cytolytic assay results (Table IV-14) mimicked those seen in the subacute study, with cell killing and subsequent ^{51}Cr release measured at levels too low ($\leq$ 4%) to detect any differences between N_2O dosing groups.

Relative thymus and adrenal gland weights of animals challenged ip with SRBCs were significantly decreased ($p < 0.05$) at 5000 ppm compared with the 500-ppm and 50-ppm groups (Table IV-15). The number of plaque-forming cells/10^6 splenic cells and per spleen was significantly less ($p < 0.05$) for 5000-ppm animals compared with control animals (Table IV-16). Anti-SRBC serum antibody titers (Figure IV-6) were reduced after 5000-ppm exposures compared to both 0-ppm and 500-ppm animals.

Hematologic Parameters

Table IV-17 shows the routine hematologic data collected from the 13-week study. Red blood cell data show no effect relative to N_2O dosing. White blood cells (wbc), however, appear to be greatly affected by animal exposures to the gas, as the total leukocytes per mm^3 of blood in the N_2O-dosed animals were significantly decreased ($p < 0.01$) compared to control animals.

Bone marrow cellularity is slightly higher in those animals dosed with N_2O than in control animals (Table IV-18). Figure IV-7 shows that bone marrow cells, as measured by the deoxyuridine suppression test, were dose responsive with regards to N_2O exposure, significantly so at the two higher

TABLE IV-13

TRITIATED-THYMIDINE UPTAKE BY CD-1 MOUSE SPLENIC LYMPHOCYTES IN MIXED CULTURE WITH MITOMYCIN-C TREATED YAC-1 CELLS FOLLOWING 13-WEEK ANIMAL EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Responders dpm/10^6 cells (X 10^{-3})	Responders/ stimulators dpm/10^6 cells (X 10^{-3})
0	16.7±3.9	25.4±3.4
50	18.2±1.9	23.6±4.5
500	25.0±3.0	43.1±5.5[b]
5000	25.8±1.2	41.0±3.5[c]

a - Data presented as the means±SE (n = 6 except for 0 ppm where n = 5).

b - Significantly different from both 0 ppm and 50 ppm at $p < 0.01$.

c - Significantly different from 0 ppm at $p < 0.05$ and from 50 ppm at $p < 0.01$.

TABLE IV-14

CELL-MEDIATED CYTOLOYSIS OF YAC-1 TARGET CELLS BY SPLENIC LYMPHOCYTES AND NON-SPECIFIC KILLER CELLS FROM CD-1 MICE EXPOSED FOR 13 WEEKS TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Percent ^{51}Cr release[a,b]		
	100:1[c]	50:1[c]	25:1[c]
0	1786±531	1467±613	1078±125
50	1103±271	711±237	294±233
500	1522±323	1000±196	742±337
5000	1717±312	861±331	728±293

a - Data presented as the means±SE (n = 6).

b - Expressed as: (dpm/10^6 target cells minus spontaneous dpm/10^6 target cells) X 10^{-2}.

c - Effector cell:target cell ratio.

Note: Maximal ^{51}Cr release was measured as 5918 dpm/10^6target cells and spontaneous as1490 dpm/10^6 target cells, both X 10^{-3}.

TABLE IV-15

ORGAN WEIGHTS[a] OF SHEEP RED BLOOD CELL-SENSITIZED CD-1 MICE[b] FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen	Thymus	Liver	Kidneys	Adrenals ($X 10^2$)
0	0.34±0.02	0.10±0.01	5.10±0.13	1.62±0.04	0.81±0.12
50	0.33±0.02	0.08±0.01	5.47±0.09	1.55±0.05	1.12±0.09
500	0.32±0.03	0.10±0.01	5.26±0.06	1.60±0.09	0.81±0.14
5000	0.33±0.02	0.08±0.01[c]	5.40±0.10	1.62±0.07	0.65±0.05[d]

a - Data presented as the means±SE (n = 6). Expressed as the organ weights (g)/100 g body weight.

b - Injected with sheep red blood cells ip 4 days prior to sacrifice.

c - Significantly different from 500 ppm at $p < 0.05$.

d - Significantly different from 50 ppm at $p < 0.05$.

TABLE IV-16

HUMORAL IMMUNE RESPONSES[a] OF CD-1 MICE FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Spleen cellularity[b]	PFC[c]/10^6 spleen cells	PFC/total spleen cells (X 10^{-3})
0	1.20±0.11	2219±206	282.8±23.5
50	1.31±0.08	1802±219	252.4±44.1
500	1.09±0.11	1803±167	210.6±31.2
5000	1.07±0.11	1277± 74[d]	134.0±20.6[d]

a - Data presented as the means±SE (n = 4 except for 5000 ppm where n = 6).

b - Number of lymphocytes recovered/spleen X 10^{-8}.

c - Plaque forming cells.

d - Significantly different from 0 ppm at $p < 0.05$.

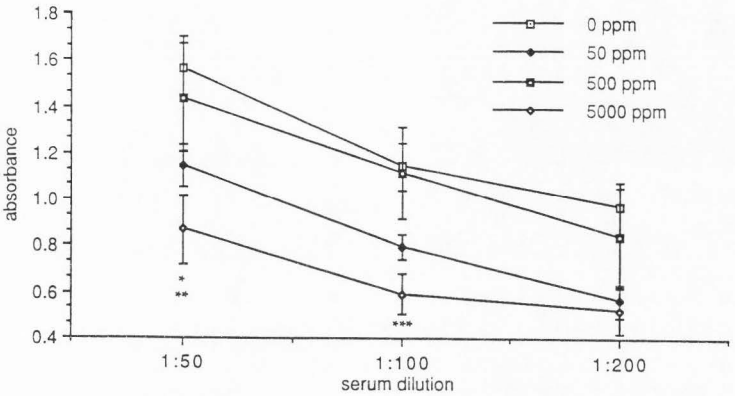


FIG. IV-6. Enzyme-linked immunosorbent assay quantitation of anti-sheep red blood cell immunoglobulin M levels from the sera of CD-1 mice exposed to nitrous oxide for 13 weeks. Titers measured in absorbance units. Presented as the means±SE (n = 6). *5000 ppm significantly different from 500 ppm at 1:50 dilution, $p < 0.05$. **5000 ppm significantly different from 0 ppm at 1:50 dilution, $p < 0.01$. ***5000 ppm significantly different from both 0 ppm and 500 ppm at 1:100 dilution, $p < 0.01$.

doses.

Endocrine Hormones

No differences were noted in either serum corticosterone or ACTH levels among the N_2O-dosing groups and control animals (Table IV-19).

TABLE IV-17

HEMATOLOGICAL PARAMETERS[a] FROM CD-1 MICE FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Erythrocytes (10^6/mm^3)	Hematocrit (%)	Hemoglobin (g/dL)	Leukocytes (10^3/mm^3)	Leukocyte differentials (%)		
					Lymphocytes	Neutrophils	Others[b]
0	10.14±0.48	44.9±0.9	16.9±0.3	8.32±0.20	71±4	23±3	6±2
50	10.30±0.31	45.3±1.0	17.8±0.4	6.44±0.24[c]	59±5	36±6	4±1
500	10.18±0.11	42.8±0.7	16.8±0.2	6.24±0.34[c]	73±2	22±2	5±1
5000	10.04±0.30	44.0±0.6	16.5±0.4	6.56±0.23[c]	57±3	37±4	6±1

a - Data presented as the means±SE (n = 6 except for luekocyte data where n = 5 for 500 ppm and 5000 ppm).

b - Includes monocytes, eosinophils, and basophils.

c - Significantly different from 0 ppm at $p < 0.01$.

TABLE IV-18

BONE MARROW CELL RESPONSES[a] OF CD-1 MICE FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Bone marrow cellularity[b]	dUdR suppression value (%)[c]	Normalized[d] dUdR suppression value (%)[c]
0	4.67±0.37	61.3±11.1	100.0±18.1
50	4.86±0.94	82.0± 9.7	133.8±15.8
500	4.90±0.16	103.8±10.4[e]	169.3±17.0[e]
5000	5.09±0.58	125.3± 3.6[f]	204.4± 5.9[f]

a - Data presented as the means±SE (n = 6).

b - Expressed as the total number of bone marrow cells collected from both femurs X 10^{-7}.

c - Expressed as the amount of [^{3}H]-TdR uptake after preincubation with deoxyuridine (dUdR) as a percentage of uptake without preincubation with dUdR.

d - Normalized to 100% for 0 ppm values.

e - Significantly different from 0 ppm at $p < 0.05$.

f - Significantly different from both 0 ppm and 50 ppm at $p < 0.01$.

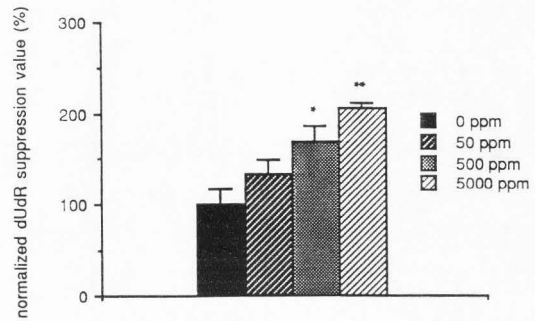


FIG. IV-7. Deoxyuridine suppression test performed on CD-1 mouse bone marrow cells following 13-week animal exposures to nitrous oxide. Presented as the means±SE (n = 6). *Significantly different from 0 ppm, $p < 0.05$. **Significantly different from both 0 ppm and 50 ppm, $p < 0.01$.

TABLE IV-19

SERUM ENDOCRINE HORMONE LEVELS[a] IN CD-1 MICE FOLLOWING 13-WEEK EXPOSURES TO NITROUS OXIDE

Nitrous oxide concentration (ppm)	Corticosterone (ng/ml)	ACTH (pg/ml)
0	65.0±27.6	286± 38
50	31.0± 3.9	268± 59
500	65.1± 2.3	374± 77
5000	64.6±21.3	298±106

a - Data presented as the means±SE (n = 6 except for corticosterone 0 ppm where n = 5).

CHAPTER V

DISCUSSION

Body and Organ Weights

During 2-week N_2O exposures only transient differences were noted in animal body weights (Table IV-2), and no differences were noted during the 13-week study period (Figure IV-5). Relative organ weights also demonstrated no differences following both exposure periods (Tables IV-3 and IV-11), with the exception of the livers. These showed transiently decreased relative weights after 2 weeks, but the weights were significantly lower for all three N_2O-dosing groups compared to control mice after 13-week gas exposures.

One possible explanation for the decreased liver weights at 13 weeks is found in studies showing that high-level (50%), usually short-term, N_2O exposures result in depressed hepatic uptake of folate (McGing *et al.*, 1978; Perry *et al.*, 1979). Furthermore, exposing rats to N_2O leads to increased levels of both plasma and urinary folate (Lumb *et al.*, 1982). As a consequence of both decreased folate uptake and its increased excretion, tissue stores of folate are markedly decreased, and this is especially true for the liver (Lumb *et al.*, 1980, 1981a), the primary folate repository organ (Nunn and Chanarin, 1985). As this substance is necessary for DNA synthesis, its absence may be responsible for decreased organ cellularity and weight.

There is inconclusive evidence that N_2O exposure results in some form of hepatotoxicity (Brodsky, 1985). One previous subchronic study (Rice et al., 1985), using a different mouse strain and higher N_2O levels for shorter (4 hr) daily time periods than were used in this study, revealed no liver weight

changes following gas exposures. Possible cytochrome P-450-mediated toxicity, however, has been ruled out following negative results from mice (Rice *et al.*, 1985) and rats (Hallen and Johansson, 1975 as cited by Brodsky, 1985).

Immunologic Assessments

The results obtained from experiments involving lymphocyte transformation suggest that N_2O appears to have a biphasic effect on duration of gas exposure. Following 2-week exposures there was a general trend toward suppressed lymphoblastogenesis, with DNA synthesis decreasing significantly at the highest N_2O exposure level (Table IV-4). This trend was also observed in the MLC assays (Table IV-5).

At the conclusion of 13-week exposures the effects were reversed. In the mitogen assays (Table IV-12) both unstimulated and PHA- and LPS-induced DNA synthesis increased in a dose-related fashion, being significantly elevated at 5000 ppm. In the presence of Con A and PWM, [^{3}H]-TdR incorporation increased by 2X and 4X, respectively; however, statistical significance was not apparent due to the variability observed in individual numbers. As with the 2-week experiments, the results of the 13-week mitogen assays were supported by the similarity of the 13-week MLC findings, with increased radioisotopic uptake being observed at the higher N_2O concentrations. While the increased [^{3}H]-TdR uptake seen in the cells from the high-dosed, subchronic animals in both the blastogenic and the MLC assays was unexpected, the occurrence was not without precedent.

Das and Hoffbrand (1970), using human peripheral blood lymphocytes from patients with B_{12} deficiency and pernicious anemia, also found that PHA-cultured cells took up more [^{3}H]-TdR than did control lymphocytes that had

no vitamin B_{12} anomaly. They further noted that dUdR failed to suppress the uptake of the radioisotope in vitamin-deficient cells and suggested that [^{3}H]-TdR added to the cell cultures skirted the blocked B_{12} pathway, allowing DNA synthesis to proceed following activation of a salvage pathway catalyzed by thymidine kinase. This enzyme has demonstrated increased activity in the bone marrow cells of people deficient in vitamin B_{12} (Nakao and Fujioka, 1968). The mechanistic postulate of Das and Hoffbrand (1970) was lent further credence by the finding of Haurani (1973), that PHA-stimulated lymphocytes from people with pernicious anemia have decreased thymidylate synthase (dTMPS) activity. More recent work by Haurani *et al.* (1984) demonstrated that *in vitro* N_2O exposure of PHA-stimulated lymphocytes also decreases dTMPS activity and that this is corrected by addition of CH_3Cbl.

The results observed here indicate that a mechanism similar to that proposed by Das and Hoffbrand (1970) may have been operating during the current experiments. If that were true it might mean that N_2O acted as a mitogen in the sense that it primed the CD-1 cells for replication, that process ensuing once [^{3}H]-TdR became available to the cells by its addition to the culturing media. The results of this research also indicate that the commonly used [^{3}H]-TdR uptake assay may not be entirely appropriate for measuring blastogenic responses to mitogenic substances following N_2O exposure. Instead, direct quantitation of cell numbers both before and after culturing with mitogenic substances may be necessary to determine whether those substances are able to induce blastogenesis following gas exposures.

The results of the cell-mediated cytolitic (CMC) assay, in which cytotoxic and helper-T-lymphocyte subpopulations undoubtedly participated,

demonstrate no immunomodulation. The low cytolysis observed in these experiments is similar to that seen in other immunotoxicologic assessments using the same animal model (Hsieh *et al.*, 1988; Srischuart *et al.*, 1987). The addition of mouse fibroblast gamma-interferon in the current experiments lent no apparent assistance to natural killer-cell (NK) activity. Based upon these results and those by Hsieh *et al.* (1988) and Srischuart *et al.* (1987), this particular animal model does not seem to be suitable for assessing NK activity.

Humoral immunocompetency following 2 weeks of anesthetic gas exposure did not seem to be compromised (Table IV-8). However, the results of the 13-week study (Table IV-16) indicate that longer-term exposures to N_2O may prove detrimental to B-cell production of antibody, inasmuch as there was a significant decrease in the number of PFCs in the mice after 5000 ppm of N_2O exposure compared to the nonexposed animals. The mice exposed to N_2O also exhibited decreased circulating anti-SRBC IgM levels after 5000 ppm gas exposures (Figure IV-6).

For SRBC hemolysis to occur using the PFC assay, there must be a cooperative interaction among antibody-secreting B lymphocytes, T-helper lymphocytes that produce the lymphokines necessary for B-cell growth and differentiation, and macrophages (antigen-presenting cells). Since macrophage function was not studied as a part of this research, the effect of N_2O on PFC capabilities through these cells cannot be addressed. Similarly, whether the gas had any disruptive influence upon the T-helper cell subpopulation cannot be addressed.

The most likely explanation for the decreased PFC activity and circulating IgM levels seen in the current experiments, other than depressed macrophage and/or helper T-cell function, is the direct effect N_2O has on

vitamin B_{12} (Banks *et al.*, 1968; Blackburn *et al.*, 1977) of hindering the B-lymphocyte replication necessary for immunoglobulin synthesis. Hall *et al.* (1981) demonstrated that human PBLs from B_{12}-deficient patients, when cultured with PWM (both a B- and T-lymphocyte mitogenic factor), produced much less IgG and total proteins than did the lymphocytes from healthy patients. They furthermore demonstrated that the addition of free, active B_{12} to the culture media results in increased production of both antibodies and total proteins from B_{12}-deficient, but not control, cells. Kafetz (1985) reported two cases wherein elderly patients with B_{12} deficiency had low immunglobulin levels prior to receiving vitamin treatment. Their antibody titers reverted to more normal levels following B_{12} therapy. Neither of these studies quantitated lymphocyte levels or cellular proliferative capacities.

Hematologic Parameters

Disruption of the dTMPS pathway, indirectly measured by the deoxyuridine suppression test (dUdRST) (Metz, 1984; Wickramasinghe, 1981), apparently occurred in the animals' bone marrows in both a time- and dose-dependent fashion subsequent to N_2O exposure, with similar trends being observed over both time periods. After 2-week exposures, dUdR failed to suppress bone marrow uptake of [^{3}H]-TdR by approximately 10% in all three dosing groups compared to controls (Table IV-10). While these differences were not statistically significant, in humans a 10% difference in uptake of [^{3}H]-TdR clinically distinguishes persons with megaloblastic anemia from those without the disease (Metz, 1984). At the end of 13-week exposures, the effect was more pronounced, with dUdR suppression of [^{3}H]-TdR uptake at the highest dose doubling that seen at 0 ppm (a 100% difference in normalized

values) (Table IV-18). These results resemble similar time- and dose-response patterns previously reported for rats (Deacon *et al.*, 1980b).

Of note are the absolute (non-normalized) dUdRST values obtained in these studies, which were higher than those observed previously in rat (Baden *et al.*, 1983; Deacon *et al.*, 1979, 1980b; Nunn *et al.*, 1982) and human (Metz, 1984; Town *et al.*, 1986; Wintrobe, 1981) models despite using a similar protocol. Nevertheless, the differences between the absolute values for cells displaying dUdR-depressed [^{3}H]-TdR uptake and those with less depressed uptake were comparable to those seen in studies with rats (McKenna *et al.*, 1980). No studies were found in which a dUdRST was performed on mouse bone marrow cells for comparison.

It was thought that perhaps the dUdR concentration (0.1 μmol/ml) used in this study was too low to suppress cellular radioisotopic uptake, but experimental observations using 10- and 100-fold higher dUdR concentrations, conducted prior to performing this study (results not shown or published), produced similar results. One study (McKenna *et al.*, 1980) indicated that rat bone marrow cells preincubated with varied concentrations of dUdR prior to [^{3}H]-TdR treatment responded by producing a wide range of dUdR suppresion values, from near 0% to near 100%. This study (McKenna *et al.*, 1980) also demonstrated that regardless of the dUdR concentration utilized, the differences in dUdRST values obtained ran parallel when comparing normal cells to those with vitamin B_{12} deficiencies.

The subchronic study by Rice *et al.* (1985) differed from the subchronic results of this study in that they reported no change in total peripheral blood leukocyte numbers when using Swiss Webster mice, despite the use of high N_2O exposure concentrations (5000 ppm to 500,000 ppm). The CD-1 mice used

in this study had markedly ($p < 0.01$) fewer leukocytes/mm^3 of blood than did their nondosed counterparts. However, based upon other N_2O-hematologic studies of varying nature (usually higher dose and shorter term) (Green and Eastwood, 1963; Kripke *et al.*, 1977), the leukopenia noted in this study was not at all unexpected. Coupled with the decreased DNA synthesis noted in both unstimulated or mitogen-stimulated lymphocytes, the observed significant leukopenia in the current experiments may indicate that a severe compromise of immune function is possible at higher levels of N_2O exposure over subchronic periods of time.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

This research has demonstrated that in a mouse model N_2O, at exposure levels to which humans may be exposed and for time periods which mimic occupational settings, may be immunotoxic; although consistent, significant effects were observed only at 5000 ppm, with no change at 50 ppm and occasional changes at 500 ppm. It is not certain whether there is a cell-mediated immune (CMI) effect or not, but it appears that N_2O may suppress antibody-mediated immunity (AMI). Nitrous oxide probably has no effect on nonspecific immunocompetency, but this animal model may not be appropriate for making such a determination. Whether the effect of N_2O upon immune system cells is direct or is the result of bone marrow suppression or both cannot be determined from these results, although the possibility of the latter occurrence seems most likely. There appears to be no effect upon circulating hormone levels following gas exposure.

Because of the variable results at the lower gas-exposure levels, it seems that N_2O at or below 50 ppm may be devoid of immunotoxic properties even after 13 weeks of exposure. These low levels of N_2O exposure are routinely observed in well-scavenged hospital operatories but are less often achieved in unscavenged hospital operatories or in dental operatories. There should, therefore, be some concern regarding N_2O's immunotoxicologic potential for personnel exposed to the gas in the latter situations. It should also be considered that occupational exposures to N_2O may last for longer periods of time (years) than were observed in these experiments. Such human

exposures may also represent a greater proportion of a person's life span (perhaps as much as 30 years or 40% for career anesthesiologists) than were observed in the CD-1 mice (approximately 15% of their life expectancy). Conversely, most human exposures are not expected to be as continuous as the exposure regimens used in these experiments, which would allow for longer and more frequent recovery periods from gas exposures.

Several recommendations seem appropriate based upon these results. First, the use of an outbred strain of mouse resulted in a large amount of intragroup variation. This perhaps masked N_2O exposure effects that otherwise might have been apparent. It is therefore recommended that any further immunotoxicologic research with this gas (or any other potentially immunotoxic substance, for that matter) be performed with inbred animal strains. Second, since there is evidence that N_2O is potentially immunotoxic, especially with regard to AMI, it is suggested that further immunotoxicologic assessment be performed to determine if there is a gas effect. This would include the performance of bacterial or viral antigenic challenge assays, macrophage-function assessment, T- and B-lymphocyte enumeration, and so on. These assays may better delineate whether N_2O has an effect upon CMI. Third, if further immunotoxicologic testing is performed, the use of an animal model that better demonstrates non-specific immune responsiveness should be considered. Last, in addition to performing further immunotoxicologic tests, it may be desireable to perform dUdRSTs on lymphoid cells as well as bone marrow cells to better determine their DNA synthetic capabilities. Such studies performed in the presence and absence of exogenous vitamin B_{12}, folinic acid, and/or methyltetrahydrofolic acid may help to define the mechanistic immunotoxicity of N_2O.

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APPENDICES

APPENDIX A

TABLE A-1

NOMINAL AND OBSERVED CONCENTRATIONS OF NITROUS OXIDE IN THE INHALATION CHAMBERS

Nominal concentration (ppm)	Observed concentration[a] (mean ppm±SE)
0	0.0± 0.0
50	50.6± 0.3
500	502.0± 1.4
5000	4990.0±19.0

a - The observations are based upon approximately 295 readings taken 4 times daily throughout the exposure periods. Measurements of nitrous oxide concentrations were taken at the level of the animal cages in the inhalation chambers and were obtained by infrared spectrophotometry.

APPENDIX B

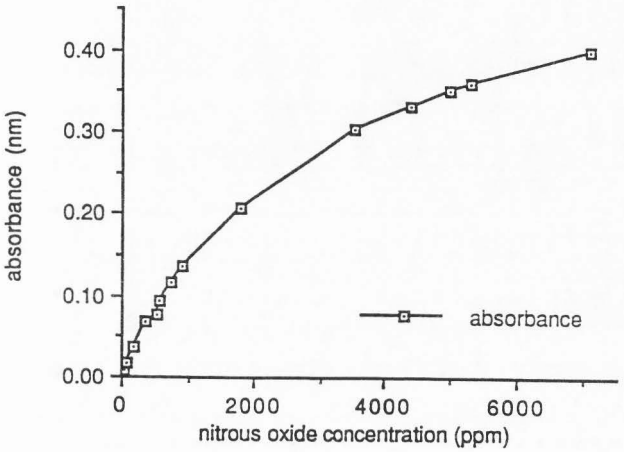

FIG. B-1. Calibration curve utilized for determining infrared analyzer absorbance reading versus inhalation chamber nitrous oxide concentration.

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