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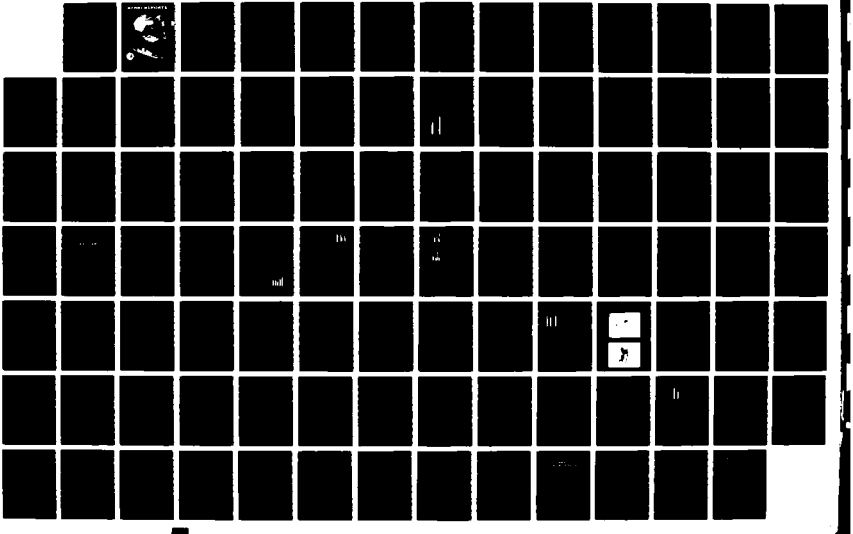

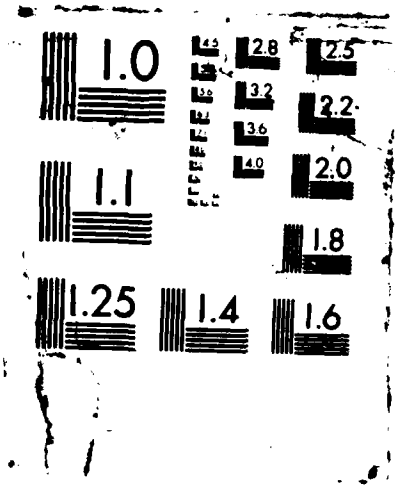
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CONTENTS

Scientific Reports

MOTOR PERFORMANCE EFFECTS OF PROPYLENE GLYCOL DINITRATE IN THE RAT

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Propylene glycol dinitrate (PGDN), a major constituent of a liquid torpedo propellant, produces incoordination and impairment of balance in humans. This study was conducted to evaluate the rat as a model for PGDN-induced motor performance decrement, and to determine if direct application of PGDN onto neural tissue is a useful alternative to other routes of exposure. PGDN was injected onto the cisterna magna (ic) of adult Sprague-Dawley rats trained on the accelerod, a test of motor performance. Three groups of 13–14 male rats each received a single dose of either 5 or 10 µl PGDN or 25 µl sterile saline (control) while anesthetized with halothane. Accelerod performance was measured 12 min after ic injection, then hourly for 6 h, and at 24 h. Injections were evaluated using a five-stage screening criterion to eliminate grossly traumatized subjects, to verify the accuracy of the injection, and to determine the extent of mechanical damage. Eighteen out of 41 subjects passed the five-stage screen. A significant decrease in performance occurred during the first 2 h following injection of 10 µl PGDN compared to the control and the 5 µl groups. No significant differences were seen between the 5 µl and control groups. These data confirm previous findings of PGDN-induced changes in human motor performance, suggesting that the rat may be a useful model for further PGDN neurobehavioral assessment. The data also indicate that ic injection may be an effective alternative to other routes of exposure for materials with appropriate chemical and biological properties if an evaluation screen is used.

INTRODUCTION

Propylene glycol dinitrate (PGDN) is a major constituent of a volatile liquid torpedo propellant, Otto Fuel II, used by the U.S. Navy.

The authors gratefully acknowledge the assistance of C. Boward and G. G. Kessell in training and testing the experimental subjects and B. Jackson in statistical analyses.

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Based on animal research, the major toxic effects of PGDN are hemosiderin deposits in the liver and kidneys, hypotension, and methemoglobin formation (Anderson et al., 1976; Clark and Litchfield, 1969; Jones et al., 1972). Human studies have shown that PGDN also produces headaches, nasal congestion, dizziness, impairment of motor coordination and balance, eye irritation, disruption in the organization of the visual evoked response, and changes in oculomotor function (Stewart et al., 1974; Horvath et al., 1981). In the work place, inhalation is the main route of exposure for personnel handling the propellant, although considerable potential also exists for percutaneous absorption. An occupational exposure survey by Carmichael and Lieben (1963) of explosive workers suggests serious PGDN-induced physiologic consequences, i.e., headaches, nausea, vomiting, lowered blood pressure, and increased pulse rate. No animal research has been done to follow up these clinical observations. Developing alternative animal models to test substances such as PGDN might be important due to restrictive regulations regarding human research. The primary objective of this study was to determine the neuromotor effects of PGDN using the rat/accelerod model, which has been shown to be a good test of motor performance following neurotoxic insult (Bogo et al., 1981; 1984).

Human inhalation studies conducted to date have been exposures to Otto Fuel II, which includes stabilizers, dyes, and desensitizers, as well as PGDN (Horvath et al., 1981; Stewart et al., 1974), or mixtures of PGDN and unidentified inert diluents (Anderson and Mehl, 1973; Jones et al., 1972; Mattsson et al., 1981), but none have used the nitrate ester alone. This is primarily because PGDN has a significant vapor pressure at room temperature and is highly flammable and explosive. Considering these characteristics of PGDN, intracisternal (ic) administration might be preferred over inhalation exposure for procedural reasons because it is a direct route of exposure, which makes it easier to control the accuracy and precision of the dose, and because the very small quantities of material necessary to conduct the experiment contribute to a safer work environment for the investigator. Further experimental advantages accrue because systemic degradation to the mononitrate by interaction with hemoglobin or enzymes is reduced and the blood–brain barrier is circumvented (Clark and Litchfield, 1969; Needleman and Hunter, 1965; Chasseaud et al., 1978). Therefore, the second objective of this study was to determine the feasibility of the ic route of exposure in the rat for modeling human inhalation response to PGDN.

METHODS

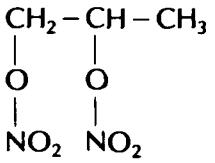
Subjects

Subjects were 41 male Sprague-Dawley rats that weighed 425 ± 9 g. Subjects were individually housed in polycarbonate cages and main-

tained in keeping with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council. Food and water were available ad libitum, and room temperature was kept at 22 ± 1°C.

Test Material

Propylene glycol dinitrate (PGDN) in ethanol solution was obtained from the Naval Propellant Plant, Indian Head, Md., and refrigerated until ready for use. PGDN or 1,2-propanediol has the following chemical structure:



Portions of 100 μl were precipitated out of alcohol stock solution using ice-cold sterile saline. The PGDN droplets were washed three times with saline to remove the remaining alcohol. Purity of the neat PGDN was verified by gas–liquid chromatography and electron-capture detection as >99.995%.

Dosing Procedures

Anesthesia was induced by a halothane-saturated atmosphere in a desiccating jar. The semiconscious subject was placed in the anesthetic-filled cylinder at the top of the resting block (Fig. 1). A rubber band looped behind the upper incisors and secured at the front of the resting block to hold the subject snugly in the cylinder. Once the tail-pinch reflex ceased, the following occurred. The tip of the guide needle was angled freehand (as shown in the left of Fig. 2), and it was inserted through the shaved skin into the previously marked ligaments between the occipital bone and the first vertebra. As the guide-needle holder (1-cc tuberculin syringe) was rotated down 90°, the tip of the needle was used to locate the upper edge of the foramen magnum. When the needle tip pierced the dura, the 33-gauge internal cannula was used as a probe to gently slide in and out of the guide needle to determine when the needle tip was no longer occluded by the dura. The guide needle was then rotated further down to lift the cerebellum and allow the cannula to be inserted 1.0–1.5 mm into the cisternal space (as shown in the right of Fig. 2). The cannula position relative to the guide needle and thus the depth of the cannula insertion were determined by using the graduations on the 1-cc glass tuberculin syringe referenced to a mark on the cannula. In preliminary tests of the procedure, injection of methylene blue demonstrated penetration and depo-

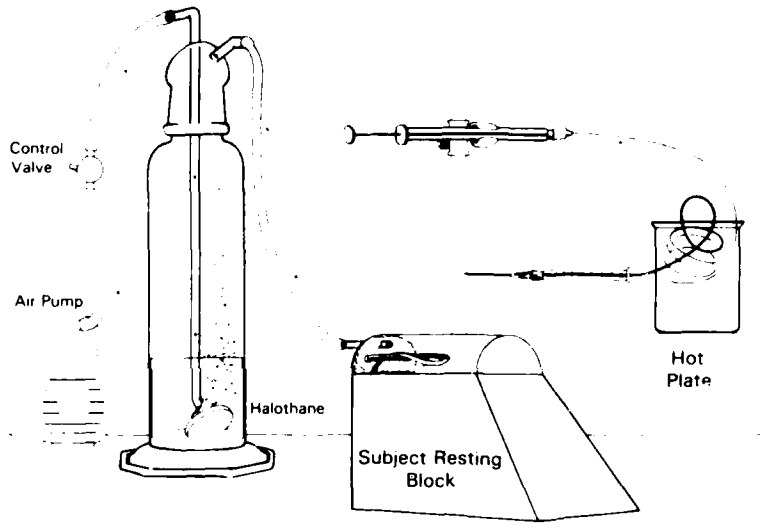


FIGURE 1. Anesthesia generation and ic injection apparatus. The anesthetic was generated by an air pump and halothane-filled glass container shown on the left. Halothane was pumped to the gauze-filled end on top of the rat resting block. The 1-cc glass tuberculin syringe was mounted on a ring stand. The polyethylene tube (0.015 in. inside diameter and 20 in. long) passed through the hot-plate-heated water maintained at 37°C to the cannula (33 gauge, 4 in. long) and the rat on the resting block.

sition within the cisterna magnum, as opposed to the ventricles and solid tissue.

The dose groups were 5 and 10 μl PGDN and 25 μl sterile saline (control). Sufficient saline was added to the PGDN injections to produce a total volume of 25 μl. The test solutions were flushed from the preloaded polyethylene tube shown in Fig. 1 into the cisterna magna over a 30-s interval.

Task

The accelerod apparatus and procedure have been described in detail elsewhere (Bogo et al., 1981). Rats were trained to maintain balance for as long as possible on a gradually accelerating 2-in.-diameter rod elevated 6 in. above a grid-shock floor. The rotational velocity of the rod was increased at an average rate of 0.9 rev/min/s. A trial began by placing a subject on the stationary rod, and it lasted until the subject fell to the grid floor. Each training session lasted from 5 to 15 min (4–20 trials). It took an average of 9 d for a subject to learn the task. In the final stages of training, shock was given only for performance that lasted less than 30 s. The task was scored as performance duration, in seconds. The average performance time before receiving PGDN was 51 ± 3 (standard error) s, with performance averaged over three trials/ses-

sion/subject. PGDN testing began after stable daily performance of 30 s or above had been established.

Initial testing began 12 min after ic injection. Selection of this test time was based on a preliminary study with sham injections. In the pilot study, animals were rendered unconscious in a halothane-saturated atmosphere and were maintained on 3% halothane for about 2 min in order to shave, mark, and sham-inject them. For sham injections, the guide needle pierced the dura but the cannula was not extended (Fig. 2). As shown in Fig. 3, rats could perform at baseline levels within 6 min after removal from anesthesia. However, the performance trend did not return to the mean baseline performance until about 12 min after injection. Therefore, this latter time was used in the actual experiments. Beyond the 12-min interval, testing occurred hourly 6 times and at 24 h.

Analysis

The performance measure was time spent on the accelerod. Multiple one-way analyses of variance (ANOVA) were done to assess the effects of time after PGDN administration (Winer, 1962). The analyses were based on the difference between pre- and post-PGDN performance. A Bonferroni allocation for each ANOVA was in effect to compensate for potential multiple analyses errors (Miller, 1981).

Injection Evaluation Procedure

Prior experience with manual ic injection techniques and preliminary experiments with the current method indicated that trauma from

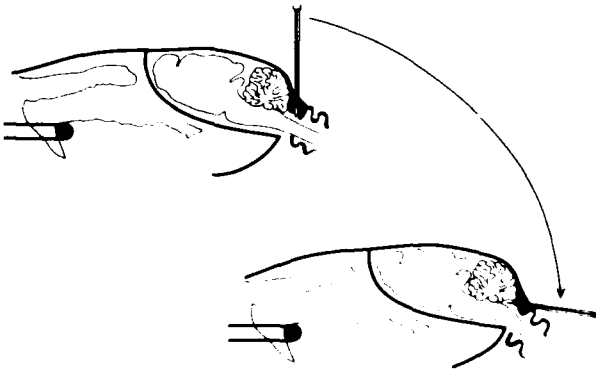


FIGURE 2. Detail of the ic injection procedure showing a side cutaway view of a rat head located on the resting block in Fig. 1. The left panel shows the incisor attached to a rubber band that maintained the head in the halothane saturated cylinder, with the needle and retracted cannula oriented perpendicular to the skull. In the right panel, the needle has been rotated 90° with the cannula inserted into the cisternal magnum.

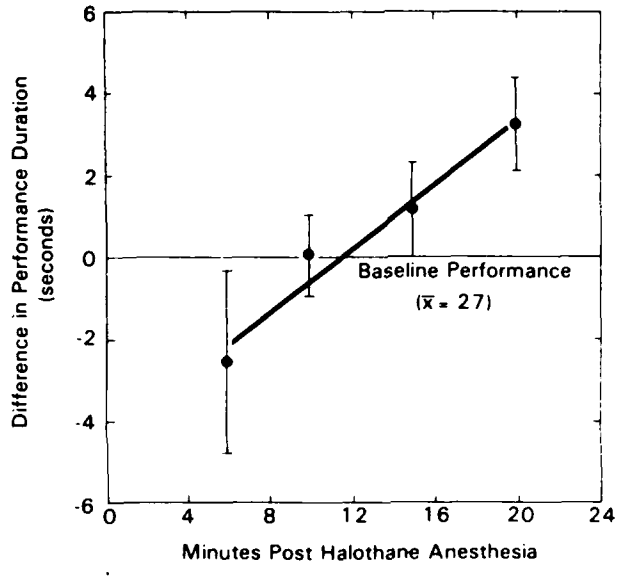


FIGURE 3. Mean ± SE of the difference in performance duration compared to baseline (N = 6). None of the performance trial periods were significantly different from baseline.

the injection could produce major aberrations in the performance data. Therefore, each injected animal was evaluated in a multistage, single-blind process to eliminate grossly traumatized subjects, verify the accuracy of the injection microscopically, and estimate the extent of any mechanical damage. A five-stage screen was used to reduce the likelihood of any aspect of the injection procedure contributing to the performance effects. The criteria were:

1. Subjective evaluation of the injection was judged satisfactory.
2. Subject recovered to baseline performance at 24 h.
3. Gross pathology of the brain at necropsy was normal. Any visible lesion, especially in the rhombencephalon and rostral section of the spinal cord, was cause for rejection.
4. Phase 1 histopathology examination. All surviving subjects (N = 39) were sacrificed 4–5 d after injection to allow sufficient time for a tissue response to chemical or mechanical insult. Serial 6-μm coronal sections of the rhombencephalon and rostral portion of the spinal cord were sampled at 30- or 90-μm intervals and stained with hematoxylin and eosin. Sections were evaluated for the appearance of needle or cannula tracks and/or related mechanical/hydraulic trauma without knowledge of the exposure conditions by a board-certified veterinary pathologist (J. Nold). Lesions were classified in descending order of severity as follows: (a) probably clinically sig-

nificant (subject rejected); (b) possibly clinically significant (subject rejected); (c) probably not clinically significant (subject accepted)

5. Phase 2 histopathology examination. Tissues were classified as in stage 4, based on the degree and location of hemorrhage, necrosis, or inflammatory response. Even though some lesions may not have affected performance, they served as useful indicators of the locus of injection in the absence of significant mechanical trauma. Some lesions classified as "possibly clinically significant" were considered acceptable if the mechanical trauma that led to the lesion was considered "probably not clinically significant," i.e., the lesion was chemically rather than mechanically induced and was appropriately located. Although the five criteria become progressively more definitive, they are not weighted, i.e., rejection occurred at any stage.

Dose Groups

Subjects were assigned to the three groups over the course of the study so that baseline accelerod performance was equal. Subjects were added to the three groups until six trained subjects per group existed that were acceptable based on the injection evaluation procedure.

RESULTS

Of the 41 animals injected in 7 groups, 23 were excluded from the final analysis based on the five-stage screen as follows: (1) 2 subjects died during injection; (2) 7 subjects failed to recover behaviorally by 24 h; (3) even though behaviorally normal at 24 h, 14 subjects manifested cannula-placement errors or mechanical trauma sufficiently severe to be classified as "possibly clinically significant," and they were rejected.

Figure 4 depicts the performance profiles of the control and PGDN-dosed subjects (N = 18, 6 subjects/group) that recovered satisfactorily from the injections and were judged acceptable by the five-stage screening process. The performance profiles are the mean ± SE of the difference between the pre- and posttreatment performance duration. The 10-μl subjects show a significant decrement in performance on the 12-, 60-, and 120-min trials. Recovery occurred at about h 4 or 5. No significant differences in motor performance were seen in the 5-μl or saline-control groups.

DISCUSSION

The physiological manifestations of hypotension, vasodilation, and debilitating headaches produced by nitrate esters like PGDN have been recognized for some time based on clinical reports on explosive-industry workers (Carmichael and Lieben, 1963). Comparable vascular effects in laboratory animals have been demonstrated only in rats (Clark

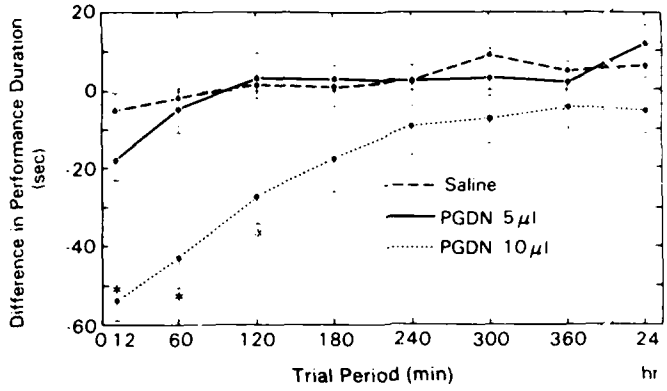


FIGURE 4. Performance profiles of 18 subjects in the 3 treatment groups (N = 6/group). Performance profiles are the mean ± SE of the difference between pre- and posttreatment performance duration. Significant effects are shown with asterisks ($p < 0.01$).

and Litchfield, 1969). More recently, Stewart et al. (1974) reported that exposure of people to PGDN vapor from Otto Fuel II at concentrations too low to induce hypotension produced motor performance and neurophysiological effects. Specifically, a 3-h exposure to 1.5 ppm PGDN induced significant changes in coordination and balance as observed in a modified Romberg test of balance and a coordination test of heel-to-toe walking. Horvath et al. (1981) conducted an occupational evaluation in humans exposed to PGDN by assessing motor performance and oculomotor function. In the latter study, both peak exposure concentrations of less than 0.25 ppm and short exposure periods (30–60 min) probably prevented the observation of significant decrements in motor performance or meaningful changes in oculomotor function.

The present study confirms in a rat model the human motor performance changes of Stewart et al. (1974). PGDN injected ic induced severe motor-performance decrement in the rat within 12 min of dosing at the 10-μl dose level, but not at 5 μl. Recovery from the high dose (approximately 33 mg/kg) was essentially linear with a gain in performance time of about 13 s/h over the 4 hourly posttreatment test periods. The effect of the 5-μl injection was not significantly different from the saline treatment, but the performance curve during the first hour parallels the high-dose recovery curve. Since severe limitations exist in the use of human volunteers to establish toxicity threshold limits, the rat/accelerod findings suggest that it may be a useful alternative model in which to conduct future PGDN neurotoxicity research.

Although we observed motor deficits in the rat suggestive of those seen in humans, the absolute dose to the target neural tissue in our study was probably greater than in the human inhalation exposures because we used a direct application of the neat chemical unaffected

by systemic distribution and metabolism (Anderson and Smith, 1973; Stewart et al., 1974). In addition, the disparity in dose-response between the two species could be a function of the difference in the physical nature of the respective motor tests. In the human study, the modified Romberg test is performed on one leg, while no comparable restriction exists in the rat. Alternatively, if only the PGDN in solution is effective, saturation of the 200-μl volume of cerebrospinal fluid in the rat would yield an effective dose level of approximately 1 mg/kg. Under this circumstance, the dose difference between the human and rat study may be reduced as much as 30-fold. Finally, the rapid onset of effect and the rapid recovery of the rats imply that the bulk of the PGDN is removed fairly quickly and that the recovery process involves a small residual amount of material locally absorbed in the tissue. The human rate of recovery from ataxia was not reported by Stewart et al. (1974).

We conducted related research with the rat/accelerod model using a 230-mg/kg dose of PGDN given subcutaneously (sc). The sc route was used so that the release of PGDN into the systemic circulation would occur over a longer duration than by oral administration, for instance, and immediate metabolism by the liver could be avoided. PGDN had a profound effect on performance over 6 h in the sc study. However, unlike the ic study, the sc effect may have been secondary to hypotension and methemoglobinemia-induced hypoxia. At ic-equivalent doses (i.e., 16 and 32 mg/kg, respectively, for the two dose levels in the present study), methemoglobin levels would not be expected to rise above control levels, and blood pressure would be likely to decrease by approximately 50 mm Hg for only a short period of time (Clark and Litchfield, 1969; Litchfield, 1971; Wyman et al., 1985). We chose the ic administration route to reduce the potential for secondary effects and to eliminate the rapid degradation that occurs in the liver following any route of administration that permits immediate access of major portions of the dose to systemic circulation (Kylin et al., 1964). Elimination of immediate systemic metabolism would also reduce the probability that degradation products would be a significant factor in the acute phase of neurotoxicity.

For acute dosing studies with volatile or flammable materials like PGDN, ic injection may offer several advantages over inhalation exposure, the route generally used to model human exposure. For example, the ic route is more direct and thus permits more quantitatively accurate dosing; the demanding technology and expense of running an inhalation system is eliminated; and research is possible with much smaller, safer quantities of the material. However, the ic technique has its own special problems. We found during preliminary work that simply discarding subjects with asymmetric locomotor behavior after injection did not exclude all subjects that had sustained significant injec-

tion trauma (Schanberg et al., 1967). In fact, three subjects that recovered fully within 24 h after injection were found at autopsy to have gross needle-puncture wounds in the dorsal spinal cord.

Any surgical rejection is undesirable, and this is more true for trained and conditioned animals, which are more valuable than naive subjects. The ic injection screen rejected 56% (23/41 subjects), suggesting that the screen is conservative; however, a closer look indicates that this may not be the total picture. Our 41 subjects were injected in 7 groups over 3 wk. In the first 4 groups, the rejection rate was 70% (16/23); however, in the last 3 groups, the rejection rate was almost halved to 39% (7/18). Further, 43% of the first 23 subjects were rejected based on stages 1–3 of the screen, which required little special expertise—i.e., death, failure to recover behaviorally, and gross pathology evaluation. However, of the last 18 subjects, only 2 were rejected based on stages 1–3. Thus, as the study progressed, the more sophisticated stages of the screen of histopathology evaluation for mechanical damage and necrosis were necessary to reject unsuitable animals, suggesting that technique refinement and improved accuracy is possible with practice.

It is interesting that 82% (32/39) of the surviving subjects performed normally, while 44% (14/32) of the subjects sustained lesions severe enough for rejection. A survey of recent literature on ic technique applications located 25 journal articles that used a manual injection methodology. When given, the descriptions of the injection technique were minimal, and only one article set any criteria for successful injections. If we had used a criterion for successful injection of "return to pretest performance within 24 h" (the criterion frequently reported), less than 25% of our rats would have been excluded. For these reasons, an evaluation screen is an important, if not crucial, adjunct in studies using an ic injection technique.

CONCLUSIONS

The present study confirms previously reported research of decrements in human motor performance produced by PGDN. The rat appears to be a useful animal model for assessing motor performance changes produced by PGDN. A selection criteria may be necessary to confirm the integrity and verify the accuracy of ic injections. In terms of directness, ic injections may be a reasonable alternative to inhalation administration.

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Exposure to γ-Irradiation Increases Phorbol Myristate Acetate–Induced H_2O_2 Production in Human Macrophages

By Elaine K. Gallin and Spencer W. Green

Cell number, protein, and phorbol myristate acetate (PMA)-induced H_2O_2 production were measured in cultured human peripheral blood monocytes for six days after exposure to varying doses of γ-radiation. Both the number of adherent cells and the protein per dish decreased with increasing radiation doses. The dose of radiation decreasing the number of adherent cells by 37% on days 4 and 6 postirradiation was 29 Gy. Four hours postirradiation there was a small decrease in PMA-induced H_2O_2 production for doses of 7.5 Gy or greater; levels returned to normal by eight hours and increased at 24 hours postirradiation. By day 4 postirradiation significant increases in PMA-induced H_2O_2 production were noted at all radiation doses (2.5 to 50 Gy). This increase was not due to a shift in the PMA dose-response curve, a change in the time course of the PMA response, or an effect of decreased cell density on the assay system. Superoxide levels were not significantly changed in cells exposed to 20 Gy. Catalase, glutathione peroxidase, and superoxide dismutase levels also were unchanged. Culturing irradiated cells with γ-interferon increased PMA-induced H_2O_2 release, which indicated that irradiated cells retained their capacity to respond to γ-interferon. These data demonstrate that irradiation affects the PMA-induced H_2O_2 production of human monocytes in a time- and dose-dependent manner. An increase in the release of reactive oxygen intermediates by the macrophage may play a role in enhancing the deleterious effects of radiation in vivo.

MACROPHAGES, although relatively insensitive to the effects of radiation compared with lymphocytes, exhibit time-dependent changes in cell function and survival after exposure to ionizing radiation in doses of 7.5 to 20 Gy. At four days postirradiation, mouse peritoneal macrophages exhibit a decrease in Fc-mediated phagocytosis[1] and a decrease in Ia expression.[2] The acid phosphatase activity of mouse peritoneal macrophages exposed to 5 Gy or more is unaffected three hours postirradiation but increases by 24 hours.[3] In human peripheral blood monocytes, exposure to 25 or 50 Gy decreased the 6-week survival of blood monocytes in culture and diminished the rate of microbial killing of the surviving cells.[4] In this paper, we show that exposure of human peripheral blood monocytes to γ-radiation decreases the number and size of adherent cells within 48 hours postirradiation. However, the surviving cells exhibit an increase in PMA-induced H_2O_2 production that is evident 24 hours postirradiation and maintained throughout the six-day culture period.

The production of H_2O_2 by macrophages is associated with both extracellular cytotoxicity and inhibition of intracellular pathogens.[5,6] Thus, the ability of macrophages to produce H_2O_2 in response to phorbol esters or particulate stimuli is associated with cell activation. However, H_2O_2 and other reactive oxygen intermediates, in addition to protecting against pathogens, can themselves produce extensive tissue damage.[7,8] It is possible that tissue damage postirradiation is due in part to augmentation of the production of H_2O_2 by the tissue macrophage.

MATERIALS AND METHODS

Cells. Human monocytes were isolated from leukopaks supplied from the National Institutes of Health blood bank by using lymphocyte separation media (Litton Bionetics, Bethesda, MD). All blood donors were advised of procedures and attendant risks in accordance with institutional guidelines. The cellular band containing lymphocytes and monocytes was washed with phosphate-buffered saline without calcium and magnesium (PBS/0 Ca,Mg) and centrifuged through fetal calf serum (FCS) (Sterile Systems, Logan, UT) at 100 g for ten minutes. The cells were resuspended in PBS/0 Ca,Mg containing 10% FCS, and placed on Percoll gradients (Pharmacia Fine Chemicals, Piscataway, NJ). The gradients were centrifuged at 1,000 g for 20 minutes at 4°C; the monocyte band was isolated and washed twice with PBS/0 Ca,Mg. The monocytes were resuspended in tissue culture medium consisting of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10 U/mL of penicillin (Difco, Detroit), 10 μg/mL streptomycin (Difco), 0.03% L-glutamine (Sigma Chemical Co, St Louis), and 10% heat-inactivated AB human serum (Bio-bee, Boston). The mononuclear cell layer obtained after Percol isolation contained approximately 90% monocytes as assessed by esterase staining. In most experiments, cells were plated on cluster-6 tissue culture dishes at concentrations of 1 to 2×10^6 cells/well. Cells were allowed to adhere for two hours and then were washed twice to remove nonadherent cells and refed with complete tissue culture medium. In some instances cells were placed in Teflon jars after isolation and were cultured for varying periods of time before plating and irradiating.

Cell number and protein. Cell number was determined on adherent populations of cells by counting the number of adherent cells in three separate 250× fields of the culture dish with inverted phase microscopy. The total number of cells in the dish was then determined from the area of the microscope field and the surface area of the dish. For protein measurements cells were washed three times with PBS to remove serum proteins, and 1 mL of 0.2% Triton X-100 (New England Nuclear, Boston) was added to the dishes. Cell lysates were measured by using the Bradford method (Bio-Rad, Richmond, CA). Bovine serum albumin (Bio-Rad) was used as the protein standard. Radiation does not result in any measurable serum

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protein sticking to the surface of the culture dishes. Protein per cell was obtained by dividing the total protein by the number of cells. All experiments were done in triplicate or greater.

Radiation. Cells were irradiated at varying times after isolation bilaterally by using a cobalt 60 γ-radiation source at a constant dose rate of 5 Gy/min.

H_2O_2 measurements. H_2O_2 release was assayed by measuring the decrease in fluorescence associated with the oxidation of scopoletin by H_2O_2.[9] Adherent cells were washed twice with PBS/Ca, Mg and 2 mL of reaction mixture containing PBS, 10 mmol/L glucose, and 10 to 80 nmol/L scopoletin (Sigma), and 6 purpurogallin units of horseradish peroxidase/mL (Sigma) at pH 7.1 was added to each well. The stimulus, phorbol myristate acetate (PMA) (Consolidated Midland, Brewster, NY) at a concentration of 100 ng/mL (0.01% final dimethyl sulfoxide concentration), was added to some of the wells. In a few studies 1 mmol/L sodium azide was also added to the reaction mixture. The cells were incubated with the reaction mixture at 37°C for one hour, after which the solution was removed from the cells. The fluorescence of each sample was measured by exciting at 350 nm and recording at 460 nm. Reaction mixture not exposed to cells was used as the control. Scopoletin levels was adjusted so that the scopoletin concentration was not the limiting factor, ie, the fluorescence levels were never reduced by more than 60%. H_2O_2 levels were calculated by comparing the fluorescence decrease produced by the cells with the fluorescence decrease caused by known amounts of ethyl peroxide (Polysciences, Warrington, PA).

Superoxide anion measurements. Superoxide was assayed by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c.[10] Cells were washed twice with Krebs-Ringer phosphate-containing glucose (KRPG), and then 2 mL of reaction mixture (KRPG containing 50 μmol/L ferricytochrome c [Sigma] with or without 0.1 mg/mL of SOD) was added to each dish. PMA was added at 100 ng/mL to the dishes. Dishes were incubated at 37°C for one hour, at which time the absorbance of the supernatant at 550 nm was measured. The value of the absorbance with SOD was subtracted from the value of the absorbance without SOD to determine superoxide levels. Superoxide levels were computed by using an extinction coefficient of 21,000.

SOD, glutathione peroxidase, and catalase measurements. For SOD and glutathione peroxidase measurements cells were rinsed twice with PBS and exposed to 0.2% Triton X-100 for 30 minutes at room temperature. Cell lysates were assayed for SOD activity by using the method of McCord and Fridovich.[11] This method assays the inhibition by SOD of the reduction of ferricytochrome c by superoxide. Xanthine and xanthine oxidase were used to generate the superoxide. Glutathione peroxidase was measured on 5 to 10 × 10^6 cells/mL according to the method of Paglia and Valentine.[12] Catalase was measured on cell fractions obtained by scraping adherent cells and sonicating them on ice for 40 seconds. Cell sonicates were then assayed for their ability to break down H_2O_2 by following the destruction of H_2O_2 spectrophotometrically at 230 nm.[13] All enzyme assays were done at room temperature.

γ-Interferon. In a few studies cells were cultured in the presence of recombinant human γ-interferon (γIFN) (AmGen, Thousand Oaks, CA) for varying periods of time before assaying PMA-induced H_2O_2 release.

RESULTS

Effect of radiation on cell number and cell size. Figure 1 shows the data from one representative experiment examining the effects of radiation on the number of adherent macrophages in culture on days 2, 4, and 6 postirradiation. Exposure to 2.5 Gy did not significantly decrease the cell number on any of the days assayed. However, with increasing doses of radiation, the cell number decreased significantly. The data from days 4 and 6 from this experiment and others are plotted in Fig. 2. The data are expressed as a percentage of control (nonirradiated) values. The line drawn through the points represents a least-squares fit to a single exponential function. The D_0 (dose of radiation decreasing the cell number by 37%) determined from this plot was 29 Gy.

Total protein per dish, shown in Table 1, also decreased in a dose- and time-dependent manner in irradiated cells. By days 4 and 6 postirradiation, protein levels decreased by 50% or more in dishes exposed to 20 and 50 Gy. The protein-per-cell values calculated from the protein and cell number data indicated that the surviving cells were smaller than control cells. That is, the protein-per-cell levels decreased by 18% to 37% on days 4 and 6 postirradiation.

H_2O_2 production. PMA-induced H_2O_2 release was measured in both control and irradiated cells at varying times postirradiation. Data from a representative experiment are shown in Fig 3A. As previously reported by Nakagawara et al,[9] the levels of PMA-induced H_2O_2 released by control (nonirradiated) macrophages decreased with time in culture. With the exception of the 5-Gy, two-day data point, all radiation doses tested produced a significant increase in H_2O_2 release compared with the control. With increasing radiation doses the decrease in H_2O_2 production, which occurred in control cells with time in culture, was abrogated. Since our data demonstrated that radiation produces a decrease in the protein per cell, the increase in H_2O_2 expressed in terms of protein could result from cells with a reduced protein content producing the same amount of H_2O_2. To determine whether this was the case, the data were expressed in terms of cell number rather than milligrams of protein. The results are shown in Fig 3B. The data are qualitatively similar and show a dose- and time-dependent increase in H_2O_2 release after irradiation.

Table 2 summarizes a number of experiments measuring H_2O_2 levels at additional time points. Because of the variability in the H_2O_2 production between different groups of cells,

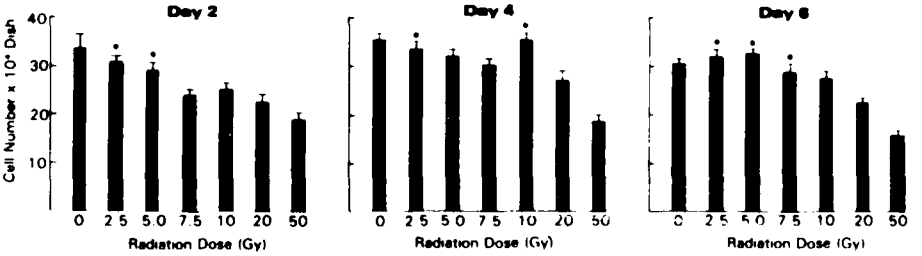

Fig 1. Dose response of effect of radiation on number of cells per dish at 2, 4, and 6 days postirradiation. Cells were irradiated one day after isolation. Each point is the average of four measurements ± SE. *, Groups not significantly different from control ($P > .01$).

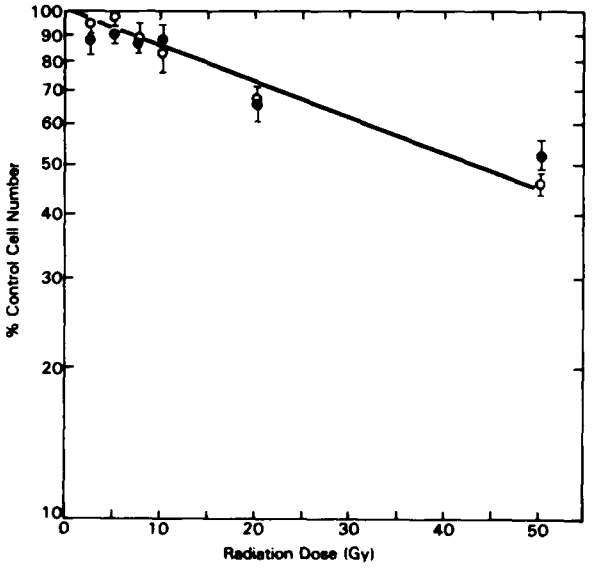

Fig 2. Dose response of effect of radiation on the number of adherent cells in culture at four (●) and six (○) days postirradiation. Cells were irradiated one day after isolation. Each data point represents the average ± SE of three separate studies, each done in triplicate or greater. The line drawn through the data is a least-squares fit (R^2 = .997).

the data are expressed as a percentage of control values. At four hours postirradiation there was actually a small but significant decrease in PMA-induced H_2O_2 production for cells exposed to 7.5 Gy or greater. Eight hours postirradiation, H_2O_2 release in cells irradiated with 10 Gy or more was still slightly reduced. It should be noted that the H_2O_2 levels obtained at four and eight hours were probably reduced by the myeloperoxidase activity present in monocytes 24 to 48 hours after isolation.[9] However, the data from cells cultured for 48 hours or longer (24 hours or longer postirradiation) was not influenced by myeloperoxidase activity (see the following section on sodium azide effects). At 24 hours postirradiation, exposure to 5 Gy produced only a 16% decrease in the protein per dish (Table 1) but a 45% increase in H_2O_2. This increase was even greater on day 2 postirradiation, was maintained on day 4, and increased further on day 6 postirradiation.

In the aforementioned studies, cells were exposed to 100 ng/mL PMA. To ensure that the dose of PMA was supramaximal for both control and irradiated cells, PMA dose-response curves were obtained for both control and irradiated (20 Gy) cells four days postirradiation. As shown in Fig 4, the dose-response curves for control and irradiated cells differed only in the magnitude of the H_2O_2 released.

The preceding studies were done on human peripheral blood monocytes that were irradiated 24 hours after isolation. To determine whether similar changes occur in more mature macrophages, cells were cultured in Teflon dishes for six to 14 days, plated on tissue culture dishes, washed to remove nonadherent cells, and irradiated with 20 Gy. PMA-induced H_2O_2 was measured on days 2 and 4 postirradiation. These studies indicated that mature human macrophages also exhibit an increase in PMA-induced H_2O_2 release after exposure to γ-radiation.

H_2O_2 measurements at a constant protein content per dish. Since the radiation doses (20 and 50 Gy) that produce the largest increase in H_2O_2 production on days 4 and 6 also decreased the cell protein content and cell number by 50% or greater, experiments were done to investigate the effects of cell density on PMA-induced H_2O_2 release. Human monocytes were plated at varying densities, and cultured for 1, 4, or 6 days; then the PMA-induced H_2O_2 release was assayed. In two out of three experiments, H_2O_2 levels increased as the protein per dish decreased below 15 μg/dish. To ensure that the increases in H_2O_2 were due solely to an effect of radiation and not simply a decrease in the protein per dish, a series of experiments were done in which monocytes were plated at varying densities before irradiation to compensate for the decrease in cell number and protein content after irradiation. The open circles in Fig 5 plot the data from cells assayed four days postirradiation. These data represent the average of three different experiments expressed as a percentage of control H_2O_2 release. It is evident that, even under conditions in which the protein per dish did not vary, radiation produced significant increases in H_2O_2 production. The line drawn through the points represents a least-squares fit to a bioexponential function. The closed circles in Fig 5 represent the data from Table 2 that were obtained from studies in which irradiated cells were plated at the same density as control cells so that the protein per dish was less than control values at the time of the measurements (Table 1). With the exception of the 20-Gy and 50-Gy points, the two sets of data are quite similar, indicating that the increase in H_2O_2 release by irradiated cells is not an artifact of decreased cell density.

To determine whether the time course of PMA-induced

Table 1. Time Course of Change in Protein per Dish Postirradiation

Time Postirradiation (h)	Radiation Dose (Gy)						n*
	2.5	5.0	7.5	10	20	50	
4	115 ± 7	109 ± 7	98 ± 7	102 ± 9	89 ± 10	95 ± 7	12
8	95 ± 5	92 ± 5	80 ± 5	93 ± 7	93 ± 5	100 ± 6	12
24	94 ± 5	83 ± 4	83 ± 5	80 ± 6	72 ± 11	86 ± 5	15
48	86 ± 5	91 ± 4	85 ± 4	79 ± 5	72 ± 8	54 ± 3	12
96	77 ± 4	71 ± 3	70 ± 2	67 ± 3	52 ± 3	35 ± 2	12
144	87 ± 4	81 ± 3	70 ± 3	68 ± 6	46 ± 4	43 ± 4	12

Data are expressed as a percentage of control (unirradiated) values. Human mononuclear cells were irradiated 24 hours after isolation.

*Number of data points for each time point.

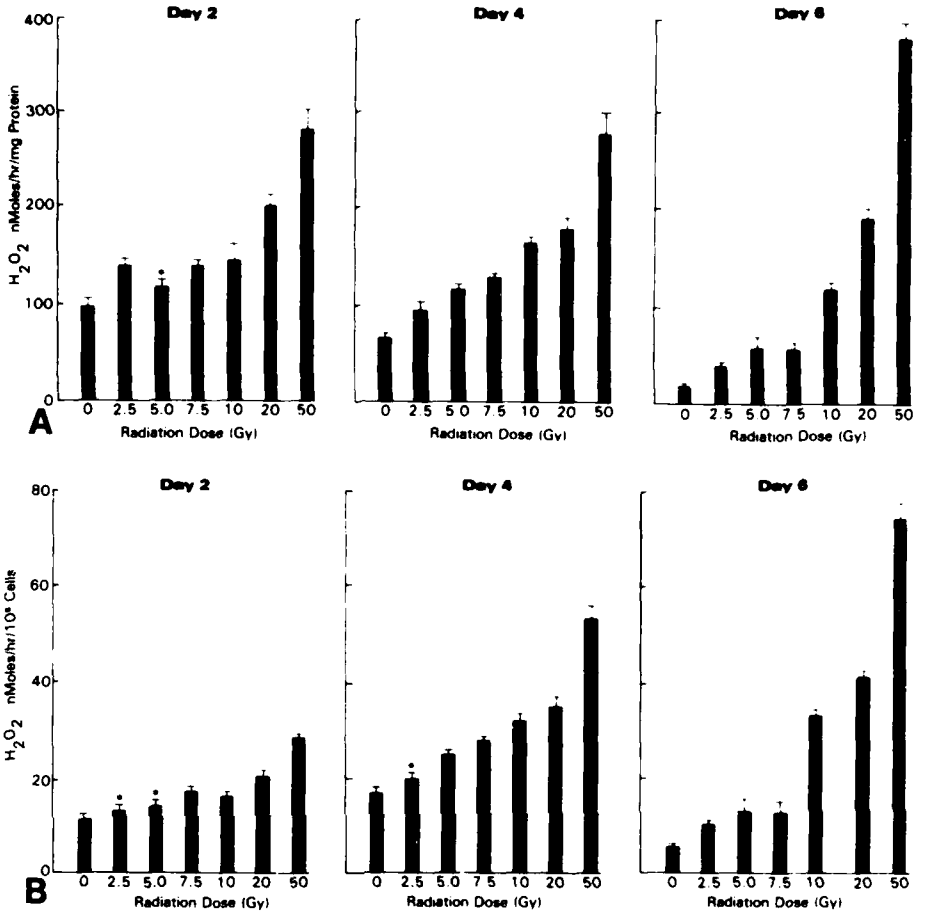

Fig 3. Dose response of effect of radiation on PMA-induced H_2O_2 release on days 2, 4, and 6 postirradiation. (A) H_2O_2 release (nmol/h/mg protein). (B) H_2O_2 release (nmol/h/10^6 cells). Data (mean ± SE of four measurements) are from the same experiment as in Fig 1. *, Groups not significantly different from control ($P > .01$).

H_2O_2 release in irradiated and control cells was similar, supernatants from PMA-treated wells were harvested at various times after the addition of PMA (100 ng/mL). Figure 6 shows the data from a typical experiment in which control and irradiated (20 Gy) cells were examined. It is evident that the time course of H_2O_2 release in both groups of cells was similar even though the total H_2O_2 released by the irradiated cells was doubled. In this experiment irradiated cells were plated at a higher density so that the protein per dish was similar in both groups of cells.

Effect of sodium azide. H_2O_2 release by freshly isolated human monocytes that contain high levels of myeloperoxidase is increased fourfold by the addition of sodium azide (which inhibits myeloperoxidase) to the reaction mixture.[9] Myeloperoxidase levels decrease in monocytes during the first two days in culture so that sodium azide has little effect on H_2O_2 production after day 2.[9] To confirm that the H_2O_2 levels measured in these studies were maximal (ie, that they could not be further enhanced by myeloperoxidase inhibitors), several studies were done in the presence of 1 mmol/L sodium azide. The addition of sodium azide to cultures ten minutes previous to and during exposure to PMA increased (3.8-fold) H_2O_2 levels in macrophages assayed four hours after isolation but had no effect on cells assayed either two or

Table 2. Time Course of Change in H_2O_2 Production Postradiation

Time Postirradiation (h)	Radiation Dose (Gy)						
	2.5	5.0	7.5	10	20	50	n*
4	90 ± 7	97 ± 11	80 ± 5	68 ± 4	76 ± 4	66 ± 7	9
8	89 ± 9	92 ± 6	95 ± 10	82 ± 11	81 ± 11	67 ± 7	9
24	114 ± 6	145 ± 8	167 ± 9	152 ± 8	163 ± 12	153 ± 10	9
48	149 ± 5	179 ± 18	233 ± 30	330 ± 70	326 ± 48	358 ± 28	12
96	139 ± 5	163 ± 6	185 ± 5	250 ± 11	296 ± 13	373 ± 20	12
144	181 ± 13	262 ± 31	239 ± 33	463 ± 62	810 ± 94	1183 ± 290	12

Data are expressed as a percentage of control (unirradiated) values. Cells were irradiated 24 hours after isolation and assayed for PMA-induced (100 ng/mL for 60 minutes) H_2O_2 production at indicated times.

*Number of data points for each time point.

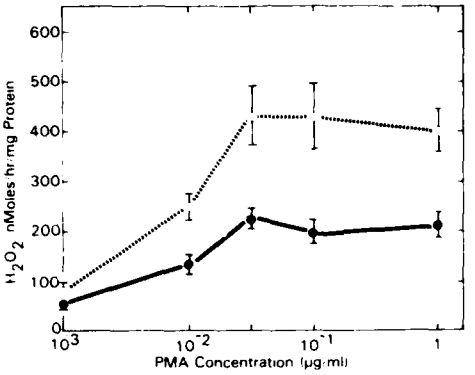

Fig 4. PMA dose-response curve of H_2O_2 release for control (●) and irradiated (○) cells. Cells were irradiated one day after isolation and assayed four days postirradiation. Each point represents the average of three different experiments ± SE.

five days after isolation (corresponding to day 1 and day 4 postirradiation).

Catalase, glutathione peroxidase, and SOD measurements. Three enzymes that play an important role in H_2O_2 metabolism are SOD, glutathione peroxidase, and catalase.[13] To determine whether in H_2O_2 release in irradiated macrophages was due to either an increase in SOD activity or a decrease in glutathione peroxidase or catalase activity, the activity of these enzymes in cell homogenates was measured. As shown in Table 3, the activities of all three of these enzymes were identical in control and irradiated (20 Gy) cells.

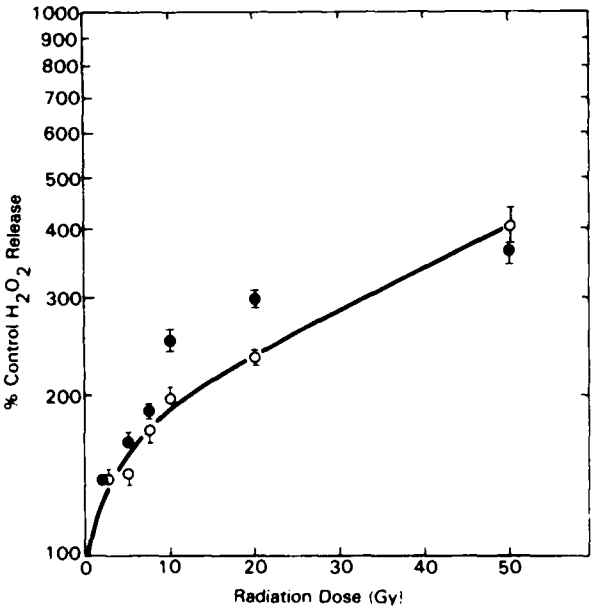

Fig 5. Effect of radiation on PMA-induced H_2O_2 release. (○) Data were obtained at four days postirradiation and expressed as a percentage of control. Points represent the average ± SE of three separate experiments. Cells were plated at different densities so that the protein per dish remained constant. (●) Data from the same experiments as in Fig 2.

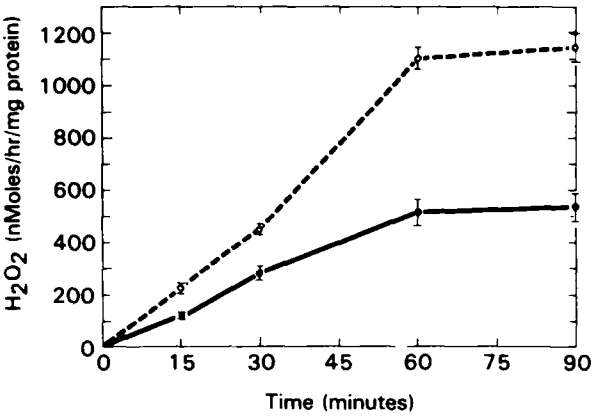

Fig 6. Time course of PMA-induced H_2O_2 release for control (solid line) and irradiated (20 Gy, dashed line) cells assayed at four days postirradiation. Cells were irradiated one day after isolation. Each point represents the mean ± SE of five measurements.

Superoxide measurements. The H_2O_2 released after PMA stimulation is produced by the dismutation of superoxide formed from the activation of an NADPH oxidase.[14] If the increase in H_2O_2 levels postirradiation were due to an increase in the oxidase activity, the levels of superoxide released by irradiated cells might be increased as well. PMA-induced superoxide release was measured on cells four days postirradiation (20 Gy). In these studies, irradiated cells were plated at a higher density than were the control cells since Johnston et al[10] demonstrated that superoxide release in mouse peritoneal macrophages varies inversely with cell density. As reported previously in human macrophages,[9] the levels of superoxide released in response to PMA were lower (two- to fourfold) than the corresponding H_2O_2 levels in all groups of cells. The superoxide data showed more variability than the H_2O_2 data (possibly due in part to the lower levels). In five of eight studies, irradiated cells showed an increase in superoxide production over control cells (with increases ranging from 10% to 200%). The data shown in Table 4 summarize the data of the eight studies. The average percent changes of irradiated cells compared with control cells for superoxide, H_2O_2, and protein per dish are given. It is evident that exposure to 20 Gy of γ-radiation did not produce a significant increase in superoxide release.

Table 3. SOD, Catalase, and Glutathione Peroxidase Activity in Radiated and Control Macrophages

	SOD*	Catalase†	Glutathione Peroxidase‡
Control	36 5 · 11	1 02 · 0 12	13 9 · 0 6
Radiated	37 9 · 11	1 23 · 0 31	15 9 · 1 1

Values are means · SEM of three different experiments

*Values are units per milligram protein. Cells were irradiated with 20 Gy one day after isolation and assayed four days later.

†Values are in nanomoles H_2O_2 degraded per minute per milligram protein. Cells were irradiated with 20 Gy five to six days after isolation and assayed four days later.

‡Values are in nanomoles NADPH consumed per minute per milligram cell protein. Cells were irradiated with 20 Gy one day after isolation and assayed four days later.

Table 4. Superoxide and H_2O_2 Production in Radiated Macrophages

	Control (%)	Protein per Dish, Control (%)*
O_2	137 ± 31	110 ± 6
H_2O_2	216 ± 33†	93 ± 7

Cells irradiated with 20 Gy 24 hours after isolation and assayed four days later. Data are means ± SE from eight different experiments.

*Dishes containing radiated cells were plated at a higher cell density (1.3 × control).

†$P < .008$.

Effects of γIFN. Experiments were carried out to determine whether the PMA-induced H_2O_2 release in irradiated cells could be further increased by pretreating the cells with γIFN. The γIFN studies were done by irradiating cells at varying times after isolation, culturing cells with γIFN (100 U/mL) for one to four days, and measuring the PMA-induced H_2O_2 release from γIFN-treated and control cells. Figure 7 shows the data from one study in which cells were cultured with γIFN immediately after irradiation and then assayed four days later. γIFN increased the PMA-induced H_2O_2 levels in all three groups of cells but had no effect on H_2O_2 levels in the absence of PMA. The combined data from this and four additional studies show that γIFN increased PMA-induced H_2O_2 levels by 402%, 263%, and 199% in the control, 7.5-Gy, and 20-Gy groups, respectively. Thus, the irradiated cells responded to γIFN. However, the levels of H_2O_2 produced by irradiated cells in the presence of γIFN were the same as for the nonirradiated cells exposed to γIFN.

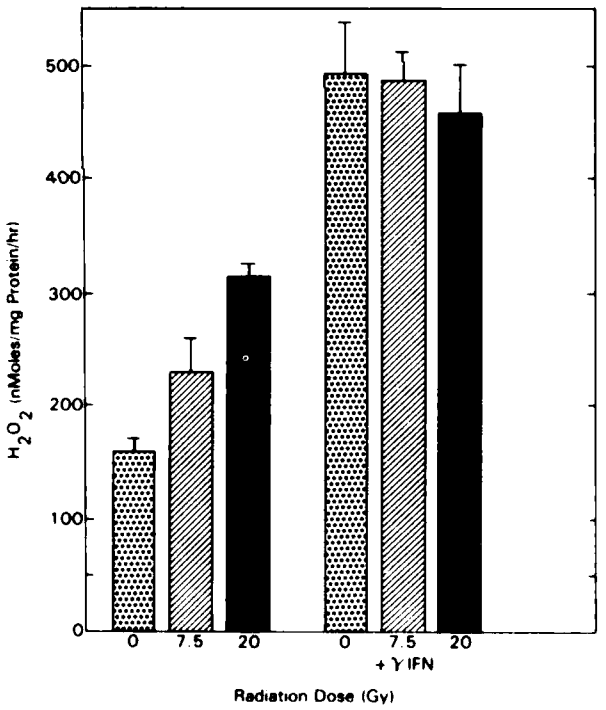

Fig 7. Effects of γIFN PMA-induced H_2O_2 release by control and irradiated macrophages. Cells were irradiated one day after isolation. (A) Cells were incubated in 100 U/mL of γIFN from days 0 to 4 postirradiation and assayed on day 4. Values represent the mean ± SE of four measurements.

Effects of supernatant from irradiated cells. To determine whether radiated cells release a factor(s) into the supernatant that increases PMA-induced H_2O_2 release by macrophages, cell-free supernatants from irradiated cells were harvested on days 1 and 3 postirradiation and added to unirradiated cells. The average of six different studies indicated that PMA-induced H_2O_2 release in control cells refed irradiated supernatant was no different (111% ± 18%) from control cells refed fresh media on days 1 and 3.

DISCUSSION

It is generally thought that macrophages are relatively radioresistant cells.[15] However, recent studies indicate that time-dependent changes in bacterial killing,[4] phagocytosis,[1] production of colony-stimulating factor,[16] and Ia expression[2] occur after in vitro exposure to radiation. In most of these studies it was not possible to determine whether radiation was directly affecting the macrophage. In this paper, the effects of γ-irradiation on Percoll- and adherence-purified human peripheral blood monocytes (~95% esterase-positive) were assessed so as to minimize the possibility that radiation is affecting a different cell type that is then influencing the macrophage.

Doses of γ-radiation as low as 2.5 Gy produced significant decreases in the number of adherent cells and the protein per dish by 48 hours postirradiation. The D_0 for the number of adherent cells remaining on days 4 and 6 postirradiation was 29 Gy. These results confirm previous studies[15] demonstrating that, in terms of the number of viable cells, macrophages are relatively radioresistant. Our data differ from those of Kwan and Norman[17] who calculated two D_0 values, 0.55 and 6.5 Gy for human monocytes four days postirradiation. However, in their studies, a mixed monocyte-lymphocyte fraction was irradiated, the cells were cultured in plastic tubes for four days, and then the number of large cells (or cells ingesting latex beads) was taken as a measure of the number of macrophages. The discrepancy between our findings and those of Kwan and Norman[17] may be due to some of these differences.

In contrast to the effects of radiation on cell number, these studies demonstrate that doses of γ-radiation as low as 2.5 Gy produce a significant increase in PMA-induced H_2O_2 release. This increase was not due to a shift in the PMA dose response because (as shown in Fig 4) both control and irradiated cells had qualitatively similar dose-response curves. No detectable H_2O_2 was released from either irradiated or control cells in the absence of PMA, which indicated that radiation had no effect on basal H_2O_2 levels. It has been shown previously that there is an inverse relationship between the level of superoxide, another reaction oxygen intermediate, and the cell density of cultured macrophages.[10] Therefore, experiments were done to (*a*) determine whether cell density affects H_2O_2 production and (*b*) examine the effects of radiation in the absence of any change in cell protein per dish. It should be noted that in the studies where the cell protein per dish was constant, the cell density in the irradiated cells will increase slightly since the protein per cell

decreased by 18% to 37% depending on the radiation dose. These studies demonstrated that, although PMA-induced H_2O_2 release was affected somewhat by cell density (when the protein per dish was less than 15 μg), radiation still produced significant increases in H_2O_2 release when the protein per dish was unchanged (Fig 6). Comparison of Figs 2 and 5 shows not only that PMA-induced H_2O_2 release is more sensitive to radiation than is cell number but also that the relationship between PMA-induced H_2O_2 release and γ-irradiation is more complex than the relationship between cell number and γ-irradiation. The curve in Fig 6, obtained from data with comparable protein values, fits a two-exponent function, thereby indicating that radiation has multiple effects on the cells or that more than one population of cells (in terms of the H_2O_2 response) exists.

To investigate the possibility that factors released from the irradiated macrophages (or possibly a contaminating cell type) were responsible for the increase in PMA-induced H_2O_2 release postirradiation, experiments were done in which control cells were exposed to supernatant from irradiated cells. These studies indicated that the effects of radiation were not due to the release of a stimulatory factor into the media since supernatants from irradiated cells had no effect on control cells. In addition, this finding supports the view that radiation is probably acting directly on the macrophage and not on a contaminating cell type.

To determine the site in the oxidative pathway responsible for the increase in the H_2O_2-releasing capacity of irradiated macrophages, studies were done to assess the release of superoxide by PMA-stimulated cells after radiation and to measure SOD, glutathione peroxidase, and catalase levels in these cells. Those experiments indicated that at four days postirradiation (20 Gy) the SOD, glutathione peroxidase, and catalase levels as well as the PMA-induced superoxide release were not significantly different from control values. Further studies will need to be done that measure the NADPH oxidative activity in radiated and control cells to elucidate the site(s) of action of radiation on the oxidative pathway.

Interestingly, γIFN, which enhances PMA-induced H_2O_2 release in macrophages,[6,18] also increases H_2O_2 levels in irradiated (20 Gy), cells. Thus, irradiated cells are still responsive to γIFN. However, γIFN has a larger effect on the control cells so that the levels of H_2O_2 released by both control and irradiated cells in the presence of γIFN are identical. A possible interpretation of these results is that there is a maximum amount of H_2O_2 that can be released by the macrophage. Therefore, irradiated cells that release more H_2O_2 in the absence of γIFN produce less H_2O_2 in response to γIFN.

The production of reactive oxygen intermediates by phagocytes is associated with increased activation since it has been linked to tumor cell cytotoxicity, the killing of bacteria, and intracellular pathogens.[5,6] However, chronic stimulation of macrophages can lead to tissue damage and inflammation. It is possible that the stimulatory action of radiation on the oxidative burst of macrophages is partly responsible for the tissue damage that occurs postirradiation. For example, the mutagenic effects of ionizing radiation are due in great part to the direct formation of active oxygen intermediates,[19] and mutagenicity may be further enhanced by additional free radicals produced by phagocytic cells. Recent studies have shown that reactive oxygen intermediates produced by stimulating neutrophils with phorbol esters are mutagenic (transforming mouse fibroblasts into malignant cells).[20]

Several studies have demonstrated an increased activation in macrophages harvested from irradiated animals. Therefore, the in vitro increases in H_2O_2 production reported in this study may have in vivo correlates. For example, Schultz et al[21] have shown that doses of x-irradiation of 1 to 8 Gy increase the ability of macrophages to suppress the proliferation of MBL-2 target cells. In these studies, 6 Gy increased the cytostatic ability of macrophages from three hours to seven days postirradiation. Similar doses of whole body radiation also increased the lysosomal enzyme content of macrophages.[22] In in vivo studies, the effects of damaged lymphocytes, infection, etc, on macrophage function cannot be separated from the direct effect of radiation on the macrophage, which makes it difficult to compare in vivo studies with the in vitro radiation data presented in this paper. However, it is evident that, if an increase in H_2O_2 production occurs postirradiation, it is likely to enhance the deleterious effects of radiation.

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Actions of Ethanol on Voltage-Sensitive Sodium Channels: Effects of Acute and Chronic Ethanol Treatment[1]

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ABSTRACT

The effects of acute and chronic ethanol treatment on neurotoxin-stimulated $^{22}Na^+$ uptake and [^{3}H]batrachotoxinin A20-α-benzoate binding to neuronal sodium channels were studied in rat forebrain synaptosomes. Fluorescence measurements were used to assess the intrinsic order or fluidity and the sensitivity to ethanol of rat forebrain synaptic plasma membranes at various intervals during and after chronic ethanol treatment. Acute ethanol administration had no significant effect on neurotoxin binding in the absence or presence of ethanol *in vitro* or on sodium uptake in the absence of ethanol *in vitro*. However, a single dose of ethanol produced a dose and time-dependent attenuation of the inhibitory effect of ethanol on sodium uptake, suggestive of acute tolerance. Chronic ethanol treatment reduced the influx of $^{22}Na^+$ in the presence of batrachotoxin and diminished the inhibitory effect of ethanol *in vitro* on sodium uptake for up to 20 days after withdrawal, but the specific binding of the neurotoxin in the presence or absence of ethanol was unchanged. Synaptic plasma membranes from chronic ethanol-treated rats showed no change in intrinsic order but the disordering effect of ethanol was significantly smaller for up to 20 days after withdrawal. Results of this study demonstrate that brain tissue from ethanol-treated rats can adapt rapidly to some effects of ethanol and that chronic ethanol administration can reduce the effects of ethanol on physical and functional properties of neurons for a prolonged period of time.

Ethanol-induced changes in the physical properties of biological membranes are thought to be involved in the diverse pharmacological actions of ethanol (Goldstein, 1984; Harris and Hitzemann, 1981). Changes in the physical properties of neuronal membranes may induce alterations in the functional properties of nervous tissues (*e.g.* enzymatic activity, ion transport and neurotransmitter regulation) resulting in impaired signal transduction and information processing and, ultimately, the behavioral manifestations of intoxication. At the present time, it is thought that ethanol and related membrane perturbants modulate the fluidity of nerve membranes by disordering the lipid portions of brain membranes (Chin and Goldstein, 1977a; Harris and Schroeder, 1981, 1982; Crews *et al.*, 1983).

Chronic exposure to ethanol has been shown to produce tolerance and physical dependence (Majchrowicz and Hunt, 1976; Ritzmann and Tabakoff, 1976). Furthermore, neuronal membranes derived from ethanol-tolerant animals are resistant to the disordering effect of ethanol *in vitro* (Chin and Goldstein, 1977b; Harris *et al.*, 1984). The mechanisms involved in the observed tolerance to the disordering effect of ethanol *in vitro* are not yet known but adaptive changes in the lipid composition of neuronal membranes have been examined for possible involvement. Membrane cholesterol content has been reported to be increased (Chin *et al.*, 1978; Smith and Gerhart, 1982), decreased (Harris *et al.*, 1984) or unchanged (Johnson *et al.*, 1979; Lyon and Goldstein, 1983) after chronic ethanol treatment. However, the reported change in cholesterol content is rather small in magnitude. Likewise, chronic ethanol treatment has been reported to produce only slight changes in the acyl composition of synaptosomal phospholipids (Sun and Sun, 1979; Alling *et al.*, 1982; Crews *et al.*, 1983; Harris *et al.*, 1984). Recently, it was shown that the membrane ganglioside content and acyl composition of gangliosides were unchanged in ethanol-tolerant mice (Harris *et al.*, 1984). Thus, it appears that chronic ethanol treatment consistently alters membrane physical properties in the absence of any marked changes in lipid composition.

The results of a number of recent studies suggested that some of the properties of voltage-sensitive sodium channels of brain

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ABBREVIATIONS: BTX-B, batrachotoxin A 20-α-benzoate; BTX, batrachotoxin; HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

synaptosomes are sensitive to the physical properties of membranes. In a group of chemically diverse membrane perturbants, the magnitude of the inhibition of neurotoxin-stimulated sodium influx was proportional to the degree of lipid disordering in the membrane core (Harris and Bruno, 1985a,b). Furthermore, for a series of aliphatic alcohols, there was an excellent correlation between the potency for inhibition of neurotoxin-stimulated sodium influx and the membrane/buffer partition coefficient, suggesting that a hydrophobic site in the membrane was involved in the action of the alcohols (Mullin and Hunt, 1984, 1985). In the present study we sought to determine whether acute or chronic ethanol treatment altered some of the properties of voltage-sensitive sodium channels in rat brain synaptosomes. We also monitored the physical properties of brain membranes at various intervals during and after chronic ethanol treatment.

Methods

Animals and chemicals. Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and were housed two per cage with free access to water and standard laboratory chow. Chemicals and suppliers were as follows: scorpion (Leiurus quinquestriatus) venom, tetrodotoxin and veratridine from Sigma Chemical Co. (St. Louis, MO); [benzoyl-2,5-^{3}H]BTX-B (51 Ci/mmol) and carrier-free ^{22}NaCl were from New England Nuclear (Boston, MA). Fluorescent probes were obtained from Molecular Probes, Inc. (Junction City, OR). BTX was kindly supplied by Dr. John Daly (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of synaptosomes. For measurement of [^{3}H]BTX-B binding and BTX-stimulated $^{22}Na^+$ influx a crude synaptosomal (P_2) fraction was prepared by a modification of the method of Gray and Whittaker (1962). Immediately after decapitation the whole brain was removed, the cerebellum and brainstem were discarded and the tissue was homogenized in 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4 (10 ml/g wet weight), with 10 strokes of a motor driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1000 × *g* for 10 min. The resulting supernatant was then centrifuged at 17,000 × *g* for 60 min. The final pellet was resuspended in ice-cold buffer containing (millimolar): KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; HEPES-Tris (pH 7.4), 50; and choline chloride, 130. Ten strokes of a loose fitting glass-glass homogenizer were used to resuspend the final pellet. Synaptosomes were kept on ice and were used immediately after preparation.

Measurement of $^{22}Na^+$ uptake. Neurotoxin-stimulated uptake of $^{22}Na^+$ was determined by a modification of the method of Tamkun and Catterall (1981). Aliquots (50 μl) of the synaptosomal suspension were preincubated at 36°C for 2 min with buffer only or buffer containing the indicated concentration of ethanol. Immediately after the preincubation with ethanol, the indicated concentration of BTX was added and the samples were incubated for 10 min at 36°C. After 10 min, the samples were diluted with a solution containing (final concentration, millimolar): KCl, 5.4; $MgSO_4$, 0.8; glucose, 5.5; HEPES-Tris (pH 7.4), 50; choline chloride, 128; NaCl, 2; ouabain, 5; 1.3 μCi of carrier-free ^{22}NaCl per ml and the indicated concentration of ethanol and BTX (micromolar). After a 5 sec incubation, the uptake of $^{22}Na^+$ was terminated by the addition of 3 ml of an ice-cold wash solution containing (millimolar): choline chloride, 163; $MgSO_4$, 0.8; $CaCl_2$, 1.8; HEPES-Tris (pH 7.4), 5; and bovine serum albumin, 1 mg/ml. The mixture was filtered rapidly under vacuum through a 0.45-μm cellulose filter (Amicon, Lexington, MA or Schleicher and Schuell, Keene, NH) and the filters were washed twice with 3 ml of wash solution. Filters were placed in scintillation vials with 15 ml of scintillation cocktail and filter radioactivity was determined by liquid scintillation spectrometry. The data are presented as corrected specific uptake after subtraction of nonspecific uptake (tetrodotoxin, 1 μM present in buffers).

Measurement of [^{3}H]BTX-B binding. The binding of [^{3}H]BTX-B was measured by a modification of the method of Catterall *et al.* (1981) as described in detail in the accompanying manuscript (Mullin and Hunt, 1987).

Fluorescence measurements. A HH-1 T-format polarization spectrofluorimeter (BHL Associates, Burlingame, CA) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel and perpendicular to the polarization phase of the exciting light (Harris and Schroeder, 1982). Polarization of fluorescence and intensity of fluorescence were calculated by an on-line microprocessor. Similar instrumentation is presented in more detail by Johnson *et al.* (1979). The fluorescent probes DPH and TMA-DPH were used. The excitation wavelength was 362 nm, a 03FCG001 filter (Melles Griot, Irvine, CA) was used in the excitation beam and KV 389 filters (Schott Optical, Duryea, PA) were used for emission. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermocouple inserted into the cuvette to a level just above the light beam.

Synaptic plasma membranes (SPM-2) were used for all fluorescence measurements. The cerebellum and brainstem were removed from the brain and SPM-2 were prepared by Ficoll and sucrose density centrifugation (Fontaine *et al.*, 1980; Harris and Schroeder, 1982). Membranes were resuspended in phosphate-buffered saline containing (millimolar): NaCl, 136; KCl, 2.7; KH_2PO_4, 1.5; $Na_2HPO_4\ 7H_2O$, 4.3; HEPES, 2.0; pH 7.4, at a concentration of 1 to 3 mg of protein per ml and were frozen and kept at −80°C before analysis. SPM-2 were diluted to 0.05 mg of protein per ml and fluorescent probes were incorporated at 35°C for 15 min with frequent vortexing. DPH was dissolved in tetrahydrofuran and TMA-DPH was dissolved in tetrahydrofuran-water (1:1). The probes were added in a volume of 0.3 to 0.5 μl/ml to give a probe concentration of 40 to 80 ng/ml. After incorporation of probe, samples were placed in the fluorimeter and maintained at 35°C. Control levels of fluorescence (base line) were determined and an aliquot of ethanol solution was added to the cuvette; fluorescence was determined 3 to 5 min later. The samples were coded and the experimenter was not aware of the source of the membranes.

Chronic ethanol treatment. Male Sprague-Dawley rats (200–300 g) were rendered ethanol-dependent by the method of Majchrowicz (1975), which entailed administering multiple doses (6–10) over a 24-hr period totaling 9 to 11 g/kg. Ethanol was administered as a 20% (w/v) solution intragastrically using a pediatric feeding tube. The animals were dosed with ethanol for 4 days, after which they were allowed to withdraw. Behavioral assessments were made hourly and the degree of intoxication or withdrawal signs were rated as described previously (Majchrowicz, 1975; Majchrowicz *et al.*, 1976).

Other methods. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the protein standard. Statistical analysis was performed using Student's *t* test for paired or unpaired samples. Multiple comparisons with a control were done by analysis of variance and Dunnett's test (Dunnett, 1964). Concentration-effect curves for membranes from control and ethanol-treated animals were compared by an analysis of variance for repeated measures.

Results

Acute ethanol administration. The effects of acute administration of ethanol on BTX-stimulated $^{22}Na^+$ uptake and the resulting blood ethanol concentrations are shown in figure 1. Ethanol was administered by intragastric intubation as a 20% (w/v) solution and the animals were sacrificed 2 hr later. At all doses studied, there was no significant effect on the uptake of $^{22}Na^+$ in the presence of BTX alone. However, at doses of 3, 4.5, and 6 g/kg there was a significant ($P < .05$) diminution in the inhibitory effect of ethanol *in vitro*. Blood

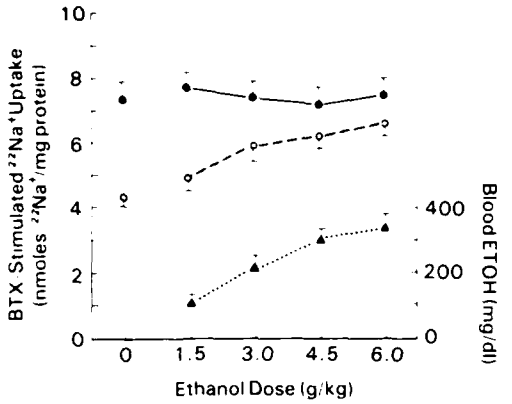


Fig. 1. Dose-response relationship for the effect of acute ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Ethanol was administered by intragastric intubation and the animals were sacrificed 2 hr later. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (●) or presence (○) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μM. Values are the means ± S.E.M., n = 4–6 rats. Effect of ethanol *in vitro* was significantly ($P < .05$) smaller at doses of 3 g/kg and greater.

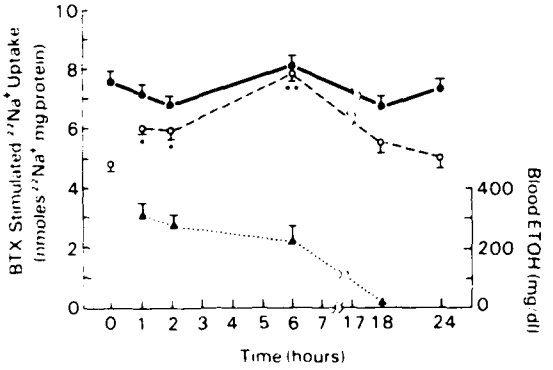


Fig. 2. Time course of effect of acute ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Ethanol (4.5 g/kg) was administered by intragastric intubation and animals were sacrificed at the indicated time. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (●) or presence (○) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μM. Values are the means ± S.E.M., n = 4–6 rats. *$P < .05$; **$P < .01$ compared to corresponding value at time zero.

ethanol concentrations associated with the effective doses of ethanol range from 47 to 75 mM. Thus, it appeared as though the administration of a single dose of ethanol resulted in tolerance to the inhibitory effect of ethanol *in vitro* on BTX-stimulated $^{22}Na^+$ uptake. To further characterize this effect, animals were administered a single dose (4.5 g/kg) of ethanol and were sacrificed at various intervals after the dose. As shown in figure 2, as quickly as 1 hr after the dose there was a significant ($P < .05$) reduction in the inhibitory effect of ethanol *in vitro*. Six hours after the dose, when the blood ethanol concentration was 50.5 ± 8.3 mM, ethanol added *in vitro* had no effect on BTX-stimulated $^{22}Na^+$ uptake. Twenty-four hours after the dose of ethanol, the inhibitory effect of ethanol *in vitro* was similar in the control and treated groups.

In order to determine whether the observed reduction in the inhibitory effect of ethanol on BTX-stimulated $^{22}Na^+$ uptake was due to alterations in the binding of the neurotoxin, experiments were performed to measure the binding of [^{3}H]BTX-B. A single dose (4.5 g/kg) of ethanol was administered and the animals were sacrificed 6 hr later. This time point was chosen based on the results of the time course experiments (Fig. 2). The results shown in table 1 illustrate the lack of any effect of acute ethanol administration on the specific binding of [^{3}H] BTX-B in the absence or presence of ethanol *in vitro*. Similar results were obtained in experiments in which a higher concentration (75 nM) of [^{3}H]BTX-B was used (data not shown).

Chronic ethanol administration. Rats treated with repeated doses of ethanol according to the method of Majchrowicz (1975) become tolerant to, and physically dependent on, ethanol (Majchrowicz *et al.*, 1976; Kynch and Prohaska, 1981). Accordingly, animals received multiple daily doses of ethanol as described under "Methods" and were sacrificed after completing 2 days of treatment, on the day of withdrawal and at 5, 10, 20 and 35 days after withdrawal. The results from ion flux measurements are shown in figure 3. There was a significant

TABLE 1

Effect of acute ethanol administration on [^{3}H]BTX-B binding

Rats received ethanol (4.5 g/kg) or saline by intragastric intubation and were sacrificed 6 hr later. Duplicate samples of forebrain synaptosomes were incubated with [^{3}H]BTX-B (10 nM) and scorpion venom (150 μg/ml) in the absence or presence of ethanol *in vitro*. Blood ethanol concentrations were 279 ± 22 mg/dl. Values are the means ± S.E.M., n = 4–6. Values in parentheses are the percentage of inhibition of binding by ethanol *in vitro*.

Group	Ethanol	[^{3}H]BTX-B Bound
	mM	*fmol/mg protein*
Control	0	330.1 ± 16.6
Control	400	208.8 ± 10.7* (36.7%)
Acute ethanol	0	336.6 ± 16.1
Acute ethanol	400	217.6 ± 9.1* (35.4%)

* Significantly ($P < .01$) different compared to binding in the absence of ethanol *in vitro*.

TREATMENT INTERVAL		B E C (mg/dl)
A	2 days Induction	241 ± 27
B	Dependent intoxicated	289 ± 19
C	Dependent withdrawing	14 ± 6
D	5 days Post withdrawal	
E	10 days Post withdrawal	
F	20 days Post withdrawal	
G	35 days Post withdrawal	

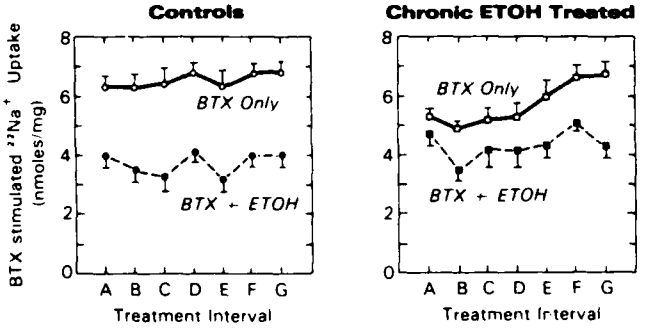


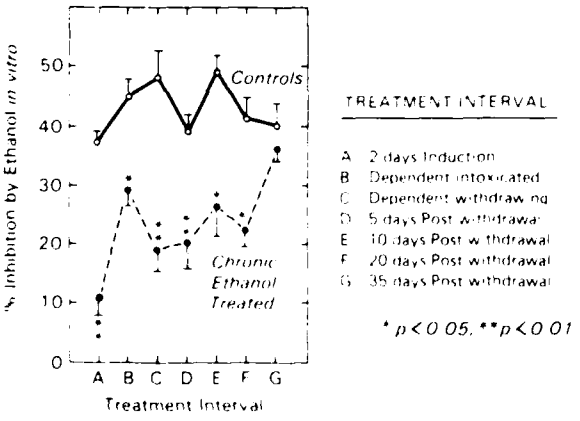
Fig. 3. Effect of chronic ethanol administration on BTX-stimulated $^{22}Na^+$ uptake. Multiple daily doses of ethanol were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated interval. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the absence (open symbols) or presence (filled symbols) of ethanol (400 mM) *in vitro*. The concentration of BTX was 1.5 μm. Values are the means ± S.E.M., n = 6–8 rats. In the chronic ethanol group, the effect of BTX only was significantly ($P < .05$ or less) smaller than corresponding control value at A, B, C and D. Also, in the chronic ethanol group, uptake in the presence of BTX and ETOH was significantly ($P < .05$) greater than the corresponding control values at E and F. B.E.C., blood ethanol concentration.

($P < .05$) decrease in the uptake of $^{22}Na^+$ in the presence of BTX alone in the ethanol-treated group after completion of 2 days of treatment, on the day of withdrawal and at 5 days after withdrawal (fig. 3, A, B, C and D). At the same time points, the addition of ethanol *in vitro* resulted in a significantly smaller degree of inhibition of BTX-stimulated $^{22}Na^+$ uptake. In the control group, the degree of inhibition by ethanol *in vitro* ranged from 36.6 ± 2.6% (fig. 3A) to 48.5 ± 4.6% (fig. 3C) whereas in the ethanol-treated group the degree of inhibition ranged from 11.2 ± 3.3% (fig. 3A) to 28.8 ± 2.0% (fig. 3B). The inhibitory effect of ethanol *in vitro* was significantly smaller in the ethanol-treated group for as long as 20 days after withdrawal (fig. 4). At 35 days after withdrawal the effect of ethanol *in vitro* was equivalent in both groups. It is of interest to note that the inhibitory effect of pentobarbital (450 μM) *in vitro* was also significantly smaller in the ethanol-treated group on the day of withdrawal and at 5 and 10 days after withdrawal (data not shown). Similar to the effect of a single dose of ethanol, chronic ethanol treatment resulted in tolerance to the inhibitory effect of ethanol *in vitro* and this tolerance was evident long after ethanol had been cleared from the body. In addition, chronic ethanol treatment also reduced the response to BTX in the absence of ethanol *in vitro*. This effect could be due to a reduced number of binding sites for BTX.

The binding of [^{3}H]BTX-B in control and ethanol-treated groups is shown in figure 5. There was no significant difference in the binding of [^{3}H]BTX-B in the absence or presence of ethanol *in vitro* at any of the treatment intervals. Similar results were obtained when a saturating concentration of [^{3}H]BTX-B (80 nM) was used (data not shown). It appears that the alterations in BTX-stimulated $^{22}Na^+$ uptake in the chronic ethanol-treated group were not due to an effect on the binding of the neurotoxin to its receptor site in the channel.

Chronic ethanol administration and membrane lipid order. The effects of chronic ethanol administration on the order or fluidity of rat brain synaptic membranes (SPM-2) were determined by measurements of the fluorescence polarization of the fluorescent probes TMA-DPH and DPH. DPH is a probe of the lower, methyl terminal portion of the lipid acyl groups (Sklar *et al.*, 1977; van Blitterswijk *et al.*, 1981) whereas TMA-DPH is a probe of the more rigid glycerol backbone regions and the carboxyl portions of the acyl groups of the membrane (Prendergast *et al.*, 1981; Harris *et al.*, 1984). The base-line fluorescence polarization of TMA-DPH and DPH was essentially identical for membranes from control and ethanol-treated rats at all times during and after the chronic ethanol treatment period (data not shown). However, chronic ethanol administration did alter the sensitivity of synaptic membranes to the disordering effects of ethanol *in vitro*. The effects of ethanol *in vitro* on the fluorescence polarization of DPH on the day of withdrawal and 5 days after withdrawal are shown in figures 6 and 7, respectively. The effect of ethanol *in vitro* on the polarization of TMA-DPH was not studied as previous studies have demonstrated that TMA-DPH is less sensitive than DPH to the effects of ethanol *in vitro* and *in vivo* (Harris *et al.*, 1984).

Chronic ethanol administration shifted the concentration effect curve to the right, resulting in a membrane tolerance on the order of 1.5- to 2-fold. Tolerance was evident after completion of 2 days of treatment and was still present at 20 days after withdrawal. The concentration of ethanol required to decrease the polarization of DPH



TREATMENT INTERVAL	BEC (mg dl)
A 2 days Induction	241 ± 27
B Dependent intoxicated	289 ± 19
C Dependent withdrawing	14 ± 6
D 5 days Post withdrawal	
E 10 days Post withdrawal	
F 20 days Post withdrawal	
G 35 days Post withdrawal	

$^*p < 0.05$, $^{**}p < 0.01$

Fig. 4. Chronic ethanol administration and percentage of inhibition of BTX-stimulated $^{22}Na^+$ uptake by ethanol (400 mM) *in vitro*. Multiple daily doses of ethanol (●) or an equivalent volume of vehicle (○) were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated intervals. Forebrain synaptosomes were prepared and BTX-stimulated $^{22}Na^+$ uptake was measured at 5 sec as described under "Methods" in the presence of BTX (1.5 μM) and ethanol (400 mM). Values are the means ± S.E.M., n = 6-8 rats per group.

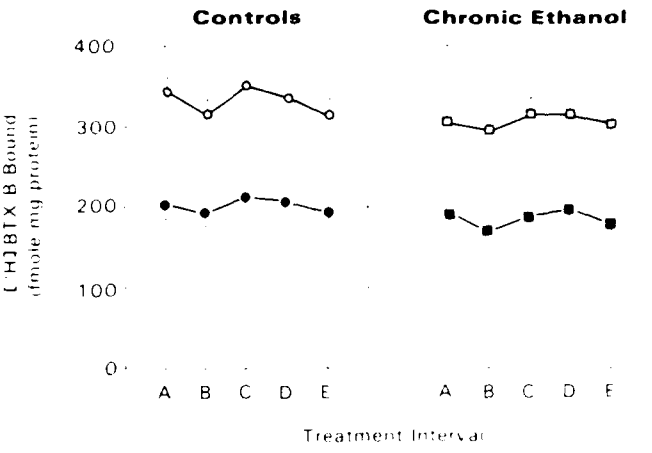


Fig. 5. Effect of chronic ethanol administration on the specific binding of [^{3}H]BTX-B. Multiple daily doses of ethanol were administered using the method of Majchrowicz (1975) and animals were sacrificed at the indicated interval (treatment interval as in fig. 8). Duplicate samples of forebrain synaptosomes were incubated with [^{3}H]BTX-B (10 nM) and scorpion venom (150 μg/ml) in the absence (open symbols) or presence (filled symbols) of ethanol (400 mM) *in vitro*. Samples were incubated at 36° for 30 min. Values are the means ± S.E.M., n = 6 per group.

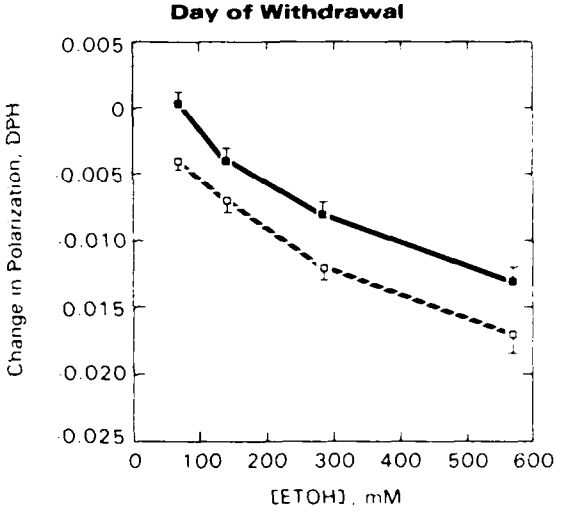


Fig. 6. Effects of ethanol *in vitro* on the fluorescence polarization of DPH. Rat forebrain synaptic plasma membrane-2 were prepared from control (□) and chronic ethanol-treated (■) animals on the day of withdrawal, approximately 6 hr after the last dose of ethanol. After DPH was incorporated, the samples were placed in the fluorimeter and maintained at 35°C. Base-line levels of fluorescence were determined and an aliquot of ethanol solution was added. Fluorescence was then determined as described under "Methods." Values are the means ± S.E.M. for six membrane preparations per group. An analysis of variance for repeated measures indicated that the curves are significantly different (F = 11.30, dF = 1,10; $P < .010$).

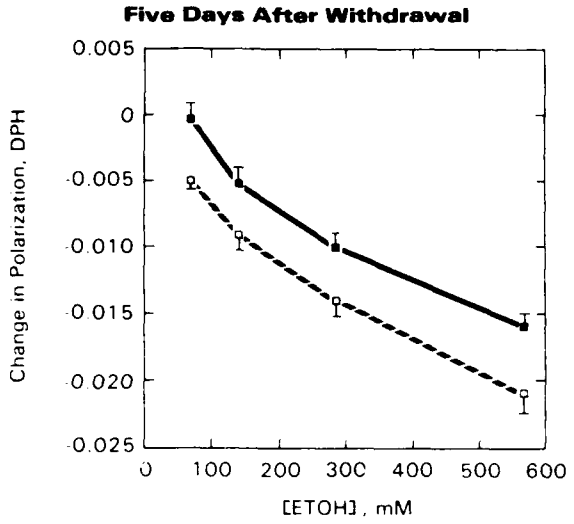


Fig. 7. Effects of ethanol *in vitro* on the fluorescence polarization of DPH 5 days after withdrawal. Rat forebrain synaptic plasma membrane-2 were prepared from control (□) and chronic ethanol-treated (■) animals 5 days after withdrawal. After DPH was incorporated, the samples were placed in the fluorimeter and maintained at 35°C. Base-line levels of fluorescence were determined and an aliquot of ethanol solution was added. Fluorescence was then determined as described under "Methods." Values are the means ± S.E.M. for six different membrane preparations per group. An analysis of variance for repeated measures indicated that the curves are significantly different ($F = 8.94$, $dF = 1,10$; $P < .025$).

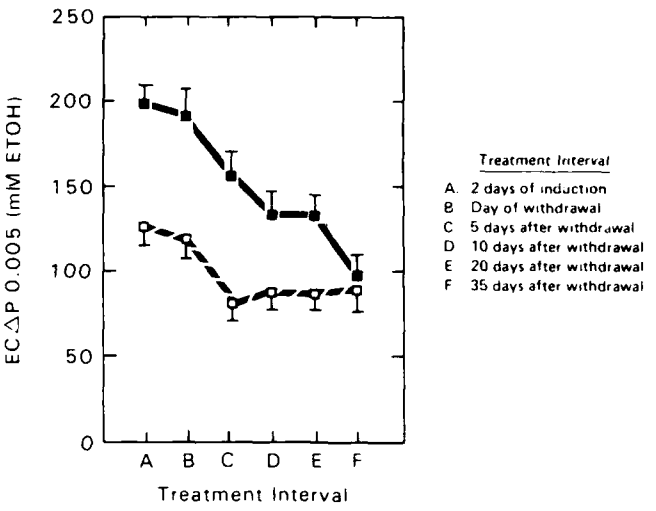


Fig. 8. Time course of altered membrane disordering by ethanol *in vitro* during and after chronic ethanol treatment. The concentration of ethanol required to decrease the fluorescence polarization of DPH by 0.005 U (ECΔ P .005) was determined by linear regression analysis of the individual concentration-effect curves at the indicated intervals during and after chronic ethanol treatment. Four concentrations of ethanol were used. Rat forebrain synaptic plasma membranes-2 from control (□) and chronic ethanol-treated (■) animals were prepared and fluorescence polarization measured as described under "Methods." Values represent the means ± S.E.M. from five to six different membrane preparations per group. Experimental group values are significantly different ($P < .05$ or less) compared to corresponding control at all intervals except F.

by 0.005 U (EC Δ P 0.005) was determined by linear regression analysis and the values obtained at each interval are shown in figure 8. The degree of tolerance to the membrane disordering effect of ethanol was estimated from the ratio of EC Δ P 0.005 ethanol-treated:EC Δ P 0.005 control. It was interesting to note that ratio of EC Δ P 0.005 values on the day of withdrawal (1.62) was remarkably similar to the values at 10 (1.48) and 20 (1.47) days after withdrawal. Thus, the tolerance produced by the chronic ethanol treatment was relatively stable and persisted for several weeks after the cessation of ethanol treatment.

Discussion

The results of the present study demonstrate that both acute and chronic ethanol treatment alter the inhibitory effect of ethanol *in vitro* on neurotoxin-stimulated $^{22}Na^{+}$ uptake. The effects of a single dose of ethanol were dependent on the dose administered and the time elapsed after the dose. Acute ethanol administration did not alter the action of BTX on the sodium channel in the absence of ethanol *in vitro* or the binding of [^{3}H]BTX-B, but it appeared that tolerance to the inhibitory effect of ethanol *in vitro* on BTX-stimulated sodium uptake was present. The reduced effectiveness of ethanol *in vitro* after a single dose of ethanol may be analogous to the finding of acute tolerance in whole animal studies (for review, see Cicero, 1980).

Chronic ethanol administration produced somewhat different results than the single dose studies. There was a significant reduction in BTX-stimulated sodium uptake in the absence of ethanol *in vitro* after 2 days of induction, on the day of withdrawal and at 5 days after withdrawal. Results of binding studies with [^{3}H]BTX-B in the chronic ethanol group suggest that the diminished response to BTX was probably not due to changes in the binding of BTX to its receptor site in the channel. Furthermore, the inhibitory effect of ethanol *in vitro* on sodium uptake was significantly attenuated for up to 20 days after withdrawal.

The fluidity or order of brain membranes from the chronic ethanol group was assessed by the fluorescence polarization of the probes DPH and TMA-DPH. There was no change in the fluorescence polarization of either probe at anytime during or after chronic ethanol treatment, indicating that the basal or intrinsic fluidity of brain membranes was unchanged. Intrinsic membrane fluidity has been reported to be decreased (Rottenberg *et al.*, 1981; Lyon and Goldstein, 1983; Harris *et al.*, 1984) or unchanged (Chin and Goldstein, 1977b; Beauge *et al.*, 1984) after chronic ethanol treatment. Brain membranes derived from the chronic ethanol group were resistant to the disordering effect of ethanol *in vitro* for up to 20 days after withdrawal, as measured by the change in the fluorescence polarization of DPH. Thus, adaptation to the effects of ethanol *in vitro* on sodium uptake and membrane fluidity followed a similar time course after chronic ethanol treatment.

The mechanisms responsible for the attenuated effects of ethanol *in vitro* reported here are not known. Acute *in vivo* administration of ethanol resulted in a marked attenuation of the inhibitory effect of ethanol on sodium uptake but does not appear to alter ethanol-induced disordering of reconstituted (Johnson *et al.*, 1979) or intact synaptic membranes (R. A. Harris, unpublished observation). Although it is merely speculation, these findings could be interpreted as an uncoupling of the effects of ethanol on the physical and functional properties of neurons. An analogous situation was reported recently by Mitchell *et al.* (1985) who demonstrated that brain membranes prepared from barbiturate tolerant-dependent mice were resistant to the inhibitory effect of ethanol and pentobarbital on sodium uptake but that the disordering effects of the drugs

were unchanged. In the present study 18 to 24 hr after a single dose of ethanol the response to ethanol *in vitro* was restored to control levels suggesting that the adaptive response was transient. This uncoupling effect may be an initial but short lived component of membrane tolerance. Alternatively, it is possible that a single dose of ethanol alters the physical properties of rather specific membrane lipids that are functionally important but are undetected by existing methods used to assess the properties of bulk lipids in neuronal membranes (Taraschi and Rubin, 1985). A lipid species of this type might be present in relatively small quantities or be required in a given state of order at a critical area of the membrane in order to provide optimal conditions for efficient activity of membrane proteins.

During chronic ethanol treatment when animals are maintained in a state of prolonged intoxication, it is likely that a variety of adaptive changes occur. Indeed, after 2 days of chronic ethanol administration tolerance to most of the acute effects of ethanol has been demonstrated (Majchrowicz and Hunt, 1976; Ritzmann and Tabakoff, 1976; Goldstein and Zaechelein, 1983). At the completion of 2 days of treatment we found that brain membranes were resistant to the inhibitory effect of ethanol on sodium uptake and the disordering effect of ethanol. These data are in agreement with the data of Lyon and Goldstein (1983) which demonstrated resistance to the disordering effect of ethanol in mouse synaptic plasma membranes after 3 days of continuous exposure to ethanol vapor. Thus, like functional tolerance to the behavioral effects of ethanol, membrane tolerance to ethanol can develop rather quickly during chronic ethanol treatment.

Tolerance to ethanol in the intact animal has been reported to dissipate over a wide range of time, from a few days (Goldstein and Zaechelein, 1983; Ritzmann and Tabakoff, 1976) to weeks or months (LeBlanc *et al.*, 1969; Kalant *et al.*, 1971; Begleiter *et al.*, 1973) after withdrawal from chronic ethanol. It is apparent that the persistence or duration of tolerance in the animal is sensitive to a number of variables including the method and length of ethanol administration and the characteristics of the test used to assess tolerance. Most studies of tolerance to the membrane disordering effect of ethanol have examined a limited number of time points, usually on the day of withdrawal, although Johnson *et al.* (1980) reported that membrane tolerance was no longer evident at 12 days after withdrawal. We found evidence of membrane tolerance, assessed by the effects of ethanol *in vitro* on sodium uptake and membrane order, for up to 20 days after withdrawal. In contrast to our findings, Taraschi *et al.* (1986) reported recently that although 4 to 5 weeks of chronic ethanol treatment were required to develop tolerance to the disordering effect of ethanol in liver microsomes and erythrocytes, the tolerance was lost after 1 or 2 days of withdrawal. Similar to the findings for behavioral tolerance to ethanol it is likely that the persistence of membrane tolerance is dependent on a number of variables including the length and method of treatment, the nature of the molecular probe and the source and type of membrane preparation. In the case of the central nervous system there is a growing body of evidence that some of the functional properties of neurons are altered for prolonged periods of time after withdrawal from chronic ethanol treatment (Begleiter and Porjesz, 1977; Walker *et al.*, 1981; Eckardt *et al.*, 1986). The relationship of persistent changes in the brain to the nature of human alcoholism remains to be established.

A number of factors suggest that alterations in the functional properties of sodium channels might be involved in some of the actions of ethanol: 1) ethanol inhibits sodium influx at concentrations achieved *in vivo*, an effect that is fully reversible (Mullin and Hunt, 1984, 1985); 2) there is an excellent correlation between membrane disordering and inhibition of sodium influx (Harris and Bruno, 1985a); 3) inhibition of sodium influx by ethanol is reduced in tissue from ethanol-treated rats (this study); and 4) tolerance to inhibition of sodium influx and membrane disordering by ethanol share a common time course after chronic ethanol treatment (this study). However, further research is necessary to define in detail the involvement of neuronal ion channels in the actions of ethanol.

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Actions of Ethanol on Voltage-Sensitive Sodium Channels: Effects on Neurotoxin Binding[1]

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ABSTRACT

Exposure of rat forebrain synaptosomes (P_2) to ethanol *in vitro* reduced the specific binding of [^{3}H]batrachotoxinin A 20-α-benzoate ([^{3}H]BTX-B) to voltage-sensitive sodium channels. This effect of ethanol was concentration-dependent and was affected by the membrane potential. Under depolarizing conditions ethanol was significantly more potent at inhibiting [^{3}H]BTX-B binding. Scatchard analysis of [^{3}H]BTX-B binding revealed that ethanol increased the equilibrium binding constant without affecting the apparent maximum number of binding sites. The rate of formation of the [^{3}H]BTX-B/receptor complex was unchanged in the presence of ethanol whereas the rate of dissociation was accelerated by ethanol. These findings are consistent with an indirect allosteric mechanism for inhibition of [^{3}H]BTX-B binding. The binding of [^{3}H]saxitoxin was unaffected by ethanol suggesting that the specific receptor sites in the channel display differential sensitivity to the inhibitory effect of ethanol. These data, in conjunction with ion flux measurements, provide further evidence that ethanol can affect the voltage-sensitive sodium channels in neuronal membranes.

The molecular mechanisms underlying the pharmacological actions of ethanol are at present unknown. There is, however, an abundance of experimental evidence which indicates that intoxicant-anesthetic agents alter the physical properties of biological membranes (Goldstein, 1984). In particular, ethanol has been shown to disorder the lipid portions of intact synaptic membranes (Chin and Goldstein, 1977a; Harris and Schroeder, 1981, 1982), resulting in an increase in membrane fluidity. In this regard, the term "membrane fluidity" is used to describe the degree of mobility of various membrane components (Goldstein, 1984).

The suggestion that intoxication and membrane disorder are causally related is supported by numerous studies utilizing various experimental approaches. Lyon *et al.* (1981) reported a strong correlation between the hypnotic potencies of aliphatic alcohols and their potencies for disordering neuronal synaptosomal plasma membranes. Brain membranes derived from ethanol-tolerant mice are also resistant to the fluidizing effect of ethanol *in vitro* (Chin and Goldstein, 1977b; Harris *et al.*, 1984). In addition, differential sensitivity to the disordering effect of ethanol may occur in membranes derived from mice that have been bred selectively for differential sensitivity to the hypnotic effect of ethanol (Goldstein *et al.*, 1982; Perlman and Goldstein, 1984). However, it has not yet been demonstrated that membrane disordering *per se* causes any known behavioral effect. It is likely that identification of specific membrane functions that are sensitive to changes in membrane order will be useful in the formulation of a mechanism that explains intoxication and/or anesthesia.

A number of studies have examined the effects of ethanol and other intoxicant-anesthetic agents on the movement of ions across excitable membranes (Hunt, 1985). Ethanol, in concentrations that occur *in vivo* (25–100 mM), has been shown to inhibit the neurotoxin-stimulated influx of sodium ions (Mullin and Hunt, 1984, 1985; Harris and Bruno, 1985a) and the potassium-stimulated influx of calcium (Harris and Hood, 1980; Leslie *et al.*, 1983) ions in brain synaptosomes. In addition, the calcium-dependent efflux of rubidium, a model for the calcium-activated efflux of potassium, has been shown to be increased in the presence of ethanol (Yamamoto and Harris, 1983).

Recently, Harris and Bruno (1985b) have studied the effects of a series of chemically diverse membrane perturbants on the lipid order of synaptic plasma membranes and the sodium and calcium fluxes in mouse brain synaptosomes. The results demonstrated that the degree of inhibition of veratridine-dependent sodium influx was proportional to the degree of lipid disordering, in particular the degree of lipid disordering deep in the membrane. The effects of the drugs on calcium uptake were not clearly related to increased membrane disorder.

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ABBREVIATIONS: BTX-B, batrachotoxin A 20-α-benzoate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STX, saxitoxin.

Thus, it appears that the inhibitory effect of ethanol on sodium influx is a consequence of the increase in membrane fluidity that occurs in the presence of ethanol. To characterize further the action of ethanol on the voltage-sensitive sodium channel, the present study examined the effect of ethanol on the binding of radiolabeled neurotoxins to specific sites in the sodium channels of synaptosomes in which electrophysiological methods are impractical.

Methods

Animals and chemicals. Male Sprague-Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and were housed two per cage with free access to water and standard laboratory chow. Chemicals and suppliers were as follows: scorpion (Leiurus quinquestriatus) venom, tetrodotoxin and veratridine from Sigma Chemical Co. (St. Louis, MO); [benzoyl-2,5-^{3}H]BTX-B (51 Ci/mmol) from New England Nuclear (Boston, MA). Batrachotoxin was kindly supplied by Dr. John Daly (National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD); and [^{3}H]STX (9.3 Ci/mmol) was a generous gift from Dr. Stephen Davio (Pathophysiology Division, U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD). All other chemicals were obtained from commercial sources and were of analytical grade.

Preparation of synaptosomes. A crude synaptosomal (P_2) fraction was prepared by a modification of the method of Gray and Whittaker (1962). Rats were decapitated and the forebrains (cerebellum and brainstem discarded) were removed and homogenized in ice-cold 0.32 M sucrose and 5 mM K_2HPO_4, pH 7.4 (10 ml/g wet weight), with 10 strokes of a motor driven Teflon-glass homogenizer. The homogenate was then centrifuged at 1000 × g for 10 min. The resulting supernatant was then centrifuged at 17,000 × g for 60 min. The final pellet was resuspended in the indicated volume of the appropriate buffer as described below.

Measurement of radiolabeled neurotoxin binding. The binding of [^{3}H]BTX-B was determined by a modification of the method of Catterall *et al.* (1981). The P_2 pellet was resuspended in binding medium containing the following (millimolar): choline chloride, 130; HEPES-Tris (pH 7.4) 50; glucose, 5.5; $MgSO_4$, 0.8; and KCl, 5.4. Incubations were carried out in a total volume of 300 μl containing 1 μM tetrodotoxin, 45 μg of scorpion venom, [^{3}H]BTX-B and an aliquot of synaptosomes (150–200 μg of protein) in the absence or presence of the indicated concentration of ethanol. Samples were incubated at 36°C for 30 min. The binding reactions were terminated by diluting the samples with 3 ml of ice-cold wash solution and collecting under vacuum on a glass-fiber filter (Whatman GF/C). The filters were then washed three times with 3 ml of wash solution consisting of (millimolar): choline chloride, 163; HEPES (adjusted to pH 7.4 with Tris base), 5; $CaCl_2$, 1.8; and $MgSO_4$; 0.8. Filters were placed in scintillation vials with 15 ml of scintillation cocktail and the tritium content was measured by liquid scintillation spectroscopy with a counting efficiency of 48%. Nonspecific binding was determined in the presence of 300 μM veratridine. Specific binding was calculated by subtracting nonspecific from total binding values. The addition of tetrodotoxin (to inhibit ion flux) and scorpion venom (to increase ligand affinity) to the assay mixture increases markedly the specific component of binding (Willow and Catterall, 1982).

[^{3}H]STX binding was measured by a modification of the method of Krueger *et al.* (1979). The P_2 pellet was resuspended in ice-cold buffer consisting of (millimolar): NaCl, 145; KCl, 5; $MgCl_2$, 1.4; $CaCl_2$, 1.0; NaH_2PO_4, 1.2; glucose, 10; and Tris-HEPES, 20, pH 7.4 to give a protein concentration of 3 mg/ml. Aliquots (100 μl) of the synaptosomes were incubated with [^{3}H]STX in a total volume of 1 ml. After incubation on ice for 60 min the samples were diluted with 5 ml of ice-cold wash solution (150 mM NaCl in 20 mM Tris-HEPES, pH 7.4) and the mixture was collected on glass-fiber filters (Whatman, GF/F). The filters were washed twice with 5 ml of wash solution and the tritium content was measured by liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 10 μM tetrodotoxin.

Other methods. Statistical analysis was performed using Student's t test. Multiple comparisons with a control were done by analysis of variance and Dunnett's test (1964). Linear segments on Scatchard plots and kinetic experiments were computed by linear regression. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the protein standard.

Results

The effect of increasing concentrations of ethanol on the specific binding of [^{3}H]BTX-B (10 nM) to rat brain synaptosomes is shown in figure 1. Specific binding of [^{3}H]BTX-B to synaptosomal sodium channels in the absence of ethanol was 320 ± 21 fmol/mg of protein. The nonspecific component of binding was unaffected by the presence of ethanol and was 20 to 25% of total [^{3}H]BTX-B binding. The lowest concentration of ethanol that caused a significant inhibition of [^{3}H]BTX-B binding was 75 mM. Ethanol did not completely inhibit the specific binding of [^{3}H]BTX-B as approximately 35 to 40% of [^{3}H]BTX-B binding was unchanged at an ethanol concentration of 800 mM.

To determine if the effect of ethanol on [^{3}H]BTX-B binding was dependent on membrane potential, binding studies were performed in buffer containing 5 or 135 mM KCl. Under these experimental conditions the membrane potential should be approximately −55 and 0 mV, respectively (Blaustein and Goldring, 1975). As the affinity of the polypeptide toxin present in scorpion venom is markedly potential-dependent (Catterall, 1977), the concentration of scorpion venom was increased 10-fold to 1500 μg/ml to ensure essentially complete receptor occupancy at both membrane potentials (Catterall *et al.*, 1981; Willow and Catterall, 1982). Ethanol was somewhat more potent at inhibiting [^{3}H]BTX-B binding under depolarizing conditions as compared with nondepolarizing conditions, as shown in figure 2.

[^{3}H]BTX-B has been shown to bind to a single class of high-affinity receptor sites in voltage-sensitive sodium channels in neuronal membranes (Catterall *et al.*, 1981; Creveling *et al.*, 1983). The equilibrium binding properties of [^{3}H]BTX-B were

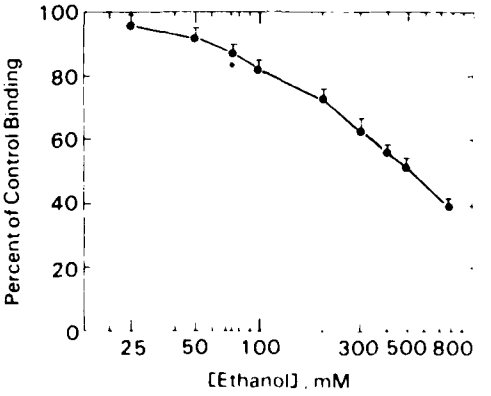

Fig. 1. Concentration-effect curve for inhibition of [^{3}H]BTX-B binding. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (in the presence of scorpion venom, 150 μg/ml) in the presence of increasing concentrations of ethanol for 30 min. Binding was then measured as described under "Methods." The ordinate represents the binding as a percentage of the binding in the absence of ethanol. Values are mean ± S.E.M., n = 4. *Lowest concentration producing a significant ($P < .05$) inhibition of binding.

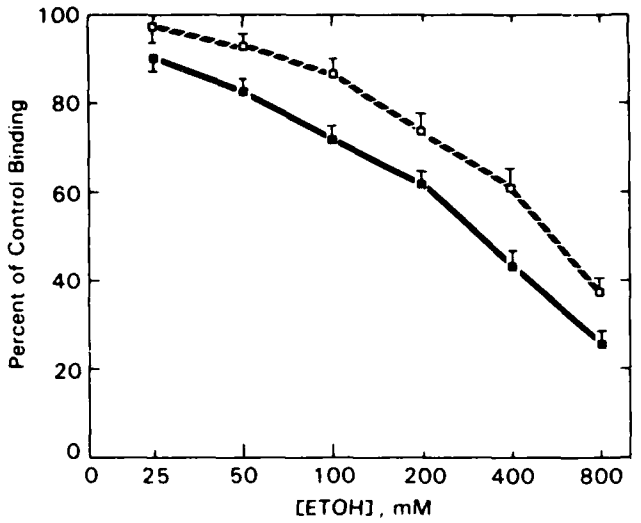

Fig. 2. Effect of ethanol on the binding of [^{3}H]BTX-B under depolarizing and nondepolarizing conditions. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (in the presence of scorpion venom, 1500 μg/ml) and increasing concentrations of ethanol in medium containing 5 mM KCl (□) or 135 mM KCl (■) for 30 min. Binding was measured as described under "Methods." The ordinate represents the binding as a percentage of the binding in the absence of ethanol. Values are the mean ± S.E.M., $n = 3$. The curves are significantly different ($F = 13.54$, dF = 1,4; $p < .025$) by analysis of variance for repeated measures.

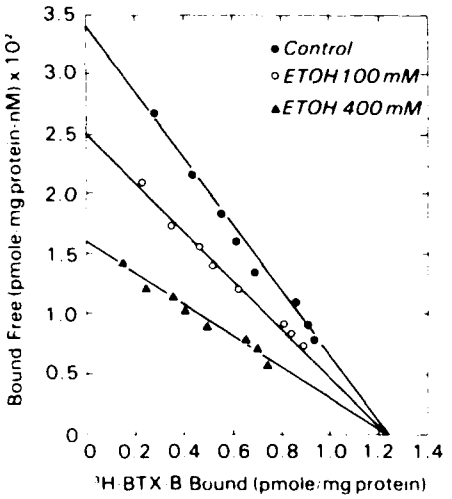

Fig. 3. Scatchard analysis of [^{3}H]BTX-B binding. Duplicate samples of forebrain synaptosomes were incubated with increasing concentrations of [^{3}H]BTX-B under standard assay conditions (scorpion venom concentration was 150 μg/ml) in the presence of the indicated concentration of ethanol for 30 min. Binding was then measured as described under "Methods". Values are the means from four experiments. The S.E.s ranged from 5 to 9% of the mean.

examined over a range of ligand concentrations. Scatchard analysis of data from four experiments yielded a straight line with an apparent maximum number of binding sites equal to 1.24 ± 0.02 pmol/mg of protein. Ethanol, at 100 and 400 mM, increased the K_d from 37.5 ± 3.1 to 50.9 ± 3.2 nM ($P < .05$) and 85.8 ± 6.0 nM ($P < .01$), respectively (fig. 3).

Ethanol (200 mM) did not alter the rate of association of [^{3}H]BTX-B to the binding site on the sodium channel (fig. 4). The dissociation rate constant (k_{-1}) was estimated by incubat-

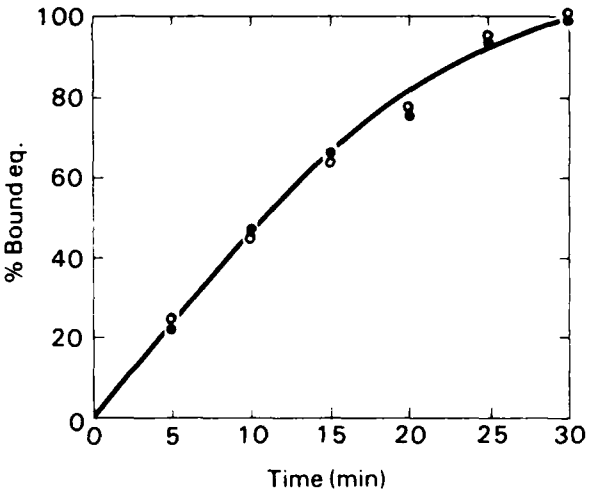

Fig. 4. Time course of formation of the [^{3}H]BTX-B receptor complex. Synaptosomes were incubated for the indicated times with 10 nM [^{3}H]BTX-B (scorpion venom concentration was 150 μg/ml) in the absence (○) and presence (●) of 200 mM ethanol. At each time point, binding was measured as described under "Methods." Values are the means from three experiments. The data are expressed as the percentage of specifically bound [^{3}H]BTX-B at equilibrium (eq) (30 min).

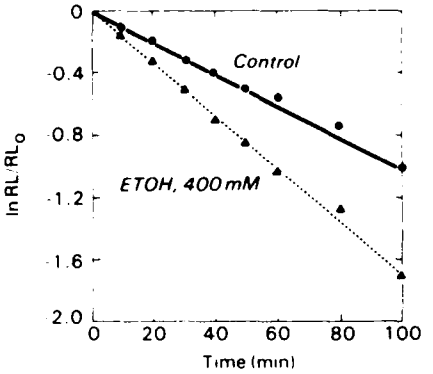

Fig. 5. Effect of ethanol on the dissociation of the [^{3}H]BTX-B/receptor complex. Duplicate samples of forebrain synaptosomes were incubated with 10 nM [^{3}H]BTX-B (scorpion venom concentration was 150 μg/ml) for 30 min. At zero time, veratridine (300 μM) in the absence or presence of ethanol (400 mM) was added and at the indicated times, samples were filtered and binding was measured as described under "Methods." Values are the means from four separate experiments. The standard errors ranged from 4 to 10% of the mean.

ing samples under standard conditions with [^{3}H]BTX-B (10 nM) for 30 min after which veratridine (300 μM) in the absence or presence of ethanol (400 mM) was added. At various intervals, the samples were filtered and bound [^{3}H]BTX-B was determined. Figure 5 illustrates the accelerated time course of dissociation of [^{3}H]BTX-B from the steady-state complex in the presence of ethanol. Ethanol (400 mM) increased the dissociation rate constant (k_{-1}) from 0.0094 to 0.0169 min^{-1}. These kinetic data indicate that ethanol is an indirect allosteric competitive inhibitor of [^{3}H]BTX-B binding.

Voltage-sensitive sodium channels also contain a receptor for STX and tetrodotoxin which is separate from and does not interact with the receptor for batrachotoxin and veratridine (Catterall, 1980). Results from a Scatchard analysis of the binding of increasing concentrations of [^{3}H]STX in the absence and presence of ethanol are shown in table 1. At a concentration of ethanol which inhibited [^{3}H]BTX-B binding by approxi-

TABLE 1

Scatchard analysis of [^{3}H]STX binding

Values are the means ± S.E.M.; n = number of membrane preparations. Duplicate samples of synaptosomes were incubated on ice for 60 min with increasing concentrations (0.10–10 nM) of [^{3}H]STX in the absence or presence of ethanol *in vitro*.

Group	n	B_{max}*	K_d
		pmol/mg protein	*nM*
Control	4	2.79 ± 0.10	1.45 ± 0.13
Ethanol, 400 mM	4	2.87 ± 0.11	1.53 ± 0.15

* B_{max}, maximum number of binding sites.

mately 40%, there was no effect on [^{3}H]STX binding. These data agree with results from ion flux studies that showed no effect of ethanol on the concentration of tetrodotoxin needed to reduce neurotoxin-stimulated $^{22}Na^+$ influx by 50% (Mullin and Hunt, 1985; Harris and Bruno, 1985a).

Discussion

The present results demonstrate that ethanol inhibits the specific binding of [^{3}H]BTX-B to sodium channels in rat forebrain synaptosomes. Using the intoxication assessment scale of Majchrowicz (1975) and the data in figure 1, ethanol inhibited [^{3}H]BTX-B binding by approximately 9 and 20% at concentrations associated with moderate intoxication (50 mM) and anesthesia (100 mM), respectively. However, under depolarizing conditions in which ethanol is significantly more potent (fig. 2), an intoxicating concentration (50 mM) of ethanol inhibited [^{3}H]BTX-B binding by 19% and an anesthetic concentration (100 mM) caused a 29% reduction in [^{3}H]BTX-B binding. In addition, brain regions differ in sensitivity to the inhibitory effect of ethanol on channel-mediated sodium influx (Harris and Bruno, 1985a). Thus, it may be possible that neuronal sodium channels are involved in some aspects of central nervous system depression associated with intoxication and anesthesia.

Analysis of the effect of ethanol on the equilibrium binding of [^{3}H]BTX-B revealed a concentration-dependent increase in the K_d with no change in the apparent maximum number of binding sites, consistent with a mechanism of competitive inhibition (Tallarida and Jacob, 1979). However, for a number of reasons it is highly unlikely that ethanol interferes with [^{3}H]BTX-B binding by a mechanism of simple competitive inhibition at a single, common site. It is more likely that ethanol inhibits the binding of [^{3}H]BTX-B through an allosteric mechanism. This conclusion is supported by the data from the studies on the kinetic binding properties of [^{3}H]BTX-B. A simple direct competitive inhibitor would be expected to alter the rate of formation of the [^{3}H]BTX-B/receptor complex, whereas ethanol had no effect on this process (fig. 4). In addition, ethanol increased the rate of dissociation of the [^{3}H]BTX-B/receptor complex in the presence of a saturating concentration of veratridine. These findings are compatible with a proposed mechanism of allosteric competitive inhibition.

It is interesting to note that ethanol had no effect on the binding of [^{3}H]STX to rat forebrain synaptosomes. The receptor site for STX is thought to be located in a highly polar, hydrophilic area at the extracellular side of the sodium channel (Narahashi *et al.*, 1966; Angelides and Nutter, 1983, 1984). Thus, the STX receptor site is in an area of the membrane in which the disordering effect of ethanol is rather weak (Chin and Goldstein, 1981; Harris and Schroeder, 1981, 1982). Additionally, although the binding of [^{3}H]STX is inhibited by exposure of the membrane to phospholipases (Baumgold, 1980), the binding of [^{3}H]BTX-B is 10 to 100 times more sensitive to this particular membrane modification (M. J. Mullin, unpublished observation). The binding site for [^{3}H]BTX-B appears to be sensitive to the physical properties of lipid components in the membrane microenvironment.

Recent observations on the covalent binding of a derivative of batrachotoxin suggest that the batrachotoxin binding site is located at the membrane lipid/channel protein interface (Brown, 1985). By disordering lipid domains in the neuronal membrane, ethanol may interfere with specific lipid-protein interactions that are necessary for the binding of batrachotoxin and the subsequent activation of the channel.

A number of drugs that depress neuronal excitability have been shown to inhibit neurotoxin-stimulated sodium uptake and [^{3}H]BTX-B binding (Willow and Catterall, 1982; Creveling *et al.*, 1983). Ethanol at concentrations that inhibit neurotoxin-stimulated sodium uptake also inhibited the binding of [^{3}H]BTX-B, presumably by an indirect allosteric mechanism. However, it is somewhat difficult to directly compare the ion flux and binding assays as there are major differences in the manner in which the assays are performed, including the composition of buffers, the presence or absence of scorpion venom and tetrodotoxin and the time scale of measurement. For example, in the ion flux assays, ethanol reduced the maximal effect of batrachotoxin (Harris and Bruno, 1985a; Mullin and Hunt, 1985) whereas in the binding studies ethanol decreased the affinity of BTX-B for the binding site.

Changes in the physical properties of neuronal membranes due to the presence of ethanol can alter the function of sodium channels which in conjunction with other altered processes may be involved in the depressant effect of ethanol.

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Radioprotection with Interleukin-1: Comparison with Other Cytokines

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Murine IL-1α and human IL-1α and IL-1β protect mice from lethal effects of radiation-induced hematopoietic syndrome. A single 100 ng dose of human IL-1α conferred protection, provided it was administered to C57BL/6 and DBA/1 mice 20 hr before irradiation with an $LD_{(100)}$ dose, with a DRF of 1.25 and 1.2, respectively. Studies of possible mechanisms of radioprotection by IL-1 showed that radioprotection of $LD_{(100)}$ irradiated mice could not be induced with IL-2, IFN-γ, or GM-CSF. Radioprotection with IL-1 did not depend on the release of prostaglandins, since indomethacin did not diminish survival of IL-1-treated mice. Unlike C57BL/6 and DBA/1 mice, C3H/HeJ mice were not protected from lethal irradiation by IL-1. Nevertheless, both high-responder C57BL/6 and low-responder C3H/HeJ strains, treated with IL-1, develop similarly enhanced levels of acute phase proteins, metallothionein and ceruloplasmin, with known radioprotective abilities. Therefore, the observed differences in radioprotection with IL-1 in murine strains probably cannot be attributed to differences in levels of these scavenging metalloproteins. In contrast, striking differences were observed in the recovery of bone marrow cells after lethal and midlethal irradiation, in that the recovery of total nucleated bone marrow cells and specifically CFU-Es was much greater in C57BL/6 than in C3H/HeJ mice 9 to 12 days following irradiation. However, the radioprotective effect of IL-1 was similar following sublethal irradiation of the two strains when recovery of endogenous hematopoietic splenic colonies was compared. Consequently, IL-1 can protect C3H/HeJ mice against sublethal but not lethal doses of irradiation. Although much remains to be learned about the mechanism by which IL-1 exerts its radioprotective effect, IL-1 treatment, as is the case for many radioprotective agents, induces recovery of erythropoiesis.

I. Introduction

Bacterial endotoxin and other agents with immunostimulating and inflammatory effects are radioprotective when administered before irradiation (Smith *et al.*, 1957; Ainsworth and Chase, 1959; Mori *et al.*, 1975). Although the mechanisms for radioprotection with such inflammatory agents are not well understood, pretreatment with these agents results in accelerated postirradiation recovery of the hematopoietic and immune systems (Hanks and Ainsworth, 1965;

Smith *et al.*, 1966a,b; Behling, 1983). Because proliferation and differentiation of hemopoietic and lymphoid cells are mediated by endogenously produced growth and differentiation factors, it could be hypothesized that the radioprotective effect of lipopolysaccharide (LPS) and similar agents depends on induction of cytokines. We have, therefore, initiated studies to test directly for the radioprotective effect of a number of purified recombinant cytokines.

II. Radioprotective Effects of IL-1

In recent communications we have shown that recombinant murine IL-1α and human IL-1α and IL-1β are radioprotective for C57BL/6 and DBA/1 mice (Neta *et al.*, 1986a,b,c). Maximal radioprotection, measured by percentage survival of mice irradiated with a lethal dose of gamma radiation ($LD_{100/30}$ = dose that kills 100% of mice within 30 days), was obtained when IL-1 was administered 20 hr before irradiation. The radioprotective effect of IL-1 in mice was reduced when IL-1 was administered at 4 hr before irradiation, and was absent if administered 45 hr before or 1 hr after irradiation. The radioprotective effect of IL-1 observed in C57BL/6 and DBA/1 mice was similar in magnitude to that reported for LPS with a DRF (dose reduction factor, defined as the ratio of $LD_{50/30}$ for IL-1-treated mice to $LD_{50/30}$ for the control mice) of 1.2–1.25. This lends further support to the view that IL-1 may be a primary mediator for radioprotection induced with inflammatory substances such as LPS.

III. Role of Other Cytokines

The necessity of a 20-hr lag period to obtain radioprotection with IL-1 suggested that IL-1 may not be directly radioprotective. Since IL-1 has a multiplicity of biological effects many possible mechanisms can account for its radioprotective activity.

First of all, induction with IL-1 of other cytokines may be responsible for the radioprotection. Therefore, we investigated the induction of certain cytokines by IL-1 as well as the capacity of several cytokines that might be produced in response to IL-1 to induce radioprotection. Recombinant IFN-γ, granulocyte–macrophage colony-stimulating factor (GM-CSF), and IL-2 were selected because (1) previous studies conducted with impure preparations of interferon (IFN) or CSF implicated these cytokines in radioprotection (Khaltowich *et al.*, 1974; Urbaschek and Urbaschek, 1983), (2) IFN and CSF are induced in high titers following LPS challenge (Neta and Salvin, 1981; Metcalf, 1971), and (3) induction of IL-2 with IL-1 was demonstrated *in vitro* (Smith *et al.*, 1980).

The titers of IFN and CSF in the sera of IL-1-treated mice were determined.

TABLE I

The Effect of Administration of Five Recombinant Cytokines on the Survival of $LD_{100/30}$ Irradiated Mice[a]

Time of delivery (hr before irradiation)	Treatment	(Number of mice)	Survival + SD (%)
20	IL-1α (100 ng)	65	86 ± 8.8
	IL-1β (200 ng)	49	75 ± 12
20	GM-CSF		
	1 μg	29	0
	5 μg	24	20 ± 17
	10 μg	41	0
3	5 μg	10	0
20	IL-2		
	0.4 μg	8	0
	1.2 μg	36	6.8 ± 12
	4.0 μg	17	11.0 ± 15.5
3	4.0 μg	8	0
20	IFN-γ		
	0.2 μg	12	0
	1.0 μg	20	0
	10.0 μg	20	0
3	1.0 μg	12	0
	Radiation control	108	0

[a]C57BL/6 mice, 10–12 weeks old, received ip injections of cytokines in doses as specified at 20 or 3 hr before irradiation. The radiation dose, 950 cGy, killed 100% of mice in 16 ± 1 days.

Administration of 100 ng of human IL-1α, a dose which is radioprotective in mice, resulted in the appearance of high serum titers of CSF at 3 and 6, but not 24 hr following injection of IL-1. Assays for interferon in the same sera were negative (Neta *et al.*, 1986c). These results suggested that CSF, but not IFN, may be of importance in IL-1-mediated radioprotection. However, direct administration of recombinant IFN-γ, GM-CSF, or IL-2 under conditions comparable to those previously used with IL-1 (*i.e.*, ip administration of a wide range of doses at 20 or 3 hr before irradiation) had no significant effect on survival of $LD_{100/30}$ irradiated mice (Table I). These results argue against the possibility that various CSFs or IL-2 produced in response to IL-1 or that IFN-γ alone can be the sole initiators of radioprotection.

IV. Role of Prostaglandins

Another recognized activity of IL-1 that may contribute to its radioprotective effect is this cytokine's enhancement of the release of prostaglandins *in vivo* and

TABLE II

Effect of Indomethacin on Radioprotection with IL-1 (Evaluated by Mice Survival)[a]

Group	Survival (%)	Mean survival time (days)
A	63	—
B	82	—
C	0	12.8 ± 36
D	0	11.5 ± 4.8

[a]Groups of 16 C57BL/6 mice received 100 ng of IL-1 ip (A), 100 ng of IL-1 + 2 im injections of 40 μg of idomethacin at 20 and 3 hr prior to irradiation (B), indomethacin alone (C), or saline (D). Twenty hours following IL-1 injections mice were irradiated with 950 cGy.

in vitro (Dinarello and Bernheim, 1981; Dinarello, 1985). Furthermore, prostaglandins were recently demonstrated to exert radioprotection if administered 5–60 min before irradiation in doses of 1–40 μg/mouse (Hanson and Thomas, 1983; Walden *et al.*, 1986). We therefore assessed the effects of indomethacin, an inhibitor of prostaglandin synthesis, on the radioprotection conferred by IL-1. C57BL/6 mice were treated with (1) 100 ng/mouse of IL-1 (group A), (2) IL-1 + indomethacin (two injections of 40 μg each administered 20 and 3 hr prior to irradiation) (group B), (3) indomethacin alone (group C), or (4) saline (group D). The mice were then exposed to 950 cGy ^{60}Co γ irradiation (an $LD_{100\ 30}$ dose for this strain). The mice were either evaluated for survival or their bone marrow cells were examined at different days after irradiation. The survival of mice receiving indomethacin and IL-1 (group B) was similar to that of mice that received IL-1 alone (group A) (Table II). Similarly, administration of indomethacin alone did not affect the survival as evident from the finding that groups C and D were dead within 18 days after irradiation (with a mean survival time of 12.8 ± 3.6 and 11.5 ± 4.8 days, respectively). Based on the results of these survival studies the radioprotection induced with IL-1 is not prostaglandin mediated. Furthermore, indomethacin treatment did not prolong survival, consequently the physiological levels of prostaglandins do not contribute to duration of survival.

A modest radioprotective effect of physiological levels of prostaglandins could be inferred, however, based on the effect of indomethacin on the recovery of nucleated bone marrow cells. Treatment with indomethacin alone significantly reduced the numbers and the size of bone marrow cells recovered at 7 days after irradiation compared to irradiated control mice (Table III). This observation attests to the effectiveness of treatment with indomethacin. However, administration of IL-1 together with indomethacin resulted in proportional increases in

TABLE III

Effect of Indomethacin on Radioprotection with IL-1 Evaluated by Recovery of Bone Marrow Cells at 7 Days after Irradiation[a]

Treatment group	Total cells (channels) 1–100	Channel 9–25	Channel 25–100
A	24333	8900	12428
B	20173	8011	9370
C	10280	3880	3568
D	15404	7580	4762

[a]C57BL/6 mice were treated as specified in Table II. At 7 days after irradiation 4 mice in each group were sacrificed and the femoral bone marrow cells were obtained. Nucleated cell counts and sizing profiles were performed with the aid of Coulter Channelyzer. The cells ranged in sizes from 80.75 to 620 fl in channels 1 to 100.

the numbers of recoverable cells (groups B vs A), further supporting the view that the radioprotective effect of IL-1 is not due to prostaglandins.

V. Genetic Differences of the Response to Radioprotection with IL-1

We also evaluated the radioprotective effect of IL-1 in C3H/HeJ mice, because their thymocytes comitogenic responses to IL-1 are much greater than that of C57BL/6 mice (Neta *et al.*, 1985). In addition, other responses to IL-1 were also reported for this strain (Kampschmidt *et al.*, 1980; Sipe *et al.*, 1979). It was therefore surprising that under experimental conditions similar to those employed with C57BL/6 or DBA/1 mice, IL-1 had no significant radioprotective effect on the survival of lethally irradiated C3H/HeJ mice. The DRF for this strain was 1.01.

This genetically determined difference in response to radioprotection with IL-1, i.e., the existence of responsive C57BL/6 and DBA/1 and nonresponsive C3H/HeJ strains of mice, presents a means of studying the critical events leading to radioprotection. We have, therefore, compared various responses of normal and irradiated C57BL/6 and C3H/HeJ strains such as hematopoiesis and acute phase responses to IL-1.

The efficacy of radioprotectants can also be evaluated using an endogenous stem cell assay which employs sublethally irradiated mice (Till and McCulloch,

TABLE IV

Endogenous CFU-s on Day 9 after 700 cGy Co-60 Irradiation

Treatment (hours)	C57BL/6		C_3H/HeJ		
IL-1	Exp 1	Exp 2	Exp 1	Exp 2	Exp 3
−20	7.0 ± 3.8*	4.3 ± 2.5	8.8 ± 4.2	4.4 ± 3.1	9.3 ± 3.0
−4		3.6 ± 1.3		5.2 ± 3.3	6.6 ± 4.3
+1		3.2 ± 1.8		5.5 ± 3.1	5.0 ± 1.0
Control protein					
−20	1.4 ± 1.7	0.4 ± 0.5	0.2 ± 1.0	0.6 ± 0.5	0.3 ± 0.6
−4		0.8 ± 0.8		0.4 ± 0.6	2.3 ± 2.3
+1		1.0 ± 0.7		2.0 ± 1.0	0.6 ± 1.1
Radiation control	1.8 ± 0.8	1.2 ± 1.0	1.0 ± 1.0	1.2 ± 1.3	1.0 ± 1.0

*The numbers represent mean counts of grossly visible colonies from 5 spleens ± S.D.

1963; Smith *et al.*, 1966b). This method is based on the demonstration that the number of spleen colonies detected 8 to 12 days after irradiation are indicative of the number of surviving stem cells with proliferative capacity: colony-forming units (CFU).

As seen in Table IV, administration of IL-1 to mice of both C57BL/6 and C3H/HeJ strains, followed 20 hr later by sublethal doses (700 cGy) of irradiation, led to enhanced recovery of endogenous splenic colonies at 9 days after irradiation. In addition the recovery of endogenous colonies also was equally enhanced in mice that received IL-1 at 4 hr before or 1 hr after irradiation. This stimulatory effect of IL-1 on hematopoietic endogenous colonies recovery in both C57BL/6 and C3H/HeJ strains suggests that IL-1 can be radioprotective in C3H/HeJ mice sublethally irradiated. In addition a radioprotective effect of IL-1 administration 1 hour after irradiation observed in both strains with radiation with sublethal, but not with lethal doses, indicates that a multitude of radioprotective mechanisms may operate, some of which however do not suffice for animal survival.

In contrast, IL-1 pretreatment of midlethally or lethally irradiated C56BL 6 vs C3H/HeJ mice resulted in a considerable difference in the recovery of nucleated bone marrow cells observed from 9 to 12 days following radiation for doses ranging from 7.50 to 9.50 cGy. For example, the numbers of nucleated bone marrow cells and erythroid colony units (CFU-E) were 10 and 1.5% of normal values, respectively, in C3H HeJ mice. In contrast both were 50% of normal values in IL-1-treated C57BL 6 mice (Neta *et al.*, 1986d). The observation that IL-1 has a similar radioprotective effect in sublethally irradiated C3H HeJ and C57BL 6 mice and that this effect is reduced in C3H HeJ but not C57BL 6 mice

at midlethal or lethal radiation doses suggests the presence of a radiosensitive component affecting hematopoietic cell recovery in C3H/HeJ mice.

Another response to IL-1 that may contribute to radioprotection is induction of increased release of acute phase proteins. IL-1 has been shown *in vivo* and *in vitro* to enhance the induction of a number of acute phase proteins (Ramadori *et al.*, 1985; Dinarello, 1984). Two such proteins, ceruloplasmin (CP) and metallothionein (MT), are metalloproteins which may act as free radical scavengers due to their high cysteine content (Goldstein and Charo, 1983; Karin, 1985). CP has been recognized as a scavenger of oxygen radicals and MT as a putative scavenger of free radicals. Formation of such radicals following irradiation causes extensive tissue damage. Both MT and CP have been reported to be radioprotective (Guttenridge and Stocks, 1981; Thornalley and Vajak, 1985). CP has been previously shown to be induced by IL-1 *in vivo* (Pekarek *et al.*, 1972) and the induction of MT gene expression by IL-1 has been recently demonstrated *in vitro* (Karin *et al.*, 1985). We have therefore compared the magnitude of induction of these two acute phase reactants in C3H/HeJ and C57BL/6 mice (Figs. 1 and 2). Both strains responded with a very similar increase in the levels of MT mRNA observed both in the livers and spleens at 6 hr following IL-1 administration. However, basal and the induced levels of MT mRNA are much higher in liver than in spleen. A similar increase in plasma levels of CP was noted in both strains. Since differences were not observed between the two strains in the levels of induction of the two scavenging proteins, we cannot attribute the observed difference in radioprotective effects of IL-1 to these acute phase reactants. Nevertheless, MT and CP may contribute to IL-1-mediated radioprotection if their effectiveness is greater in the C57BL/6 than C3H/HeJ strain, i.e., more free radicals are produced in C3H/HeJ than C57BL/6 mice.

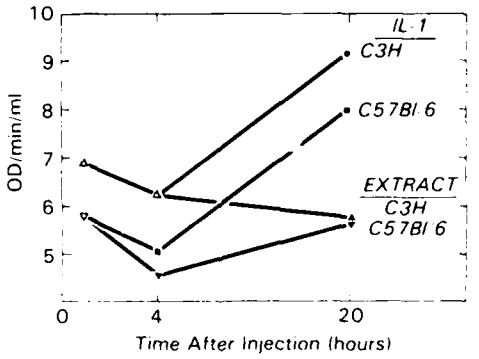

Fig. 1. Ceruloplasmin induction with IL-1. Ceruloplasmin oxidase was measured by monitoring the change in optimal density due to oxidation of the substrate, *o*-dianisidine dihydrochloride. One unit is defined as a change in 1.0 OD unit/min.

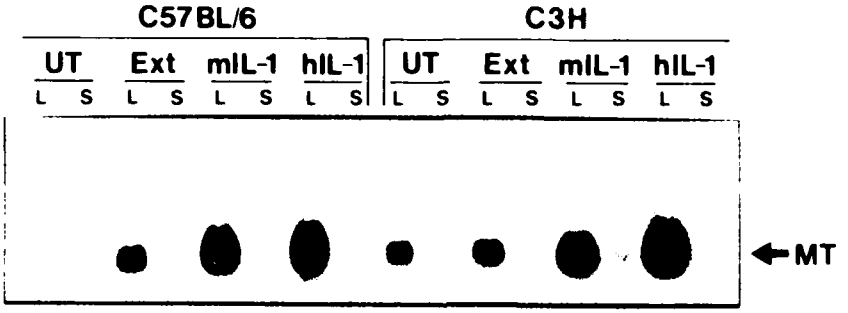

Fig. 2. Induction of MT RNA by IL-1. Total cellular RNA was extracted from livers (L) and spleens (S) of C57BL/6 or C3H/HeJ mice injected with control protein from bacterial extract (Ext), recombinant murine IL-1 (mIL-1), or recombinant human IL-1 (hIL-1). Untreated mice (UT) served as additional control. The hybridization was carried out with 170 Gp *Bam*HI-*Pvu*II human MT-IIA probe.

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THE IN VIVO EFFECTS OF INTERLEUKIN 1

I. Bone Marrow Cells Are Induced to Cycle after Administration of Interleukin 1[1]

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We have previously reported that interleukin 1 (IL-1) administration 20 hr before irradiation protects mice from lethal effects of radiation. The recovery of total nucleated bone marrow cells and of hematopoietic progenitor cells was enhanced in IL-1 treated, as compared to untreated, irradiated mice. This suggested that IL-1 administration may affect the cells in the bone marrow of normal mice. Intraperitoneal administration of recombinant IL-1 resulted in bone marrow cell enlargement and increased cycling of these enlarged cells. In addition, the capacity of bone marrow cells from IL-1 treated mice to proliferate in response to granulocyte macrophage-colony-stimulating factor (GM-CSF) in cell suspension cultures was enhanced. The above effects were not genetically restricted as C57BL/6, B6D2F$_1$, C3H/HeN, and C3H/HeJ mice showed similar responses. A comparative study showed that 100 ng of IL-1 was much more effective in stimulating bone marrow cells by the above criteria than 5 μg GM-CSF. Since IL-1, unlike CSF, can not be demonstrated to have a direct in vitro stimulatory effect on bone marrow cells, the aforementioned in vivo effects of IL-1 are presumably mediated by other hematopoietic growth factors. We have previously shown that IL-1 induces the appearance of high titers of CSF in the serum. Consequently hematopoietic growth factors that are generated at local sites following IL-1 administration may mediate the observed cell cycling effect.

It has been known for over 30 yr that adjuvants which stimulate cells of the reticuloendothelial system (RES), can protect against deleterious effects of radiation. The observation that lipopolysaccharide (LPS) administered before irradiation is radioprotective was first reported in 1953 by Mefferd et al. (1), and was later examined in greater detail by Ainsworth and Chase (2), Perkins et al. (3), and Smith et al. (4). Their studies revealed that the time of hemopoietic recovery was the critical parameter in permitting survival from midlethal doses of radiation. The patterns of hemopoietic recovery in endotoxin treated animals, as measured by increases in bone marrow cellularity, and circulating granulocytes, lymphocytes, and platelets, closely resemble the hematopoietic recovery in animals receiving syngeneic bone marrow transplants. Consequently, the accelerated restoration of functional immunocytes and hematopoietic cells to normal levels in endotoxin treated mice is believed to be a major factor in the survival of lethally irradiated mice.

The proliferation and differentiation of cells of the hemopoietic system depends directly on growth and differentiation factors which are produced endogenously in response to immunomodulatory stimuli (5). The hypothesis that the radioprotective effects of adjuvants such as LPS is mediated by the production of endogenous cytokines has been evaluated. Direct administration of one such substance, interleukin 1 (IL-1)[2] prior to lethal doses of radiation, has recently been demonstrated to have a radioprotective effect similar to that of immunomodulators (6).

Mice given IL-1 20 hr before lethal irradiation, by 5 days after irradiation have greatly enhanced numbers of nucleated bone marrow cells when compared to untreated irradiated mice (7). Similarly, the number of endogenous splenic colonies (CFU-s) was greatly enhanced in IL-1 treated irradiated mice (8).

These observations suggested that IL-1 administration may stimulate bone marrow cells. To further evaluate the above phenomenon, we investigated the effect of IL-1 administration on normal murine bone marrow cells. Our evaluation included assessing the effect of IL-1 on bone marrow cell size, cell cycling, and on the generation of granulocyte macrophage-colony-stimulating factor (GM-CSF) responsive bone marrow cells. We chose to examine changes in the myeloid cell lineage because of the importance of these cells in controlling infections, a primary cause of death in radiation-induced hematopoietic syndrome. The effects of the cytokines were evaluated in four different strains of mice to ensure that the

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[2] Abbreviations used in this paper: IL-1, interleukin 1; BFU-E, burst forming units-erythroid; GM-CFU, granulocyte macrophage colony-forming units; GM-CSF, granulocyte macrophage-colony-stimulating factor; HU, hydroxyurea.

phenomenon was not genetically restricted. The results of these experiments show that within 20 hr IL-1 administration leads to an enhanced proportion of bone marrow cells entering the cell cycle. The effect on bone marrow cell cycling of IL-1 and GM-CSF was compared. We observed that use of 100 ng IL-1 was much more effective than 5 μg GM-CSF.

MATERIALS AND METHODS

Mice. Inbred strains of mice, C57BL/6J, C3H/HeJ, and B6D2F$_1$ were obtained from The Jackson Laboratory, Bar Harbor, ME. C3H/HeN mice were also used (Animal Genetics and Production Branch, NCI, Frederick, MD). The mice were housed in the Veterinary Department Facility at the Armed Forces Radiobiology Research Institute in cages of 10 to 12 mice with filter lids. Female mice, 8 to 12 weeks of age were used for all experiments. Standard laboratory chow and HCl acidified water (pH 2.4) were given ad libitum. All cage cleaning procecures and injections were carried out in a microisolator.

Lymphokines. Purified human recombinant IL-1 (specific activity 7.5×10^8 U/mg protein) as assessed by the co-mitogenic effect in the thymocyte proliferation assay (9) and murine recombinant GM-CSF supplied in sucrose (lot 344-061-47 specific activity of 4×10^7 U/mg protein) were obtained from Immunex Corp., Seattle, WA. Both lymphokines were diluted in 0.5% bovine serum albumin in normal saline (sterile-filtered) to a concentration of 10 μg/ml and 100 μg/ml, respectively, and stored at −20°C until just before i.p. injection. At that time, the lymphokines were diluted in pyrogen-free saline and administered at doses of 100 ng/0.5 ml per mouse for IL-1 and 5 μg/0.5 ml per mouse for GM-CSF. All preparations contained less than 0.06 μg of LPS per injection as assessed by the Limulus amebocyte assay.

Treatment schedule. IL-1 or GM-CSF were administered to three or four mice per experimental group which were sacrificed by cervical dislocation. In some experiments hydroxyurea (HU) was injected (900 mg/kg body weight i.p.) 2 hr before sacrifice of the mice.

Recovery of bone marrow cells. Femurs were removed and placed on ice in Hanks' balanced salt solution (HBSS) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (GIBCO, Grand Island, NY). Single cell suspensions were prepared by washing each cavity of the femur with 3 ml of HBSS with a sterile syringe and 26-gauge needle. Cell counts were obtained using a hemocytometer. Viability, as assessed by trypan blue exclusion method, was always found to be >95%. Cytospins were prepared on a Shannon Cytospin II (250 × G, 6 min) using 10^5 cells and 0.1 ml fetal calf serum per slide. They were stained with a modified Wright's Giemsa stain (Diff-Quick, ASP, McGaw Park, IL), and evaluated by light microscopy.

In vitro bone marrow cell proliferation assay. GM-CSF was diluted in complete media containing RPMI 1640, 10% fetal calf serum (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine. GM-CSF was added to 96-well microtiter plates (Falcon) in 0.1 ml volumes so that the final concentrations were 10, 1, and 0.1 ng/ml. Next, the bone marrow cells were added in 0.1 ml volumes at concentrations of 1×10^5 and 5×10^4 cells/well. The cultures were incubated for 2 days at 37°C with 5% CO_2 in air. At this time, the cells were pulsed with 1 μCi [^{3}H] thymidine per well and harvested 18 hr later (Skatron Cell Harvester, Sterling, VA) onto glass fiber filters which were then counted in Beta-Count scintillation fluid on a Mark'III Scintillation Counter.

Cell sizing procedure. The bone marrow cells were resuspended at a 1:100 dilution of the original cell concentration in Isoton II, an isotonic solution specific for the Coulter Sizing system. Cell profiles were obtained at several cell concentrations (ranging from 1:500 to 1:10), with the use of different media and at different time points (1, 2, or 4 hr after recovery) with similar results.

The cells were collected by the Coulter Sampling Stand equipped with a manometer. Since the manufacturer recommended that the aperture is most efficient when the sized cells are 2 to 20% in diameter of the aperture size and since the bone marrow cells range in diameter from 1 to 10 μm (10), a manometer with 70 μm diameter aperture was utilized. The relevant Coulter ZM settings were amplification −8 and current −100. For the Coulter Channelyzer C-1000, the base channel threshold was set at 15 to exclude red blood cells and the count range switch was set at 4K. (Similar results were obtained at 1K and 10K.) These settings were determined after calibrations of the manometer with 10-μm beads. Using this calibration procedure and a shape correction factor of 1.38 for lymphocytes, the size or the threshold factor of each channel was found to be 5.39 μm and the range of volumes analyzed was 80.87 to 620 μm.

After a sample of cells was sized, the frequency distribution as determined by the Channelyzer was stored and analyzed using the Accucomp software package and an Apple IIe personal computer.

Flow cytometric analysis of DNA content and cell size. Flow cytometric analysis of DNA content was performed using the propidium iodide (PI) staining technique as previously described (11). Briefly, bone marrow cells obtained from 2 to 4 mice per group (approximately 2×10^6 cells) were fixed in 70% (v/v) ethanol and kept at 4°C overnight. After centrifugation, cells were resuspended in 1 ml of a mixture containing 40 μg/ml ribonuclease A (Sigma, St Louis, MO) and 18 μg/ml PI (Calbiochem, La Jolla, CA) and were incubated for 20 min at room temperature. The cell size distribution (as determined by forward light scattering) and their cycle position (as determined by PI fluorescence) were analyzed on a FACS IV flow cytometer (Becton Dickinson, Sunnyvale, CA). In selected experiments, cell size distributions, as determined by forward light scattering, were performed on freshly obtained bone marrow cell suspensions.

Statistical analysis of the results were performed using the Mann Whitney and Wilcoxon Sign Ranks tests.

RESULTS

Effect of IL-1 on bone marrow sizing profile. Our preliminary examination revealed that by 20 hr after administration of IL-1 the total nucleated cell numbers in the bone marrow were not different from the control numbers. Therefore we looked for a qualitative rather than quantitative change in bone marrow cells. For this reason changes in the overall size distribution of the entire nucleated bone marrow cell population were examined. Figure 1 depicts a representative experiment showing that IL-1 treatment of mice results in an enlargement in bone marrow cell size. Figure 2 presents a summary of 20 experiments in which four different mouse

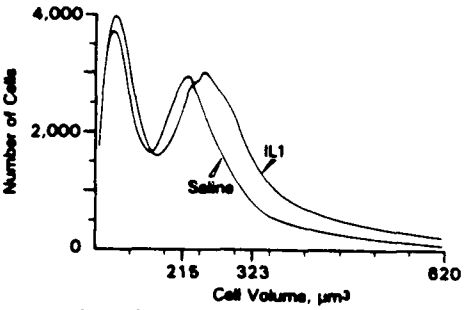

Figure 1. The effect of IL-1 on the size of bone marrow cells. The size of bone marrow cells from C57BL/6 mice treated with IL-1 or saline 20 hr before cell harvest was assessed using a Coulter Channalyzer as described in *Materials and Methods*. The data show a representative experiment of nine experiments performed with this mouse strain.

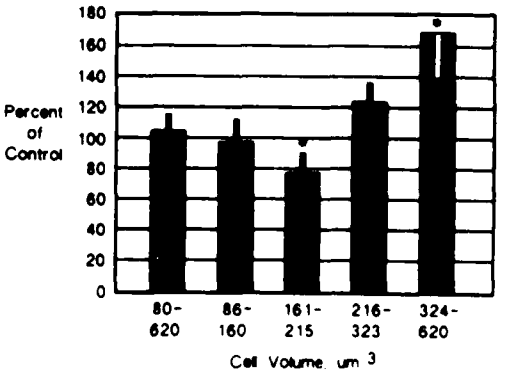

Figure 2. Effect of IL-1 administration on bone marrow cell size distribution. Bone marrow cells from C57BL/6, B6D2F$_1$, C3H/HeN, and C3H HeJ mice treated with IL-1 or saline 20 hr before cell harvest were sized using a Coulter Channalyzer as described in *Materials and Methods*. The mean percent of control cell volume ± SEM for different cell size compartments of 20 separate experiments is depicted. * $p < 0.01$ compared to control

strains were assessed in this manner. Since bone marrow from all four mouse strains behaved similarly, the data was pooled. The major changes observed in bone marrow cell populations following IL-1 administration include a significant reduction ($p < 0.01$) in the number of cells that range from 160 to 215 μm^3 in volume and a significant increase ($p < 0.01$) in the number of cells ranging from 215 to 620 μm^3 in volume. These data were confirmed by forward light scattering analysis of fresh and ethanol-fixed bone marrow cells using flow cytometry. In five separate experiments, treatment with IL-1 resulted in an average increase of 25.3 ± 4.1% in the numbers of large cells in the bone marrow. Many of the larger cells were determined by light microscopy of cytospin preparations to be blast cells. Therefore, 20 hr after the administration of IL-1 there was an increase in the size of bone marrow cells in all four strains of mice. Consequently, the effect of IL-1 was not genetically restricted.

Relationship of cell cycling to bone marrow cell size following IL-1 administration. To examine the basis for enhanced numbers of large cells in the bone marrow, the effect of HU was tested in IL-1 treated and control, saline treated mice. This cytotoxic drug has been established to be stage specific in that it is lethal to cells in the S phase and arrests cells in the late G_1 phase of the cell cycle (12, 13). Following HU treatment of IL-1 treated and saline treated control mice, bone marrow cells were analyzed on the basis of their size to determine which of the cell fractions were most affected by such treatment. Figure 3, *A* and *B*, presents representative experiments showing that HU treatment resulted in a significant reduction in the bone marrow populations of medium and large cells in IL-1 treated mice, but had no effect on the size distribution of cells of saline treated mice. Figure 4 presents a summary of 20 experiments using four different mouse strains which behaved similarly. The percent decrease in bone marrow cell size from IL-1 and HU treated mice,

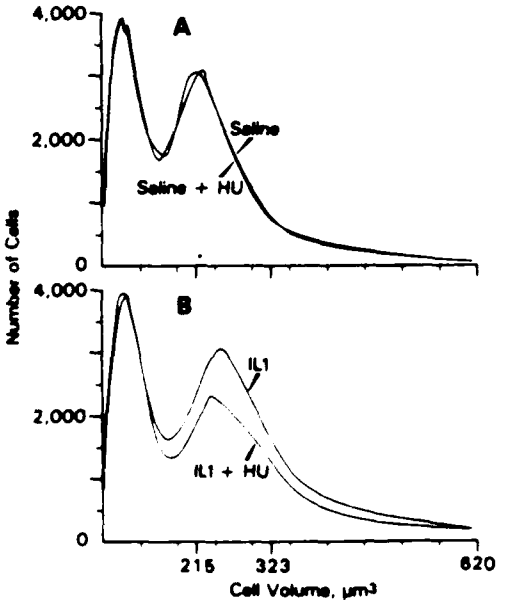

Figure 3. Effect of IL-1 with and without HU on bone marrow cell size distribution. Bone marrow cells from C57BL/6 mice treated with saline (*A*) or IL-1 (*B*) 20 hr before cell harvest, were sized with a Coulter Channalyzer. Three C57BL/6 mice were used in each group. This experimental result is representative of nine similar experiments performed with this mouse strain.

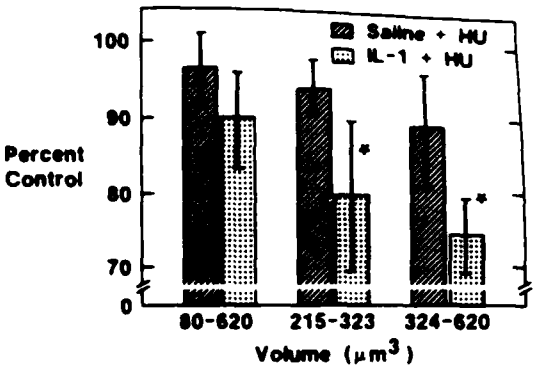

Figure 4. Effect of administration of HU on bone marrow cell size. Bone marrow cells from C57BL/6, B6D2F$_1$, C3H/HeN and C3H/HeJ mice 20 hr after saline or IL-1 administration and 2 hr after saline or HU administration were sized using a Coulter Channelyzer. The results are expressed as percent change in cell numbers in different compartments following HU treatment of IL-1 treated mice in comparison to mice treated only with IL-1. The effect of HU treatment of saline treated mice was compared to mice given only saline. The mean ± SEM of 20 experiments is depicted (three to four mice were utilized in each group). * $p < 0.01$.

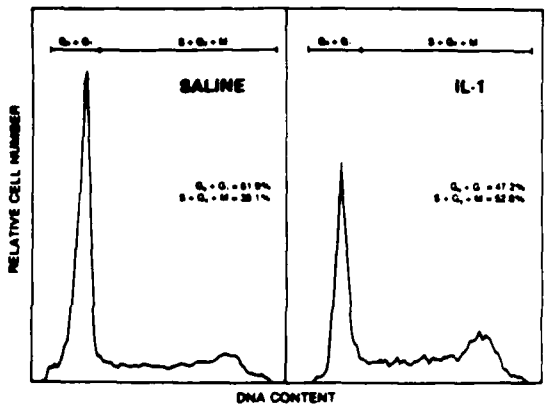

Figure 5. Cell cycle analysis of bone marrow cells obtained from C57BL/6 mice treated with saline or 100 ng IL-1. Bone marrow cells obtained from mice treated with IL-1 or saline (control group) were fixed, stained with propidium iodide and the cell cycle profile determined by flow cytometric analysis as described in *Materials and Methods*. Each histogram is based on the DNA analysis of 15×10^3 large bone marrow cells. The experiment is representative of five studies performed.

compared to bone marrow of mice given only IL-1, was greatest for populations of cells ranging from 323 to 620 μm^3 in volume ($p < 0.01$). The proportions of smaller cells were not affected by HU treatment. The fact that HU treatment results in greater reduction in cell numbers in IL-1 treated than control bone marrow cells lends support to the hypothesis that more cells were cycling in IL-1 treated than in control mice.

In order to directly examine the effect of IL-1 on cell cycling we analyzed the cell cycle distribution of bone marrow cell populations after treatment with IL-1 or saline (control group). Large and small cell populations were independently analyzed for DNA content by thresholding based on forward light scattering. Significant differences between IL-1 or saline-treated groups were not observed in the small cell populations (data not shown). However, there was a significant increase in the percent of large cells in the S + G_2 + M phases of the cell cycle in the IL-1-treated as compared to the control group. A representative experiment is presented in Figure 5. Similar results were observed in four additional experiments. These results clearly indicate that 100 ng of IL-1 increased the number of bone marrow cells going through

the cell cycle. This effect of IL-1 was dose dependent, since a 1 ng dose did not change the proportion of large cells in cycle, 10 ng increased their cycling only in one of two experiments by 10%, and 100 ng increased their cycling by 14 ± 2%.

Effect of IL-1 on the generation of GM-CSF responsive bone marrow cells. In view of the finding that IL-1 leads to enlargement and cycling of bone marrow cells, we next examined the capacity of these cells to respond in culture to a growth factor GM-CSF. We chose to study GM-CSF in order to obtain more information on the effect of in vivo IL-1 treatment on the GM-CSF responsive myeloid lineage. GM-CSF is known to stimulate the proliferation of macrophage granulocyte progenitor cells (granulocyte macrophage-colony-forming units: GM-CFU) in a semi-solid agar colony assay and in suspension cultures (14, 15). The proliferation by bone marrow cells in response to CSF in a suspension culture was reported to be directly proportional to the increase in units of colony stimulating activity (14). We assessed the proliferative response of bone marrow cell suspensions cultured for 72 hr in the presence of GM-CSF. The culture of 1.5×10^7 cells with either 10 ng/ml, 1 ng/ml of GM-CSF, or with media alone, resulted in the presence of $(3.2 \pm 0.1) \times 10^7$, $(1.8 \pm 0.1) \times 10^7$, or $(6.6 \pm 0.6) \times 10^6$ viable cells, respectively. The cells grown with GM-CSF had much greater proportion of large cells. Whereas in control cultures 5% of cells ranged in size from 320 to 620 μm^3, in cultures with 1 ng of GM-CSF 40% and with 10 ng GM-CSF 50% of cells were in this range. Furthermore, cytospin preparations from such cultures has shown that control populations consisted of 65% of polymorphonuclear (PMN) cells and 20% macrophages, and GM-CSF containing cultures consisted of 35% PMN cells and 60% macrophages.

Administration of IL-1 in increasing doses of: 1, 10, and 100 ng/mouse resulted in a gradual increase in the proportion of GM-CSF responsive cells obtained from such marrows. Thus, thymidine uptake in response to GM-CSF of 1×10^5 cells/well from mice receiving 1 ng/mouse of IL-1 was 101 ± 18% that of the controls, 10 ng—125 ± 37%, and 100 ng—188 ± 36% that of the control cells (mean ± SD, results of two experiments). The results in Table I indicate that the proportion of GM-CSF responsive cells present in bone marrow following IL-1 administration was estimated to be double that in the bone marrow of saline-treated mice. This estimate was based on the similar thymidine uptake obtained using 5×10^4 bone marrow cells from IL-1 treated mice, and 1×10^5 cells from saline treated control mice at all doses of GM-CSF used (Table I). In contrast, addition of IL-1, alone or in combination with GM-CSF directly to bone marrow cell cultures, did not result in proliferation or in enhanced proliferation of these cells (data not shown). Consequently, although IL-1 can not be shown to have a direct effect on bone marrow cells in vitro, in vivo treatment with IL-1 can be shown to enhance the subsequent response of cultured bone marrow cells to GM-CSF.

IL-1 induces GM-CSF responsive cells to cycle. To examine the basis for the enhanced numbers of GM-CSF responsive cells, the effect of HU was tested in IL-1 treated and control, saline treated mice. Figure 6 presents a summary of 15 experiments which were performed to test bone marrow cells from four different strains of mice, C57BL/6, B6D2F$_1$, C3H/HeN, and C3H/HeJ. Because similar responses were obtained with cells of different mouse strains, all the results were pooled. Bone marrow cells from mice treated with IL-1 had significantly higher ($p < 0.003$) proliferative responses to GM-CSF in culture than cells from control mice. Treatment of mice with HU resulted in significantly greater ($p < 0.001$) reduction in GM-CSF responsive bone marrow cells from IL-1 treated than from saline treated mice in all four strains examined. These results support the conclusion that administration of IL-1 induces a greater proportion of GM-CSF responsive cells to enter the replicative S phase of the cell cycle.

Comparison of in vivo effects of IL-1 and GM-CSF on bone marrow cells. In vivo administration of GM-CSF to mice was recently reported to result in increased cycling of GM-CFU and of other progenitor cells (16–18). This effect was best observed when mice were pretreated with lactoferrin, an agent that renders progenitor cells more sensitive to GM-CSF (16). We compared the effect of IL-1 with GM-CSF on bone marrow generation of GM-CSF responsive cells, cell size and cell cycle. Figure 7 summarizes seven experiments in which the effect of GM-CSF administered intraperitoneally to normal C57BL/6 mice in doses of 5 μg was compared to the effect of 100 ng of IL-1. Administration of IL-1 had a much greater stimulatory effect than GM-CSF on both the induction of GM-CSF responsive cells ($p < 0.01$) and on their cycling ($p < 0.001$). The proportion of large cells in the bone marrow was not significantly increased following GM-CSF administration, and was considerably less than the cell enlargement induced by IL-1 (results not shown). Similarly, the inhibitory effect of HU treatment on bone marrow cells was less following GM-CSF administration than following IL-1 administration. There was no difference in the proportion of large bone marrow cells recovered from control or GM-CSF treated mice subsequently exposed to HU. Therefore, administration of IL-1 in doses 50-fold lower on a weight basis than GM-CSF resulted in much greater induction of bone marrow cell cycling.

TABLE I

Effect of treatment with IL-1 on bone marrow cell proliferation in response to GM-CSF

Treatment of Mice	Cells/Well	Response (cpm)*			
		Control	GM-CSF		
			0.1 ng	1.0 ng	10 ng
IL-1	1×10^5	869 ± 109	3,674 ± 256	18,500 ± 1,594	33,360 ± 2,131
	5×10^4	556 ± 70	3,326 ± 699	11,212 ± 261	20,456 ± 1,195
Saline	1×10^5	662 ± 98	3,272 ± 243	12,868 ± 728	19,852 ± 1,911
	5×10^4	534 ± 89	3,009 ± 383	8,715 ± 593	13,611 ± 1,145

* Representative of 15 experiments

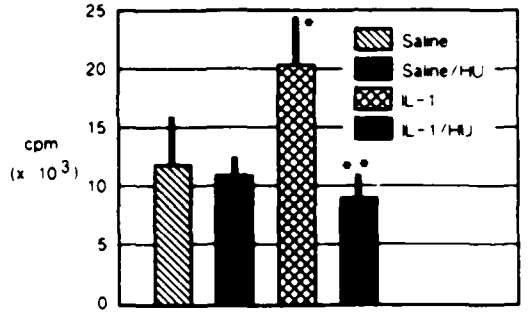

Figure 6. The effect of administration of IL-1 and HU to C57BL/6, B6D2F$_1$, C3H/HeN, and C3H/HeJ mice on the proliferative responses of bone marrow cells to GM-CSF. Groups of six mice were given 100 ng of IL-1 or saline intraperitoneally. Two hours prior to obtaining bone marrow cells half of the mice in each group (three mice/group) were given 900 mg/kg HU or saline intraperitoneally. The cells from every group were cultured for 72 hr with GM-CSF as described in *Materials and Methods*. The results represent the averages of the mean of triplicate cpm of [^{3}H]TdR incorporation derived from 15 experiments ± SEM. * $p < 0.003$—in comparison with saline treated group. ** $p < 0.001$—in comparison with IL-1 treated group.

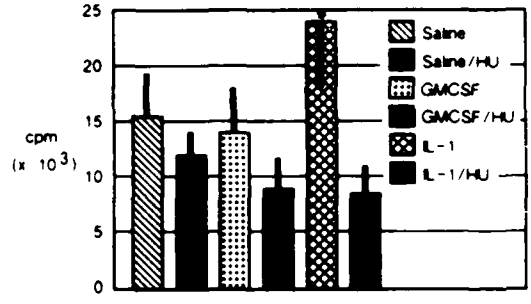

Figure 7. Comparison of the effect of GM-CSF and IL-1 with and without HU treatment on the proliferation of bone marrow cells. Bone marrow cells of C57BL/6 mice (obtained 20 hr after ip administration of saline, 5 μg GM-CSF or 100 ng of IL-1 and 2 hr after HU or saline administration) were cultured with increasing concentrations of GM-CSF (as presented in Table I). The results represent the mean of triplicate counts of [^{3}H]TdR incorporation of seven separate experiments. * $p < 0.01$ compared to GM-CSF treatment; ** $p < 0.01$ compared to GM-CSF vs GM-CSF/HU treatment.

DISCUSSION

IL-1 has been recognized to be a cytokine with multiple in vivo and in vitro effects (19–21). It has stimulatory effects on a variety of cells. For example, IL-1 was documented to induce T cells to progress from G_0 into the G_1 phase of the cell cycle (22). However, before the present report, the effect of IL-1 on bone marrow cells has not been evaluated. This lack of interest may be based on previous negative findings, including our own, showing that IL-1 alone did not promote the in vitro growth of bone marrow cells (see *Results*) or of GM-CFU (23). Furthermore, as an endogenous pyrogen and mediator of the inflammatory response, IL-1 has been thought of as a noxious rather than a beneficial cytokine. Lately, however, IL-1 has been implicated as a differentiation and maturation-inducing agent for a variety of cells (24). Furthermore, IL-1 has been proposed to participate in wound healing (25) and to increase protection from infections (26). Our own studies demonstrated IL-1 to be a radioprotector (6–8). In addition, the enhanced levels of IL-1 detected in amniotic fluid (27), in the circulation after exercise (28), and postovulation (29), suggest that this cytokine may play a constructive role in normal function, as well as in the development of host defenses.

In the present study administration of IL-1 to mice has several dramatic effects on bone marrow cells. The shifts in cell sizing and light scattering profiles (Figs. 1 and 2) observed at 20 hr after administration of IL-1 are probably the net result of at least two competing events: an early mobilization of neutrophils and other cells out of the marrow compartment (30) and of bone marrow cell proliferation. The increase in the proportion of large bone marrow cells can be shown to be due to the induction of blastogenesis and proliferation of cells remaining in the bone marrow following IL-1 administration. The evidence that IL-1 stimulates bone marrow cells is based on our data showing an increase in GM-CSF responsive cells in the bone marrow (Table I and Figs. 6 and 7), increases in the proportion of large bone marrow cells as measured by Coulter Volume Channelyzer and by the light scattering (Figs. 1 and 2), increased sensitivity to HU treatment (Figs. 3, *A* and *B*, 4, 6, and 7) and increases in proportion of cells in S and G_2 + M phases of cell cycle (Fig. 5).

The late S phase of the cell cycle was reported in numerous studies to be the most radioresistant phase of the cell cycle (31, 32). Therefore, our finding of enhanced cell cycling with IL-1 suggests that the radioprotective effect of this cytokine may be related to the induction of larger numbers of bone marrow cells into the radioresistant late S phase. This was substantiated using HU, an agent that is selectively lethal to cells in the S phase of the cell cycle (12, 13). Treatment with HU depleted a greater proportion of bone marrow cells in IL-1 treated than in saline treated mice (Figs. 3, *A* and *B*, 4, 6, and 7). This provides further experimental support for the view that IL-1 induces BM cells to cycle. We have previously observed that GM-CSF, unlike IL-1, has no radioprotective effects (7). In this study we observed that GM-CSF, in the doses used, unlike IL-1, had negligible effects on bone marrow cell cycling. This contrasts with reports that GM-CSF, provided it is given in conjunction with lactoferrin, has marked stimulatory effects on bone marrow cell cycling (16).

Although these results describe the relative effects on bone marrow of the administration of the two above cytokines, IL-1 and GM-CSF, conclusions as to the mechanisms, i.e., direct effect of IL-1 as a cycling signal, can not be drawn from these in vivo experiments. In fact we have observed that following intraperitoneal administration of a single dose of recombinant IL-1, high titers, 2 to 3×10^7 U per ml of CSF, appeared in the circulation within 2 hours and persisted for up to 6 hours (23). In all likelihood these titers are higher than levels of CSF achieved following intraperitoneal administration of a single 20,000 unit dose of GM-CSF. Furthermore, additional evidence exists that IL-1 can stimulate CSF production by stromal cells (33). Similarly, fibroblasts stimulated with IL-1 release supernatant factors that support CFU-GM, burst-forming units-erythroid (BFU-E), and CFU-GEMM colony formation (34). Treatment of the same IL-1 stimulated fibroblasts cultures with anti-IL-1 inhibited the release of such stimulatory activities. It is therefore probable that in addition to CSF, IL-1 may stimulate the release of other hematopoietic growth factors. IL-1 treatment may result in CSF being present in greater abundance at the appropriate local sites and is therefore, more effective in inducing bone marrow cells to cycle and proliferate than exogenously administered GM-CSF.

Although the existence of separate specific growth factors for different lineages of hematopoietic progenitor cells is well documented, administration of each of these agents in vivo stimulated a broader than anticipated spectrum of progenitor cells. For example, high doses of GM-CSF stimulated CFU-GEMM, CFU-E, and BFU-E as measured by colony formation in addition to the expected myelogenous cells (16–18). Therefore, these in vivo findings suggest that a cascade of effects may be generated in vivo to permit stimulation of cells not responsive under in vitro culture conditions. The most recent observation that B cell-stimulating factor (BSF), a molecule which stimulates B cell growth, also acts synergistically with the hematopoietic growth factors IL-3, erythropoietin, and GM-CSF (35) presents another possible mechanism operating in vivo. Thus, interactions between any of these different mediators may be required for induction of hematopoiesis.

Our observation that single 100 ng dose of IL-1 is more effective than 5 μg GM-CSF in induction of bone marrow cell cycling and cell proliferation may prove to be of clinial significance. Pyrogenicity of IL-1 probably may be separated from other systemic effects such as radioprotection. CSF and fibrinogen release since treatment with indomethacin did not diminish these three aforementioned responses (8) (R. Neta and S. N. Vogel, unpublished results). Administration of IL-1 in doses higher than 100 ng/mouse did not produce toxic effects. For example, increasing doses of IL-1 up to 1 μg/mouse did not reduce its radioprotective effect (R. Neta, unpublished results). Similarly, doses of up to 30 μg/mouse did not result in measurable toxicity (36). Therefore, IL-1 may provide an effective agent for treatment of immune and hematopoietic dysfunction.

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Glucan:
Mechanisms Involved in Its "Radioprotective" Effect

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It has generally been accepted that most biologically derived agents that are radioprotective in the hemopoietic-syndrome dose range (eg, endotoxin, Bacillus Calmette Guerin, Corynebacterium parvum, etc) exert their beneficial properties by enhancing hemopoietic recovery and hence, by regenerating the host's ability to resist life-threatening opportunistic infections. However, using glucan as a hemopoietic stimulant/radioprotectant, we have demonstrated that host resistance to opportunistic infection is enhanced in these mice even prior to the detection of significant hemopoietic regeneration. This early enhanced resistance to microbial invasion in glucan-treated irradiated mice could be correlated with enhanced and/or prolonged macrophage (but not granulocyte) function. These results suggest that early after irradiation glucan may mediate its radioprotection by enhancing resistance to microbial invasion via mechanisms not necessarily predicated on hemopoietic recovery. In addition, preliminary evidence suggests that glucan can also function as an effective free-radical scavenger. Because macrophages have been shown to selectively phagocytize and sequester glucan, the possibility that these specific cells may be protected by virtue of glucan's scavenging ability is also suggested.

Key words: radioprotection, macrophages, hemopoiesis, free-radical scavengers

INTRODUCTION

The exposure of mammals to a single whole-body dose of ionizing radiation results in a complex set of symptoms whose onset, nature, and severity are a function of both total radiation dose and radiation quality [3,10,17]. In general, radiation injury can be classified into three syndromes which become evident at progressively higher radiation doses: the hemopoietic syndrome, the gastrointestinal syndrome, and the central nervous system syndrome [3,10,17].

The hemopoietic syndrome occurs at the lowest radiation doses (< 10 Gy) and is manifest by hemopoietic stem cell depletion [8,10,58] and ultimately by depletion of mature hemopoietic and immune cells [2,7,26,29, 42,52], which (whether destroyed directly by the radiation insult or lost naturally through attrition) cannot be regenerated without hemopoietic stem cells. In turn, the loss of mature hemopoietic and immune cells severely impairs antimicrobial immunity, and ultimately death ensues owing to invasive opportunistic infections [5,22,33,53].

Most biological agents previously shown to be specifically radioprotective in the hemopoietic-syndrome dose range (eg, endotoxin, Bacillus Calmette Guerin, *Corynebacterium parvum*, etc), have also been shown to be hemopoietic stimulants [20,31,32,50]. Thus, it has generally been assumed that the "radioprotection" afforded by these agents results from enhanced hemopoietic recovery and, subsequently, from enhanced resistance to microbial invasion. During the past several years we have evaluated the radioprotective properties of glucan, a *Saccharomyces*-derived immunostimulant [14,23], and have shown that it also enhances survival in mice exposed to radiation in the hemopoietic-syndrome dose range [37,39,40]. As with other agents that are radioprotective in this dose range, glucan was shown to significantly accelerate hemopoietic recovery in sublethally irradiated (6.5 Gy) mice [38,39,41]. Specifically, pluripotent hemopoietic stem cells (CFU-s), granulocyte-macrophage progenitor cells (GM-CFC), pure macrophage progenitor cells (M-CFC), erythroid progenitor cells (CFU-e, BFU-e), and peripheral blood cells were all shown to recover more rapidly in glucan-treated mice than in radiation controls. Hemopoietic regeneration also ultimately oc-

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curred in lethally irradiated (9 Gy) glucan-treated mice (this report). However, since at day 15 postirradiation (when most radiation control mice had already died) hemopoietic elements in glucan-treated mice had recovered to less than 5% of normal levels, it appeared that hemopoietic recovery alone was unlikely to account for glucan's ability to radioprotect animals in the first weeks after radiation exposure. Thus, the possibility that early postirradiation glucan may enhance survival by enhancing and/or prolonging the function and/or survival of already existing cell populations important in host defense against microbial invasion (ie, granulocytes and macrophages) was also evaluated.

MATERIALS AND METHODS

Mice

In all experiments, 20- to 25-g C3H/HeN female mice were used. Approximately 3% of all mice entering the AFRRI animal facility were sacrificed for representative quality-control histopathology, bacteriology, and serological viral pathogen screens. While waiting for these results, the remaining mice were quarantined and acclimated to the 6:00 A.M. to 6:00 P.M. light cycle, Wayne Lab Blox rodent chow, and acidified (pH 2.5) water used in the animal facility. Only shipments of healthy mice were released for experimentation.

Glucan

Particulate endotoxin-free glucan, prepared as described by DiLuzio et al [14], was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY). Based on nuclear magnetic resonance, this glucan preparation was reported to be 99.9% chemically pure (N.R. DiLuzio, personal communication), and consisted of 1–3-μm glucan particles suspended in sterile saline. As indicated in the specific experiments, 1.5 mg of glucan (in a 0.5-ml volume) was administered either intravenously (i.v.) via the lateral tail veins, or intraperitoneally (i.p.). Control mice were injected with an equivalent volume of sterile saline or, where specified, a 1.5-mg dose of D-glucose (Sigma Chemical Co., St. Louis, MO) dissolved in sterile saline.

Irradiation

All mice were bilaterally exposed to total-body irradiation administered from the AFRRI cobalt-60 gamma-ray source at a dose rate of 0.4 Gy (1 Gy = 100 rads) per minute. Samples for in vitro free-radical scavenging experiments were exposed to irradiation administered from the same source at a dose rate of ~40 Gy/min. Dosimetry was determined by ionization chambers [49], and all irradiations were performed at room temperature.

Peritoneal Exudate Cell Collection and Macrophage Isolation

Mice were anesthetized with halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) and injected i.p. with 5 ml of calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing 2 units/ml of preservative-free heparin (Abbott Laboratories, North Chicago, IL). Following gentle massage, the peritoneal lavage fluid was withdrawn through a 20-gauge needle inserted into the cavity, and the cell content of the lavage fluid was determined by counting the cells on a hemocytometer. For macrophage cell isolation, the peritoneal cells were cultured in 35-mm petri dishes (Corning, Corning, NY) in Dulbecco's modified essential medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at a concentration of 2.5×10^6 cells/ml per dish. Macrophages were allowed to attach for 2 h at 37°C in 5% CO_2. The plates were then washed three times with DMEM to remove the nonadherent cells, and the adherent macrophages used as described for the 5'nucleotidase assay.

Survival Assays

Mice used in survival studies were exposed to 9 Gy of total-body irradiation, and their survival was checked daily for 30 days. Statistical differences between control and glucan-treated mice were determined by the Kruskal Wallis test.

Hemopoietic Cell Assays

The hemopoietic assays used have been described in detail elsewhere [39]. Pluripotent hemopoietic stem cells were evaluated by the spleen colony (CFU-s) assay using 9-Gy irradiated mice as transplant recipients [54]. Twelve days after transplantation, the recipients were sacrificed and their spleens removed. The spleens were fixed in Bouin's solution, and the number of grossly visible colonies were counted. Committed granulocyte-macrophage hemopoietic progenitor cells (GM-CFC) were assayed by a modification [31] of the semisolid agar technique originally described by Bradley and Metcalf [9] and Pluznik and Sachs [43]. Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 7.5% CO_2. The cell suspensions used for these assays represented the pool of tissues from 3–12 normal, irradiated, or glucan-treated irradiated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A Medium (MC5A) containing 5% heat-inactivated fetal bovine serum (HIFBS). Spleens were pressed through a stainless steel mesh screen, and the cells were washed from the screen with 6 ml of MC5A plus 5% HIFBS. The total number of nucleated

cells in each suspension was determined by counting the cells on a hemocytometer.

Bacteriological Assays

To evaluate the occurrence of opportunistic infections in irradiated mice, the liver, spleen, and peripheral blood of animals were monitored for bacterial translocation. Individual mice were halothane-anesthetized, and ~0.5 ml of blood was removed by cardiac puncture. A 0.1-ml sample of blood was then streaked onto two enriched BHI agar plates (DIFCO, Detroit, MI). The liver and spleen of these mice were also aseptically removed, homogenated, and streaked onto two BHI plates. One plate of each tissue was incubated for 48 hours at 37°C and 5% CO_2 to isolate aerobic bacteria. The other plate was incubated for 96 h in an anaerobic jar to isolate anaerobic bacteria. Microorganisms were identified by means of standard criteria [30,51].

Granulocyte Chemiluminescence Assay

Oxidative burst activity was used as an indicator of peripheral blood granulocyte function [27,47,48] and was measured by chemiluminescence [1,28]. To perform these studies, mice were halothane-anesthetized and ~0.5 ml of blood was removed by cardiac puncture and immediately mixed with 1 drop (1 unit) of preservative-free heparin. Twenty microliters of each blood sample was mixed with 200 μl of opsonized zymosan particles suspended in luminol (ZAP, Packard Instruments, Chicago, IL) and light emissions were counted immediately and at 5-min intervals over a 45-min period with the aid of a PICOLITE 6112 Luminometer (Packard Instruments, Chicago, IL). Total white cell counts (Unopettes, Becton Dickinson, Rutherford, NJ) and differential white cell counts were also performed on each blood sample.

Reticuloendothelial-System Carbon Clearance Assay

The rate of removal of colloidal carbon from the circulation was used to measure the phagocytic activity of various macrophage populations comprising the reticuloendothelial system (RES) [24]. To perform this assay, individual mice were injected i.v. with 0.25 ml of diluted colloidal carbon (~160 mg/kg) (C11/143a, Gunther Wagner, Hanover, West Germany), and its removal from the circulation was measured. At 1, 2, 4, 6, 8, and 10 min after carbon injection, ~0.2 ml of blood was removed from halothane-anesthetized mice by cardiac puncture, and 50 μl was immediately dispersed into 4 ml of 0.1% sodium carbonate solution. At the end of the blood collections, the absorbance of each sample was spectrophotometrically measured against a 0.1% sodium carbonate blank at 650 nm. Time (in minutes) was semilogarithmically plotted against absorbance. A regression line was calculated, and the time required for the absorbance to be halved (T1/2) was determined.

Macrophage 5'Nucleotidase (5'N) Assay

Macrophage 5'nucleotidase activity, which has been shown to decrease as macrophage activation increases [34], was used to assess peritoneal macrophage activation in irradiated mice. In this assay, washed dishes of adherent macrophages were lysed with 200 μl of 0.05% Triton X-100 (Sigma Chemical Company, St. Louis, MO) in distilled water, and the cell lysate was used to measure 5'N activity. Specifically, 5'N was assayed with 0.15 mM ^{3}H-adenosine monophosphate (AMP) as substrate in 50 mM Tris buffer (pH 9.0) containing 12 mM magnesium chloride [15]. The specific activity is expressed as nmol AMP hydrolyzed per minute per mg cell protein at 37°C. The protein content of macrophage cell lysates was determined by the Bio-Rad procedure (Bio-Rad Labs, Rockville Center, NY) with bovine gamma globulin as a standard. Triplicate determinations were routinely performed for each assay.

Free-Radical Scavenger Assay

The ability of glucan to act as a free-radical scavenger was assayed by evaluating its reactivity with water radiolysis products in competition with methional as an alternate free-radical trap. In this system, the interaction of methional with free radicals results in the production of ethylene gas. A decrease in ethylene gas production can be interpreted as an agent's ability to compete with methional in the scavenging of free radicals. Ethylene measurements were performed according to the method initially described by Beauchamp and Fridovich [4] and later modified by Pryor and Tang [44]. The radical-assay solution contained 0.1 mM methional (Sigma Chemical Co. St. Louis, MO) in 50 mM phosphate buffer (pH 7.8), and 0.45% sodium chloride. Three milliliters of this solution were pipetted into 10-ml glass reaction vessels, and specified amounts of either D-glucose (Sigma Chemical Co., St. Louis, MO) or glucan were added to each vessel. The vessels were then sealed with Teflon-lined caps and irradiated. After irradiation, 50 μl of gas above each reaction solution was removed with a gas syringe, and the content of ethylene gas was measured by gas chromatography using a Porapac-Q 6-ft column with a Sigma 3B gas chromatograph coupled to a flame ionization detector (Perkin-Elmer, Norwalk, CN). The flow rate was typically 20 ml/min, with the injection chamber maintained at 140°C. Ethylene gas standards were obtained from Scott Speciality Gases (Plumsteadville, PA).

RESULTS

Effect of Glucan on Survival in Lethally Irradiated Mice

Figure 1 illustrates that 1.5 mg of glucan administered i.v. 1 day before a 9-Gy irradiation enhanced survival by 63% ($p < .05$, with respect to i.v. saline or D-glucose controls). This glucan dose and injection route were used to evaluate the effect of glucan on hemopoietic cells, bacterial translocation, granulocyte function, and RES clearance capacity in irradiated mice. However, to perform the 5'N assay in irradiated mice, i.p. injections were used to obtain the number of macrophages needed for this assay more easily. Thus, evaluation of the survival-enhancing effect of i.p. glucan administration was also necessary. In the same figure it can be seen that 1.5 mg of glucan administered i.p. also enhanced survival in otherwise lethally irradiated mice ($p < .05$, with respect to i.p. saline or D-glucose controls). However, i.p. glucan administration was less effective than i.v. administration and enhanced survival by only 26%. As expected, no survival enhancement was observed with either i.v. or i.p. administration of either saline or 1.5 mg of D-glucose.

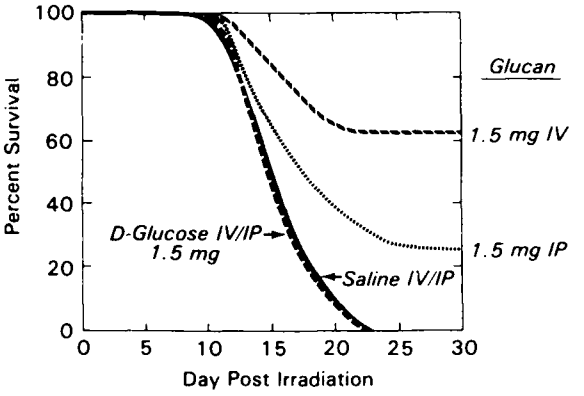

Fig. 1. Effect of route of glucan injection on survival in 9-Gy irradiated C3H/HeN mice. Saline, D-glucose, or glucan was administered 1 day before irradiation, and survival was monitored for 30 days. Data represent cumulative survival data obtained from three separate experiments and a total of 28–33 mice in each treatment group. No difference was observed between i.v. and i.p. saline and D-glucose treatments; thus data from both injection routes were pooled. These groups had a total of 56 and 61 mice, respectively.

Effect of Glucan on Hemopoietic Recovery in Lethally Irradiated Mice

Table 1 describes the bone marrow and splenic CFU-s and GM-CFC recovery observed in mice administered either saline or 1.5 mg of glucan 1 day before a 9-Gy irradiation. Both CFU-s and GM-CFC recovery commenced earlier in glucan-treated mice than in controls. However, hemopoietic activity was not detected in glucan-treated mice until day 13 postirradiation, and even by day 15 postirradiation (when ~65% of control mice were already dead) CFU-s and GM-CFC bone marrow

TABLE 1. Effect of Glucan on Hemopoietic Recovery in Lethally Irradiated Mice (Percent of Normal Control Values)*

	Day postirradiation				
	11	13	15	18	21
Bone marrow					
CFU-s femur[a]					
Radiation	0	0	0	[b]	[b]
Glucan + radiation	0	0	0.15 ± 0.09	0.96 ± 0.16	2.56 ± 0.31
GM-CFC femur[c]					
Radiation	0	0	0	[b]	[b]
Glucan + radiation	0	0.14 ± 0.11	0.49 ± 0.14	0.92 ± 0.26	1.22 ± 0.29
Spleen					
CFU-s spleen[d]					
Radiation	0	0	0.02 ± 0.02	[b]	[b]
Glucan + radiation	0	0.51 ± 0.08	0.68 ± 0.12	12.94 ± 0.96	20.58 ± 2.06
GM-CFC spleen[e]					
Radiation	0	0	0	[b]	[b]
Glucan + radiation	0	1.34 ± 0.22	4.22 ± 0.39	48.49 ± 3.67	262.25 ± 11.33

*Values represent means ± standard errors obtained by averaging data from 3–4 individual experiments
[a]CFU-s per femur for normal control mice = 1,655.4 ± 55.1
[b]Not enough animals surviving to test at these time points
[c]GM-CFC per femur for normal control mice = 6,526.1 ± 118.3
[d]CFU-s per spleen for normal control mice = 3,314.6 ± 96.5
[e]GM-CFC per spleen for normal control mice = 1,592.4 ± 77.3

values were only 0.15% and 0.49% of normal and splenic values were only 0.68% and 4.22% of normal.

Effect of Glucan on Bacterial Translocation in Lethally Irradiated Mice

Figure 2 illustrates the effect of glucan on bacterial translocation in lethally irradiated mice. Bacterial organisms could be detected in the spleens and liver of 10%–13% of both control and glucan-treated mice at 7 days postirradiation. The percent of mice with bacterial flora in these organs increased for both treatment groups through day 11 postirradiation. However, by day 11, significantly fewer glucan-treated mice exhibited splenic and hepatic bacterial flora than did control mice (25%–28% versus 42%–43%). In addition, by day 15 postirradiation, the percent of glucan-treated mice exhibiting bacterial organisms in the liver and spleen had decreased to ~10%, while it had risen to ~70%–80% in control mice. In both glucan-treated and control mice, the percent of animals exhibiting bacterial flora in the peripheral blood was less than that exhibited in the spleen and liver. In the peripheral blood, a bacterial translocation pattern similar to that observed in the spleen and liver was observed on days 11–15 postirradiation (ie, less translocation in glucan-treated than in control mice). However, at earlier time points (days 7 and 9), a greater percent of glucan-treated mice exhibited peripheral blood bacterial flora than did control mice (9%–13% versus 0%–7%). The most commonly observed organisms were *Proteus mirabilis*, *Staphylococcus aureus*, and *Escherichia coli*, and no species difference was observed in the organisms detected in the spleen, liver, or peripheral blood. In some instances, multiple types of bacterial organisms were observed in a single mouse. However, as illustrated in Figure 3, this consistently occurred more frequently in control mice than in glucan-treated mice.

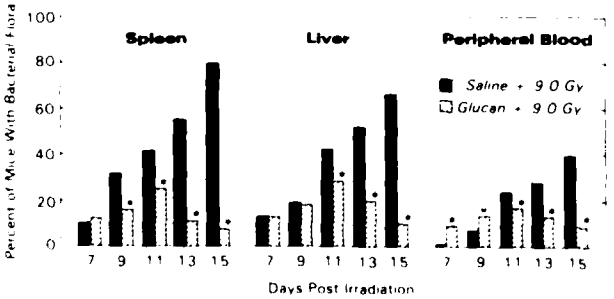

Fig. 2. Effect of glucan on bacterial translocation in 9-Gy irradiated C3H/HeN mice. Saline (control) or glucan (1.5 mg) was administered i.v. 1 day before irradiation. Data from three separate experiments were pooled and represent a total of 31–35 mice in each treatment group at each time point. Statistical differences were assayed by Student's t-test. $^{*}p < 0.05$, with respect to control mice.

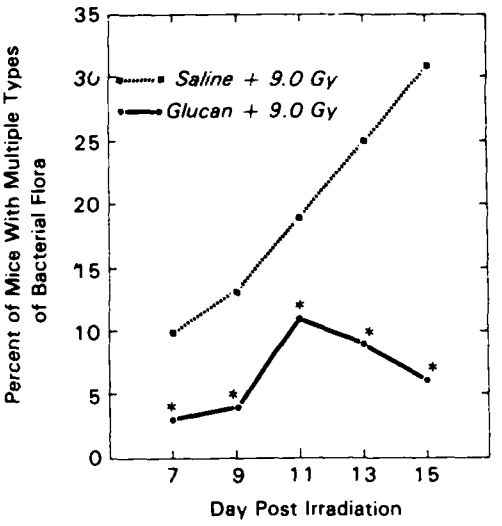

Fig. 3. Effect of glucan on multiple bacterial translocations in 9-Gy irradiated C3H/HeN mice. Saline (control) or glucan (1.5 mg) was administered i.v. 1 day before irradiation. Data from three separate experiments were pooled, and represent a total of 31–35 mice in each treatment group at each time point. Data represent the percent of bacterially infected mice that presented with more than one bacterial species. Statistical differences were assayed by Student's t-test. $^{*}p < 0.05$, with respect to control mice.

Effect of Glucan on Granulocyte Chemiluminescence in Lethally Irradiated Mice

To evaluate the effect of glucan on granulocyte function, peripheral-blood granulocyte oxidative burst activity was measured by chemiluminescence. As can be seen in Figure 4, no significant differences were observed

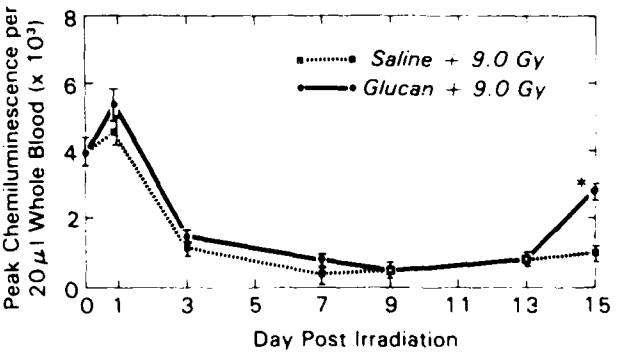

Fig. 4. Effect of glucan on opsonized zymosan-induced peripheral-blood granulocyte chemiluminescence in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of data obtained from 8–9 individual mouse blood samples. Statistical differences were assayed by Student's t-test. $^{*}p < 0.05$, with respect to control mice.

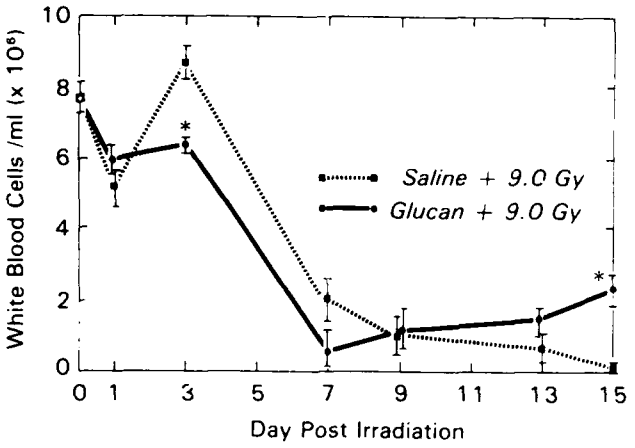

Fig. 5. Effect of glucan on white blood cellularity in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of cell counts obtained from 8–9 individual mouse blood samples. Statistical differences were evaluated by Student's t-test. *p < 0.05, with respect to control mice.

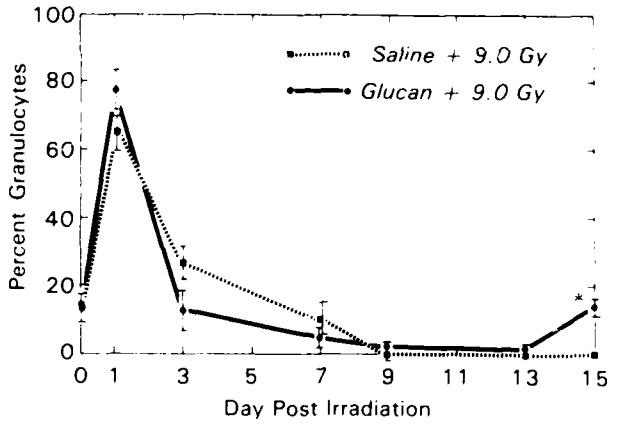

Fig. 6. Effect of glucan on peripheral blood granulocytes in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of values obtained from 8–9 individual mouse blood samples. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

between the oxidative burst activity of granulocytes obtained from glucan-treated and control mice until day 15 postirradiation, when the granulocyte response from glucan-treated mice was 140% of that from control mice. Likewise, peripheral-blood white cell numbers and the percent of peripheral-blood granulocytes were significantly elevated in glucan-treated mice 15 days postirradiation (Figs. 5 and 6).

Effect of Glucan on Carbon Clearance in Lethally Irradiated Mice

To evaluate the effect of glucan on RES clearance capacity, mice were assayed for their ability to clear intravenously injected colloidal carbon (Fig. 7). At day 1 postirradiation, glucan-treated mice cleared one-half of the injected carbon in just 6.8 min, compared to 8.2 min in control mice. However, at days 3 and 5 postirradiation, carbon clearance in glucan-treated mice was dramatically slower than in control mice (9.5–9.6 min versus 6.1–6.7 min). This was followed on days 7–11 postirradiation by a period when the T1/2 values of glucan-treated and control mice were approximately equal. However, by days 13 and 15 postirradiation, the carbon clearance T1/2 in control mice had increased dramatically, whereas that in glucan-treated mice had decreased. On day 15 postirradiation, control mice cleared half of the injected carbon in 9.3 min, while glucan-treated mice cleared the same amount in only 5.5 min.

Effect of Glucan on Macrophage 5'Nucleotidase Activity in Lethally Irradiated Mice

To evaluate the effect of glucan on macrophage activation, peritoneal macrophage 5'N activity was assayed. Figure 8 illustrates that macrophages from both control and glucan-treated mice exhibited a rapid and dramatic decrease in 5'N activity (indicative of macrophage acti-

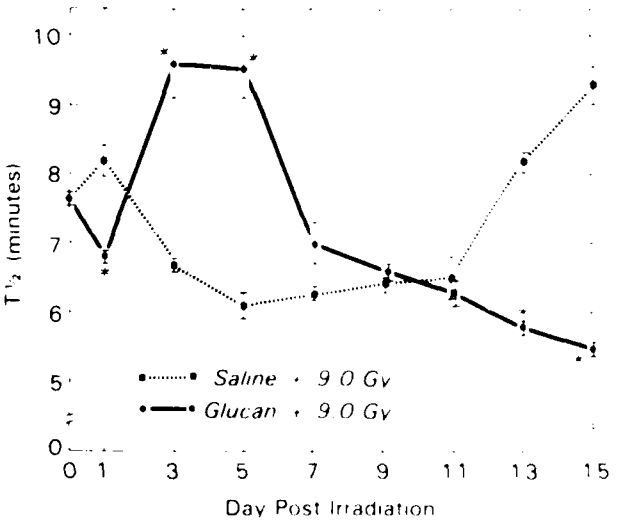

Fig. 7. Effect of glucan on reticuloendothelial-system carbon clearance in 9-Gy irradiated C3H/HeN mice. Mice were injected i.v. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the mean ± standard error of T1/2 values obtained from 7–9 individual mice. Statistical differences were assayed by Student's t-test. *p < 0.05, with respect to control mice.

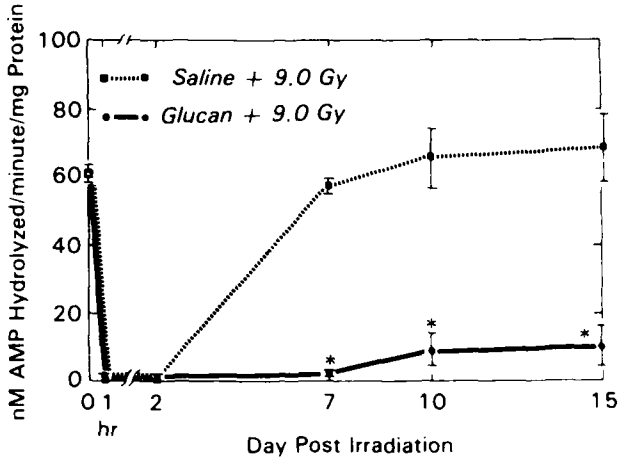

Fig. 8. Effect of glucan on peritoneal macrophage 5'N activity. Mice were injected i.p. with saline (control) or glucan (1.5 mg) 1 day before irradiation. Each data point represents the *mean ± standard error of the specific activities from 3–4 separate experiments.* In each experiment, peritoneal exudate cells were pooled from 8–10 mice. Statistical differences were determined by Student's t-test. *$p < 0.05$, with respect to control mice.

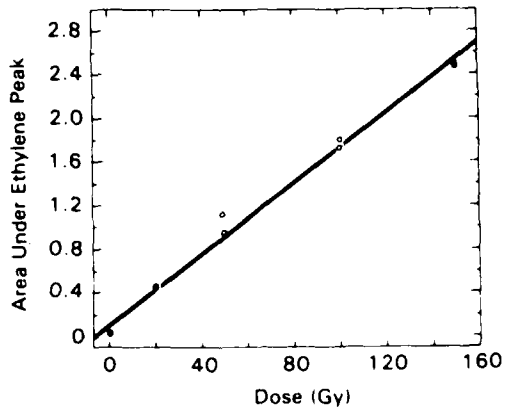

Fig. 9. Effect of cobalt-60 gamma rays on ethylene yields in a 0.1 mM methional/50 mM phosphate buffer solution. The ethylene in a 50-μl aliquot of gas above the reaction solution was analyzed by gas chromatography ~24 hours after irradiation. Each symbol represents an individual sample. Based on least-squares line of fit, $p < 0.0001$.

vation) which persisted through day 2 postirradiation. At these times, 5'N activity was barely detectable in either treatment group. Macrophage 5'N activity in control mice returned to preirradiation levels at days 7, 10, and 15 postirradiation (~60 nmol AMP/min/mg). However, macrophage 5'N activity in glucan-treated mice remained significantly reduced (2–10 nmol AMP/min/mg), indicating macrophage activation through day 15 postirradiation.

Ability of Glucan to Scavenge Radiation-Produced Free Radicals

To address the possibility that once inside macrophages glucan may protect these cells through chemical means (and hence enhance host resistance by enhancing the survival of macrophages), glucan's ability to scavenge free radicals was assayed. A standard curve for the test system used in these experiments, ie, the production of ethylene gas following the irradiation of a methional solution, is shown in Figure 9. In repeated experiments, the dose response for ethylene production from methional was linear over the broad radiation dose range of 0 to 150 Gy. Figure 10 illustrates the effect of glucan or D-glucose (control) on ethylene gas yields in this system. Experiments were performed with a 100-Gy radiation exposure, and data were normalized to the ethylene gas produced by methional after exposure to 100 Gy with no test agent present. These results indicate that glucan is more effective than D-glucose in competing with methional for radiolysis products. Scavenger activity with D-glucose was observed only at D-glucose concentrations greater than 0.083 mg/ml, whereas scavenger activity with glucan was observed at glucan concentrations as low as 0.0075 mg/ml.

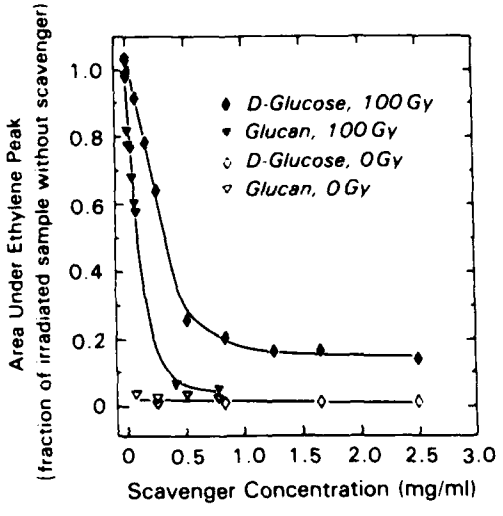

Fig. 10. Effect of glucan or D-glucose (control) on ethylene yields in nonirradiated and 100-Gy irradiated methional solutions. Ethylene in a 50-μl aliquot of gas above each methional-based reaction solution was determined as described in the legend of Figure 9. Data were normalized to the area under the *ethylene peak for the 0 Gy or 100 Gy irradiated samples* with no test agent present.

DISCUSSION

It has been postulated that most biologically derived agents that are radioprotective in the hemopoietic-syndrome dose range function by enhancing hemopoietic recovery and, hence, by regenerating mature cells necessary for the irradiated host to resist otherwise life-threatening opportunistic infections [5,20,22,31-33, 50,53]. Recently, we demonstrated radioprotection by yet another biologically derived agent, glucan, and reported glucan's ability to not only enhance survival specifically in the hemopoietic-syndrome dose range [37,40], but also to enhance hemopoietic stem cell regeneration in sublethally irradiated mice [38,39,41]. Although there is no doubt that hemopoietic recovery is absolutely necessary for long-term survival following otherwise lethal irradiation [3,10,17], significant hemopoietic regeneration in lethally irradiated glucan-treated mice did not appear to occur early enough to account for the increased survival observed in these animals in the first few weeks postirradiation (Fig. 1; Table 1). Thus, further studies were initiated to evaluate additional mechanisms through which glucan may mediate its radioprotective effects prior to detectable hemopoietic recovery.

The bacteriological studies presented here clearly demonstrated enhanced host resistance to microbial invasion in glucan-treated mice as early as 9–11 days postirradiation (Fig. 2). Not only were glucan-treated mice more resistant to microbial invasion, but in addition they were actually able to arrest the progressive increase in opportunistic infections seen in control mice. This ability to arrest opportunistic infections successfully became clearly apparent after day 11 postirradiation, when glucan-treated mice not only exhibited a progressive decrease in bacterial infections, but also generally presented with single, as opposed to multiple, bacterial species (Fig. 3). Correlated with this increased ability to resist opportunistic infections was the fact that 63% of glucan-treated mice (compared to 0% of control mice) survived the radiation insult (Fig. 1).

The fact that glucan-treated irradiated mice exhibited enhanced host resistance to microbial invasion prior to the detection of significant numbers of new hemopoietic elements prompted investigation into glucan's ability to enhance and/or to prolong the function of mature cell populations present at the time of irradiation. Because both granulocytes and macrophages have been shown to play critical roles in nonspecific host resistance against microbial invasion [21,27], and because these cells have been shown to be relatively radioresistant in comparison to lymphocytes and hemopoietic stem cells [2,7,26, 29,42,52], glucan's effect on these specific cells was evaluated.

Experiments with granulocytes (Figs. 4–6) demonstrated that in both control and glucan-treated mice, granulocyte numbers and granulocyte oxidative burst activity were critically reduced by 1 week postirradiation. In control mice, these parameters showed no signs of recovery prior to death while in glucan-treated mice, both granulocyte numbers and granulocyte oxidative burst activity commenced recovery at day 15 postirradiation. These recoveries, however, occurred too late to explain the enhanced resistance to microbial invasion observed in glucan-treated mice prior to day 15 postirradiation.

While the studies presented in this paper did not suggest that granulocytic mechanisms were involved in glucan's immediate ability to enhance resistance to microbial invasion in irradiated mice, they did strongly implicate the involvement of macrophage mechanisms in this phenomenon. This is perhaps not surprising since, in normal animals, glucan has been shown to enhance macrophage function dramatically [12,13,57] and to increase nonspecific host resistance to a variety of bacterial, viral, fungal, and parasitic infections [for review see reference 12]. Although it appeared that in irradiated mice glucan may also enhance host resistance to infections via macrophage activation, the interpretation of these studies is complicated by the fact that radiation alone can activate macrophages [2,6,7,11,36].

The carbon clearance studies presented in this paper confirmed this phenomenon in that an increased clearance capacity was observed in control mice on days 3–11 postirradiation (Fig. 7). While it was suspected that glucan-treated mice would exhibit an even faster carbon clearance capacity than control mice, on days 3, 5, and 7 postirradiation, they actually cleared carbon less efficiently than controls (Fig. 7). This phenomenon seemed paradoxical; however, it could be explained if following irradiation a RES blockade was established in glucan-treated mice. The glucan used in these experiments was particulate in nature [14] and has been shown to be selectively taken up by macrophages [19]. In addition, even under normal circumstances, macrophages have been shown to phagocytize necrotic lymphocytes and nuclear debris within hours after irradiation [2,25]. Thus, a RES blockade could have resulted in glucan-treated mice owing to the phagocytosis of not only a large number of glucan particles, but also large quantities of radiation-induced debris. Such a RES blockade may also have contributed to the enhanced hemopoietic regeneration ultimately observed in glucan-treated irradiated mice (Table 1) since RES blockade has previously been reported to aid in hemopoietic repopulation in irradiated mice by preventing the phagocytosis of slightly injured, yet still functional, hemopoietic stem cells [35]. Similarly, a RES blockade may have prevented the phagocytic loss of mature functional hemopoietic cells capable of effective defense against microbial invasion in the first few weeks postirradiation. The glucan-induced RES blockade, how-

ever, only persisted through the first week postirradiation. After this time, macrophages in glucan-treated mice appeared to regain their phagocytic capacity. It is known that within a week a large portion of intravenously administered glucan is broken down and metabolized [19]. Thus, the ability of macrophages in glucan-treated irradiated mice to regain their phagocytic activity in the second week postirradiation coincided with a time frame in which most intracellular glucan should have been broken down and metabolized and suggested that once "emptied," glucan-activated macrophages could again commence phagocytosis. By day 9 postirradiation, the clearance capacities of control and glucan-treated mice were identical and at days 11, 13, and 15 postirradiation, the clearance capacity of glucan-treated mice continued to increase while that of control mice progressively decreased. Interestingly, the day 11 time point at which glucan-treated mice began to surpass control mice in their clearance capacity coincided with the time point at which glucan-treated mice also began to arrest microbial invasion by opportunistic pathogens. In addition, at day 15 postirradiation, when opportunistic pathogens were detected in ~80% of the control mice and only ~10% of the glucan-treated mice, glucan-treated mice were capable of clearing carbon twice as rapidly as controls. In support of these RES studies, the 5'N studies presented in this paper also demonstrated that macrophage activation differed in control and glucan-treated mice in the first few weeks postirradiation. In these studies, peritoneal macrophage activation was observed in both control and glucan-treated mice immediately after irradiation (Fig. 8). However, in control mice this activation disappeared by day 2 postirradiation and in glucan-treated mice it persisted throughout the 15-day postirradiation observation period. Thus, both the RES and the 5'N data presented here demonstrated glucan's ability to enhance macrophage function profoundly in irradiated mice, and supports the contention that, in the first few weeks after irradiation, glucan may enhance resistance to opportunistic pathogens (and hence survival) via macrophage-mediated mechanisms.

Although glucan appeared to mediate its radioprotective effects by enhancing and/or prolonging macrophage function, the possibility that even prior to this phenomenon glucan may radioprotect by traditional radical-scavenging mechanisms could not be excluded. On the cellular level, injury following exposure to ionizing radiation has been shown to be attributed to hydroxyl radicals [4], as well as secondarily produced organic radicals [44,56] and hydrogen peroxide reaction products [55], which can result in damage to the cellular DNA, enzymes, and membranes [16,18,45,46]. The possibility that glucan may act to prevent such radiation damage was suggested by the experiments presented in Figure 10 in which glucan's ability to trap free radicals chemically was clearly demonstrated. Since glucan has been shown to be selectively taken up by and sequestered in macrophages [19], once inside these cells glucan may maintain its ability to scavenge free radicals and, thus, selectively protect these cells. If this occurs, it may explain why macrophages in glucan-treated mice function longer and better than macrophages in control mice after irradiation. Studies to determine realistic macrophage intracellular glucan concentrations at the time of irradiation and to elucidate the exact radioprotective potential of glucan once inside purified macrophage cell populations are currently in progress in our laboratories.

In conclusion, the results of these studies suggest the critical role of macrophages (but not granulocytes) in mediating glucan's antimicrobial and hence early survival-enhancing effects in irradiated mice. Because macrophage activation and enhanced macrophage function could be detected as early as 1–24 h after irradiation, it appeared that the macrophages responsible for these responses were "radiation survivors" and did not arise from glucan-induced hemopoietic repopulation which did not become evident until days 13–15 postirradiation. However, from these studies it was impossible to discern if at such later times (eg, day 15 postirradiation), "old surviving," "newly produced," or both types of macrophages were responsible for the responses measured. In spite of this, these studies shed new light on additional mechanisms by which "hemopoietic stimulants" may enhance survival in irradiated mice. Whether additional cellular and/or chemical mechanisms are also involved in glucan's radioprotective effect remains to be determined.

ACKNOWLEDGMENTS

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Interactions Between Radiation and Amphetamine in Taste Aversion Learning and the Role of the Area Postrema in Amphetamine-Induced Conditioned Taste Aversions

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RABIN, B. M., W. A. HUNT AND J. LEE. *Interactions between radiation and amphetamine in taste aversion learning and the role of the area postrema in amphetamine-induced conditioned taste aversions.* PHARMACOL BIOCHEM BEHAV 27(4) 677–683, 1987.—Three experiments were run to assess the role of the area postrema in taste aversion learning resulting from combined treatment with subthreshold unconditioned stimuli and in the acquisition of an amphetamine-induced taste aversion. In the first experiment, it was shown that combined treatment with subthreshold radiation (15 rad) and subthreshold amphetamine (0.5 mg/kg, IP) resulted in the acquisition of a taste aversion. The second experiment showed that lesions of the area postrema blocked taste aversion learning produced by two subthreshold doses of amphetamine. In the third experiment, which looked at the dose-response curve for amphetamine-induced taste aversion learning in intact rats and rats with area postrema lesions, it was shown that both groups of rats acquired taste aversions following injection of amphetamine, although the rats with lesions showed a less severe aversion than the intact rats. The results are interpreted as indicating that amphetamine-induced taste aversion learning may involve area postrema-mediated mechanisms, particularly at the lower doses, but that an intact area postrema is not a necessary condition for the acquisition of an amphetamine-induced taste aversion.

Conditioned taste aversion Amphetamine Area postrema Dose-dependent Radiation Combined treatment

A conditioned taste aversion (CTA) is produced when a novel tasting solution is paired with an unconditioned stimulus (UCS), such that the organism will avoid ingestion of that solution at a subsequent presentation. In addition to toxic unconditioned stimuli, such as ionizing radiation and lithium chloride (LiCl), taste aversions can also be produced by pairing the novel stimulus with a variety of compounds that an organism will self-administer, such as amphetamine [4,12].

Taste aversions produced by toxic stimuli such as ionizing radiation or LiCl depend upon the integrity of the area postrema (AP) [7, 9, 14, 17], the brainstem chemoreceptive trigger zone for emesis [2]. In contrast, lesions of the AP have been reported to have no effect on the acquisition of an amphetamine-induced CTA [1,17]. The results of the lesion studies seem to be in accord with the results of the more behaviorally-oriented studies which have shown that the behavioral responses of rats to flavors paired with LiCl differ from the responses to flavors paired with amphetamine [10,11]. Thus, the acquisition of taste aversions following treatment with toxic unconditioned stimuli may involve different mechanisms than those produced by nontoxic stimuli [12].

In the preceding report [16], it was shown that subthreshold doses of radiation could be combined with subthreshold doses of LiCl to produce a CTA. This finding was interpreted as being consistent with the hypothesis that similar mechanisms underlie the acquisition of taste aversions produced by both radiation and LiCl. Since both radiation and LiCl unconditioned stimuli require that the AP be intact

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TABLE 1

FLUID INTAKE (ml) FOLLOWING COMBINED TREATMENT WITH SUBTHRESHOLD RADIATION AND AMPHETAMINE

Delay Interval (hr)	Conditioning Day		Test Day	
	Water	Sucrose	Water	Sucrose
Control	4.20±1.70*	24.20±1.85	3.20±0.83	20.20±2.02
0.25	3.42±0.96	18.83±1.43	11.25±2.14	9.75±1.72
0.50	4.30±1.27	18.50±1.25	11.90±1.64	9.00±1.67
1.00	4.50±0.92	16.40±1.18	4.50±1.21	15.20±1.41
1.50	4.58±0.78	16.00±1.59	11.42±1.51	13.25±1.61
2.00	7.64±1.52	21.18±1.51	9.27±2.04	15.36±2.23

*Mean ± standard error.

for CTA learning to occur [7, 9, 12, 17] it seems reasonable to assume that this brainstem structure may be involved in the observed interaction. If the common reliance of both radiation and LiCl on the AP provides the basis for the interactions observed in the preceding experiment, then combining radiation or LiCl with a UCS that does not require the mediation of the area postrema for CTA learning should not result in the acquisition of a CTA.

GENERAL METHOD

Subjects

The subjects were male Sprague Dawley rats weighing 300–375 g at the start of the experiment. The rats were housed in individual cages in a room with a 12:12, light:dark cycle. Food and water were continually available, except as required by the experimental protocol.

Taste Aversion Training

Taste aversions were produced using a two-bottle design in which the animal was given a choice between tap water and a 10% sucrose solution on both conditioning and test days. The rats were first placed on a 23.5 hr water deprivation schedule for 10 days. On the conditioning day (day 10) all rats were presented with two calibrated drinking tubes containing tap water and 10% sucrose solution for 30 min. Immediately following the drinking period, the rats were given the appropriate treatment and returned to their home cages for 24 hr. On the test day (day 11), the rats were again given a choice between tap water and sucrose solution and intake of each solution recorded. Relative intake of tap water and 10% sucrose solution were transformed into preference scores: sucrose intake divided by total fluid intake.

EXPERIMENT 1

The first experiment of this series was designed to determine whether or not subthreshold doses of ionizing radiation could be combined with subthreshold doses of amphetamine to produce a CTA. As indicated above, since the AP has been reported not to mediate the acquisition of an amphetamine-induced CTA [1,17], it should not be possible to combine subthreshold amphetamine with subthreshold radiation, in contrast to combinations of radiation and LiCl [16], to produce a CTA if the AP serves to integrate the combined treatments.

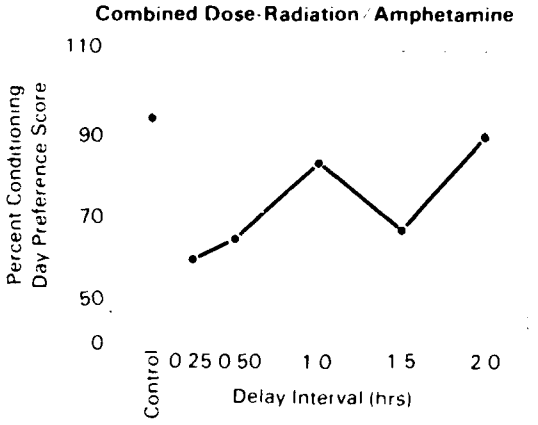

FIG. 1. Effects of combined treatment with subthreshold radiation (15 rad) and subthreshold amphetamine (0.5 mg/kg, IP) as a function of the delay interval between treatments. Control was given a single injection of amphetamine. Test day sucrose preference is expressed as the percentage of the conditioning day preference. Error bars indicate the standard error of the mean.

Method

The subjects were 63 male albino rats divided into 6 groups of 7–12 subjects/group. Immediately after ingestion of a 10% sucrose solution, the experimental rats were placed in a plastic restraining box and exposed to 15 rad at a dose rate of 20 rad/min using a ^{60}Co source. Dosimetry was performed using thermoluminescent detectors (LiF TLD 100's) and a 3.3 ml Victoreen chamber. Following delay intervals of 0.25, 0.5, 1.0, 1.5 or 2.0 hr, independent groups of rats were given an IP injection of 0.5 mg/kg d-amphetamine. The control animals were given a single IP injection of 0.5 mg/kg amphetamine. Preliminary experiments had indicated that this dose of amphetamine was just below threshold for producing CTA learning.

Results and Discussion

The effects of combined amphetamine and radiation treatment are presented in Table 1. These data are summarized in Fig. 1, which presents the test day sucrose preference as a percentage of the conditioning day preference for the sucrose solution. The data from the control group confirms that a single dose of 0.5 mg/kg amphetamine does not produce a CTA. The results from the groups given combined treatment with radiation (15 rad) and amphetamine (0.5 mg/kg, IP) show that treatment with subthreshold doses of ionizing radiation and amphetamine can be combined to produce a CTA. Statistical analysis of the data using a one-way analysis of variance followed by planned comparisons [6] showed that significant differences from the controls were observed at delay intervals of 0.25 hr, $F(1,57)=4.94$, $p<0.01$, 0.5 hr, $F(1,57)=3.95$, $p<0.01$, and 1.5 hr, $F(1,57)=3.06$, $p<0.05$. The other delay intervals, 1.0 and 2.0 hr, did not differ significantly from control.

These results show that a subthreshold dose of radiation can be combined with a subthreshold dose of amphetamine to produce a CTA. This observation means that amphetamine is similar to LiCl because subthreshold doses of both drug stimuli can be combined with irradiation to produce a CTA. For both sets of drug unconditioned stimuli,

the effective delay intervals were relatively short, lasting for only 1.0 to 1.5 hr [16].

As such, these results are not consistent with the hypothesis proposed above that, because of presumed differences in the role of the AP in the acquisition of taste aversions produced by these unconditioned stimuli, subthreshold radiation exposure would not combine with subthreshold amphetamine to lead to the acquisition of a CTA. The observation that combined radiation and amphetamine treatment does produce a CTA would suggest either that the basis for the interaction of combined subthreshold unconditioned stimuli involves brain structures other than the AP, or that amphetamine, like radiation and LiCl, may also have effects on the AP.

EXPERIMENT 2

Although the lesion studies cited above [1,17] indicate that destruction of the AP does not prevent the acquisition of an amphetamine-induced CTA, the preceding results, which show an interaction between radiation and amphetamine, suggest that radiation and amphetamine may be producing similar effects within the organism. Otherwise, it should not be possible for the two unconditioned stimuli to combine to produce an effect on behavior. The basis for this interaction between radiation and amphetamine is not certain. Garcia *et al.* [5] have proposed that a treatment-produced malaise or illness experienced by the organism is the proximal UCS leading to the acquisition of a CTA. Because amphetamine can produce a CTA, it must produce an experienced illness within the organism which would form the basis for the observed interaction between radiation and amphetamine. Rabin and Rabin [13], on the contrary, have shown that CTA learning can occur in anesthetized animals which cannot experience a treatment-induced illness. They have proposed that the proximal UCS for CTA learning is the activation of specific neural circuits, independently of any experiential effects resulting from the treatment. According to this theory, the basis for the observed interaction between radiation and amphetamine would be in the capacity of these stimuli to excite similar neural circuits. Because a radiation-induced CTA requires the mediation of the AP, there is the possibility that treatment with amphetamine may also produce effects in the AP.

This experiment was designed to determine whether or not the AP may be involved in mediating the acquisition of a CTA produced by combined treatment with subthreshold doses of a UCS. Because it is already well-established that the radiation-induced CTA depends upon the integrity of the AP, this experiment utilized combined treatment with subthreshold doses of amphetamine in rats with lesions of the AP and in intact rats.

Method

The subjects were 28 rats divided into 3 groups. In the first group were 8 rats with AP lesions and treated with two combined injections of amphetamine (0.5 mg/kg, IP) separated by a delay interval of 30 min. The second group consisted of 10 intact control rats treated with the combined amphetamine injections. The third group of 10 intact rats, who were administered a single injection of amphetamine (0.5 mg/kg, IP) followed by an equivolume injection of isotonic saline 30 min later, served as a comparison group for the combined treatment groups.

Lesions were made in the AP of 8 rats using procedures detailed previously [14]. Briefly, all rats were anesthetized with sodium pentobarbital (35 mg/kg, IP). The AP was exposed and thermal lesions were made using a cautery probe under direct visual control. After surgery, the rats were given a prophylactic injection of bicillin (60,000 units) and allowed to recover in their home cages for a period of 2–4 weeks before beginning behavioral testing.

The general procedure was similar to that detailed in Experiment 1. Immediately following ingestion of the 10% sucrose solution on the conditioning day, all rats were given a single injection of amphetamine (0.5 mg/kg, IP). Thirty min later, without further access to the sucrose solution, the two amphetamine combined groups were given a second injection of amphetamine (0.5 mg/kg, IP), while the comparison group was given an injection of isotonic saline. All rats were tested for a CTA 24 hr later.

At the conclusion of the testing, all operated rats were anesthetized with sodium pentobarbital (50 mg) and perfused intracardially with isotonic saline followed by 10% formalin saline. Sections were cut through the brainstem at the level of the AP at 50 μm and stained with thionin. Representative sections of an intact animal and an animal with AP lesions are presented in Fig. 2. Examination of the histological material indicated that for the most part the lesions were restricted to the AP, although they did occasionally affect the dorsal parts of the nucleus of the solitary tract.

Results and Discussion

Mean conditioning day water intake showed a range of 4.30 to 9.40 ml and sucrose intake ranged from 14.40 to 22.60 ml. For both water and sucrose intake, the largest amounts were consumed by the rats with AP lesions.

As shown in Fig. 3, treatment with either a single injection of amphetamine followed by isotonic saline in intact rats, or treatment with combined injections of amphetamine in rats with AP lesions, did not produce a CTA. In contrast, a CTA was observed in the intact rats given the combined injections of amphetamine. A mixed analysis of variance for the groups receiving the combined amphetamine injections indicated that the main effect for condition for the comparison between the intact rats and rats with AP lesions was significant, $F(1,16)=11.92$, $p<0.01$, while the main effect for day was not significant, $F(1,16)=0.01$, $p>0.10$. The condition-by-day interaction, $F(1,16)=5.70$, $p<0.05$, was significant, thereby indicating that the test day preference scores of the two groups were significantly different, with the intact rats showing a reduction in sucrose preference, while the rats with AP lesions showed an increase in preference.

The implication of the present results, which show that lesions of the AP can block the acquisition of a CTA produced by combined treatment with two subthreshold doses of amphetamine, is that the AP is somehow involved in the acquisition of a CTA following treatment with amphetamine. As such, these results would support the hypothesis that the AP serves to integrate the combined effects of treatment with radiation and amphetamine. However, this finding would run counter to the results of previous research [1,17] which suggests that the AP is not involved in the acquisition of an amphetamine-induced CTA. These apparently discrepant findings regarding the possible role of the AP in amphetamine-induced CTA learning may derive from the fact that the studies which reported that AP lesions did not disrupt the acquisition of an amphetamine-induced CTA

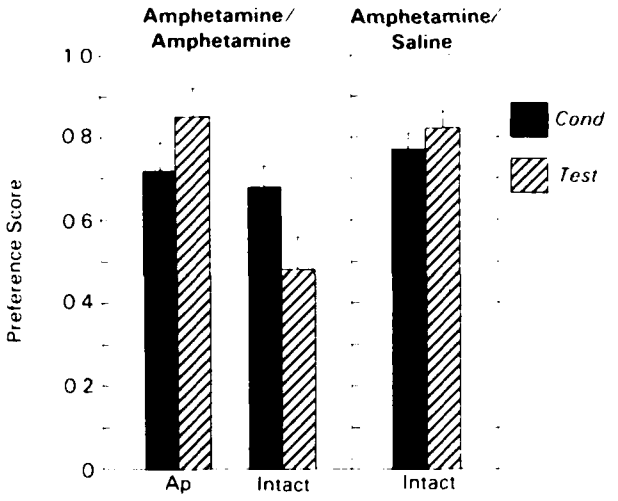

FIG. 3. Effects of area postrema lesions on the acquisition of a conditioned taste aversion produced by two subthreshold amphetamine injections separated by 30 min. Error bars indicate the standard error of the mean.

used a suprathreshold dose of amphetamine together with a single-bottle test in contrast to the subthreshold dose utilized in the present experiment which was combined with the more sensitive two-bottle procedure.

EXPERIMENT 3

Although the research cited above strongly indicates that lesions of the AP do not prevent the acquisition of an amphetamine-induced CTA [1,17], there are some findings that are difficult to reconcile with such an hypothesis. First, since dopaminergic terminals have been reported in the AP [8], it seems reasonable to assume that treatment with amphetamine would affect these terminals and, consequently, AP activity and taste aversion learning. Second, it has been reported that microinjection of amphetamine into the vicinity of the AP will produce a CTA [3]. This finding raises the question of why peripherally-administered amphetamine would not affect the AP to produce a CTA. These findings, in combination with the results of the preceding two experiments, would seem to be consistent with the hypothesis that the AP is, in some way, involved in CTA learning following combined treatment with amphetamine and ionizing radiation.

There is, therefore, evidence to suggest both that the AP is not involved in the acquisition of an amphetamine-induced CTA and that it is. It may be possible that the importance of the role of the AP in amphetamine-induced taste aversion learning is a function of the dose of amphetamine that is used to produce the CTA. Such a dose-related role for the AP in mediating CTA learning has been reported in studies of taste aversions produced by the toxic compound WR-2721 [15]. The present study was designed to examine the role of dose and AP lesions in the acquisition of an amphetamine-induced CTA.

Method

The subjects were 117 male Sprague Dawley-derived rats weighing 300–375 g at the start of the experiment. Lesions were made in the AP of 54 rats, while the remaining rats served as intact controls. The lesion and histological procedures were identical to those described in the preceding experiment. Examination of the histological material at the conclusion of the experiment indicated that most rats had lesions restricted to the AP, although the extent of tissue damage did include the dorsal parts of the nucleus of the solitary tract in some of the animals (see Fig. 2).

After a 2–3 week period to allow for recovery from the surgery, the behavioral testing was begun as detailed above. Immediately after the drinking period on the conditioning day, independent groups of control rats and rats with AP lesions were given IP injections of a single dose of amphetamine. The doses of amphetamine were 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/kg. With the exception of the AP group receiving the lowest dose of amphetamine (n=5), there were between 9–11 subjects in each group. The rats were then returned to their home cages for 24 hr before testing for the acquisition of a CTA.

Results and Discussion

For the intact animals, conditioning day water intake averaged 5.28±0.41 ml and sucrose intake averaged 17.79±7.26 ml across all dose levels. For the rats with AP lesions, the corresponding intakes were 5.66±0.70 ml for water intake and 25.17±0.85 ml for sucrose intake. Although the development of a CTA in the intact rats was reflected as an increase in water intake which was paired with a corresponding decrease in sucrose intake such that total fluid intake remained relatively constant across all tested doses, the rats with AP lesions given the three highest doses of amphetamine showed a decrease in sucrose intake that was not completely balanced by the corresponding increase in water intake. As a result, these three groups of rats showed an average decrease in total fluid intake of approximately 10 ml.

The results are summarized in Fig. 4, which presents test day sucrose preference as the percentage of the conditioning day preference score. An analysis of variance showed that both the main effect for dose, $F(5,105)=7.48$, $p<0.001$, and the main effect for condition for the comparison between control and lesion rats, $F(1,105)=11.77$, $p<0.001$, were highly significant. The significant main effects would indicate that test day sucrose preference was a function both of the dose of amphetamine and of the presence of an AP lesion. The dose by condition interaction, $F(5,105)=0.81$, $p>0.10$, was not significant, indicating that both lesion and intact rats showed a reduction in sucrose preference in response to treatment with amphetamine across the various doses. These data indicate, therefore, that AP lesions attenuate an amphetamine-induced taste aversion, particularly at the lower doses, but do not prevent CTA learning following treatment with higher doses of amphetamine. As such, the present results would be concordant with previous research using the higher amphetamine doses which reported that lesions of the AP do not disrupt the acquisition of an amphetamine-induced CTA [1,17].

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FIG. 2. Photomicrographs of the brainstem of the rat showing an intact area postrema (A, arrow) and a representative lesion (B).

A

B

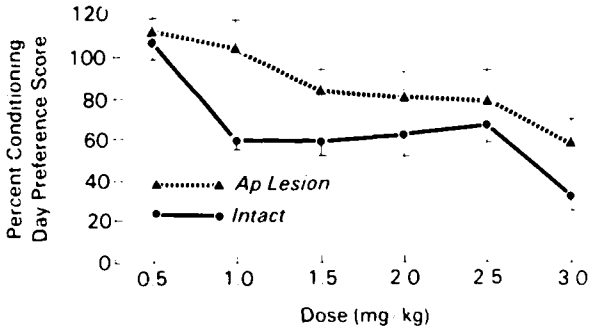

FIG. 4. Effect of dose and area postrema lesions on the acquisition of an amphetamine-induced taste aversion. Test day sucrose preference is expressed as the percentage of the conditioning day preference. Error bars indicate the standard error of the mean.

These data indicate that the major effect of AP lesions on the amphetamine-induced CTA is at the lowest effective doses, 1.0 and 1.5 mg/kg, although the intact animals seem to show a generally reduced preference compared to the animals with AP lesions at all tested doses. This finding is consistent with the observation of a dose-dependent effect of AP lesions on the CTA produced by treatment with WR-2721 [15].

The data presented in Fig. 4 seem to suggest a two-stage process underlying the acquisition of an amphetamine-induced CTA. In intact animals, there is an initial sharp decrease in sucrose preference between a dose of 0.5 and 1.0 mg/kg. Sucrose preference then seems to stabilize until a dose of 2.5 to 3.0 mg/kg, at which point there is another sharp decrease in sucrose preference. The responses of the rats with AP lesions generally parallel those of the intact animals. Given that the major effect of AP lesions seems to be at the lowest doses of amphetamine, at 1.0 and 1.5 mg/kg, it may be that the CTA produced by lower doses of amphetamine may involve some AP-mediated mechanisms, but as the dose of amphetamine increases, there is an increasing involvement of other mechanisms such that the AP becomes relatively less important for CTA learning at the higher doses.

GENERAL DISCUSSION

The results of these experiments indicate that the AP is involved in the acquisition of an amphetamine-induced CTA in a dose-dependent manner, such that the importance of the AP-mediated mechanisms decrease as the dose of amphetamine is increased. In contrast to taste aversions produced by LiCl or ionizing radiation [7, 9, 14, 17], an intact area postrema is not, therefore, a necessary condition for the acquisition of an amphetamine-induced CTA. Since lesions of the dorsal tegmentum disrupt the acquisition of an amphetamine-induced CTA in animals with an intact AP [18], an intact AP may not even be a sufficient condition for such learning. However, when an intact AP is present, it does contribute to the acquisition of a CTA produced by amphetamine. The present results indicate, therefore, that the role of the AP in amphetamine-induced taste aversion learning is a relatively complex one, which varies as a function of the dose of amphetamine.

As such, the results of the present studies are generally consistent with the results of previous research [1,17] in showing that destruction of the AP does not prevent the acquisition of an amphetamine-induced CTA following treatment with high doses of amphetamine. However, the observation that the AP can contribute to the development of a CTA following treatment with low doses of amphetamine is consistent with the observation that microinjection of amphetamine in the vicinity of the AP produces a CTA [3]. Similarly, the present observation that lesions of the AP can modulate the intensity of an amphetamine-induced taste aversion provides a potential physiological basis for the previous finding that a subthreshold dose of amphetamine can be combined with a subthreshold exposure to ionizing radiation to produce CTA learning [16].

ACKNOWLEDGEMENTS

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Taste Aversion Learning Produced by Combined Treatment With Subthreshold Radiation and Lithium Chloride

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RABIN, B. M., W. A. HUNT AND J. LEE. *Taste aversion learning produced by combined treatment with subthreshold radiation and lithium chloride.* PHARMACOL BIOCHEM BEHAV **27**(4) 671–675, 1987.—These experiments were designed to determine whether treatment with two subthreshold doses of radiation or lithium chloride, either alone or in combination, could lead to taste aversion learning. The first experiment determined the thresholds for a radiation-induced taste aversion at 15–20 rad and for lithium chloride at 0.30–0.45 mEq/kg. In the second experiment it was shown that exposing rats to two doses of 15 rad separated by up to 3 hr produced a taste aversion. Treatment with two injections of lithium chloride (0.30 mEq/kg) did not produce a significant reduction in preference. Combined treatment with radiation and lithium chloride did produce a taste aversion when the two treatments were administered within 1 hr of each other. The results are discussed in terms of the implications of these findings for understanding the nature of the unconditioned stimuli leading to the acquisition of a conditioned taste aversion.

Conditioned taste aversion Radiation Lithium chloride Combined treatment

WHEN a novel tasting solution is paired with an unconditioned stimulus (UCS) such as exposure to ionizing radiation or injection of lithium chloride (LiCl), an organism will avoid ingestion of that solution at a subsequent presentation. This avoidance behavior, called a conditioned taste aversion (CTA), is typically acquired in a single pairing of the novel conditioned stimulus (CS) and the UCS.

Although both the radiation- and LiCl-induced CTA seem to share some mechanisms in common [9], they differ in terms of their capacity to produce a CTA utilizing a "backwards" conditioning procedure in which the UCS is presented before the CS. In the typical conditioning experiment the CS is followed by the presentation of the UCS. When the UCS is administered before the CS, the conditioned response is much weaker or may not occur at all. As a UCS for CTA learning, LiCl follows this pattern, such that a CTA is not acquired if the UCS is presented as little as 5 min before the CS [1]. With the radiation UCS, in contrast, CTA learning will occur even when the organism is irradiated up to 6–24 hr before the presentation of the CS [1, 2, 15]. These findings suggest that exposing an organism to ionizing radiation, in contrast to injection of LiCl, produces some change within the organism that remains active over an extended period of time, and that serves to produce the temporal contiguity between UCS and CS necessary for conditioning to occur.

If, as suggested above, exposing an organism to ionizing radiation does produce some long-lasting change within the organism, it may be possible that successive exposures will cumulate to the extent that normally ineffective irradiations will have an effect on the behavior of the organism. Conversely, the observation that LiCl does not produce a CTA when administered before the CS would suggest that LiCl does not produce a similar long-lasting change within the organism and therefore, that its effects would not cumulate over successive treatments.

In addition, because experimental manipulations that affect the acquisition of a radiation-induced CTA have similar effects on the acquisition of a LiCl-induced CTA, Rabin and Hunt [9] have proposed that similar mechanisms underlie the CTA learning produced by treatment with these apparently disparate stimuli. In general, the more similar different stimuli are, the greater the probability that the organism will

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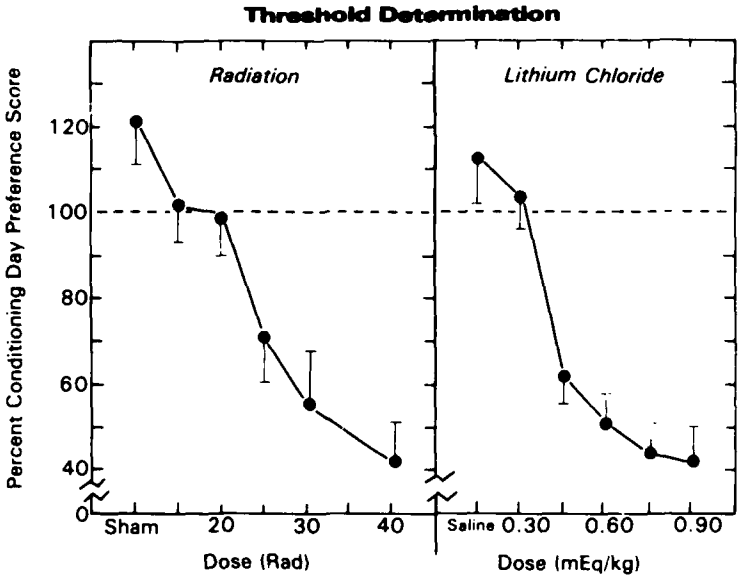

FIG. 1. Threshold determinations for producing a taste aversion following treatment with radiation or LiCl. The test day sucrose preferences are presented as the percentage of the conditioning day preference score. Error bars indicate the standard error of the mean.

make similar responses to them. Under these conditions, if similar mechanisms do mediate the acquisition of a CTA produced by irradiation and by LiCl treatment, then it should be possible to produce a CTA by combining subthreshold irradiation with injection of a subthreshold dose of LiCl.

The present experiments were designed to evaluate these hypotheses by examining the effects of repeated subthreshold exposures to ionizing radiation or LiCl, either separately or in combination, on the acquisition of a CTA.

GENERAL METHOD

Subjects

The subjects were male Sprague-Dawley-derived rats weighing 300–400 g at the start of the experiment. The rats were housed in individual cages in a room with a 12:12 light: dark cycle. Food and water were continually available except as required by the experimental protocol.

Taste Aversion Training

All taste aversions were produced using a two-bottle design. The rats were first placed on a 23.5 hr water deprivation schedule for 10 days during which water was available for 30 min a day during the early light phase of the diurnal cycle. On the conditioning day (day 10), the rats were presented with two calibrated drinking tubes for the 30 min drinking period, one tube containing tap water and the other containing a 10% sucrose solution, and the intake of each was recorded. In order to make certain that each rat sampled a sufficient quantity of the novel sucrose solution, any rat that did not show a greater sucrose than water intake was discarded from the experiment. Where possible, rats were assigned to the various experimental or control conditions following presentation of the CS to minimize the possibility that this procedure would selectively affect one treatment group.

TABLE 1

FLUID INTAKE (ml) AS A FUNCTION OF DOSE OF RADIATION OR LITHIUM CHLORIDE

	Conditioning Day		Test Day	
Dose	Water	Sucrose	Water	Sucrose
Radiation (Rad)				
Sham	6.25 ± 1.37*	17.40 ± 1.55	2.20 ± 0.79	18.20 ± 1.88
15	5.45 ± 1.18	20.36 ± 1.52	5.45 ± 1.35	21.82 ± 1.39
20	5.45 ± 1.22	17.00 ± 1.38	6.55 ± 1.49	18.18 ± 1.56
25	6.45 ± 1.40	16.82 ± 1.23	10.36 ± 1.98	10.82 ± 2.04
30	4.40 ± 1.06	17.50 ± 1.98	11.60 ± 2.11	8.20 ± 1.89
40	4.36 ± 1.00	14.27 ± 1.21	14.00 ± 1.23	6.45 ± 1.29
Lithium Chloride (mEq/kg)				
Sal	3.40 ± 0.85	18.00 ± 1.23	3.30 ± 0.98	19.00 ± 1.19
0.30	4.82 ± 1.13	17.55 ± 1.73	3.82 ± 1.19	18.36 ± 4.05
0.45	3.09 ± 1.00	18.45 ± 1.09	10.00 ± 1.55	9.27 ± 0.93
0.60	3.55 ± 1.02	19.55 ± 1.24	12.45 ± 2.11	11.09 ± 1.56
0.75	3.73 ± 0.85	19.09 ± 1.05	12.45 ± 1.66	7.09 ± 1.13
0.90	3.44 ± 0.71	18.33 ± 0.80	11.33 ± 1.79	7.56 ± 1.89

*Mean ± standard error.

Over the course of the experiment, approximately 10% of the animals tested failed to show an initial preference for the sucrose CS. Immediately following the drinking period, the subjects were given the appropriate UCS. On the test day (day 11) the rats were again presented with the two calibrated drinking tubes and their intake of water and sucrose recorded.

Data Analysis

Water and sucrose intakes were transformed into preference scores: sucrose intake divided by total fluid intake. Test day preference is presented as the percentage of the conditioning day preference score. Statistical analyses were initially performed using two-way analyses of variance. Comparisons between specific groups were made using orthogonal comparisons, and the Scheffe correction applied to take into account the fact that the comparisons were made on a *post hoc* basis [6].

EXPERIMENT 1

The first experiment was designed to establish the thresholds for radiation- and LiCl-induced CTA learning under the specific conditions for the current experiments.

Method

For the determination of the radiation-induced CTA threshold, 64 rats were divided into 6 groups of 10–11 rats per group. One group of rats served as a sham irradiated control group and was placed in a clear plastic restraining box and then carried to the source, but not exposed. The remaining 5 groups of rats were exposed to one of a successively lower dose of radiation until a dose was reached that did not produce a test day decrease in sucrose preference. The tested doses, provided by a ^{60}Co source, were 40, 30, 25, 20, and 15 rad administered at a dose rate of 20 rad/min. Dosimetry was performed using thermoluminescent detectors (LiF TLD 100's) and a 3.3 ml Victoreen chamber.

For the determination of the threshold for an LiCl-induced CTA, 65 rats were divided into 6 groups of 10–11 rats per group. The sham-treated control group was given an IP injection of isotonic saline. The remaining 5 groups of rats were given an IP injection of one of successively lower doses of 0.3 M LiCl. The tested doses were 0.90, 0.75, 0.60, 0.45, and 0.30 mEq/kg.

Results and Discussion

The determination of the threshold doses for radiation- and LiCl-induced taste aversions is presented in Table 1 and Fig. 1. Increasing the dose of both stimuli produced non-linear increases in the intensity of the CTA. As shown in Table 1, the increasing intensity of the CTA is reflected as a test day increase in water intake combined with a decrease in sucrose intake, such that total fluid intake remained relatively constant over the range of LiCl and radiation treatments.

For ionizing radiation, exposure to a dose of approximately 25 rad produced a decrease in test day sucrose intake relative to conditioning day intake. Further increases in the dose of radiation to 40 rad produced corresponding increases in the strength of the aversion. This threshold level for the radiation-induced CTA is somewhat higher than that reported by Garcia *et al.* [4], although these variations may reflect differences in the quality of the radiation and in the conditions associated with the behavioral testing [13,14].

The threshold for a CTA induced by treatment with LiCl is between 0.30 and 0.45 mEq/kg. Further increases in the dose of LiCl produced a non-linear increase in the strength of the aversion. This figure is in general agreement with the threshold of 0.15 mEq/kg determined by other investigators (e.g., [3,4]). As with the radiation threshold, these differences probably reflect differences in the specific testing conditions.

Although treating rats with a 15-rad dose of radiation and a 0.30-mEq/kg dose of LiCl seems to limit the test day increase in sucrose preference observed with the control animals, the animals given these doses showed no test day decrease in sucrose preference relative to their conditioning day preference. Therefore, these doses were taken as the threshold doses for the following experiments using combined subthreshold treatments with radiation and LiCl, either alone or in combination.

EXPERIMENT 2

If, as suggested above, irradiation does produce a long-lasting change within the organism, then it should be possible to present two subthreshold doses of radiation, neither of which would by itself produce a CTA, such that the combined doses would result in the acquisition of a CTA following the single conditioning trial. For LiCl, on the contrary, combining subthreshold doses in a single conditioning trial should not have a similar additive effect leading to the acquisition of a CTA. In addition, if the mechanisms by which both LiCl and irradiation produce a CTA are similar [9], then combined subthreshold doses of LiCl and radiation should also lead to CTA learning.

The present experiment was designed to evaluate these hypotheses by examining the effects of successive treatments with radiation or LiCl alone or in combination using a single conditioning trial. In addition, a series of delay intervals between the presentation of the successive treatments was tested in order to determine the time course of these effects.

Method

The subjects were 312 rats divided into groups of 9–13 animals per group. For the subjects exposed to the successive radiation doses, independent groups of experimental and control animals were tested at delay intervals (time between successive exposures) of 0, 0.25, 0.50, 1.0, 1.5, 2, 3, 4 and 5 hr. The subjects given successive injections of LiCl or radiation exposure combined with a LiCl injection were tested at delay intervals of 0.25, 0.50, 1.5 and 2 hr.

The control subjects were treated identically to the experimental groups, except that they were not given the second subthreshold treatment. For the radiation controls this meant that the rats were kept in the exposure box for the delay interval or returned to it at the appropriate time and carried to the radiation source, but not exposed. For the LiCl groups or the combined radiation-LiCl controls, the rats were given a second injection of isotonic saline at the end of the appropriate delay interval. At the delay intervals of 1.0, 1.5 and 2.0 hr, the same animals were utilized as controls for both the dual radiation exposure and the combined radiation-LiCl groups by keeping the rats in the restraining box for the delay interval and giving them an injection of isotonic saline when they were removed from the box.

The general procedure was as follows. Immediately following ingestion of the sucrose solution on the conditioning day, the rats were treated with the appropriate UCS, either irradiation (15 rad at a dose rate of 20 rad/min) or LiCl (0.30 mEq/kg, IP). At the end of the delay interval, the subjects were given the second exposure to the UCS without further access to the sucrose solution. In all cases, the radiation UCS was administered before the LiCl UCS in the combined treatment groups.

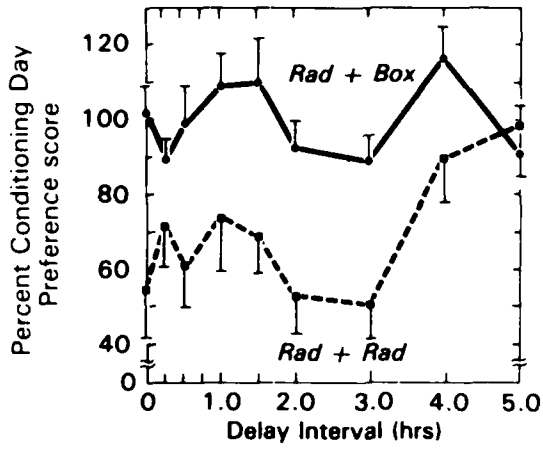

FIG. 2. Effect of treatment with two subthreshold (15 rad) exposures to radiation (dashed lines) or a single exposure and a sham treatment in which the rats were maintained in, or returned to, the exposure box after the appropriate delay interval (solid lines) on sucrose preference as a function of the delay interval between the two successive exposures. Test day preference is expressed as the percentage of the conditioning day sucrose preference. Error bars indicate the standard error of the mean.

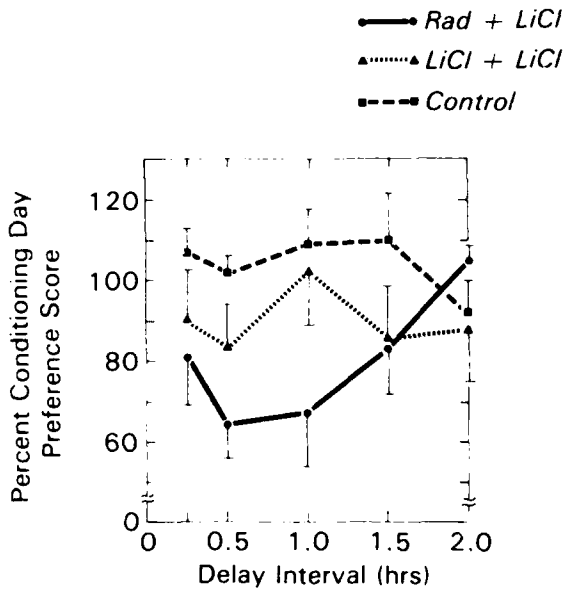

FIG. 3. Effect of treatment with subthreshold (0.3 mEq/kg) injections of LiCl (dashed lines) or with combined subthreshold radiation and LiCl (solid lines) on sucrose preference. Test day sucrose preference is expressed as the percentage of the conditioning day preference. Error bars indicate the standard error of the mean.

Results and Discussion

Conditioning day water intake across all groups showed a range of 3.00–7.50 ml while the range of sucrose intakes was 16.00 to 24.00 ml. As in the previous experiment (Table 1), the acquisition of a CTA was reflected in a change in the relative intakes of water and surcose while total fluid intake remained constant across the various treatment groups.

The effects of combining two subthreshold doses of radiation following a single presentation of the CS are summarized in Fig. 2. The analysis of variance showed that the main effect for condition for the comparison between the experimental and control subjects was highly significant, $F(1,174)=44.94$, $p<0.001$, as was the comparison for delay interval, $F(8,174)=2.61$, $p=0.01$. These results indicate that it is possible to combine two subthreshold doses of radiation, neither of which has an effect when administered separately, to produce a CTA. The effective time period for the combined effects is over the range of delay intervals from 0.25 to 3.0 hr. An inspection of Fig. 2 suggests that the two combined doses of 15 rad have an effect equivalent to that of a single dose of 30 rad (0 hr delay interval) over the entire range of effective delay intervals (0.25–3.0 hr). The observation that the interaction was not significant, $F(8,174)=1.51$, $p>0.10$, suggests that the general trends in both experimental and control subjects were similar, although the experimental animals, given the two radiation exposures, showed a consistently greater change in test day sucrose preference than the control animals, which had been given only the single exposure.

As shown in Fig. 3, combined subthreshold injections of LiCl were not effective in producing a CTA, whereas the combined treatment with radiation and LiCl was effective over delay intervals of 1 hr or less. The analysis of variance indicated that the main effect for the comparison between conditions was significant, $F(2,138)=6.76$, $p<0.01$. Neither the main effect for delay interval, $F(4,138)=0.54$, $p>0.10$, nor the condition by interval interaction, $F(8,138)=1.40$, $p>0.10$, was significant. Since the main effect for interval was not significant, the scores for each condition were combined across intervals and comparisons between the different treatment conditions were run using orthogonal comparisons with the Scheffe test [6]. These comparisons showed that the sucrose preference of the combined radiation/LiCl group was significantly different than that of the control group, $F(1,138)=13.18$, $p<0.05$, while the group given the two successive subthreshold injections of LiCl did not differ significantly from the controls, $F(1,138)=4.55$, $p>0.05$.

In general these results are concordant with the hypotheses proposed above. The observation that it is possible to combine two subthreshold doses of radiation to produce a CTA suggests that exposing the organism to ionizing radiation produces some change in the organism that remains active for the 3-hr delay interval over which the combined effects were obtained. In addition, the apparent similarity in the strength of the CTA between the single 30 rad dose and the two combined 15 rad doses suggests that the two subthreshold doses are combining additively over the interval to produce an effect on behavior. The differences in the time scale over which these effects were obtained in the present experiment and the longer intervals over which a radiation-induced CTA can be observed using the backwards conditioning paradigm [2,15], may be related to the dose of radiation used in the two experiments. In contrast to the marginal

dose used in the present experiments (a total of 30 rad), the radiation doses used in the backwards conditioning experiments (100 rad and greater) are sufficient to produce a nearly maximal avoidance of the CS.

LiCl, in contrast, is a relatively short-acting agent as shown by its inability to produce a behavioral effect when used in a backwards conditioning design [1]. As such, it was proposed above that treatment with two subthreshold injections of LiCl would not combine to produce a CTA. The present results support this hypothesis. Although these results do suggest that the effects of combined LiCl injections may act to reduce CS intake compared to controls given only a single injection of LiCl, these differences were not significant.

Also, the present results, which show that combined treatment with a single subthreshold exposure to radiation and a single subthreshold injection of LiCl is sufficient to produce a CTA, are consistent with the hypothesis that similar mechanisms mediate taste aversions produced by radiation and LiCl. However, in contrast to the duration of the effects produced by two radiation exposures (approximately 3 hr), the time course of the combined radiation/LiCl interaction (approximately 1 hr) is much shorter. Although the present data do not allow a determination of the reasons for this difference in the effective interval of the combined effects, it may be possible that the longer action of the radiation-induced change permits a greater summation of effects when two doses of radiation are used in contrast to a lesser summation following the use of both radiation and the shorter-acting LiCl.

GENERAL DISCUSSION

Overall, the results are consistent with the hypotheses proposed above. Exposing the organism to ionizing radiation produces a relatively long-lasting change in functioning that serves to mediate both the acquisition of a CTA in a backwards conditioning paradigm [1, 2, 15] and when two subthreshold doses are given within a 3 hr interval. With LiCl, in contrast, two subthreshold doses cannot be combined to produce a CTA, which is concordant with the observation that LiCl does not produce a CTA when administered in a backwards conditioning design [1].

The observation that a subthreshold dose of LiCl can be combined with a subthreshold dose of radiation to produce a CTA is consistent with the hypothesis proposed by Rabin and Hunt [9] that similar mechanisms underlie the capacity of both radiation and LiCl to produce a taste aversion. At some level, either peripheral or central, both of these unconditioned stimuli must be producing similar effects on organismic functioning in order for the combined treatments to produce an effect. The finding that a subthreshold dose of radiation can be combined with a subthreshold dose of LiCl to produce a CTA may indicate that the organism fails to discriminate between these stimuli, but instead responds to them as if they were related stimuli. The present data, however, do not allow a determination of the mechanisms underlying the combined interaction of these apparently disparate stimuli. It may be that both radiation and LiCl chloride produce a common "malaise" [5] such that the combined treatment is sufficient to bring it above the threshold level for a behavioral response. Alternatively, it is possible that both radiation and LiCl similarly affect specific neural circuits [10], and that the combined treatment is capable of producing a sufficient change in neural activity to produce a corresponding change in behavior leading to the acquisition of a CTA. In the latter case, it seems likely that interaction involves the area postrema either directly or indirectly, because the integrity of the AP is necessary for CTA learning to occur following treatment with either radiation or LiCl [8, 11, 12].

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ENHANCED HEMATOPOIETIC RECOVERY IN IRRADIATED MICE PRETREATED WITH INTERLEUKIN-1 (IL-1)[2,3]

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ABSTRACT

Data in this report compare the number of colony-forming cells (CFC) in bone marrow from irradiated and pre-irradiated C57Bl/6J mice injected with saline or recombinant interleukin-1-alpha (rIL-1). Eight to 12 days after sublethal or lethal irradiation, there were more CFU-E (colony-forming units-erythroid), BFU-E (burst-forming units erythroid), GM-CFC (granulocyte-macrophage colony-forming cells), and day 8 CFU-S (colony-forming units-spleen) in bone marrow from rIL-1 injected mice than from saline injected mice. Prior to irradiation, there was no increase in number of CFC in bone marrow from rIL-1 injected mice. However, as determined by sensitivity to hydroxyurea, rIL-1 injection stimulated GM-CFC into cell cycle. These results demonstrate that rIL-1 injection increases the number of CFC that survive in irradiated mice and may be a consequence of the stimulation of CFC into cell cycle prior to irradiation.

INTRODUCTION

The physiological mechanisms determining sensitivity to ionizing radiation as determined by LD50/30 radiation doses in mice (i.e., dose at which 50% of the mice die within 30 days) and the rate of hematopoietic recovery in the surviving animals are not well understood. Various chemical and biological substances,

as well as physiological manipulations, can alter the effect of radiation on these parameters (1-7). For example, administration of inflammatory agents such as bacterial endotoxins to mice prior to their exposure to ionizing radiation results in an increase in the number of mice that survive beyond 30 days (1,3). Also, in the surviving animals there is an earlier recovery of mature cells in the peripheral blood and of hematopoietic colony-forming cells (CFC)[1] in the bone marrow and spleen (1-5). The decrease in radiosensitivity of endotoxin treated mice is partially attributed to mobilization of CFC to the spleen (8-10), release of hematopoietic stimulatory factors into the circulation (8,11), and stimulation of CFC into cell cycle (4,5).

A broad spectrum of metabolic, hematological, and immunological changes occur in mice within a few hours to a few days after the injection of endotoxin (7-11). Interleukin-1 (IL-1) is one of many factors released after injection of endotoxin and is one of the primary mediators of many of its effects (reviewed in 12-14). In addition, IL-1 has been shown to induce the production of other cytokines such as GM-CSF (15,16) and stimulators of erythroid and multipotential progenitor cells (16). Recently, Neta et al. (17) demonstrated that more mice survived lethal doses of gamma radiation when pretreated with murine or human recombinant IL-1-alpha (rIL-1). With the availability of rIL-1 it is now possible to test which effects of endotoxin are responsible for decreasing the sensitivity of hematopoietic tissues to ionizing radiation.

Previous studies demonstrated that rIL-1 injection increased the number of ECFU-S (endogenous colony-forming units spleen) in irradiated mice (18). Pretreatment with endotoxin also produces an increase in the number of surviving ECFU-S (1,3,7). However, an increase in the number of ECFU-S is not always correlated with earlier bone marrow recovery (7,8) or increased survival (3,7). Therefore, in the present studies, the effect of rIL-1 injection on recovery of CFC in bone marrow and spleen of sublethally and lethally irradiated C57Bl/6 mice was determined. After irradiation, there were significantly more CFC in bone marrow and spleen from rIL-1 pretreated mice than from saline pretreated controls. Prior to irradiation (i.e., pre-irradiated mice) an increase in the number of CFC or an increase in the number stimulated into cell cycle were investigated as possible mechanisms for the enhanced hematopoietic recovery observed in rIL-1 injected irradiated mice.

MATERIALS AND METHODS

Mice

C57Bl/6J female mice were purchased from Jackson Laboratories, Bar Harbor, ME and housed 5-10 mice per cage. Animals were maintained on a 12 hour light/dark cycle and were allowed food (Wayne Rodent Blox) and HCl acidified water (pH 2.4) ad libitum. Ten to 16 week old mice were used for these studies. Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by

the Institute of Laboratory Animal Resources, National Research Council.

Treatment with Interleukin-1

Administration of 100-150 ng rIL-1 to C57Bl/6 mice 18-24 hours before their exposure to gamma radiation was previously determined to be the optimal regimen for radioprotection (18). Both human and murine recombinant alpha interleukin-1 (rIL-1) were used in these studies. Murine rIL-1 was provided by Drs. W. Benjamin and P. Lomedico of Hoffman-LaRoche, Nutley, NJ. The stock solution was supplied in 5 M guanidine hydrochloride. A similar preparation of a purified protein from a bacterial extract containing the plasmid without IL-1 cDNA was used as a control solution. Human rIL-1 (a generous gift from Dr. Steven Gillis of Immunex) was supplied in a solution of phosphate buffered saline at pH 7.2. Immediately before use, stock solutions of rIL-1 were diluted with pyrogen free saline (McGaw), and 0.5 ml containing 100-150 ng rIL-1 was administered to normal mice by intraperitoneal injection 20-24 hours before their exposure to gamma radiation. Control animals were administered 0.5 ml saline or diluted extract solution at the same time. Endotoxin (LPS) contamination in rIL-1 solutions was measured by the Limulus lysate assay. Based on these results less than 0.2 ng of LPS was administered per injection.

Irradiation

Mice were exposed bilaterally to gamma radiation at a dose rate of 0.40 Gy/min from a cobalt-60 radiation source. Twenty to

24 hours after rIL-1 administration, mice were exposed to 7.5, 8.5, 9.0, 9.5, or 10.0 Gy total body irradiation. The percentage of animals surviving more than 30 days at each radiation dose was used to calculate the LD50/30 radiation dose (i.e., the radiation dose at which 50% of the mice died $\leq$ 30 days).

Preparation of Cell Suspensions

Mice were sacrificed by cervical dislocation, and the femurs and spleens were excised. Cells were flushed from the tissues with Hanks' balanced salt solution (GIBCO) and dispersed through a 25 gauge needle until a single cell suspension was obtained. Cell concentrations were determined by hemacytometer counts.

Hematopoietic Colony-Forming Assays

CFU-S (colony-forming unit-spleen) determinations were done basically as described by Till and McCulloch (19). Recipient mice were exposed to 10.0 Gy cobalt-60 radiation. This dose was sufficient to reduce background day 8 macroscopic colonies to an average of less than 1 per spleen. Cell suspensions were diluted to give $5x10^4$-$5x10^5$ cells in 0.2 ml media and injected into a caudal vein of each mouse. After 8 days, the spleens were removed, placed into Bouin's fixative, and the number of macroscopic colonies was counted.

CFU-E (colony-forming unit-erythroid) and BFU-E (burst-forming unit-erythroid) determinations were made using a plasma clot culture system as described by Weinberg et al. (20). Cells

were plated at a final concentration of $2x10^5$ to $1x10^6$ cells/ml with 0.25 U/ml (CFU-E) or 3.0 U/ml (BFU-E) anemic sheep plasma, step III erythropoietin (Connaught Labs, Inc., Lot # 3088-2 and 3092-2) as 0.4 ml plasma clots in 4 well Nunclone culture dishes (Nunc). Cultures were incubated at 37°C with 5% CO_2. After 2.5 days for CFU-E and 8 days for BFU-E the plasma clots were harvested, fixed, stained, and evaluated as described by McLeod et al. (21).

GM-CFC (granulocyte-macrophage-colony forming cell) were assayed using the double layer agar technique basically as described by Hagan et al. (22). The culture medium was double strength CMRL-1066 culture medium (Connaught Medical Research Laboratory) containing 10% (vol/vol) fetal calf serum, 5% (vol/vol) horse serum, 5% (wt/vol) trypticase soy broth, 20 g/ml L-asparagine, and penicillin-streptomycin. In the bottom layer of 35 mm plastic Petri dishes was 1 ml of a 1:1 mixture of culture medium and 1.0% agar (Bactoagar, Difco) containing 50 ul PMUE (pregnant mouse uterine extract) as a source of colony-stimulating activity (23). The top layer contained 1 ml of a 1:1 mixture of culture medium and 0.66% agar containing $5x10^4$ bone marrow cells for assay. Cultures were incubated at 37°C in 5% humidified CO_2 in air. After 10 days of culture colonies greater than 50 cells were scored as GM-CFC.

The number of CFC in S phase of the cell cycle was determined basically as described by Rickard et al. (24). Mice were administered 900 mg/kg body weight hydroxyurea (Sigma) in

Dulbecco's Phosphate Buffered Saline (GIBCO) by intraperitoneal injection. Two to 2.5 hours later the bone marrow was assayed for surviving CFC. The percentage of CFC sensitive to HU was calculated by subtracting the percentage of CFC surviving after HU treatment from 100%.

Statistics

Two-tailed Student's t-test was used to test for significant differences in colony-forming units per tissue between groups of mice. LD50/30 radiation doses were calculated using a weighted logit-log method for dose interpolation (25).

RESULTS

Survival of Irradiated Mice Pretreated with Saline or IL-1

The percentage of mice surviving beyond 30 days after exposure to gamma radiation was compared for rIL-1 and saline pretreated mice (Figure 1). At lethal doses of radiation, more rIL-1 pretreated mice survived than saline pretreated animals. For example, after 8.5 Gy radiation, 30% of the saline pretreated mice died within 30 days, while none of the rIL-1 pretreated animals died. The LD50/30 radiation dose for rIL-1 pretreated mice was 9.6 $\pm$ 0.07 Gy. This was significantly greater ($p<0.01$) than 8.7 $\pm$ 0.07 Gy calculated for the saline controls (Table 1). In these studies, pretreatment of C57Bl/6J mice with rIL-1 increased the LD50/30 radiation dose by 0.9 Gy.

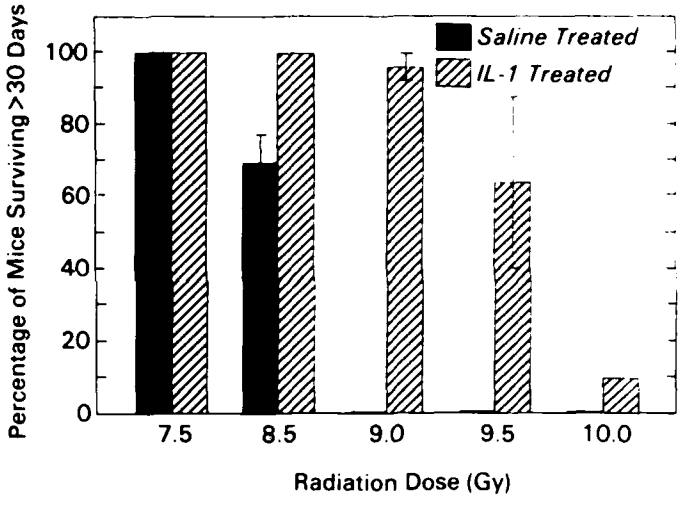

FIG. 1 Effect of Radiation Dose on Survival of Saline or IL-1 Pretreated C57Bl/6J Mice. Mice were administered murine or human rIL-1 by intraperitoneal injection 24 hours before their exposure to cobalt-60 radiation. The percentage of mice that survived beyond 30 days after irradiation was calculated. The number of saline pretreated/rIL-1 pretreated mice was: 26/12 at 7.5 Gy (n=1 study), 28/36 at 8.5 Gy (n=2), 19/23 at 9.0 Gy (n=2), 123/103 at 9.5 Gy (n=4), and 20/10 at 10.0 Gy (n=1).

TABLE 1

Sensitivity of rIL-1 and Saline Injected Mice to Co-60 Radiation[a]

Calculations	Saline Injected (n)[b]	rIL-1 Injected (n)[b]
LD50/30 Dose[c]	8.7 ± 0.07 Gy	9.6 ± 0.07 Gy
%Surviving >30 Days After 9.5 Gy		
Murine rIL-1	2 ± 4.1 (53)	77 ± 19.5 (26)
Human rIL-1	0 ± 0 (67)	58 ± 19.8 (71)
Combined	1 ± 2.6 (120)	67 ± 21.0 (97)
Mean Survival Time[d]	13 ± 2.8 days	15 ± 5.2 days

a. IP injection 24 hours prior to irradiation
b. Total number of mice followed for survival
c. Radiation dose at which 50% of mice died ≤ 30 days
d. For mice that died in ≤ 30 days after 9.5 Gy irradiation

The mean survival time of mice that died within 30 days was not significantly different for saline, extract, human rIL-1, or murine rIL-1 pretreated mice exposed to 9.5 Gy gamma radiation. For rIL-1 injected mice there was no significant difference ($p=0.26$) in survival of mice injected with human or murine rIL-1 (Table 1). Since the effect on survival was not significantly different, hematopoietic studies in mice pretreated with human rIL-1 or murine rIL-1 were combined in the following sections.

Effect of IL-1 on Recovery of CFC After Lethal Radiation

In one study, the number of hematopoietic colony-forming cells recovered in the bone marrow and spleen was compared for rIL-1 and saline pretreated mice various times after 9.5 Gy irradiation. In this study, 9.5 Gy was lethal for 100% of the saline pretreated mice, while 63% of the rIL-1 pretreated animals survived beyond 30 days. One day after irradiation, in both saline and rIL-1 injected mice, bone marrow cellularity (cells/femur) was reduced to 40% of normal and was further decreased to less than 5% by day 4 (Figure 2). At these same time points, CFU-E, BFU-E, and GM-CFC were undetectable in bone marrow and spleen from either group of mice. At day 8, bone marrow cellularity of saline pretreated mice was still less than 5% of normal, and CFU-E were 0.5% of normal. In contrast, cells and CFU-E per femur had increased to 25% and 8%, respectively, in rIL-1 pretreated mice. Other CFC were detectable in bone marrow from both groups of animals by day 12 (Table 2). At day 12, the number

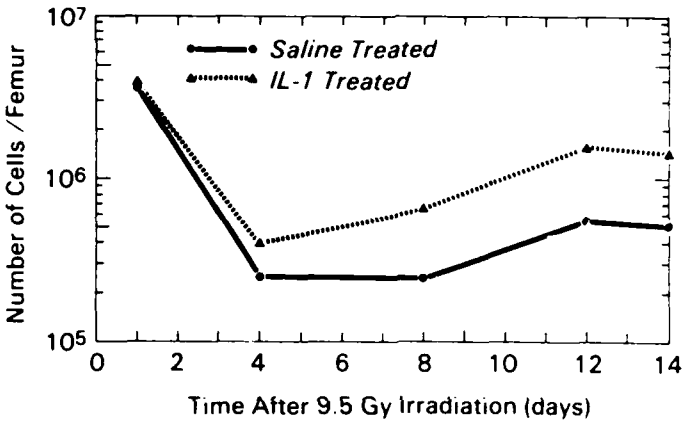

FIG 2 Recovery of Bone Marrow Cells in Mice after 9.5 Gy Irradiation. Mice were administered 150 ng human rIL-1 24 hours before their exposure to cobalt-60 radiation. Cells were pooled from 8 mice per group at each time point.

TABLE 2

Recovery of Colony-Forming Cells Twelve Days After 9.5 Radiation[a]

Assay	n[b]	Saline Injected	rIL-1 Injected
		Number/Femur (% Normal)[c]	
Cells ($x10^6$)	6	0.7±0.18 (4±0.6)	3.0±1.73 (23±16.7)[e]
CFU-E ($x10^3$)	4	0.4±0.50 (3±3.4)	3.0±0.47 (32±17.2)[e]
BFU-E ($x10^3$)	4	0.0±0.00	0.2±0.08 (32±10.2)[e]
GM-CFC	2	6.5±0.14 (<1)	177±55.1 (1±0.3)[e]
Day 8 CFU-S	1[d]	0.6±1.20 (<1)	3.2±6.40 (<1)
		Number/Spleen (%Normal)[c]	
Cells ($x10^6$)	3	6.7±2.03 (6±1.0)	7.7±2.30 (7±1.1)
CFU-E ($x10^3$)	3	0±0	0.1±0.20 (2±3.9)
BFU-E	3	0±0	0±0
GM-CFC	2	21±30.4 (1±1.6)	137±194 (7±10.2)
Day 8 CFU-S	1	0±0	87±7.7 (4)

a. Co-60 (0.40 Gy/min.) irradiation 24 hrs after rIL-1 or saline injection
b. Number of studies
c. Cells pooled from 8-10 mice per group for each study
d. Mean±S.D. of colonies from 5 spleens per group
e. Significantly different at $p<0.05$ (absolute numbers)

of CFU-E, BFU-E, GM-CFC, and day 8 CFU-S in bone marrow from rIL-1 pretreated mice was more than 2 fold greater than recovered in bone marrow from saline pretreated mice. Also, more CFU-E, GM-CFC, and day 8 CFU-S were recovered in spleens from rIL-1 pretreated mice than from saline pretreated animals.

Effect of IL-1 on Recovery of CFC after Sublethal Irradiation

After 7.0 or 7.5 Gy irradiation, 100% of both saline and rIL-1 pretreated mice survived beyond 30 days (Figure 1). However, pretreatment of mice with rIL-1 24 hours prior to sublethal irradiation promoted an earlier recovery in bone marrow cellularity. For example, 10 days following 7.5 Gy irradiation, cells and CFU-E per femur were 3-4 fold higher in rIL-1 injected mice than in saline injected mice (Table 3). BFU-E were undetectable in bone marrow from saline injected mice, but were detectable in rIL-1 injected mice after 7.0 to 8.5 Gy irradiation. These studies demonstrate that rIL-1 injection increases the number of CFC that survive in sublethally and lethally irradiated mice.

Effect of rIL-1 on CFC in Pre-Irradiated Mice

The effect of rIL-1 injection on CFC was also determined in bone marrow and spleens from mice prior to irradiation (i.e., pre-irradiated mice). Twenty-four hours after rIL-1 injection, bone marrow cellularity was approximately 75% of bone marrow cellularity from saline injected mice (Table 4).

TABLE 3

Effect of rIL-1 on Recovery of Erythroid Progenitor Cells in Bone Marrow of Mice after Sublethal Irradiation

Treatment[a]	Number/Femur[b] Cells($\times 10^6$)	CFU-E ($\times 10^3$)[c]	BFU-E[c]
7.0 Gy (Day 8)			
Saline	2.1	2.2±0.58	0±0
rIL-1	12.0	15.7±4.79	32±15.0
	6.4	11.6±3.21	64± 0.0
7.5 Gy (Day 10)			
Saline	2.3	6.9±0.52	0±0
rIL-1	7.8	30.3±0.69	160±54.9
8.0 Gy (Day 10)			
Saline	3.1	4.4±0.70	0±0
rIL-1	4.5	16.0±4.12	220±50.0
8.5 Gy (Day 10)[d]			
Saline	2.5	0.8±0.27	0±0
rIL-1	5.9	2.9±0.27	29±0.0

a. Mice injected with 100-150 ng rIL-1 24 hours prior to irradiatio (7.5 and 8.0 Gy from same study)
b. Cells pooled from both femurs of 5-8 mice per group
c. Mean±S.D. of 2 cultures each
d. 100% of rIL-1 injected and 75% of saline injected mice survived >30 days

TABLE 4

Colony-Forming Cells in Bone Marrow from Pre-irradiated Mice after Saline or rIL-1 Injection[a]

Assay[b]	Saline Injected Mice Number/Femur	n	rIL-1 Injected Mice % Saline Injected Mice
Cells	2.7±0.77 ($\times 10^7$)	9	75±11.0[d]
CFU-E	3.3±1.77 ($\times 10^4$)	9	59±20.5[d]
BFU-E	1.6±0.94 ($\times 10^3$)	8	93±36.9
GM-CFC	1.9±0.30 ($\times 10^4$)	4	90±20.0
CFU-S	2.8 ($\times 10^3$)	1	92

a. Mice were administered 100-150 ng human rIL-1 24 hours earlier
b. Cells were pooled from both femurs from 3-5 mice per group
c. n=number of studies
d. Significantly different ($p<0.05$) from saline injected values

CFU-E were significantly reduced in number per femur in rIL-1 injected mice. However, there was no significant reduction in the number of GM-CFC, BFU-E, or day 8 CFU-S per femur. These results demonstrate that rIL-1 injection did not increase the number of CFC in the bone marrow of pre-irradiated mice.

Splenic cellularity was determined for individual mice in one study (3 mice/group). Total cellular, CFU-E, and CFU-S content were not significantly different in rIL-1 or saline injected mice. However, when compared to saline injected mice, there was a significant increase in GM-CFC per spleen from 0.6 ± 0.21 ($x10^3$) in saline injected mice to 1.9 ± 0.91 ($x10^3$) in rIL-1 injected mice. This increase in splenic GM-CFC may result from the mobilization of cells from bone marrow to the spleen after rIL-1 injection.

Effect of rIL-1 on Number of CFC Sensitive to Hydroxyurea (HU)

The stimulation of CFC into cell cycle in pre-irradiated mice after rIL-1 injection was investigated as an alternate mechanism that may lead to enhanced hematopoietic recovery after irradiation. The percentage of CFU-E and BFU-E from bone marrow sensitive to HU (i.e., in S-phase of the cell cycle) was not significantly different ($p<0.05$) in bone marrow from saline and rIL-1 pretreated mice (Table 5). However, compared to saline injected mice, there was a significant increase in the percentage of GM-CFC sensitive to HU in bone marrow from rIL-1 injected mice.

TABLE 5

Colony-Forming Cells Sensitive to Hydroxyurea (HU) in Pre Irradiated Mice after Saline or rIL-1 Injection[a]

Assay[c]	n[d]	Percentage Killed by HU[b] Saline Injected Mice	rIL-1 Injected Mice
CFU-E	5	76±14.9	73± 7.7
BFU-E	4	59±18.3	70±24.7
GM-CFC	3	33±11.5	64± 5.4[e]

a. Mice given i.p. injections of human rIL-1 24 hours before assay
b. Hydroxyurea (900 mg/kg body weight) by i.p. injection 2.5 hours before assay
c. Cells pooled from 3-5 mice per group
d. Number of studies
e. Significantly different from Saline Injected Mice ($p<0.05$)

DISCUSSION

Administration of rIL-1 to mice prior to their exposure to gamma radiation resulted in an increase in the number of mice that survived beyond 30 days (17). Data presented in this report demonstrate that after irradiation, there is a greater number of CFU-E (colony-forming units-erythroid), BFU-E (burst-forming units-erythroid), GM-CFC (granulocyte-macrophage colony-forming cells), and day 8 CFU-S (colony-forming units-spleen) in bone marrow from rIL-1 injected mice than from saline injected animals. For example, ten days after 7.5 Gy irradiation, the number of cells and CFU-E per femur of rIL-1 injected mice was 3 to 4 fold higher than from saline injected mice. Also, after lethal irradiation, the number of CFU-S and *in vitro* colony-forming cells (CFC) in bone marrow and spleen from rIL-1 injected mice was greater than from saline injected mice. These data demonstrate that

administration of rIL-1 to C57Bl/6 mice prior to irradiation has an effect similar to endotoxin injection in promoting an earlier recovery of CFC in lethally and sublethally irradiated mice (1,2,4).

Similar to pretreatment with endotoxin (2,10), rIL-1 injection results in an increase in the size of the stem cell pool surviving the immediate and delayed effects of radiation damage to hematopoietic tissues. The decrease in radiosensitivity of hematopoietic tissues in endotoxin pretreated mice has been partially attributed to mobilization of CFC from bone marrow to the spleen (8,9), the release of humoral and colony-stimulating factors (9,10), and stimulation of CFC into cell cycle (4,5,10) in pre-irradiated animals. In the present studies, there was some evidence of cell mobilization after administration of rIL-1. Twenty-four hours after rIL-1 injection, the number of CFU-S, BFU-E, and GM-CFC per femur was not significantly different from saline pretreated mice. However, bone marrow cellularity was reduced to 75% of saline injected mice. These results along with an increase in GM-CFC per spleen, and the previously reported increase in ECFU-S (18) demonstrate that some CFC and differentiated cells were mobilized from the bone marrow into the circulation after injection of rIL-1.

CFU-E were decreased by almost 40% in mice after injection of rIL-1. Since there was no increase in numbers in the spleen, this decrease did not appear to be a result of mobilization to the spleen. A similar decrease in marrow CFU-E was previously

observed in endotoxin pretreated mice (26). For example, CFU-E recovered from bone marrow of mice administered 20 ug endotoxin 24 hours earlier were reduced by almost 50%. These results suggest, that similar to pretreatment with endotoxin, there is a suppression of erythropoiesis after injection of rIL-1.

Studies suggest that CFC in S phase of the cell cycle are less sensitive to radiation damage than CFC in other phases of the cell cycle (27). In the present studies, there is evidence that in pre-irradiated mice, rIL-1 stimulates some CFC compartments into cell cycle. After rIL-1 injection, as determined by sensitivity to hydroxyurea, there was an almost 2 fold increase in the percentage of GM-CFC in S phase of the cell cycle. Similarly, endotoxin injection stimulates GM-CFC (11,22), as well as CFU-S (10), into cell cycle. Further studies on cell kinetics of CFU-S are needed to determine the possible direct or indirect effects of rIL-1 on CFU-S and how these may be related to the observed enhanced hematopoietic recovery in irradiated mice.

Results from the present studies demonstrate that survival and responses of CFC in bone marrow of irradiated and pre-irradiated mice are similar to responses after endotoxin injection. For example, injection of rIL-1 does not increase the mean survival time of lethally irradiated mice. However, there is an increase in the number of mice that survive after lethal irradiation when pretreated with murine or human rIL-1. Both human and murine rIL-1 promote earlier hematopoietic recovery after lethal and sublethal irradiation. In addition, in bone

marrow of pre-irradiated mice, rIL-1 injection decreases the number of CFU-E and stimulates GM-CFC into cell cycle. Thus, comparison of similarities and differences in response of CFC to endotoxin and rIL-1, and other cytokines induced by them,may be useful in delineating the physiological mechanisms determining radiation sensitivity and the recovery of irradiated stem cell populations.

FOOTNOTES

[1]Colony-forming cells (CFC); colony-forming units-erythroid (CFU-E); burst forming-units-erythroid (BFU-E); granulocyte-macrophage colony-forming units (GM-CFC), colony-forming units-spleen (CFU-S); recombinant interleukin-1 alpha (rIL-1); hydroxyurea (HU)

[2]Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

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Selecting Bedding Material

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PHYSIOLOGICAL FUNCTIONS IN an animal are the complex manifestations of the dynamic interaction of its genetic and environmental background. These functions are integrated into a complete but delicately balanced biological system, which is directly influenced by the physical, chemical, and microbial aspects of the animal's environment (*1*). Bedding material is one of the important controllable environmental factors which can have a profound effect on data generated in studies that utilize laboratory animals. Just as housing should provide animals with a physiologically and psychologically stress-free environment, bedding material, which is an integral part of housing, should be given the same consideration. Consultation between the investigator and the animal facility staff is therefore essential to ensure that the bedding material selected does not interfere with the organ system, physiological function, biochemical mechanism, or micro-molecular event being studied.

Many studies have been carried out on the specifications and criteria for bedding selection (*2–7*), and species preference studies have been conducted as well (*8–10*). Studies have also been conducted on some of the specific effects of bedding materials; for example, the factors causing endogenous effects (*11–28*), effects on reproduction (*29–33*), tumor incidence and carcinogenesis (*34–39*), and the effects of contamination (*40–43*). In addition, the inhalation of wood dust has been described as a possible cause of nasal cancer in woodworkers (*44, 45*). The biological effects of both cage cleanliness (cage changing frequency) and crowding (number of animals per cage) have also been described (*14, 23, 24*). All of these variables must be considered when examining the overall effectiveness of bedding materials. In addition, consideration must be given as to whether the bedding material is to be used directly or indirectly (in direct use the animal has intimate contact with the bedding material on a solid cage-bottom, whereas in indirect use the bedding is placed on a tray underneath a wire cage-bottom). Trade-offs between various desirable characteristics of bedding material will have to be made, and some of these characteristics may exclude certain materials.

Important evaluative criteria for the selection of bedding material are:

1. Type of laboratory animal: The material selected depends on the type of animal used and the type of caging that suits it best.
2. Compatability with research objectives: The material should not introduce into the study additional variables.
3. Availability: The material should be readily available throughout the year. A change in the type of bedding material used may add an unwanted variable to the study.
4. Non-nutritive/nonpalatable: The material should not be attractive for the animal to ingest, thus avoiding a source of nutrient variability.
5. Harmless to animals: The material should be free of toxic chemicals and pathogenic organisms as well as splinters that could cause traumatic injury.
6. Absorbent: The bedding material should be capa-

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This research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute for Laboratory Animal Resources, National Research Council.

ble of soaking up urine and fecal moisture to provide a dry, odor-free environment.

7. Comfort: The material should be a good heat insulator and a good nesting material.

8. Dust-free: The material should not have small particles that could act as fomites for disease transmission or irritate the respiratory system.

9. Disposability: The bedding material should be easily removed and replaced, thus preventing it from becoming a health hazard.

10. Cost: The material should be low-priced to help minimize overall animal maintenance costs.

11. Storage and transportability: The bedding material should be packaged in a manner that prevents vermin infestation and promotes easy handling by laboratory personnel.

Wood and wood byproducts are the most commonly used bedding materials. Softwoods, such as white pine, ponderosa pine, cedar, and aspen, and hardwoods, such as birch, maple, and beech, are used to produce bedding material that is available either as chips or shavings. These forms are available in a variety of sizes, including sawdust, which is usually composed of mixed woods. Beddings made from wood byproducts include paper chips, paper strips, plain, polylined, or deotized paper sheets, and waxed and unwaxed paper trays. Nonwood-derived bedding materials include corncobs ground to several different sizes, pellets made from various combinations of peanut hulls, sunflower seed hulls, ground corn cob, and alfalfa, liquids, foams, straw, and hay. The most commonly used bedding materials have been listed by type in Table 1. The table, which is based on the authors' experiences, shows what is available and whether one type of bedding material might be more desirable than another.

It is imperative that as much information as possible be collected on the characteristics of each type of bedding material. As technological advances in the animal bedding industry come about, questions must be asked of the manufacturers about these materials and the techniques for processing them. In addition, independent guaranteed analysis studies should be done. This literature review shows that much research has been done on the effects of certain bedding materials; there is, however, much more work to be done. Research and industry should work together to perform this additional needed testing.

Each of us has the capability within our respective institutions to study further the effects of these bedding

Table 1. Comparison of the characteristics of bedding materials used in biomedical research animal facilities.

	Absorbency		Dust-free		Endogen effect		Cost		Nesting material		Ease of disposal	
Application: Direct or Indirect	Dir	Ind	Dir	Ind	Dir	Ind	Dir	Ind	Dir	Ind	Dir	Ind
Hardwood chip	A	A	P	P	A	A	G	P	P	NA	A	P
Hardwood chip (heat-treated)	A	A	P	P	G	G	G	P	P	NA	A	P
Softwood chip	G	G	A	A	NR	NR	G	P	A	NA	A	P
Softwood chip (heat-treated)	G	G	G	G	G	G	A	P	A	NA	A	P
Cedar shavings	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Softwood shavings	G	G	A	A	NR	NR	A	P	G	NA	P	P
Hardwood shavings (heat-treated)	A	A	P	P	A	G	P	P	A	NA	P	P
Sawdust	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Paper chip	G	G	G	G	G	G	P	P	A	NA	A	P
Paper sheet	NA	G	NA	G	NA	G	NA	G	NA	NA	NA	A
Polylined paper sheet	NA	G	NA	G	NA	G	NA	P	NA	NA	NA	G
Deotized paper sheet	NA	G	NA	G	NA	A	NA	A	NA	NA	NA	A
Paper tray	NA	G	NA	G	NA	G	NA	P	NA	NA	NA	A
Waxed paper tray	NA	NE	NA	G	NA	G	NA	P	NA	NA	NA	A
Newspaper (printed)	NR	A	NR	A	NR	NR	NR	G	NR	NA	NR	A
Newsprint paper (not printed)	A	A	A	A	A	A	A	A	G	NA	P	A
Ground corncob	G	G	A	A	G	G	P	P	P	NA	A	P
Pellet	NA	G	NA	P	NA	A	NA	P	NA	NA	NA	P
Deotized pellet	NA	G	NA	P	NA	P	NA	P	NA	NA	NA	P
Bentonite (clay)*	NR	G	NR	P	NR	A	NR	A	NR	NA	NR	A
Liquid	NA	NE	NA	NE	NA	U	NA	A	NA	NA	NA	G
Foam	NA	NE	NA	NE	NA	U	NA	P	NA	NA	NA	G
Straw	A	A	A	A	A	A	G	G	G	NA	P	P
Hay	P	P	P	P	A	A	A	A	G	NA	P	P

Legend: G = good; A = average; P = poor; NR = not recommended; NA = not applicable; NE = not evaluated; U = effect unknown

*Bentonite should be used as a direct bedding only in litter boxes

materials on different species and strains of laboratory animals. Collaborative efforts, through information exchange, will help define further which material will do the best job and will have the least detrimental effect on the research data and on the animals themselves.

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