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SHORT COMMUNICATION

Induction of Marrow Hypoxia by Radioprotective Agents

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The ability of thiol and non-thiol radioprotectors to induce hypoxia was determined using the binding of [1H]misonidazole by bone marrow cells as a measure of hypoxia. When administered at maximally radioprotective doses, four drugs (WR-2721, cysteamine, 5-hydroxytryptamine, and 16,16-dimethyl prostaglandin E₂) significantly increased the amount of [1H]misonidazole bound by marrow cells, while no significant increase in binding was observed with three other agents (endotoxin, AET, superoxide dimutase). Doses of WR-2721 previously shown to provide suboptimal radioprotection did not significantly increase [1H]misonidazole binding. These results suggest that the physiological effects of some radioprotectors, that is, their ability to induce marrow hypoxia, may contribute to their efficacy in vivo.

INTRODUCTION

Many compounds which possess sulfhydryl groups have been shown to protect bone marrow from radiation injury. The most effective thiol radioprotective agent is ethiofos [S-2-(3-aminopropylamino)ethylphosphorothioic acid or WR-2721] (1). Several mechanisms of action have been postulated. These include hydrogen atom donation, OH radical scavenging, formation of mixed disulfides, metal chelation, and the production of hypoxia (2). Actual radioprotection may occur through a combination of these mechanisms. Studies using model biomolecule systems show that sulfhydryls do interact with radiation-induced free radicals, but direct evaluation in animal models has been more difficult to achieve because of the short half-life of various radical species. Nonetheless, it is believed that chemical mechanisms play a major role in the radioprotective effects of these drugs in vivo systems.

The possibility that the production of tissue hypoxia by radioprotectors contributed to the protective effects observed with these drugs has also been considered. Some radioprotectors produce profound physiological changes including hypertension, apnea, brachycardia, and altered blood flow (3). Many of these altered physiologies can be induced by WR-2721 (4) and also by 16,16-dimethyl prostaglandin E₂ (DiPGE₂) (5, 6). The biological responses to these radioprotectors may have an impact on marrow oxygenation. WR-2721 has been postulated to reduce the peripheral
oxygen tension (4). Tissue hypoxia may also explain the protection observed with certain non-thiol compounds, including 5-hydroxytryptamine (5-HT).

Direct evaluation of the role of marrow hypoxia in the radioprotection of the hematopoietic system has been hampered by the lack of a suitable method for quantifying tissue hypoxia in vivo. With current microelectrode technology, the $pO_2$ cannot be accurately determined in the marrow of small rodents. Attempts to correlate hypoxia with other physiological parameters such as blood flow or production of lactic acid are complicated since they may be influenced by changes unrelated to hypoxia. To overcome these problems and directly evaluate the role of marrow hypoxia in radioprotection we have used the sensitizer–adduct technique to assess the extent of marrow hypoxia following treatment with radioprotective agents. This system is based on the metabolic reduction and subsequent binding of misonidazole to tissues as a function of the tissue oxygen concentration (7, 8). Misonidazole (Miso) is preferentially bound by hypoxic tissue. Using Miso binding as a measure of tissue oxygenation, we have demonstrated that treatment with WR-2721, DiPGE$_2$, cysteamine (Cys), or 5-HT induces significant hypoxia in mouse bone marrow.

MATERIALS AND METHODS

Mice. Six- to twelve-week-old BALB/c female mice obtained from Jackson Laboratories (Bar Harbor, ME) were used in these experiments. Mice were housed in a Canadian Council on Animal Care accredited facility on a 12-h light/dark cycle. Mice were provided with a standard rodent pellet diet and with acidified water ad libitum.

$[^3]H$Misonidazole. $[^3]H$-labeled misonidazole ($[^3]H$Miso) (sp act 356.7 $\mu$Ci/mg) was prepared by Dr. James Raleigh as described (9). Animals received a single intraperitoneal (ip) injection of $[^3]H$Miso in a 0.5 ml volume of phosphate-buffered saline (PBS) according to the schedule detailed below. The dose of $[^3]H$Miso was calculated to achieve a peak plasma concentration of approximately 100 $\mu$M.

Drugs and treatment schedule. 16,16-Dimethyl prostaglandin E$_2$ was obtained in the free oil form as the generous gift of Dr. Douglas Morton (Upjohn Co., Kalamazoo, MI). Stock solutions were prepared by dissolution in ethanol to a concentration of 10 mg/ml and diluted to the indicated concentration in PBS prior to administration as a single subcutaneous injection into the nape of the neck in a 0.1 ml volume. WR-2721 was obtained from the Cancer Treatment Division of the National Cancer Institute. All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO) and were prepared in PBS immediately prior to use. Superoxide dimutase (SOD) was administered intravenously, while the remaining drugs were given as a single ip injection in a volume of 0.01 ml/g body wt. Mice were treated first with radioprotectors. Miso was injected at various intervals thereafter corresponding to the expected time of maximum radioprotective effect. Specifically, $[^3]H$Miso was administered 5 min after 5-HT (300 mg/kg); 10 min after DiPGE$_2$ (40 $\mu$g/mouse) or Cys (100 mg/kg); 15 min after 2-(2-aminoethyl)-2-thiopseudouridine dihydrobromide (AET) (300 mg/kg); 30 min after WR-2721 (200–400 mg/kg); 60 min after SOD (200 mg/kg); and 24 h after endotoxin (LPS) (50 $\mu$g/mouse). In each experiment, control animals received 0.01 ml/g of PBS (ip) followed by $[^3]H$Miso at intervals equivalent to those used for the radioprotectors.

Bone marrow assay. Sixty minutes after $[^3]H$Miso, animals (three to five per group) were killed by cervical dislocation and the femurs were removed. Marrow cells were isolated by flushing femurs of individual animals with ice-cold complete medium (MEM Spinner with 5% fetal calf serum). Known numbers of cells were placed in duplicate tubes and washed four times with complete medium to remove any unbound $[^3]H$Miso. Following the last wash, the acid-soluble and acid-precipitable cellular fractions were isolated according to techniques previously described (10), and the total amount of $[^3]H$Miso bound to these fractions was determined by liquid scintillation counting. The results calculated for individual animals were expressed as pmol $[^3]H$Miso bound per $10^6$ cells. Each drug was evaluated on two or three separate occasions. A Student's t test was used to determine significant differences between control and experimental groups.
RESULTS

Seven chemicals with known radioprotective properties were screened for their effects on $[^{3}H]$Miso binding to normal bone marrow cells in vivo. Cys, DiPGE$_2$, 5-HT, and WR-2721 treatment resulted in significantly increased binding to the acid-insoluble, macromolecular fraction (Fig. 1). No significant increase in marrow binding was observed following treatment with LPS, SOD, or AET.

In these studies, the dose and exposure time of each radioprotector duplicated that previously reported to produce maximum radioprotective effects (6, 11–16). We also determined the dose dependency of the degree of marrow hypoxia produced by increasing concentrations of WR-2721 by administering graded doses of WR-2721 (200–400 mg/kg) 30 min prior to $[^{3}H]$Miso. The results in Fig. 1 indicate that as the dose of WR-2721 was increased, the amount of $[^{3}H]$Miso bound to the marrow also increased. The response was statistically significant only at the optimal radioprotective dose of 400 mg WR-2721/kg of body weight.

DISCUSSION

The oxygen dependence of the metabolic reduction and covalent binding of misonidazole has been well described (7, 8). Because the $K_m$ for the oxygen inhibition of the binding is similar to the $K_m$ of the oxygen effect on radiosensitivity (8), misonidazole binding can be used as a reliable marker of radiobiological hypoxia. In this study, the binding of Miso to the macromolecular fraction of bone marrow cells was used
to indirectly assess the extent of hypoxia produced by treatment with radioprotective agents. Previous studies showed that increased binding of Miso occurs to both the small molecular weight species found in the acid-soluble fractions and to macromolecules such as DNA which are associated with the acid-precipitable fraction (10). The ratio of bound products between the acid-soluble and -precipitable fractions is dependent upon cell line and handling procedures, but the binding kinetics is similar for both fractions (10, 17). In this study, only the binding to the macromolecular fraction was considered to avoid possible problems in interpretation arising from an increased concentration of small molecular weight thiol species to which \(^{3}H\)Miso is capable of binding, although this would not be a problem for non-thiol containing compounds such as 5-HT or DiPGE₂.

Four of seven agents tested (WR-2721, DiPGE₂, Cys, 5-HT) produced significant increases in \(^{3}H\)Miso binding, suggesting that the marrow of animals treated with these drugs would be less oxygenated at times associated with peak radioprotective effects. In support of this hypothesis is evidence that various physiological and chemical properties of these drugs are such that they may contribute to the production of relative marrow hypoxia. For example, the largest uptake of \(^{3}H\)Miso occurred in bone marrow cells from animals receiving DiPGE₂. This concentration of DiPGE₂ produces a 16% increase in hematocrit (6) and a 30% reduction in breathing rate (18) 15 min after administration. A dose-dependent reduction in peripheral blood flow with blood pooling in the spleen also has been noted for WR-2721 (4). Similarly, the hypotensive and vasoactive effects of 5-HT (15) and Cys (19) have been described. Changes in blood flow distribution and respiration rate might reasonably be expected to have indirect effects on the oxygen supply of the marrow. In addition, a chemical reduction in the amount of oxygen available to the marrow may directly contribute to hypoxia. WR-1065, the dephosphorylated product of WR-2721, consumes oxygen in an in vitro system (20). Our preliminary results measuring the oxygen consumption of marrow cells treated in vivo with WR-2721 suggest that increased oxygen consumption also occurs in the host animal. Other thiol containing compounds such as Cys have been shown to undergo autooxygenation in vitro (21); however, their ability to produce the same effects in vivo have yet to be evaluated. These physiological/chemical drug effects coupled with the known dependence of Miso binding on oxygen concentration are consistent with the hypothesis that the observed increase in \(^{3}H\)-Miso binding was a consequence of a shift of marrow oxygen tension to lower values. A counterargument to this proposal is the possibility that these drugs affect the in vivo reduction of Miso which is independent of any changes in marrow \(pO_2\). However, this latter possibility is unlikely because (1) the addition of exogeneous thiols has been shown to decrease, not increase, the binding of misonidazole to cellular macromolecules (7), and (2) even if these drugs were to increase the metabolism of Miso (through the stimulation of nitroreductase enzymes, for example), the resultant reactive species would be oxidized back to the parent compound under conditions of normal marrow \(pO_2\).

In the case of WR-2721, the increase in marrow binding was found to be dose dependent, with only the optimally protective dose (400 mg/kg) producing significantly increased binding. However, other studies have demonstrated that smaller doses of WR-2721 (75–300 mg/kg) afford some marrow protection (22). This suggests
that several mechanisms contribute to the radioprotective effects of WR-2721, and that at low drug doses, chemical rather than physiological mechanisms predominate. Similarly, the failure of AET or SOD to alter \[^{[H]}\text{Miso}\] binding suggests that any physiological changes produced by these drugs were insufficient to render the marrow relatively hypoxic and that chemical and/or alternative physiological changes must be postulated to account for their radioprotective properties. In the case of LPS, less \[^{[H]}\text{Miso}\] binding was observed in drug-treated animals than in controls. The reason for this has yet to be determined. However, the possibility that LPS treatment altered the pharmacokinetics of Miso or resulted in increased oxygen delivery to the marrow should be considered.

Several investigators have drawn attention to the fact that protection with WR-2721 appears to be most effective for cells or tissues that are at intermediate oxygen tensions (23, 24). In such cases, it has been postulated that small reductions in oxygen tension are sufficient to render the tissues hypoxic. Previous studies by Meyn and Jenkins (25) which measured the induction of DNA strand breaks in vivo and in vitro have suggested that marrow resides at relatively low oxygen tensions in vivo. In such a case, small shifts in the availability of oxygen to critical hematopoietic targets may exert protective effects. Confirmation of the hypothesis that small changes in physiological oxygen concentrations produce radiobiological hypoxia awaits techniques for accurately measuring the in vivo \(pO_2\) of cell populations.

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REFERENCES


CELL-CYCLE RADIATION RESPONSE: 
ROLE OF INTRACELLULAR FACTORS

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ABSTRACT

We have been studying variations of radiosensitivity and endogenous cellular factors during the course of progression through the human and hamster cell cycle. After exposure to low-LET radiations, the most radiosensitive cell stages are mitosis and the G1/S interface. The increased activity of a specific antioxidant enzyme such as superoxide dismutase in G1-phase, and the variations of endogenous thiols during cell division are thought to be intracellular factors of importance to the radiation survival response. These factors may contribute to modifying the age-dependent yield of lesions or more likely, to the efficiency of the repair processes. These cellular factors have been implicated in our cellular measurements of the larger values for the radiobiological oxygen effect late in the cell cycle compared to earlier cell ages. Low-LET radiation also delays progression through S phase which may allow more time for repair and hence contribute to radiotolerance in late-S phase. The cytoplasmic and intranuclear milieu of the cell appears to have less significant effects on lesions produced by high-LET radiation compared to those made by low-LET radiation. The repair of lesions evident at all cell ages; however, high-LET particles cause a more profound block in G1 phase than that observed after low-LET radiation. Hazards posed by the interaction of damage from sequential doses of radiations of different qualities have been evaluated and are shown to lead to a cell-cycle-dependent enhancement of radiobiological effects. A summary comparison of various cell-cycle-dependent endpoints measured with low- or high-LET radiations is given and includes a discussion of the possible additional effects introduced by microgravity.

INTRODUCTION

The quantitative understanding of long-term effects of the components of space radiation on carcinogenesis and nervous system functions depends on the nature of the molecular injury produced by particles of various atomic numbers and energies. Of particular interest is the functional dependence of individual lesions, their repair and misreplication, and the time-dependent interactions that can occur at low dose rates.

Many types of cancer cells have chromosomal defects, frequently including band deletions or reciprocal translocations /1/. Correlations have been made between what are termed "inherited fragile sites" and the chromosomal breaks involved in the structural rearrangements associated with some neoplasms /2,3/. It has been suggested that certain genes of differentiated cells require submicroscopic rearrangements for normal functional activity, and these changes could provide a mechanism whereby unique chromosome sites become inherently susceptible to further rearrangements caused by various chemical or physical agents in specific cell types /4/. Thus, both inherited genetic susceptibility as well as normal programmed genetic functions occurring during cell division could contribute to the vulnerability of cellular targets to damage from ionizing radiations.

Of serious concern in the space environment are particulate radiations that are capable of causing an assortment of types and degrees of chromosomal damage depending on particle charge and energy, and the cellular targets at risk. Lesions produced by particles from protons to uranium can result in minor to severe chromosomal aberrations /5,6/. There is a high probability that, especially where low doses are concerned, the chances are increased for cellular survival, and therefore there is a greater likelihood for expression of transformation events.

Specific intracellular factors, including for example antioxidant enzymes like superoxide dismutase (SOD) and catalase (Catalase), are known to inhibit carcinogenic transformation by X-rays and certain chemotherapeutic drugs like bleomycin, and are also known to reduce the promotion action of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) in vitro /7/. This suggests that oncogenic action can be mediated in part by free radicals and that antioxidants can inhibit oncogenesis and late events in the progression of cell transformation associated with promotion. Additional evidence indicating these factors may contribute to variations
in cell competence to transformation is that the cell content of SOD and CAT is reported to vary from one cell type to another /8/.

We have been studying human and other mammalian cells in culture that are synchronized with respect to their stage in the cell cycle. Our goal is to investigate whether cells are most sensitive to modifications that can influence radiation survival and can also ultimately lead to mutation or transformation. We have used split-dose techniques to elucidate the significance of the cell cycle to the enhancement of particle effects. In order to understand the mechanisms underlying these effects we have begun characterizing intracellular factors that are thought to play a role in detoxifying radical-induced damage. We report here a study demonstrating cell-stage dependent enhancements of the lethal effects of split-doses of charged particle beams of low- and high-linear energy transfer (LET). We include cell age-dependent measurements of SOD enzyme activity and a speculative discussion of the relevance of this antioxidant and other intracellular factors to the ultimate effects of ionizing radiations.

**METHODOLOGY**

Human T-1 cell fibroblasts cultured in vitro were used in these experiments. The cell line was originally established by Van Der Veen et al. /9/ and was kindly provided to us by G.W. Barendsen of the TNO Radiobiology Laboratories, Rijswijk, The Netherlands. The cells were maintained in minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum and were grown at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The concentration of glutamine (0.25 g/liter) was doubled for experimental cultures which were not replenished with fresh media during incubation to measure survival. Routine screening for mycoplasma has indicated that the cultures are free of contamination.

Experiments were completed with exponentially growing cell populations, as well as those synchronized by mitotic selection. The techniques used to obtain large numbers of highly synchronized cells in various phases of the cell division cycle have been described in detail elsewhere /10/. The cell cycle time is approximately 20 hrs. In each experiment the quality of the synchrony was evaluated by cell volume distributions, and cell progression was monitored by autoradiographic analysis of cells pulse-labeled for 20 min with [3H]thymidine at various times after mitotic selection. Representative autoradiography data from a typical experiment are given in Figure 1. Cell populations at two ages post-mitotic selection were irradiated for survival studies: mid G1-phase (3 hours) and late-S phase (14 hours).

![Fig. 1. Representative autoradiography data from unirradiated human T-1 cell populations pulse-labeled for 20 minutes with tritiated thymidine at various times after synchronization. Data are from one of the experiments reported here, and the ages and incorporation levels of the synchronized populations studied are indicated.](image-url)

The level of activity of the antioxidant enzyme superoxide dismutase (SOD) was measured in unirradiated populations of synchronized cells. Mitotically-selected cells were plated into 75 cm² tissue culture flasks and were either processed immediately or were allowed to progress at 37°C to appropriate ages throughout the cell cycle. SOD activity levels are therefore reported for populations synchronized at three cell ages post mitotic selection: M phase (0 hours), G2 phase (4 hours) or mid S phase (12 hours). At the appropriate time the attached cells were trypsinized and washed twice in a phosphate buffered saline (PBS) solution containing EDTA but free of calcium and magnesium. Aliquots were counted with a Coulter Counter to determine the cell concentration. The remaining cells were transferred to 1.5 ml plastic vials and rapidly frozen to -50°C. Cell protein was extracted by the addition of digitonin (2.5 mg/ml) immediately after a rapid thawing of each sample. Aliquots of the supernatants of the digitonin-treated samples were used for protein and enzyme assays.

Total superoxide dismutase (SOD) activity, including Cu/Zn SOD and Mn SOD, was determined according to the method of McCord and Fridovich /11/. Total cellular protein was determined by the Coomassie brilliant blue dye binding method /12/ using standard protein reagents from Bio-Rad. The mean of the ratio of enzyme activity (U/mg total protein) and protein concentration (mg/10⁶ cells)
cells) from three separate measurements of both enzyme activity and protein concentration are reported in each of two experiments. The standard error of this ratio reflects the variance associated with both enzyme activity and protein concentration (mg/10^6 cells) from three separate measurements of both enzyme activity and protein concentration are reported in each of two experiments. The standard error of this ratio reflects the variance associated with both enzyme activity and protein concentration (mg/10^6 cells) 

For radiation exposures, cells were distributed into tissue culture flasks and allowed to attach as a monolayer. The cells were irradiated through the wall of the flask. Heavy-ion exposures were made at the Bevalac facility. Parallel-plate ionization chambers were used for dose measurements. A typical Bragg ionization curve is given in Figure 2. Exposures were made in the plateau (dose-average LET = 32 keV/um) and in the Bragg peak (dose-average LET = 183 keV/um) of the beam. The physical properties of the particle beams including the calculated LET distributions of the primary particles and the fragments have been described elsewhere (14,15). In the case of experiments with synchronized cells, at the appropriate culture age, flasks were removed from the 37°C incubator, were cooled down to 4°C to prevent the cells from further progression, and were transported to the Bevalac facility for irradiation. Unirradiated control cultures were handled in a similar fashion. In some experiments the cells received split doses of the plateau ions (low LET), or split doses of the Bragg peak ions (high LET), or split doses of high-LET followed by low-LET radiations. Split-repair times varied from one hour to 24 hours. Control samples were unirradiated, or received only the first dose fraction, or received both dose fractions in an immediate sequence.

When irradiations were completed, the samples were returned to 37°C and assayed for colony-forming ability by standard techniques. Cells were trypsinized, resuspended, counted, plated into MEM, and incubated at 37°C in a 95% air and 5% CO₂ incubator for 10 to 12 days. Colony-forming ability was scored by staining and fixing the cultures with 1% methylene blue in 30% ethyl alcohol. Clones containing at least 50 cells were scored as survivors. Colony counts on four or more 25-cm² tissue culture flasks were averaged for each data point. Eight or more flasks were used for control and high dose samples. Survival curves were obtained from computer-fits of the data to the linear quadratic model of cell inactivation using programs developed by N.W. Albright (16).

RESULTS

Fig. 2. Bragg ionization curve of Neon 425 MeV/u ion beam. Cellular monolayers were placed at the depths indicated in the entrance plateau and in the Bragg peak.

Split-Dose Effects of Low- or High LET Particles on Survival

Dose-response curves for exponentially growing human T-1 cell populations irradiated with single- or split-doses of either plateau or Bragg peak ions from a neon beam with an initial energy of 425 MeV/u are presented in Figure 3. The fractionation interval was 24 hours. The data indicate that there is split-dose recovery of the damage from the low LET particles, but no recovery is observed after irradiation with ions of the Bragg peak. The kinetics of split-dose recovery were studied at shorter time intervals. Figure 4 is a compilation of data from two replicate experiments to investigate split-dose effects of 425 MeV/u neon plateau or Bragg peak ions where the time between the two doses was one to twenty-four hours. The data show dose-dependent recovery in the plateau, but no recovery in the Bragg peak.

The split-dose effects of low- and high LET neon beams on synchronized cell populations are presented in Figure 5. Populations of cells in mid G1 phase or late S phase at the time of the first dose were studied. In agreement with the asynchronous experiments, the data show split-dose recovery of damage from plateau ions and a lack of recovery from Bragg peak ions.
Fig. 3. Dose-response curves for asynchronous cell populations irradiated with single doses (solid symbols) or split doses (open symbols) or either plateau (circles) or Bragg peak (triangles) ions. Different symbols represent different experiments. The fractionation interval was 24 hrs.

Fig. 4. Kinetics of split-dose recovery of asynchronous human T-1 cells irradiated with 425 MeV/u plateau or Bragg peak ions. Experiments were repeated twice. The data show dose-dependent recovery in the plateau, but no recovery in the Bragg peak.

Fig. 5. Effects of 425 MeV/u Neon plateau (•) or Bragg peak (△) ions on synchronized cell populations in mid $G_1$-phase or late S-phase at the time of the first dose.
Fig. 6. Dose-response curves for late-S-phase cells cooled to ice temperatures and irradiated with single doses of plateau ions alone (■), or single doses of Bragg peak ions alone (○). Some cells were also irradiated with Bragg peak ions at room temperature (△). Also shown are the survival data for cooled cells irradiated sequentially with 2.7 Gy Bragg peak ions followed within 2 minutes by graded doses of plateau ions (○). Survival expected from the independent action of the two radiations (---).

Fig. 7. Survival data measured with populations of cells synchronized either in mid G₁-phase or late S-phase at the time of a first dose of Bragg peak ions followed at various times by a dose of plateau ions. The stars represent the survival level expected from the independent action of each radiation.

Enhancement of Cell Killing from Sequential Doses of High- and Low-LET Radiation

Dose-response curves for late-S phase cells irradiated with plateau neon ions alone, or with Bragg peak ions alone are shown in Figure 6. The cells were cooled to ice temperature just before irradiation to inhibit cell progression. Repair processes may have also been inhibited. The survival data from cultures irradiated in the Bragg peak at room temperature also are shown however and are not different from identical cultures irradiated at the reduced temperature. The survival curves show clearly that the late-S-phase cells are resistant to radiation damage from low LET neon ion beams in the plateau of the Bragg curve, and more sensitive to doses in the Bragg peak. Included in this study are survival data for cells precooled on ice and irradiated sequentially with 2.7 Gy Bragg peak ions followed within two minutes by graded doses of plateau ions. The survival of this cell population was much less than that expected from the independent action of the two radiations (shown as a dashed line on Figure 6).

To explore further the age-dependence of the enhanced lethal effects from sequential doses of high-LET and low-LET ion beams, survival data were obtained from populations of cells synchronized either in mid G₁-phase or late-S-phase at the time of a first dose of Bragg peak ions, followed at various times by a dose of plateau ions (Figure 7). Comparisons of the measured survival with the survival expected from the independent action of each radiation were made. The data show that G₁-phase cells are more sensitive than expected and become even more radiosensitive with a 2-hr interval between the two doses as the cells move to the G₁/S border. The cells then show increasing radioresistance that levels off with an 8-hr fractionation interval at a survival fraction that is equivalent to what would be expected initially from the independent action of the two radiations. The late-S-phase cells are also much more sensitive than expected with the combined radiation treatment, but become more resistant with split-times up to 4 hrs and then more radiosensitive when the cells divide and enter G₁-phase. The cell progression was confirmed with results obtained using flow cytometry methodology (data not shown).
Cell Age-Dependent SOD Activity

In three replicate experiments, exponentially growing human T-1 cells were found to have a total (Cu-Zn- and Mn-containing) SOD enzyme activity level of 21.3 ± 1.9 U/mg total protein (mean ± std. error). Biochemical results from experiments with synchronized cell populations show high quality of synchrony with a 50% reduction in cellular protein content data with a significant peak of 30 U/mg total protein in G1 phase compared to 17 U/mg total protein in mitosis. Intermediate levels of protein content and SOD activity were measured for S-phase cells. The observation of a peak of SOD activity in G1 phase has also been found in synchronized Chinese hamster cells /17/.

DISCUSSION

Particle Split-Dose Effects

The synergistic effects of low- and high-LET radiations have been reported in sequential /18-24/, as well as simultaneous /25,26/ exposures of exponentially growing asynchronous mammalian cells to radiations of different qualities (e.g., low- and high-LET). The cell killing effects due to damage interaction after sequential irradiation with a high-LET particle beam and X-rays have also recently been reported to vary throughout the Chinese hamster cell cycle/27/. The greatest effect was observed in late-S-phase which was most resistant to either of the radiations. In this paper we report that human T-1 cells show a dose-dependent recovery from split doses of plateau 425 MeV/u neon ions (32 keV/μm). Most of the recovery is completed within 6-8 hours. In contrast, no recovery is measured in split-dose experiments with Bragg peak neon ions (183 keV/μm) even after a 24 hr fractionation time. Secondly, split-dose experiments with synchronized cell populations indicate cell-cycle dependent differences in recovery kinetics from plateau neon-ion damage, while both mid and late S-phase cells show no recovery from Bragg peak damage. Finally, we show that sequential doses of Bragg peak ions followed immediately by doses of plateau neon ions are more effective than expected from the independent action of the two radiations. This effect is dependent on the age of the cell at the time of the high-LET radiation exposure, and also on the fractionation interval before the exposure to the low-LET radiation.

Our data clearly show that there are differences in the effects of split-doses of low- and high-LET radiations and, also, that split-dose effects are processed differently by cells in G0- and in S-phase. Under certain conditions enhancing effects occur between low- and high-LET radiation dose components. The simplest explanation for the data in Figures 4 and 5 is that single high-LET particle tracks produced by ions in the Bragg peak are by themselves sufficient to kill cells. This may be because each of these tracks is capable of producing several DNA lesions in close proximity along the track, which then may interact resulting in enhanced mistargeting /28/. The lethal effects of high-LET radiation, hence, become somewhat independent of the temporal sequence of its administration. Low-LET neon ions produce more diffuse lesions. Lesions produced by the first dose are more likely to be repaired, or otherwise modified, before more lesions are produced by the second dose.

Data such as are shown in Figures 6 and 7, indicate that high-LET tracks made by particles in the neon Bragg peak produce sublethal lesions in cell nuclei. These lesions can then interact with a subsequent low-LET dose. Why there is a time-dependent structure of the effects shown in Figure 7, and why these effects are different in G0- and in S-phase is not completely understood at present. One possibility is that the high concentration of cellular factors including SOD activity in G0-phase reduces, via a decrease in oxygen radicals, the concentration of DNA lesions produced by low-LET radiations. In contrast, as shown in Figure 8, the activity of SOD is reduced in late-S-phase, and therefore, the enhancing effects of low- and high-LET radiations can be greater. Another possibility is that the first high-LET dose may perturb the cellular radioprotective factors in the period following the exposure to the neon peak. Further work is needed to explain the molecular mechanisms underlying these effects and to investigate additional cell-cycle dependent modifications of these effects with time between the two radiation doses.

Radiation Age Response: Intracellular Factors

It has been known since the early 1960s that there are variations in the radiosensitivity of cultured mammalian cells at different phases of the division cycle /29-32/. In the late 1960s and early 1970s high-LET radiations, including fast neutrons, alpha particles, pions and heavy-ion particle beams, were found to diminish the amplitude of the variation between the most sensitive and the most resistant phases (see /33/ for review). Rodent cell lines with a short G0 phase (about 2 h) were primarily used in these studies which indicated that the reduced age response was still qualitatively similar to that of low-LET radiations in that cells at (or near) mitosis and at the G1-S phase interface are more radiosensitive than late-S phase cells.
In human cells which have generally a much longer G\textsubscript{1} phase, two peaks of radioresistance to low LET radiation are usually seen, one in G\textsubscript{1} and one in S-phase /34/. Recent work has indicated that high-LET radiations eliminate the X-ray-resistant peak of survival at early G\textsubscript{1}-phase in synchronized human cells, but not the resistant peak at late-S-phase, thus suggesting a differential age dependence of radiolesions and/or repair effects depending on radiation quality /35-37/.

Early attempts to correlate a single molecular factor or activity like DNA synthesis as an explanation for the age dependence of radiosensitivity through the cell cycle proved to be inadequate since it failed to explain radioresistance in G\textsubscript{1} phase. Based on measurements of sulfhydryl variation that indicate non protein sulphydryls are greatest in G\textsubscript{1} phase, whereas glutathione levels are greatest in S-phase /13,39/, Sinclair proposed a factor "0" that may be some small fraction of the intracellular pool which varied as a function of cell cycle position to control cell survival /40/. We have recently demonstrated that cell age-dependent fluctuations in glutathione (GSH) and glutathione disulfide in human T-1 cells are correlated with changes in X-ray oxygen enhancement ratio (OER) for cell survival /41/, however, this effect may be a consequence of fluctuations in GSH and/or other cellular factors resulting in differences in the initial damage and/or the cells' ability to repair these lesions.

DNA damage, measured as DNA strand breaks, has been shown to be readily modified by GSH concentrations at oxygen levels typically found in tissues /42/. The types and yield of radiation-induced base damage are clearly dependent on the oxygen concentration as demonstrated from model system studies using nucleic acids /43/. DNA conformation also can influence the yield and type of base lesions formed from OH radical attack on DNA /44/. In cellular studies, Devey et al. /45/ examined changes in radiosensitivity and dispersion of chromatin during the cell cycle of synchronous Chinese hamster cells. They found that the decrease in X-ray radiosensitivity as cells moved from mitosis to S-phase correlates well with the dispersion of chromatin that occurs during these parts of the cell cycle. They hypothesized that based on DNA fiber dimensions and how tightly they are packed, the probability for lesion interactions between lesions could explain the similarity of sublethal and potentially lethal lesion repair kinetics. They could not account for the increase in radiosensitivity at the G\textsubscript{1}/S interface. With regard to high LET lesions it is likely that the increasing dispersion of chromatin /45,46/ and/or its association with membranes /47/ through the G\textsubscript{1}-phase nucleus as the cell ages could result in changes in the volume or distribution of critical targets irradiated with particle beams compared to X-rays /10/ and thereby explain LET-differences in relative radiosensitivity.

If we return to the concept of the age dependency of the cell's ability to repair lesions, the cellular levels of GSH may contribute to the maintenance of the cellular redox capacity, a prerequisite for normal metabolism to occur. Further studies on the importance of redox state in radiosensitization, however, are needed. The isolation of radiation repair deficient mammalian mutant cell lines, notably a Chinese hamster cell line that is extremely sensitive to killing by gamma irradiation in the G\textsubscript{1} and early S phases of the cell cycle, but which has normal resistance in late S phase /48/, has significantly enhanced our understanding of the role of GSH in radiation resistance.

Fig. 8. Human T-1 cell-cycle stage dependence of total cell protein content in mg per 10\textsuperscript{6} cells (left panel) and total SOD enzyme activity per mg of total protein (right panel). The data are from two separate experiments. Error estimates represent in each case the standard error of the mean of three replicate samples.
standing. The mutant is able to repair single-strand DNA breaks (ssb), but is deficient in the repair of double-strand DNA breaks (dsb) produced by γ-irradiation during the sensitive G2- and early S-phase. In the resistant late-S period, repair is nearly the same for both the mutant and parent cell types. This correlation between γ-ray sensitivity and repair, strongly suggested that an inability to repair double-strand DNA breaks in G2-phase is the basis for the hypersensitivity of the mutant to the lethal effects of γ-rays during this phase of the cell cycle. These researchers have stated that their work also implies that in normal cells there are at least two pathways for the repair of double-strand DNA breaks, one of which functions primarily in late-S phase, and the other, either throughout the cell cycle or only in the G2- and early-S phases. Radiosensitive mutant Chinese hamster ovary cell lines isolated by Jeggo and Kemp /49/ have also recently been shown to have a detect in double strand-break rejoining /50/. Additional work involving genomic DNA transfection experiments have shown that after genome uptake, revertant cells are able to survive γ-irradiation as well as the wild type CHO line, and in one of the cells studied, normal dsb repair and the normal reversible nature of the G2-block in division was restored /51/.

Delays in cell division from exposure to low-LET ionizing radiations are known to prolong the duration of S phase and cause a G2-phase delay. A pronounced increase in the effectiveness of high-LET radiations in delaying cells in G2-phase has been reported /52/. In space under the combined stresses of exposure to transit vibration and acceleration and radiations of different LET, alterations in cell progression could be further altered by microgravity. It has been reported that at high g, cells divide faster at the expense of reduced motility, since energy consumption remains the same, while in microgravity, lymphocytes show a dramatic reduction in proliferation rate, reduced glucose consumption, but a strong increase of energy consumption /54/. WI-38 embryonic lung cells which do not undergo differentiation steps grow and move normally at zero gravity, but they also consume less glucose /54/. This has led to the conclusion that cells are sensitive to gravity, especially those which are differentiating.

Alterations caused by weightlessness on the effect of radiation are the most part been in the form of an enhancement of the radiation effect /55/. This is especially noted in the direct genetic studies in which there is enhancement in a large number of cases involving chromosome breakage and rejoining /56-58/. Developmental anomalies are also enhanced by weightlessness, related very likely to genetic alterations /59-61/, as perhaps is also the significant loss of xanthine dehydrogenase activity in Drosophila larvae /62/.

New satellite bioexperiments to be discussed at this meeting will likely contribute to our basic understanding of the molecular mechanisms operating under weightlessness conditions which are responsible for altering chromosome damage from radiation. Further experimentation is needed to systematically evaluate the consequences of the combined stresses of weightlessness and the damage interaction of radiations of different quality on various phases of the cell division cycle. We have reported preliminary measurements of cell age-dependent activity of SOD enzyme that indicate a peak level in G2-phase prior to DNA synthesis. Gamma-radiation induction of SOD enzyme activity has been reported /63,64/ as well as the radiosensitivity of Drosophila larvae with a highly active SOD allele /65/. It may be important to explore radiation-induced alterations in SOD enzyme activity as a part of the modification of radiation response, especially under the stresses of the space environment.

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REFERENCES

8. C. Borek, R. Miller, C. Pa\textsuperscript{1} Natl. Acad. Sci. USA 76, 1800 (1979).


Characteristics of Radiation-Induced Performance Changes in Bar-Press Avoidance With and Without a Preshock Warning Cue

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Hierarchicalbold Rush and W. A. Hunt. Characteristics of radiation-induced performance changes in bar-press avoidance with and without a preshock warning cue. Pharmacol Biochem Behav 33(3): 549-554, 1989. — Rats were trained to perform one of three tasks in which responses on a lever delayed the onset of footshock for 20 sec. One task provided a warning tone beginning 15 sec after the last response on the lever and lasting for 5 sec just prior to the presentation of a shock (fixed-interval signalled avoidance), while a second task provided no external cues (unsignalled avoidance). The third task was similar to the fixed-interval signalled avoidance task, except that the warning tone preceding shock began at varying intervals after the last response on the lever (variable-interval signalled avoidance). Animals trained on the signalled avoidance paradigms received fewer shocks than those on the unsignalled avoidance paradigm. After 10 krad of gamma radiation, animals performing on either task with cues were less able to avoid shock, although they recovered somewhat over a 90-min period. The animals not provided cues also experienced more shocks during the first 10 min after irradiation but were relatively less affected in performing the task. Response rates on the bar and the patterns of responding on these tasks were not significantly different after irradiation, except that animals responded after the onset of shock more often after irradiation than before. These results suggest that rats will continue to effectively use task-related cues after irradiation, but that the relative degree of behavioral decrement may depend on the initial level of performance or possibly the complexity of the task.

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<th>Performance</th>
<th>Ionizing Radiation</th>
<th>Avoidance Cues</th>
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Behavioral deficits are commonly observed in laboratory animals after high doses of ionizing radiation and have been found as degraded performance on a number of tasks (6). Behavioral abnormalities have been observed in victims of a number of nuclear accidents, including the one at Chernobyl (4, 7, 8). Although some of these responses could have resulted from generalized trauma, they might reflect an effect of ionizing radiation on behavior. This laboratory has been studying the ability of rats to actively avoid shock and how exposure to ionizing radiation can disrupt this behavior. Initial studies involved using a task in which animals learned to jump up onto a ledge to avoid an electrical foot-shock (5). Auditory cues were provided to alert subjects to an impending shock. The results demonstrated that doses of 2.5 to 20 krad of high-energy electrons or gamma photons degraded the performance on this active avoidance task in a dose-dependent manner. Escape behavior was unaltered. Furthermore, electrons were more effective than photons in disrupting this task.

In an attempt to characterize this effect, additional experiments were undertaken to determine whether the animals were capable of performing the required movements and whether they would ask for and use visual and auditory cues to enhance performance (2). A paradigm was used that involved responses on two levers each with different consequences (1). When one lever was pressed, an electrical shock occurring at 5-sec intervals was postponed for 20 sec. Pressing the other lever activated a visual cue (overhead light) for a 60-sec period, during which an auditory cue (tone) occurred 5 sec before the presentation of each shock. The animals rapidly learned to respond for the tone and to use it to effectively avoid shock.

A 10-krad dose of gamma photons severely disrupted the ability of animals to perform this task (2). Almost immediately after irradiation, the animals received significantly more shocks than controls. However, the animals could readily execute the required movements of pressing a bar. In fact, responding on the lever to avoid shock increased, but mostly just subsequent to the onset of shock. In addition, irradiated subjects did not continue to respond to produce the visual and auditory cues. In other words,
instead of responding to the cues, the animals responded to the shocks. When subjects did use the tones after irradiation, they did so in a way which suggested that they detected the cues and were able to respond to them appropriately. In other experiments, animals were shown to receive increased shocks after doses of radiation as low as 2 krads (unpublished observations).

In the present experiments, we attempted to determine whether the presence of temporal and sensory cues influenced an animal's performance after irradiation. In one experiment, rather than require the animals to specifically respond for preshock warning cues as in the previous study (2), these auditory cues were always available (fixed-interval signalled avoidance). In another experiment, no cues other than temporal ones were available (unsignalled avoidance). A third experiment provided no predictability of the onset of shock based on temporal cues. Instead, the subject received the same average number of preshock warning cues as in the experiments using the fixed-interval signalled avoidance paradigm, except that the time of onset of the warning signal after a response was unpredictable temporally (variable-interval signalled avoidance).

METHOD

Thirty-six male Long Evans (Blue Spruce) rats (300 g) were the experimental subjects. Rats were quarantined on arrival and screened for evidence of disease by serology and histopathology before being released from quarantine. The rats were housed individually in polycarbonate isolator cages (Lab Products, Maywood, N.J.) on autoclaved hardwood contact bedding (Beta Chip Northeastern Products Corp., Warrensburg, N.Y.) and were provided commercial rodent chow (Wayne Rodent Block, Continental Grain Co., Chicago, Ill.) and distilled water (pH 2.5 using HCl) ad lib. Animal holding rooms were kept at 21 ± 1°C with 50 ± 10% relative humidity on a reversed, 12-hr, light-dark lighting cycle with no twilight.

The apparatus and experimental designs were similar to those previously described (2), except as indicated below. Prior to the first training session, animals were placed in the operant chambers for at least 2 hr to familiarize them with the apparatus. Thereafter, each experimental session lasted 4 hr. The animals then were trained to a 0.5-sec, scrambled, electrical foot-shock (1.0 ma) by responding on the left lever. Responses on the right lever had no scheduled consequence in this study. A single response postponed the onset of shock by 20 sec. In the absence of responding, shock occurred at 5-sec intervals. Twelve of the rats received a 5-sec warning tone just prior to the scheduled presentation of a shock (fixed-interval signalled avoidance) (9). In this group, the onset of the warning tone always followed the last response on the lever by 15 sec. Another 12 rats received the same preshock warning tones, except that the interval between a response on the lever and the onset of the warning cue before the next scheduled shock varied with equal probability between 0.5 and 120 sec. The mean interval was 15 sec (the same as the interval in the fixed-interval signalled-avoidance group) masking the time of the onset of the warning tone in this group effectively unpredictable (variable-interval signalled avoidance). The last 12 rats were trained similarly, except no warning tones were provided (unsignalled avoidance) (10). Training was complete when the animals could successfully avoid more than 90% of the shocks that could be presented (12 min).

During the warning tone, a response on the lever terminated the warning tone and reset the response to tone interval. Responses made during shock presentation terminated both shock and warning tones and also reset the response to tone interval response to shock interval in unsignalled avoidance. In the absence of a response in either signalled condition, shock onset followed the onset of the preshock warning tone by 5 sec. If no response was made during the shock, the tone and shock terminated simultaneously 0.5 sec after shock onset.

After training, subjects were habituated to the effects of interrupting the schedule and transporting them for irradiation. After 2 hr of performing the task on which the animals were trained, the session was suspended with the tone and response lever disabled. The animal was placed in a Plexiglas restraining tube, transported to the Co source, and returned without being irradiated. The session then resumed. This procedure was repeated daily until there was less than a 10% difference in the number of shocks received and in the number of responses made during the next hour, compared with those during the hour before removing the animals from the conditioning chambers.

After habituation, each group of animals who learned the fixed-interval signalled avoidance, unsignalled avoidance, or variable-interval signalled avoidance tasks was randomly divided into two subgroups, composed of six animals each. From each group of trained animals, one subgroup was irradiated with a single bilateral dose of 10 krads of gamma radiation from a Co source at a rate of 6.6 krads/min. Control subgroups were handled identically, except they were not irradiated. The transport time from the radiation facility to the conditioning chambers was less than 5 min. At the end of the study, all animals were euthanized with a barbiturate overdose (80 mg/kg, IP) within 48 hr after irradiation. All animals were submitted for necropsy and found to be free of concurrent disease.

For radiation dosimetry, paired 50-mIl ion chambers were used. Delivered dose was expressed as a ratio of the dose measured in a tissue-equivalent plastic phantom enclosed in a restraining tube to that measured free in air.

For the analysis of data, only the measurements made during the 60 min prior to and the 90 min after irradiation were used, periods when the performance of the animals was most consistent. The data collected were divided into six, 10-min blocks before removal from the apparatus for irradiation, and nine, 10-min blocks postirradiation. For response measures, each postirradiation block was totaled and expressed as the percentage of the mean number of responses for the six, 10-min periods immediately preceding irradiation. Responses from the sham-irradiated animals similarly were recorded. All other measures were presented as totals for each 10-min period. The data were analyzed statistically using multiple factor analyses of variance with repeated measures on one factor (11). Radiation dose (0 or 10 krads) was one factor, and the time after treatment was the repeated factor. The level of significance was 0.05.

RESULTS

Unirradiated animals performed well on both signalled and unsignalled avoidance paradigms. However, performance was better when auditory cues were available. Those animals provided warning tones typically received less than five shocks during a 10-min period (Figs. 1 and 2). However, animals provided no warning tones were less proficient in avoiding shock. They typically received about 8 shocks per 10-min period. (10, 115)° 4.23, p < 0.05 (Fig. 3).

Irradiated animals experienced an increased number of shocks, although they did not exhibit any gross abnormalities in spontaneous behavior and were able to move about freely. Animals performing either signalled avoidance task received approximately 10 times as many shocks during the first 10 min after irradiation (Figs. 1 and 2). Animals provided no warning tones were less proficient in avoiding shock. They typically received about 8 shocks per 10-min period. (10, 115)° 4.23, p < 0.05, for the fixed-interval signalled avoidance group; (10, 115)° 8.01, p < 0.05, for the variable-interval
RADIATION AND ACTIVE SHOCK AVOIDANCE

signalled avoidance group), compared to a 2.5-fold increase in shocks received by animals performing the unsignalled avoidance paradigm, \( F(1,10) = 0.025, p > 0.05 \). During the remaining 80 min of the session, performance improved, but the number of shocks received by animals performing the two signalled avoidance paradigms continued at a significantly higher level relative to controls. The number of warning tones provided to the animals performing on the signalled avoidance paradigms was unchanged after irradiation \( [F(1,10) = 2.08, p > 0.05] \) for the fixed-interval signalled avoidance group; \( F(1,10) = 0.755, p > 0.05 \) for the variable-interval signalled avoidance group (Figs. 1 and 2).

Although the animal performing on any of the three paradigms experienced more shocks after irradiation, they were still able to respond on the avoidance lever. The response rates varied depending on the paradigm used. With the fixed-interval signalled avoidance paradigm the rate was lowest \( (63.8 \pm 2.7 \text{ responses/10-min interval}) \), while that for the unsignalled avoidance paradigm was the highest \( (103.1 \pm 7.2 \text{ responses/10-min interval}) \). The response rate for the variable-interval signalled avoidance group was intermediate \( (99.4 \pm 1.4 \text{ responses/10-min interval}) \). However, after irradiation, the average number of responses during each 10-min interval was not significantly different from controls (data not shown).

Since the rate of responding remained unchanged but the number of shocks received increased, the pattern of responding may be altered by irradiation. To test this possibility, interresponse time (IRT) histograms were constructed for the fixed-interval signalled and unsignalled avoidance groups in order to determine the distribution of responses during a session. The data for the variable-interval signalled avoidance group were not suitable for

![FIG 1 Mean number of shocks and warning tones, ± SEM, received by animals trained on the fixed-interval signalled avoidance paradigm. Control values are the result of pooling the values for all subjects in the control group for the 90 min after sham irradiation. The data presented were based on observations from 6 animals.](image1)

![FIG 2 Mean number of shocks and warning tones, ± SEM, received by animals trained on the variable-interval signalled avoidance paradigm. Control values are the result of pooling the values for all subjects in the control group for the 90 min after sham irradiation. The data presented were based on observations from 6 animals.](image2)

![FIG 3 Mean number of shocks, ± SEM, received by animals trained on the unsignalled avoidance paradigm. Control values are the result of pooling the values for all subjects in the control group for the 90 min after sham irradiation. The data presented were based on observations from 6 animals.](image3)
this type of analysis because the animal's response to the warming tone was not reliably related to the subject's last response and consequently showed a flat distribution. Based on the requirements of the fixed-interval signal avoidance paradigm, the subjects, as expected, responded mostly just after the onset of the warming tone (Fig. 4A). On the other hand, the animals performing in the unsignalized avoidance paradigm often responded to the shock and continued responding for a time with short IRIs (Fig. 5A). As the IRIs lengthened, a shock eventually occurred, precipitating another period of responses with short IRIs.

The pattern of IRIs after irradiation was not greatly affected. Those animals trained on the fixed-interval signal avoidance paradigm still responded after the warming tone (Fig. 4B). However, when subjects did not avoid shock, they usually responded just after the onset of shock. Few responses occurred at longer intervals after shock. The IRIs of the irradiated animals trained on the unsignalized avoidance paradigm were essentially the same as their corresponding controls, except there were more shock-elicited responses (Fig. 5B).

In order to determine whether the irradiated animals in the fixed-interval signal avoidance group were really using the warming tones, the latencies between the presentation of the tone and responding on the avoidance lever were determined and are shown in Fig. 6. It was assumed that consistently short latencies to respond would follow the presentation of a tone. Short latencies were found in both irradiated and unirradiated animals, indicating that the animals could detect and use the tones even after irradiation.

Subjects in the variable-interval signal avoidance group performed similarly to those in the fixed-interval signal avoidance group (Fig. 5). The former group also responded with consistently short latencies to the onset of the warming tone in both irradiated and unirradiated conditions, indicating that even when the onset of the warming tone was made unpredictable, the animals continued to wait for it and use it as an aid in responding.

**Discussion**

The results from this study demonstrate again that exposure to...
Radiation and active shock avoidance

Fig. 6 A: Latency distribution of responses. SEM to the onset of the warning tone for the animals trained on the fixed-interval signalled avoidance paradigm. The control group of the control group during the 90 min after sham irradiation. B: Latency distribution of responses. SEM to the onset of the warning tone during the 90 min after irradiation. The data presented were based on observations from 6 animals.

Fig. 7 A: Latency distribution of responses. SEM to the onset of the warning tone for the animals trained on the variable-interval signalled avoidance paradigm of the control group during the 90 min after sham irradiation. B: Latency distribution of responses. SEM to the onset of the warning tone during the 90 min after irradiation. Note: In some cases the SEM was too low to be graphed. The data presented were based on observations from 6 animals.

X-irradiation can degrade performance on active avoidance paradigms and is consistent with previously published reports. Typically, irradiated animals received more shocks than the unirradiated controls. Although performance was degraded, the animals were capable of executing the movements necessary to avoid shock. The rates and patterns of responding on the avoidance lever were generally unaltered after irradiation, except that animals performing on the two signalled avoidance paradigms responded more frequently to the shock rather than to the warning tone. Even so, it appears that subjects could detect the tones and were able to respond to them appropriately, even when the tones were temporally unpredictable.

The relative degree of behavioral decrement after irradiation appears to depend on the availability of visual and auditory cues that could be used to successfully avoid shocks. Although it can be seen from Figs. 1 to 3 that the number of shocks received by irradiated animals performing on the three paradigms was roughly the same, the preirradiated levels of performance were different. Prior to irradiation, animals trained on either signalled avoidance paradigm performed significantly better than those animals trained on the unsignalled avoidance paradigm, as evidenced by the fewer number of shocks received by the former animals. These findings suggest that the number and nature of cues and the consequent level of performance (presumably better with cues) have some
bearing on the likelihood of the occurrence of a radiation-induced performance decrement.

Although not the intention of the experimental design, another way to look at the relationship between cues and performance is to consider the three paradigms as requiring of the animals different levels of performance. In order for the animals trained on the two signalled avoidance paradigms to perform as well as they did, compared to those trained on the unsignalled avoidance paradigm, they needed cues to assist them. When the animals were irradiated, for some reason they did not use as many of the cues provided. Consequently, their performance was more like that of irradiated animals trained on the unsignalled avoidance paradigm in which no external cues were provided.

Why the irradiated animals were not using the cues is not clear. They apparently could detect them because responses with short latencies were still observed after presentations of the warning tones before the onset of shock even when the onset of these warning tones was unpredictable. The response pattern is not suggestive of deafness nor stupor, and the subjects were apparently not relying more on internally based time-cues rather than the tones. Another possibility is that they could maintain only temporary selective attention, rather than a more general vigilance. In addition, irradiated rats from other experiments showed no differences in their abilities to detect and respond to warm water cues (3). Rather than these performance decrements being related to abnormalities in perception, task learning, and motor function, they may result from some cognitive deficit, possibly a lack of motivation. That is, the cues and responding to these cues might become of lower value to the subject relative to other cues and behaviors.

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REFERENCES

Norepinephrine-Induced Phosphorylation of a 25 kd Phosphoprotein in Rat Aorta is Altered in Intraperitoneal Sepsis

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An attenuation of the contractile response to norepinephrine (NE) has been previously demonstrated in rat aorta during intraperitoneal sepsis and endotoxemia. In this study, we determined whether NE-induced protein phosphorylation is altered in septic rat aorta as compared to control rat aorta. We found that the NE-induced phosphorylation of a 25 kd phosphoprotein was decreased. NE increased phosphorylation of the 25 kd band by 54% (P < .01) in the control aorta but only 12% (not significant) in the septic aorta. Pyrophosphate gel purification of phosphorylated myosin showed that this 25 kd band was not related to the myosin-phosphorylatted (P) light chain. Two-dimensional polyacrylamide gel electrophoresis analysis revealed that this 25 kd band represents two proteins with distinct isoelectric points of 6.5 and 6.2. These results further document that intrinsic alterations occur in the NE-mediated signal transduction system in rat aorta during sepsis and that such alterations could contribute to depressed aortic contractility.

Key words: protein phosphorylation, protein kinase, phosphoinositide metabolism, receptor, \(\alpha_1\)-adrenoceptor

INTRODUCTION

Decreased sensitivity of the vasculature to \(\alpha_1\)-adrenergic receptor stimulation is well described in endotoxemia and sepsis [1–3]. The rat aorta has provided a reliable

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These findings were presented at the First International Shock Congress, 1987, and published in preliminary form [23].

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model in which to study this phenomenon in cecal ligation- and puncture-induced intraperitoneal sepsis [4]. Recent investigations suggest that this insensitivity correlates with intrinsic alterations in the \( \alpha_1 \)-adrenoceptor and its phospholipase C-coupled signal transduction system. In particular, we have demonstrated a marked reduction in \( \alpha_1 \)-adrenoceptor numbers as well as in basal and norepinephrine (NE)-induced phosphoinositide turnover, and calcium metabolism in aorta from this cecal ligation and puncture model of intraperitoneal sepsis [5,6].

In this study, we further examined the NE-induced signal transduction cascade in rat aorta by investigating whether alterations in protein phosphorylation occur in sepsis. A decrease in NE-mediated phosphorylation of a 25 kd phosphoprotein was observed in aorta from septic rats, further demonstrating a derangement in the NE-mediated signal transduction system during sepsis.

**MATERIALS AND METHODS**

**Chronic Rat Sepsis Model**

Male Sprague-Dawley rats (250–300 g) were obtained from Taconic Farms, Germantown, NY. Control and experimental animals were randomly subjected to sham surgery or to cecal ligation and two-hole puncture, respectively, as previously described [7]. This procedure resulted in a mortality rate of 20–30%. 24 hours after surgery, the experimental rats displayed signs of sepsis including piloerection, a bloody mucosal and nasal discharge, bloody diarrhea, and lethargy. All animals were sacrificed by decapitation and the thoracic aorta was rapidly removed and divided into 4 mm rings.

**[\( ^{32} \)P] Labeling of Rat Aorta**

Each aortic ring was placed in a 10 × 75 mm borosilicate glass tube and incubated for 90 minutes in 0.3 ml oxygenated phosphate-free buffer (5 mM Hepes, 140 mM NaCl, 4.5 mM KCl, 1 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 10 mM glucose, pH 7.4, 37°C) in the presence of 10 \( \mu \)Ci carrier-free \( ^{32} \)Pi orthophosphate (8 mCi ml\(^{-1}\), Amersham). This resulted in equilibrium labeling of rat aorta. The rings were then treated for 10 min with 10 \( \mu \)M NE, a concentration which produced maximal aortic isometric contraction [8], or with an equivalent volume of sterile buffer solution.

The phosphorylation reaction was stopped immediately by freezing the rings in liquid nitrogen. The rings were then pulverized in liquid nitrogen and glass-to-glass homogenized in stopping solution as described by Draznin et al. [9] (100 mM NaF, 80 mM sucrose, 10 mM EDTA, pH 7.40). Protein was then determined by using the method of Bradford [10]. Ten micrograms of protein was then solubilized in 50 \( \mu \)l of a sodium dodecyl sulfate (SDS) solution (62.5 mM Tris, 10% glycerol, 100 mM DTT, 3% SDS, pH 6.8).

**Isolation of Phosphorylated Rat Aorta Myosin**

The isolation of phosphorylated rat aorta myosin was carried out as previously detailed [11]. Briefly, six rat aortae were removed, divided into 4 mm rings, and placed in oxygenated phosphate-free buffer containing \( ^{32} \)P as described above. After exposure to 10 \( \mu \)M NE, the rings were frozen in liquid nitrogen and extracted in 16 volumes of a buffer consisting of 100 mM sodium pyrophosphate, pH 8.8, 5 mM EGTA, 50 mM sodium fluoride, 10% glycerol, 15 mM 2-mercaptoethanol, and 0.2
mM dichlorvos (serine protease inhibitor). Pyrophosphate gel electrophoresis was carried out at 0°C for 16 hours at a constant 84 V. Following electrophoresis, gels were stained briefly for 15 min in 0.1% Coomassie blue R-250, 50% methanol, and 10% acetic acid and destained in cold water. The purified myosin band was removed with a razor blade and placed on one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE gel) as described below.

**Gel Electrophoresis**

One-dimensional SDS-PAGE was performed with 10 µg protein/50 µl SDS solution per lane. Electrophoresis on 10% SDS polyacrylamide gels with 3% stacking gels occurred as described by Laemmli [12]. Sigma molecular weight standards (Sigma Chemical Co., St. Louis, MO) were used to identify proteins. The use of protease inhibitors (30 µM PMSF, 50 µg ml soybean trypsin inhibitor, and 50 µg ml leupeptin) did not alter the protein or phosphorylation patterns (not shown). Therefore, they were not routinely used in this study.

Two-dimensional gel electrophoresis was performed according to modification of the procedure of Laemmli [12]. 10 µg of protein was electrophoresed in 2.5 × 110 mm area tube gels containing 3% polyacrylamide, 55% urea, 2% NP-40, and 2% ampholine (1.7% of pH 3.5–10 and 0.3% of pH 5–7). The gels were equilibrated in SDS sample buffer and the second dimension was then run on 11 × 14 cm slab gels containing 10% SDS polyacrylamide gel.

**Autoradiography and Quantitation**

Gels were dried and phosphorylation was qualitatively determined by autoradiography with Kodak X-OMAT film. The gels were scanned and quantitated with an LKB laser densitometer. The area under the curve of all protein bands represents the total protein phosphorylation. The amount of phosphorylation of the 25 kd protein was determined by dividing the area of the 25 kd by the total area of all the proteins. This allowed determination of phosphorylation to be analyzed with equalization of samples for 32P labeling.

**RESULTS**

Recently, attenuation of NE-induced phosphoinositide metabolism, calcium influx (calcium channel entry), and calcium efflux (intracellular calcium release) has been demonstrated in rat aorta from this model of sepsis [5,6]. Since protein phosphorylation can be influenced by increase in cytosolic calcium as well as by phosphoinositide-hydrolysis-derived diacylglycerol, we investigated whether changes in protein phosphorylation also occurred.

Aortae from cecal-ligation-and-punctured rats and from sham-operated rats were labelled with [32P] orthophosphate and then exposed to 10 µM NE, a dose known to elicit maximal contraction of rat aorta [8,13]. One-dimensional gel electrophoresis of smooth muscle protein revealed a decreased NE-induced phosphorylation of the 25 kd band during sepsis (Figs. 1, 2). NE increased phosphorylation by 54% (P < 0.01) in control aorta but only by 12% (not significant) in septic aorta (Table 1). There were also differences noted in equilibrium [32P] labeling in control and sham tissue. The septic tissue showed greater [32P] uptake into the 25 kd phosphoprotein during the basal condition (Table 1). However, despite this
phenomena, NE-induced phosphorylation of the 25 kd phosphoprotein was significantly greater in control rats. Finally, we observed no significant differences in the total protein phosphorylation among the control and septic groups with and without NE (not shown).

To define the identity of this 25 kd band, we determined whether this represented a component of the myosin P light chain. Myosin purification from a
Fig. 2. Scanning densitometric analysis of 25 kd protein band after phosphorylation in the presence (NE) and absence (basal, B) of 10 μM NE in control (C) and septic aortae (S), each at 10 min response.
TABLE I. \[^{12}P\] Phosphorous Labeling of 25 kd Band\(^{\dagger}\)

<table>
<thead>
<tr>
<th></th>
<th>25kd phosphorylation</th>
<th>Total phosphorylation × 100</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.55 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Control + NE</td>
<td>3.93 ± 0.37*</td>
<td></td>
</tr>
<tr>
<td>Septic</td>
<td>2.93 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Septic + NE</td>
<td>3.37 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

\(^{\dagger}\) Values are mean ± SE for 8 different preparations. Quantitation is described in Materials and Methods. *P < .01 vs. control using Student's unpaired t-test.

control phosphorylated aorta preparation revealed phosphorylation of a 20 kd but not the observed 25 kd band. This suggested that the phosphorylated protein was not the myosin P light chain. To further identify the protein band, we submitted the phosphorylated sample to two-dimensional electrophoresis. This analysis revealed that the 25 kd band represents two distinct proteins (Fig. 3). These two proteins have isoelectric points of 6.5 and 6.2. Analysis of two-dimensional gel electrophoresis of septic aortic proteins did not show a selective attenuation of basal or NE-induced labeling in either of the two spots. Therefore, the observed alteration in phosphorylation appears to occur equally in both.

**DISCUSSION**

This paper demonstrates that NE-mediated phosphorylation of a 25 kd phosphoprotein is decreased in aorta from rats after cecal-ligation-and-two-hole-puncture-induced intraperitoneal sepsis. It appears that the sepsis-induced alteration in NE-mediated phospholipase C-coupled signal transduction continues to the level of protein phosphorylation. NE binding to the \(\alpha\)\(_{1}\)-adrenergic receptor results in phospholipase C-mediated hydrolysis of phosphoinositides to the by-products, inositol triphosphate and diacylglycerol [14–16]. These second messengers, in turn, are thought to play a role in increased intracellular Ca\(^{2+}\) release and activation of phosphorylating enzymes such as protein kinase C [17]. Thus, it would be predicted that alterations in this system would result in specific changes in protein phosphorylation, as was observed in this study.

Even though the function of the 25 kd phosphoprotein is not known, phosphorylation of this protein could be involved in the regulation of vascular smooth muscle contraction. Similar to our finding, Draznin et al. [9] found an increase in the phosphorylation of a 25 kd protein following NE activation in rat aorta. This 25 kd protein is not a myosin P light chain. Recently, Foster and Chatterjee [18] reported that a 25 kd protein is phosphorylated in skinned carotid artery following stimulation of protein kinase C with phorbol dibutyrate. This phosphorylation occurred without changes in the level of phosphorylation of the myosin P light chain, suggesting a possible role for 25 kd protein in vascular smooth muscle contraction. Since increased phosphorylation from NE continues at 10 min, one could speculate that this 25 kd
Fig. 3. Two-dimensional gel electrophoresis of band shows two distinct spots (see arrows) at isoelectric points of 6.2 and 6.5 after phosphorylation of rat aorta control. Similar findings were also observed by using aortae from septic rats.

protein has a functional role in long-term tonic contraction and hence belongs to the family of filament or latch proteins [19].

The investigation of the biochemical basis for diminished sensitivity of the vasculature as well as of the liver [20,21] to α1-adrenergic agonist stimulation has shown fundamental and intrinsic alterations in the signal transduction system. In rat aorta, sepsis induces a marked reduction in α1-adrenergic receptor numbers, basal phosphatidylinositol-4,5-bisphosphate (PIP2) synthesis (PIP2 is a major substrate for the phospholipase C enzyme), basal phosphoinositide metabolism, and basal calcium efflux [5,6]. Upon α1-adrenergic stimulation, there is also a diminution of NE-induced phosphoinositide metabolism, calcium influx and efflux, and protein phosphorylation in the aorta during intraperitoneal sepsis [5,6]. These intriguing observations suggest that intrinsic alterations in the signal transduction pathway may contribute at a molecular level to the observed alteration in α1-adrenoceptor function in rat intraperitoneal sepsis.

These findings suggest that even though endotoxin and circulating mediators such as tumor necrosis factor, interleukin, and platelet-activating factor may play a
role in vasodilation and in diminished responsiveness to vasoconstricting agents in sepsis [22], intrinsic biochemical alterations in vascular tissue also exist. It is possible that a cause-and-effect relationship exists between these extrinsic mediators and the intrinsic biochemical alterations during sepsis. It is conceivable, for example, that tumor necrosis factor may induce $\alpha_1$-adrenoceptor tachyphylaxis by an as-yet-undefined mechanism (see ref. 22 for discussion). Future experiments will be needed to test this idea.

In summary, intrinsic alterations occur in the NE-mediated signal transduction system in rat aorta in sepsis. As previously demonstrated [5,6], these changes occur at each level of the receptor-mediated signal transduction cascade.

ACKNOWLEDGMENTS

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REFERENCES

Aortic Protein Phosphorylation in Sepsis


The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHEW, publication No. (NIH) 85-23.
Quantitative Measurement of Radiation-Induced Base Products in DNA Using Gas Chromatography-Mass Spectrometry

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Gas chromatography-mass spectrometry with selected-ion monitoring was used to study radiation-induced damage to DNA. Quantitative analysis of modified purine and pyrimidine bases resulting from exposure to ionizing radiation using this technique is dependent upon the selection of appropriate internal standards and calibration of the mass spectrometer for its response to known quantities of the internal standards and the products of interest. The compounds 6-azaadymine and 8-azaadenine were found to be suitable internal standards for quantitative measurement of base damage in DNA. For the purpose of calibration of the mass spectrometer, relative molar response factors for intense characteristic ions were determined for the trimethylsilyl derivatives of 5-hydroxuracil, thymine glycol, and 5,6-dihydrothymine using 6-azaadymine, and for the trimethylsilyl derivatives of 4,6-diamino-5-formamidopyrimidine, 8-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 8-hydroxyguanine using 8-azaadenine. Accurate measurement of the yield of radiation-induced modifications to the DNA bases is also dependent upon two chemical steps in which the purines and pyrimidines are released from the sugar-phosphate backbone and then derivatized to make them volatile for gas chromatography. The completeness of these reactions, in addition to assessing the stability of the modified DNA bases in acid and their trimethylsilylated derivatives over the time necessary to complete the experimental analysis, was also examined. Application of this methodology to the measurement of radiation-induced base modification in heat-denatured, nitrous oxide-saturated aqueous solutions of DNA is presented.

INTRODUCTION

The interaction of ionizing radiation with cellular water results in the formation of a number of highly reactive radical species which are then capable of interacting with cellular constituents. The hydroxyl radical is the most reactive radical species that is produced, interacting via abstraction of hydrogen atoms and addition reactions on cellular constituents (for a review see Ref. (1)). Radiation-induced cell lethality has generally been attributed to the induction of damage in the nuclear DNA (2). Radia-
tion-induced damage to nucleic acid constituents manifests itself in the formation of altered purine and pyrimidine bases, altered deoxyribose moieties, abasic sites, strand breaks, and DNA-protein crosslinks (for a review see Ref. (1)). Repair of these structural modifications is essential to maintain the integrity of the genomic message, thereby preventing the processes of mutagenesis, cell death, and carcinogenesis (2).

In an effort to examine the consequences of radiation-induced modifications to DNA at the cellular level, it is necessary to develop assays to identify and measure such damage. Several types of assays have been developed to measure the yield of radiation-induced purine and pyrimidine products in DNA which include chromatographic (3-11), immunochemical (12-16), and biochemical (17, 18) techniques. However, all of these techniques are capable of assaying only a very limited number of modified DNA products and, because of the limitations of selectivity and sensitivity, the radiation doses used exceed those used in studies measuring biological end points.

In an effort to overcome these limitations, the technique of gas chromatography-mass spectrometry with selected-ion monitoring (GC–MS/SIM) has been pursued. This technique has been used to identify a plethora of radiation-induced purine and pyrimidine products (19-23) and DNA base-amino acid crosslinks (19, 21, 24). Further, using the GC–MS/SIM technique, detection of these products has been possible at doses lower than those reported using other methods of analysis. The GC–MS/SIM technique has been well documented for quantitative measurement of organic compounds at low concentrations in complex mixtures (25). However, the determination of optimum conditions for quantitative measurement of radiation-induced DNA base products by this technique has not been established. In this respect, quantitative analysis using this technique is dependent upon a number of factors including completeness of hydrolysis and derivatization reactions, stability of the products to the conditions of hydrolysis, and stability of the derivatized products over the time necessary to complete the analysis of the derivatized products. Further, selection of appropriate internal standards and calibration of the mass spectrometer with respect to the response of the internal standards and the response of authentic samples of the radiation-modified constituents under analysis are necessary for accurate measurements. This paper addresses these issues and describes the use of GC–MS/SIM for quantitative analyses of radiation-induced base modifications in DNA.

MATERIALS AND METHODS

Chemicals The compounds 5-hydroxyuracil (isobarbituric acid), 8-bromoadenine, 5,6-dihydrothymine, 4,6-diamino-5-formamidopyrimidine (FAPy-adenine), 2,5,6-triamino-4-hydroxyprymidine, 8-azaadenine, 8-azaguanine, 8-aza-thymine, and calf thymus DNA were purchased from the Sigma Chemical Co. 2-Amino-8,8-dihydroxypurine (8-hydroxyguanine) was purchased from the Chemical Dynamics Corp. Nitrous oxide (ultrahigh purity) was purchased from Matheson Gas Products, Inc. Silylation grade acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosilane) were obtained from the Pierce Chemical Co. The ε-isomer of 5,6-dihydroxy-5,6-dihydrothymine (thymine

2 Mention of commercial products does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.
and purification by crystallization from water. 2.6-Diamino-4-hydroxy-5-formamidopyrimidine (FAP-guanine) was synthesized by treatment of 2.5,6-triamino-4-hydroxy pyrimidine with concentrated formic acid and recrystallized from water (26).

The concentrations of the stock solution of 5,6-dihydrouracil and 8-azathymine were determined gravimetrically. The concentrations of all other solutions were determined spectrophotometrically using their molar extinction coefficients (26–29). The purity of each compound was also assessed by GC-MS as described below. Appropriate corrections to the concentration of each compound were made in accordance with the HPLC and GC analyses.

Preparation of irradiated DNA samples. Calf thymus DNA (0.5 mg/ml) was dissolved in 30 mmol dm⁻¹ phosphate buffer (pH 7.0) and denatured by heating the solution to 95°C for 5 min followed by rapid cooling in ice water. Aliquots of this solution were exposed to incremented doses of ionizing radiation at a dose rate of 100 Gy/min as measured by Freke dosimeter (29) in a 60Co γ-ray source (GammaCell220). Heating of denatured DNA was used for radiolysis in an attempt to maximize product yields (30). The solutions were bubbled with nitrous oxide for 20 min prior to, and then throughout, the irradiation interval. Nitrous oxide was used to saturate the DNA solutions in an effort to produce a spectrum of different types of radiation-induced products. Irradiated and untreated solutions of DNA were then dialyzed extensively against deionized water in Spectra/por dialysis membranes molecular weight cutoff 1000 (Fisher Scientific Co.). The quantity of DNA recovered following dialysis was measured spectrophotometrically (assuming that 1.0 OD₂₆₀ = 50 μg/ml), by a modified Burton's assay (31), and gravimetrically following lyophilization. Calibration of the Burton's assay was accomplished by developing a calibration curve using ultrahigh purity DNA (Sigma). The measurement of the amount of DNA by spectrophotometric assay or the modified Burton's assay was in good agreement. Gravimetric measurements tended to be higher and were not used in the analysis of the data. Following lyophilization, 6-azathymine and 8-azadecamidine were added to the 2.5 mg DNA sample. The optimal amount of internal standard added to these samples was determined from preliminary measurements.

Chemical hydrolysis of irradiated DNA. Conditions for optimal hydrolysis of irradiated DNA samples containing modified purines and pyrimidines were determined by measuring product release as a function of time of chemical hydrolysis. In this series of experiments, 1.0 ml of 80% formic acid was added to samples of DNA which had been irradiated to 100 Gy as described above and lyophilized. One set of these samples was subjected to high-performance liquid chromatography to monitor the loss of the DNA polymer peak and the increase in the peaks corresponding to the liberated bases. A Nucleogen DNA 100 (5 x 125 mm) (Sheehy) was used for separation and the mobile phase consisted of 15 mmol dm⁻³ phosphate buffer (pH 7.2) and 5 mmol dm⁻³ urea with an exponential gradient of 0–1 mol dm⁻³ KOH following at a rate of 2.2 ml/min. Absorption spectra and the absorbance at selected wavelengths were collected using instrumentation described above. A second set of DNA samples was hydrolyzed, derivatized, and analyzed by gas chromatography–mass spectrometry as described below. In this series of samples, release of the modified purine and pyrimidine bases was monitored as a function of hydrolys time. From the data collected in these analyses, the optimal time for release of the DNA bases at 150°C was 30–40 min (see Results and Discussion).

Sample preparation for gas chromatography. In preliminary experiments, samples containing 75 nmol of 8-azadecamidine and 6-azathymine were prepared in polytetrafluoroethylene-capped hypovials (Pierce). Following lyophilization, at 0.2 ml mixture of BSTFA and acetone (1:1) was added and the mixture was heated for increasing time intervals at 150°C prior to analysis by gas chromatography with flame ionization detection (GC-FID) as described below. Once the time required for complete derivatization of the samples was determined, the optimal derivatization conditions were used to prepare the samples for gas chromatography.
the internal standards was determined. 75 nmol of each trimethylsilylated internal standard was added to a lyophilized mixture containing 75 nmol of each of the authentic purine and pyrimidine compounds. These samples were derivatized and measured by GC-FID as described below and used to determine the time course of the derivatization reaction. To assess the stability of the trimethylsilylated base products over the course of days, 75 nmol each of the authentic purines and pyrimidines, including the internal standards, were added to hypovials. Following lyophilization, a 0.2-ml mixture of BSTFA and acetonitrile (1:1) was added to the samples, the mixture was then heated for 30 min at 130°C, and aliquots of this mixture were analyzed over a 5- to 6-day interval by GC-FID as described below.

For the measurement of the relative molar response factors for use in GC-MS/SIM sample analysis, mixtures of the internal standards and the authentic reference materials were prepared which contained 1-6 nmol of each compound. Following lyophilization, these samples were trimethylsilylated in a 0.2-ml mixture of BSTFA and acetonitrile (1:1) by heating for 30 min at 130°C prior to analysis by GC-MS/SIM as described below.

For the measurement of DNA samples, lyophilized samples of hydrolyzed DNA were derivatized by trimethylsilylation in a 0.2-ml mixture of BSTFA and acetonitrile (1:1) in the hypovials by heating for 30 min at 130°C prior to analysis by GC-MS/SIM as described below.

Analyses by gas chromatography with flame ionization detection. A Varian Model 8000 gas chromatograph with a flame ionization detector, a Varian 3700 automatic sample injector, and a Hewlett-Packard Model 9000 Series 300 computer work station was used to measure completeness of the derivatization reaction and stability of the trimethylsilylated purines and pyrimidines using the identical chromatographic conditions described below.

Analyses by gas chromatography-mass spectrometry with selected-ion monitoring. A Hewlett-Packard Model 5970B Mass Selective Detector controlled by a Hewlett-Packard Model 59970C computer work station and interfaced to a Hewlett-Packard 5890A gas chromatograph was used for analysis. The injection port and the GC-MS interface were kept at 250 and 270°C, respectively. The ion source was maintained at 250°C. Separations were carried out on a fused silica capillary column (12.5 m × 0.32 mm) coated with crosslinked 5% phenyl-methyl silicone gum phase (film thickness, 0.17 μm) (Hewlett-Packard). The column temperature was increased from 120 to 250°C at a rate of 8°C/min after 2 min at 120°C. Helium was used as the carrier gas at an inlet pressure of 10 kPa. Samples were injected using the split mode. Mass spectra were obtained at 70 eV.

Determination of product yield. Calculation of product yields from each DNA sample incorporated relative molar response factors determined from slopes of calibration curves, measured peak areas of the ions representing the internal standards and the products of interest, and the measured amount of DNA in each sample. The slope of the best fit line obtained by linear regression analysis of these data was then used to assess product yield.

RESULTS AND DISCUSSION

Quantitative analysis of modified purine and pyrimidine bases by GC-MS/SIM is dependent upon completeness of two chemical reactions: (i) hydrolysis of the modified bases from the sugar-phosphate backbone of the DNA and (ii) derivatization of these products to make them volatile for gas chromatography. The analysis is also dependent upon stability of the modified purine and pyrimidine bases to the conditions of acid hydrolysis and stability of their trimethylsilylated derivatives to the conditions of derivatization. Further, stability of the trimethylsilylated products is required over the time necessary to complete the analysis of the experiment. Finally, to achieve quantitative measurement using selected ion monitoring, the mass spectrometer must be calibrated with respect to the signal generated by the internal standards and the compounds of interest. For this purpose, ideally a stable isotope-labeled analog of the analyte is used as an internal standard. A calibration plot is obtained for the response of the mass spectrometer to known quantities of both the analyte and the internal standard by monitoring an intense and characteristic ion of each
compound during the analysis (25). In the case of radiation-induced DNA base products, no isotope-labeled analogs are available. Therefore, a number of structurally similar compounds were evaluated for potential use as internal standards with the result that 6-azathymine and 8-azaadenine were found to be suitable for quantitation of DNA base products.

As an example of the analysis typical of that generated by GC–MS/SIM for the experiments presented herein, Fig. 1 illustrates a selected-ion chromatogram generated from a DNA sample γ-irradiated to 100 Gy in nitrous oxide-saturated aqueous solution. The chromatogram illustrates the time windows used to monitor the characteristic ions of the trimethylsilyl (Me₃Si) derivatives of the products, and their identification may be referenced in Table 1. The products discussed are identified by their retention time and by selection of more than one characteristic ion to monitor simultaneously in each time window (21). For this analysis, approximately 3 μg of the hydrolyzed DNA sample was applied to the column. Characteristic ions for the unmodified bases were not monitored since their response would be very large relative to the modified bases. The chromatogram illustrates that the DNA base products and the internal standards can be well separated from one another (Fig. 1). Further, the chromatogram clearly demonstrates that the responses of neither the modified bases nor the internal standards, 6-azathymine and 8-azaadenine, are adversely affected by the presence of excessive quantities of unmodified bases.

The elution positions of the Me₃Si derivatives of 6-azathymine [6-azathymine (Me₃Si)₃] and 8-azaadenine [8-azaadenine(Me₃Si)₃] (peaks 1 and 9, respectively) indicate that these compounds are ideally suited for quantitative measurement by GC–MS/SIM of the modified purines and pyrimidines investigated. The trimethylsilyl derivative of 8-azaguanine was also tested as a potential internal standard. However,
TABLE I

Peak Identification in Fig. 1

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound*</th>
<th>Peak</th>
<th>Compound*</th>
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<tbody>
<tr>
<td>1</td>
<td>6-Azathymine</td>
<td>8</td>
<td>(trans) Thymine glycol</td>
</tr>
<tr>
<td>2</td>
<td>5,6-Dihydrothymine</td>
<td>9</td>
<td>8-Azaadenine</td>
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<td>3</td>
<td>5-Hydroxy-5,6-dihydrothymine</td>
<td>10</td>
<td>8-APy-adenine</td>
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<td>4</td>
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<tr>
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<td>6</td>
<td>5-Hydroxycytosine(d)</td>
<td>13</td>
<td>8-Hydroxyguanine</td>
</tr>
<tr>
<td>7</td>
<td>(cis) Thymine glycol</td>
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</table>

* As their trimethylsilyl derivatives.

\(b\) 5-Hydroxyuracil is a product of the acid-induced deamination and dehydration of cytosine glycol (35).

\(c\) 5-Hydroxy-5,6-dihydrouracil is the deamination product of 5-hydroxy-5,6-dihydrocytosine (20).

\(d\) 5-Hydroxycytosine is a product of the acid-induced dehydration of cytosine glycol (35).

despite having an ideal elution position (approximately 9.4 min), this compound was not suitable for use as an internal standard because of its poor solubility in water. Several other reasons make these compounds a good choice for use as internal standards. These compounds are stable in acid, are available commercially, and are not formed in treated DNA. The trimethylsilylated products are stable over a period of days (data not shown) and the mass spectra of 6-azathymine(Me3Si) and 8-azaadenine(Me3Si) exhibit intense M+ and (M-Me)+ ions (m/z 271 and 256, respectively, and m/z 280 and 265, respectively).

The extent of derivatization and the stability of the trimethylsilylated derivatives of the modified purines and pyrimidines were also addressed. Derivatization of purines and pyrimidines has been known to occur over a very rapid time scale (32–34). However, the time dependence of the derivatization reaction for the radiation-induced purine and pyrimidine products has not been reported. Figure 2 reveals the time course of the derivatization reactions for the modified purines and pyrimidines reported herein and indicates that the reactions proceed to completion within 20 min. However, due to the number of the samples generated in these experiments and the finite time required to perform an analysis, stability of the trimethylsilyl products over the time course of days was determined. Figure 3 confirms the stability of those products over the number of days required to complete the analysis for a typical experiment. These data indicate that the derivatization proceeds very rapidly and that the trimethylsilylated products thus formed are indeed stable for sufficient time to complete the experimental analyses.

Having demonstrated that the derivatization reaction was optimized, calibration of the mass spectrometer using authentic purine and pyrimidine compounds was necessary. The mass spectrometer was calibrated by analyzing samples containing known molar amounts of the modified bases and internal standards. The ion current ratio of an ion of a compound and an ion of the internal standards was measured and plotted as a function of the ratio of the molar amounts of the compound and the internal standard. This yielded a calibration plot, in which the slope of the linear
MEASUREMENT OF DNA DAMAGE

Fig. 2. Time dependence of derivatization of modified purine and pyrimidine bases. The ratio of the area of the product over that of the internal standard (\(I_p/I_s\)) as measured by GC-FID was plotted as a function of time of derivatization at 130°C. (A) Modified pyrimidines using 6-azathymin as the internal standard: (•) 5,6-dihydrothymine(MeSi)\(_2\) (m/z 257); (○) thymine glycol(MeSi)\(_2\) (m/z 259); (■) 5-hydroxyuracil(MeSi)\(_2\) (m/z 329). (B) Modified purines using 8-azaadenine as the internal standard: (▲) FAPy-adenine(MeSi)\(_2\) (m/z 354); (△) 8-hydroxyadenine(MeSi)\(_2\) (m/z 352); (▼) FAPy-guanine(MeSi)\(_2\) (m/z 442); (▼) 8-hydroxyguanine(MeSi)\(_2\) (m/z 440). Each data point represents the mean (±SE) from three replicate experiments.

Fig. 3. Stability of modified purine and pyrimidine bases after derivatization. Data were obtained using GC-FID and plotted similar to that illustrated in Fig. 2. Modified pyrimidines (A) and purines (B) are also identified in Fig. 2. Each data point represents the mean (±SE) from three replicate experiments.
Fig. 4. (A) Calibration plots for the Me₃Si derivatives of the modified pyrimidine bases relative to 6-azathymine(Me₃Si). The slope of the lines represents the relative molar response factors for (●) 5,6-dihydrothymine(Me₃Si)₂ (m/z 257); (●) thymine glycol(Me₃Si)₄ (m/z 259); and (■) 5-hydroxyuracil(Me₃Si)₃ (m/z 329). (B) Calibration plots for the Me₃Si derivatives of the modified purine bases relative to 8-azaadenine(Me₃Si). The slope of the lines represents the relative molar response factors for (▲) FAP-adenine(Me₃Si), (m/z 354); (△) 8-hydroxyadenine(Me₃Si), (m/z 352); (◇) FAP-guanine(Me₃Si), (m/z 442); and (●) 8-hydroxyguanine(Me₃Si), (m/z 440). Each data point represents the mean ± SE from three replicate experiments and the data were fitted by least-squares linear regression analysis.

The Me₃Si derivatives of the modified bases used here are the molecular ion (M⁻) and the characteristic (M–Me)⁺ ion (for the mass spectra see Refs. (19) and (20)). In the case of thymine glycol(Me₃Si)₄, the intense m/z 259 ion was used. An example of the determination of the relative molar response factors for the m/z 257, 329, and 259 ions of 5,6-dihydrothymine(Me₃Si)₂, 5-hydroxyuracil(Me₃Si)₃, and thymine glycol(Me₃Si)₄, respectively, is illustrated in Fig. 4A using the m/z 256 ion of 6-azathymine(Me₃Si)₂. Figure 4B illustrates an example of the determination of the relative molar response factors of the m/z 354, 352, 442, and 440 ions of FAP-adenine(Me₃Si)₄, 8-hydroxyadenine(Me₃Si)₄, FAP-guanine(Me₃Si)₄, and 8-hydroxyguanine(Me₃Si)₄, respectively, using the m/z 265 ions of 8-azaadenine (Me₃Si)₂. These figures indicate that the response of the mass spectrometer for these compounds in the range of the ratio of concentrations used was linear. The yield of 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol), which is the actual radiation-induced product of the cytosine moiety in DNA (20), is represented by the summation of the yields of 5-hydroxyctosine and 5-hydroxyuracil. These compounds result from the acid-induced modification of cytosine glycol (35). In the case of 5-hydroxyctosine(Me₃Si)₄, the relative molar response factors were assumed to be equal to those of 5-hydroxyuracil(Me₃Si)₄ due to the lack of authentic compound. This assumption was based upon the similarity of the mass spectra of these compounds with respect to the ions and their relative intensities (for comparison of the mass spectra see Refs. (20) and (22)). Due to lack of authentic reference standards, relative molar response factors for the compounds 5-hydroxy-5,6-dihydrouracil [the acid-induced degradation product of 5-hydroxy-5,6-dihydrocytosine (20)] and 5-hy-
MEASUREMENT OF DNA DAMAGE

Fig. 5. Release of bases from DNA irradiated to 100 Gy in nitrous oxide-saturated aqueous solution as measured by HPLC as a function of time of formic acid hydrolysis at 150°C. The inset illustrates the HPLC chromatogram of irradiated DNA before (A) and after (B) 40 min of acid hydrolysis.

droxy-5,6-dihydrothymine were estimated from the mass spectra of these compounds and therefore may have an error associated with them. The measured relative molar response factors are not universal since they are dependent upon experimental and instrumental parameters and might also change according to the tuning conditions associated with the day to day operation of the mass spectrometer. Hence the relative molar response factors must be determined in each laboratory and measured routinely to ensure accurate measurements.

With respect to analyses of irradiated DNA samples, the modified purine and pyrimidine bases must first be released from the sugar-phosphate backbone of the DNA molecule. Removal of purine and pyrimidine bases from DNA in the presence of formic acid (36) is one method which has been used to assess the yield of acid-stable radiation products (3, 17). In the present work, release of DNA bases from a sample of DNA irradiated to 100 Gy as a function of time of hydrolysis in formic acid was followed by HPLC using an ion-exchange column capable of separating highly polymerized DNA from purines and pyrimidines. The chromatograms illustrated in the inset of Fig. 5, where the absorbance at 260 nm is monitored over the time of chromatography, reveal that high-molecular-weight DNA (retention time 52 min on chromatogram A) is hydrolyzed to the constituent bases (retention time 3–6 min on chromatogram B). The time course of this hydrolysis, as illustrated in Fig. 5, indicates that base release has gone to completion within 40 min. The peaks eluting between
Fig. 6. Release of base products from samples of DNA irradiated to 100 Gy in nitrous oxide-saturated aqueous solution as measured by GC-MS/SIM as a function of the time of formic acid hydrolysis at 150°C. (A) Modified pyrimidines: (●) 5,6-dihydrothymine; (■) 5-hydroxy-5,6-dihydrocytosine; (♦) 5-hydroxy-5,6-dihydrothymine; (○) cytosine glycol; (▲) thymine glycol. (B) Modified purines: (▲) FAPy-adenine; (▵) 8-hydroxyadenine; (◇) FAPy-guanine; (◇) 8-hydroxyguanine. Each data point represents the mean (±SE) from three replicate experiments.

30 and 40 min in the hydrolyzed sample of irradiated DNA (chromatogram B) are devoid of absorbance spectra characteristic of purine or pyrimidine constituents and are as yet unidentified. Analysis of these samples by GC-MS/SIM reveals that a plateau for release of the radiation-induced DNA base products occurs within 40 min. If the modified bases were labile under the acidic conditions used, then a decrease in their yield would occur over prolonged exposure. However, a distinct plateau was maintained for at least 80 min of heating at 150°C (Fig. 6). The data in Figs. 5 and 6 indicate that the release of modified purines and pyrimidines from the sugar-phosphate backbone of DNA is complete within 30–40 min of hydrolysis in acid at 150°C and that the products are stable under these conditions.

As a demonstration of this methodology for quantitative analysis of radiation-induced DNA base modification, product formation was assessed as a function of radiation dose. Figure 1 illustrates a selected-ion chromatogram generated from a DNA sample which was γ-irradiated to 100 Gy in nitrous oxide-saturated aqueous solution. Following analysis, the areas of the peaks representing those products listed in Table I were integrated and product yield from each sample was calculated. Figure 7 illustrates the product yield in each DNA sample in terms of micromoles per gram of DNA plotted as a function of radiation dose. Generally, products were detected in low yields in unirradiated samples (Fig. 7; Ref. (22)). However, product yield was found to increase linearly as a function of radiation dose in the range studied. From these data, radiation chemical yield was calculated from the slope of the best-fit lines obtained by least-squares linear regression analysis and listed in Table II. Although several studies address the yield of the radiation-induced DNA base products in air- and/or nitrogen-saturated aqueous solution (3–6, 8–15), no studies which permit di-
MEASUREMENT OF DNA DAMAGE

Fig. 7. Product formation resulting from exposure of nitrous oxide-saturated solutions of heat-denatured DNA to ionizing radiation. (A) Modified pyrimidines: (●) 5,6-dihydrothymine; (●) 5-hydroxy-5,6-dihydrocytosine; (●) 5-hydroxy-5,6-dihydrothymine; (●) cytosine glycol; (●) thymine glycol. (B) Modified purines: (▲) FAPy-adenine; (▼) 8-hydroxyadenine; (●) FAPy-guanine; (●) 8-hydroxyguanine. Each data point represents the mean (±SE) from three replicate experiments and the data were fitted by least-squares linear regression analysis.

rect comparison of the yield of these products obtained from DNA samples irradiated in nitrous oxide-saturated aqueous solution are available.

In summary, 6-azathymine and 8-azaadenine have been suggested for use as appropriate internal standards for quantitative measurement of radiation-induced DNA base damage using the GC–MS/SIM technique and the procedure used to calibrate the mass spectrometer by experimental determination of relative molar response factors has been presented. Studies which demonstrate completeness of acid hydrolysis

### TABLE II

Product Yield from DNA Samples Irradiated in Nitrous Oxide-Saturated Solutions in the Heat-Denatured Conformation

<table>
<thead>
<tr>
<th>Product</th>
<th>Product yield (µmol J⁻¹) × 10⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-Dihydrothymine</td>
<td>0.88</td>
</tr>
<tr>
<td>5-Hydroxy-5,6-dihydrothymine</td>
<td>4.05*</td>
</tr>
<tr>
<td>Cytosine glycol</td>
<td>1.63*</td>
</tr>
<tr>
<td>5-Hydroxy-5,6-dihydrocytosine</td>
<td>0.74*</td>
</tr>
<tr>
<td>Thymine glycol</td>
<td>1.43</td>
</tr>
<tr>
<td>FAPy-adenine</td>
<td>0.31</td>
</tr>
<tr>
<td>8-Hydroxyadenine</td>
<td>0.31</td>
</tr>
<tr>
<td>FAPy-guanine</td>
<td>0.41</td>
</tr>
<tr>
<td>8-Hydroxyguanine</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*DNA, 0.5 mg/ml, in 30 mmol dm⁻³ phosphate buffer, pH 7.0.

* Relative molar response factors have been estimated.
of the DNA samples and optimization of the derivatization reactions were presented. In addition, the stability of the modified DNA bases in acid and the stability of the trimethylsilylated DNA bases over the time course required to complete analysis of a typical experiment were also described. Using this methodology, the yield of radiation-induced DNA base damage was assessed in samples of heat-denatured DNA exposed to $\gamma$ radiation in nitrous oxide-saturated aqueous solution and radiation chemical yields under these conditions are reported. Quantitative measurement of a plethora of radiation-induced DNA base modifications under a variety of radiolysis conditions is now feasible.

ACKNOWLEDGMENTS

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REFERENCES

Tropism of canine neutrophils to xanthine oxidase

Dale F. Gruber, PhD, and Ann M. Farese, MA

SUMMARY

Quantitative evaluation of neutrophil chemotaxis was performed on cells obtained by hypotonic-lysis techniques from heparinized blood samples from clinically normal dogs. The techniques resulted in neutrophil recovery rates significantly greater than for random baseline medium migration. Neutrophil migrations to normal canine plasma and xanthine oxidase were quantitatively less than to zymosan-activated plasma, but were equivalent to each other and significantly greater than for random migration. Migration to xanthine oxidase was maximal at concentrations near 1 U/ml within 30 minutes.

Exposure of neutrophils to inflammatory substances initiates physiologic and metabolic changes in the cell, including ion fluxes, cellular orientation or polarization, locomotion, lysosomal secretion, and production of reactive oxygen intermediates. One important intermediate of oxygen metabolism is superoxide anion. Neutrophils reportedly release superoxide anion during phagocytosis or after activation by complement, aggregated immunoglobulins, or endotoxins. The participation, or relationship, of superoxide anion to the inflammatory process has been inferred from the anti-inflammatory effects of its antagonist, superoxide dismutase. Superoxide anion also may be produced acellularly by enzymatic action. Xanthine oxidase was the first identified biological source of superoxide anion. Widely distributed, xanthine oxidase is particularly rich in lung, liver, and intestinal tissues, where it exists primarily as xanthine dehydrogenase. Xanthine oxidase, an oxidoreductase, generates superoxide anion from xanthine, hypoxanthine, and acetaldehyde substrates. Neutrophil chemotaxis toward inflammation may be related to the presence of superoxide anion.

Despite the fact that defective inflammatory cell migration has been associated with the pathogenesis of many diseases, little information exists on operant regulatory mechanisms. Even less is known of chemotaxis regulation in dogs, despite the fact that dogs are being used for investigation of septic shock, pulmonary injury, and myocardial infarction. The study reported here was conducted to determine whether xanthine oxidase, an superoxide anion substrate, alters the directed migration of isolated canine neutrophils.

Materials and Methods

Animals—Hra Beagles (Canis familiaris) 1 to 2 years old and weighing 10 to 12 kg were quarantined and screened for evidence of disease before being released to experiments. Unless prescribed by protocol, subjects were not administered medication for 30 days prior to or during experimental participation. Dogs were provided commercial dog food and allowed access to tap water ad libitum. Animal-holding rooms were maintained at 21 C ± 1 C with 50% ± 10% relative humidity, using at least 10 air changes/hour of 100% conditioned fresh air. All holding rooms were maintained on a 12-hour light-dark full-spectrum photoperiod with no twilight.

Neutrophil isolation—Blood (5 ml, once month) was drawn from each dog's lateral saphenous vein into heparinized 10 U/ml of blood syringes. Blood was washed in Hanks balanced salt solution (400 x g, 10 minutes, 21 C). Contaminating RBC were lysed with 0.83% NH4Cl (10 minutes, 4 C) and washed in Hanks balanced salt solution. The leukocyte pellet was suspended in phosphate-buffered saline solution supplemented with 0.2% heattreated fetal bovine serum. Wright-stained blood smears reportedly release superoxide anion during phagocytosis were prepared for differential and morphologic examinations. Complete blood cell counts were obtained by automated analysis. Viability of cells isolated in this manner was > 95% when assessed by trypan blue or propidium iodide exclusion.

Chemoattractants—Zymosan-activated plasma was prepared by incubating 5 mg of zymosan 1 ml of freshly drawn plasma for 30 minutes at 37 C. Zymosan was removed by centrifugation (400 x g, 10 minutes, 21 C). Plasma or zymosan-activated plasma chemoattractants were prepared by adding plasma to buffer at a concentration of 1:100 (v/v). Xanthine oxidase was added to phosphate-buffered saline solution at 2.5, 5, 10, and 20 1,000 x v controls. Xanthine oxidase was added to phosphate-buffered saline solution at 2.5, 5, 10, and 20 1,000 x v controls. Xanthine oxidase was added to phosphate-buffered saline solution at 2.5, 5, 10, and 20 1,000 x v controls.
Chemotaxis assay—A 48-well microchemotaxis chamber assembly was used to quantitate cellular migration by counting neutrophils that migrated through a 10-µm-thick polycarbonate polystyrene-free membrane with 5-µm pores. Lower wells received chemoattractant or medium. Upper wells received a cellular mixture adjusted to contain 106 neutrophils. Chambers were incubated at 37°C for 1 hour. Filters were removed and the nonmigrating side was scraped clean of cells. Filters were fixed in 100% methanol for 1 minute and stained in differential stain. Four high-power fields across the diameter of the well were examined, and the mean cellular migration per field per hour was determined.

Statistical Analysis—All data were recorded as mean ± SEM. Differences were analyzed for statistical significance, using the Student t test.

Results

The migratory responses of neutrophils to concentrations of xanthine oxidase between 0.125 and 1 U/ml, final volume, are depicted (Fig 1). Migration to 1 U/ml was generally better than the migration to lower concentrations, but was significantly (P < 0.05) increased only at 90 minutes. The highest concentration level was selected for further examination.

The migration of neutrophils to chemotaxis medium alone was enhanced by the inclusion of 1 U of xanthine oxidase/ml of medium (Fig 2). At 30 minutes' incubation, cellular migration toward 1 U of xanthine oxidase was significantly (P < 0.01) enhanced over that toward medium alone. This level of significance was maintained through the 90-minute incubation period.

To determine whether the chemotaxis technique was affected by cellular concentration, a dilution curve was developed. Neutrophils responded to medium alone or to 1 U of xanthine oxidase/ml of medium, in almost straight-line fashion (Table 1). The cellular concentration selected for further examination was 2 × 106 cells/ml.

On the basis of the total and differential leukocyte counts obtained from the 20 counts and final cell isolates, 76% of the total available neutrophils were harvested by these techniques. Final cell isolates were almost 89% neutrophils. Viability was normally > 95% when examined by trypan blue or propidium iodide exclusion.

The mean background random migration was 79 ± 3 cells/high-power field (Table 1). The presence of 1:100 normal dog plasma in buffer increased the mean migration/high-power field to 113 ± 6 cells (P < 0.001). Similar concentrations of the same plasma aliquot activated by zymosan resulted in mean migration of 147 ± 9 cells/high-power field (P < 0.001 vs medium, P = 0.008 vs plasma). Xanthine oxidase added to buffer resulted in mean cellular migration of 118 ± 6 cells/high-power field (P < 0.001 vs medium). The migratory effect attributable to the addition of xanthine oxidase to buffer was not significantly different from the migratory effect of plasma (P > 0.05) as demonstrated by the Student t test.

![Graph](image-url)
Fig 3. Migratory effects of 1 U of xanthine oxidase ml on neutrophil concentrations ranging between 0.5 and 2.5 - 10^6 cells ml.

- 0.5528, but was less than that for zymosan-activated plasma (P < 0.02).

Discussion

The physiologic responsiveness of a host to trauma includes inflammatory processes characterized by the infiltration and accumulation of neutrophils. The amplification and perpetuation of inflammatory responses depends on the emigration of normal leukocytes into the area of injury. Trauma may alter the capacity of neutrophils to migrate, thereby exacerbating existing bacterial infections into serious, often fatal, sepsis. Neutrophils migrate to injury sites by moving along concentration gradients established by intrinsic factors, eg, complement-split products or extrinsic factors, such as those elaborated by bacteria. Inflammatory cells also have been suggested as an intrinsic chemotactic source based on suppression of leukocyte infiltration after superoxide dismutase administration in rodents and human beings. Petrone et al suggested that superoxide anion elaborated from neutrophils reacted with plasma products to generate chemotaxic factors. Superoxide dismutase administered 1 hour prior to administration of carrageenan inhibited edema and influx of neutrophils, as measured by Arthus response. Our findings suggest that xanthine oxidase, a tissue factor, has chemotaxic properties.

Trauma results in adenosine triphosphate catabolism and formation of the purine nucleotide precursors hypoxanthine and xanthine. At the same time, xanthine dehydrogenase in tissues in protelytically converted to xanthine oxidase. The aerobic enzymatic interaction of the purine nucleotides and xanthine oxidase reduces molecular oxygen to superoxide anion and H_2O_2. Tubaro et al reported a marked increase in the xanthine oxidase activity of neutrophils and macrophages from mice infected with *Staphylococcus aureus*. The addition of xanthine substrate to neutrophils elicited from the peritoneal cavities of clinically normal mice increased their phagocytic bactericidal activity.

It has been proposed that the protective effects of allopurinol, a competitive inhibitor of xanthine oxidase, may be related to its ability to inhibit the production of superoxide anion via its effects on xanthine oxidase. Allopurinol may reduce free radical tissue damage by inhibiting access to the xanthine oxidase substrate, thereby reducing inflammation. The participation of xanthine oxidase in the process of inflammation may be clinically relevant in instances of mechanical, thermal, reperfusion, radiation, or inflammatory injury.

References


Effects of Acute Sublethal Gamma Radiation Exposure on Aggressive Behavior in Male Mice: A Dose-Response Study

DONNA M. MAIER, M.A., Ph.D., and MICHAEL R. LANDAVER, M.S., Ph.D.

MATERIALS AND METHODS

Subjects: A total of 62 male Crl:CFW (SW)BR VAF/Plus Swiss-Webster mice (Mus musculus) from Charles River Breeding Laboratory (Raleigh, NC) (31-43 g) were used. The mice were 5.5 months old at the time of irradiation. All animals were quarantined on arrival, and representative animals were screened for evidence of disease before being released from quarantine. They were individually housed in plastic Micro-isolator cages on hardwood-chip contact bedding in an AAALAC-accredited facility. Commercial rodent chow (Wayne LabBloX, Wayne, OH) and acidified (pH 2.5 using HCl) water were freely available. Animal holding rooms were maintained at 70° ± 2° F, with 50°% ± 10°% relative

Avoidance responding, food and water intake, maze performance, and emesis (see 4,12,15, and 23 for reviews). In most studies, radiation produced a behavioral decrement. However, the degree and time course of the behavioral decrement varied with the behavior studied as well as with the radiation dose, quality of radiation, and animal species used (12,15,22). The complexity and physical demands of the task and, possibly, the motivation underlying its performance are involved in the different radiation-induced sequelae for various behaviors (4,8,15,19). Thus, it is necessary to assess different types of behaviors separately in order to ascertain the full range of possible radiation-induced behavioral decrement.

Our laboratory is currently exploring the consequences of radiation exposure on social behavior, an area which has not been adequately addressed in the past (19). Since mice exhibit territorial behavior under laboratory and natural conditions (2,3), a mouse model of social behavior was chosen to examine the effects of radiation on territorial aggressive behavior. The present study monitored the effects of acute, sublethal doses of gamma radiation on aggressive interactions over a 3-month period.

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humidity. The mice were on a reversed 12-h light/dark full-spectrum lighting cycle with no twilight (lights off at 0700 hours).

**Radiation exposure:** The mice were placed in ventilated, polycarbonate restraint devices for approximately 20 min during irradiation. Irradiation was accomplished with bilateral, whole-body exposure to gamma-ray photons from a cobalt-60 source. The mice received either 3, 5, or 7 Gy of gamma radiation administered at a dose rate of 1 Gy/min. These doses were chosen based on an LD50/30 (95% confidence limits) of 7.66 (7.50-7.82) Gy for male Swiss-Webster mice housed under our laboratory conditions. The sham-irradiated control mice were treated exactly like the irradiated mice, except that they were placed behind a lead shield and were not exposed to radiation.

**Procedure:** A resident-intruder paradigm, in which a resident mouse attacks an intruder that has entered its territory, was used to measure social behavior in this study (26). This paradigm has been widely used to measure offensive aggressive behavior (observed in the resident as it attacks the intruder) and defensive behavior (observed in the intruder as it defends itself from the resident) and has ethnological validity as dominant mice defend their territories in the wild (2,3). The mice were individually housed 1 month before irradiation, since this has been reported to be an effective noninvasive method for inducing offensive aggressive behavior in mice (5,7,24). After 2 weeks of individual housing, each animal was brought to the test room, paired with another weight-matched mouse, and left to fight for 5 to 10 min. The animal that dominated in this encounter was designated as the resident and the subordinate animal became the intruder. Animals remained in the same resident-intruder pairs throughout the experiment, and all further testing was conducted in the resident’s home cage. Resident animals were randomly assigned to four treatment groups: sham-radiation (N = 8), 3 Gy (N = 8), 5 Gy (N = 7), or 7 Gy (N = 8).

The mice were habituated to the testing room for 1 h before the test. Intruders were placed in the resident’s home cage (25.7 × 15.2 × 12.1 cm) for 5 min during the dark portion of the light/dark cycle, and the mice were videotaped under infrared light. After the study was completed, the videotapes were analyzed by a scorer unaware of the treatment condition. The following behaviors displayed by the resident mouse were scored: latency to attack, number and duration of roll and tumble fights, and number of bites, lunges, and chases (2,3,26). Bites did not draw blood or leave any discernible mark on the intruder. Scored behaviors displayed by the intruder mouse were duration of defensive upright postures, number of squeaks, and number of escapes from the resident (2,3,6). The mice were tested 1 week before irradiation to ensure maintenance of the dominance relationship established between them at 2 weeks preirradiation. The baseline test was performed 2 d before irradiation. Postirradiation tests were conducted 1 to 3 h postirradiation (day 0), and days 2, 5, 7, 9, 12, 15, 19, 21, 26, 29, 48, 62, and 97 postirradiation.

**Statistical analysis:** The nonparametric Kruskal-Wallis and Mann-Whitney U tests were used to analyze the data (11). If the Kruskal-Wallis test revealed a significant difference among groups (which occurred on test days 0–19 postirradiation), paired comparisons of daily scores of each irradiated group with the control group were made using the Mann-Whitney U test. This was the procedure followed for each of the eight behaviors analyzed. A one-tailed alpha level (p < 0.05) was set for all statistical comparisons, since radiation has previously been shown to result in decrements in aggressive interactions (19).

**Fig. 1.** Offensive aggressive behaviors displayed by resident mice in a 5-min resident-intruder test. Resident mice were sham-irradiated or exposed to 3, 5, or 7 Gy of gamma radiation on day 0. Data are presented as a percentage of control (sham-irradiated) group. (A) Number of fights, (B) Duration of fights, (C) Number of bites, and (D) Number of lunges and chases. N = 8 in the 3 Gy group; N = 7 in the 5 Gy group; N = 8 in the other groups; 'p < 0.05 compared with controls.
RESULTS

In general, resident mice irradiated with 3 Gy did not exhibit any significant differences in offensive aggressive behavior directed toward the intruders when compared with the sham-irradiated controls (Fig. 1). Similarly, there were no differences in the defensive behaviors of the nonirradiated intruders paired with the residents treated with 3 Gy when compared with the controls (Fig. 2).

For purposes of clarification, data are illustrated as a percentage of the sham-irradiated control group in Figs. 1 and 2. The means and standard errors of all the parameters of the control group are presented in Table 1. The control group is always considered to be 100% of the response and is depicted by a solid line.

For the 5 and 7 Gy groups, significant changes in offensive behavior in the irradiated residents occurred primarily during the second week postirradiation. In the 5 Gy group, attack latency (data not shown) increased significantly on days 7 and 9 postirradiation (U = 8.7; p < 0.05) and the number of bites decreased significantly on day 12 postirradiation (U = 46.5; p < 0.05) (Fig. 1). Defensive behavior emitted by the nonirradiated intruders paired with residents treated with 5 Gy also showed significant decreases, primarily in the second week postirradiation. The number of escapes and squeaks decreased significantly on day 7 postirradiation in intruders paired with residents irradiated with 5 Gy (U = 8.7; 54, p < 0.01; U = 8.7; 50, p < 0.01) and the number of defensive upright postures decreased significantly on day 12 postirradiation (U = 46.5; p < 0.05) (Fig. 2). Decreases in defensive behavior also occurred on day 2 postirradiation in these intruders. Both time spent in the defensive upright posture and number of escapes decreased at this time in the intruders paired with 5 Gy residents (U = 46.5; 8.7; p < 0.05) (Fig. 2). Number of escapes also decreased significantly in these intruders on day 0 (U = 46.5; 8.7; 53, p < 0.05) (Fig. 2).

In the residents treated with 7 Gy, number of fights and flight duration decreased significantly on day 9 postirradiation [U (8.8) = 55.5, p < 0.01; U (8.8) = 53.5, p < 0.05] (Fig. 1). On day 12 postirradiation, the number of lunges and chases decreased significantly [U (8.8) = 50.5, p < 0.05] (Fig. 1). Defensive behavior displayed by the nonirradiated intruders paired with residents treated with 7 Gy also showed significant decreases during the second week postirradiation. The number of escapes and squeaks decreased significantly on day 9 postirradiation [U (8.8) = 54, p < 0.05; U (8.8) = 51, p < 0.05] in intruders paired with the 7 Gy residents (Fig. 2). On day 12, both the number of squeaks and time spent in the defensive upright posture decreased significantly in these intruders [U (8.8) = 57.5, p < 0.01; U (8.8) = 49.5, p < 0.05] (Fig. 2). Time spent in the defensive upright posture also decreased significantly on day 19 postirradiation [U (8.8) = 53, p < 0.05] (Fig. 2). No significant effects were found in the 7 Gy group during the first week postirradiation.

After day 19 postirradiation, no significant differences were found between any of the irradiated groups and the sham-irradiated control group on any measure for either the residents or the intruders. Testing was stopped after day 97 postirradiation. There were no significant differences between the 5 and 7 Gy groups when the two were compared with each other.

DISCUSSION

The results of the present study indicate that gamma radiation can temporarily suppress offensive aggressive behavior during the second week postirradiation. The extent to which this finding is influenced by a generalized malaise or by reductions in spontaneous locomotor activity and energy utilization is difficult to determine. These effects may be due to reduced spontaneous activity, which has been suggested to influence cognitive function in rodents [2]. It is also important to note that the radiation doses used in this study were not necessarily representative of those used in previous studies [1].

Fig. 2. Defensive behavior displayed by nonirradiated intruder mice paired with resident mice that were sham-irradiated or exposed to 3, 5, or 7 Gy of gamma radiation on day 0. Data are presented as a percentage of control (sham-irradiated) group. (A) Number of escapes, (B) Number of squeaks, and (C) Duration of defensive upright postures. N = 7 in the 5 Gy group; N = 8 in the other groups; *p < 0.05 when compared with controls.
TABLE 1. MEANS (± S.E.M.) OF THE SHAM-IRRADIATED GROUP FOR BEHAVIOR OF RESIDENT MICE AND BEHAVIOR OF INTRUDER MICE OVER 3 MONTHS OF TESTING.

(These values were used to compute the control lines that appear in Fig. 1 and 2.)

<table>
<thead>
<tr>
<th>Offensive Aggressive Behaviors of Sham-Irradiated Residents</th>
<th>Day*</th>
<th>No. of Fights</th>
<th>Fight Time**</th>
<th>No. of Bites</th>
<th>No. of Lunges-Chases</th>
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Defensive Behaviors of Intruders Paired with Sham-Irradiated Residents

<table>
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<th>Defensive Behaviors of Intruders Paired with Sham-Irradiated Residents</th>
<th>Day*</th>
<th>No. of Escapes</th>
<th>No. of Squeaks</th>
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<td>58.9 (7.1)</td>
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<td>127.1 (22.8)</td>
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</table>

* Test day with baseline test on day (- 2) before sham irradiation, sham irradiation on day 0, and all other tests on days postirradiation (N = 8).

** Total duration of behavior in seconds.

*** Data lost because of technical difficulties.

activity is not clear. In a study using doses of 0.5 to 7 Gy gamma radiation, spontaneous locomotor activity and body weight in Swiss-Webster mice were temporarily decreased by 7 Gy cobalt-60 during the second and third weeks postirradiation but later recovered (16). In C57BL/6J mice, doses of 6 and 7 Gy cobalt-60 depressed locomotor activity during the second and third weeks postirradiation (17). In rats, temporary decrements in locomotor activity occurred 2 to 3 weeks following irradiation with 4 to 6.8 Gy X-rays (10,14); injection of a bone marrow suspension into rats irradiated with 6.5 Gy X-rays prevented these locomotor decrements (13). Behavioral decrements occurring in the second week postirradiation and beyond may be linked to radiation-induced suppression of hematopoietic tissue. Irradiation in the 5 to 10 Gy dose range has been found to cause a decrease in all blood cell elements including platelets, erythrocytes, lymphocytes, and granulocytes (9). The resulting anemia, inability to fight infection, and impairments in blood clotting that cause internal hemorrhaging (9), may lead to behavioral suppression until the blood cells have recovered.

Significant decreases in defensive behavior occurred on days 0 and 2 postirradiation in the nonirradiated intruders paired with residents irradiated with 5 Gy. Fight duration and number of bites in the 5 Gy residents also decreased on days 0 and 2 postirradiation, but these decreases, while marked, were not significant. The reason for these early decreases is unclear. A high degree of variability was noted on the baseline test day among the four treatment groups. This has previously been observed (19) and is due to the large amount of individual variation in aggressive behavior from day to day. Since aggressive behavior is a result of the interaction of two mice rather than a single animal performing a task alone, variability is further increased. In future studies, we plan to use a larger sample size in an attempt to reduce this variability.

In previous studies using 10 Gy cobalt-60 in Swiss-Webster mice, decreases were found in offensive be-
behavior beginning on day 7 postirradiation and in spontaneous locomotor activity beginning on day 5 postirradiation (18,19). These animals did not recover normal levels of behavior and died in the second week postirradiation (18,19). In the present study, which used lower doses of radiation, no animals had died by 3 months postirradiation and a behavioral dose-response relationship was observed. A dose of 3 Gy had no effect on aggressive behavior, while doses of 5 and 7 Gy produced similar behavioral decrements. It is possible that the physiological effects produced by each dose were similar enough to produce equivalent behavioral alterations.

The results of the present study indicate a temporary deficit in offensive aggressive behavior with 5 and 7 Gy of gamma radiation along with concomitant alterations in behavior in the nonirradiated conspecifics; the latter may reflect the decline in offensive behaviors observed in the irradiated animals paired with them. Irradiation with either high-energy electrons or gamma photons has been found to decrease neurotoxin-stimulated uptake of sodium into synaptosomes in doses as low as 0.1 Gy (27). Thus, lower survivable doses may have an impact on central nervous system functioning that could underlie subtle changes in complex behaviors (23).

In conclusion, the results of the present study support our earlier finding that radiation has a suppressive effect on aggressive behavior, possibly as a result of the acute radiation syndrome (18). Previous work has demonstrated that the time course of radiation-induced behavioral suppression varies as a function of the behavior investigated (12,15,22). For example, research from this laboratory has indicated that radiation-induced decrements in locomotor activity precede deficits in aggressive behavior (18). The ability to coordinate rescue efforts following a nuclear accident or radiation exposure to space crews is of concern to both the civilian and military sectors. Radiation-induced alterations in behavior are likely to interfere with the effective functioning of teams and crews and, thus, deserve further study. The use of radioprotectants to mitigate radiation-induced behavioral deficits is, therefore, currently under investigation.

Acknowledgments
The authors would like to thank H.D. Davis, M.E. Greenville, and G.J. Ruggerio for their invaluable assistance.

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References
19. Maier DM, Landauer MR. Alterations in aggressive behavior of mice following exposure to gamma radiation. [Unpublished results.]
Progressive Behavioral Changes During the Maturation of Rats With Early Radiation-Induced Hypoplasia of Fascia Dentata Granule Cells

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MICKLEY, G. A., J. L. FERGUSON, M. A. MULVIHILL AND T. J. NEMETH. Progressive behavioral changes during the maturation of rats with early radiation-induced hypoplasia of fascia dentata granule cells. Neuropathol. Teratol. 11:4:385–393, 1989. – Localized exposure of the neonatal rat brain to X-rays produces neuronal hypoplasia specific to the granule cell layer of the hippocampal dentate gyrus. This brain damage causes locomotor hyperactivity, slowed acquisition of passive avoidance tasks and long bouts of spontaneous turning (without reversals) in a bowl apparatus. Here we report how these behavioral deficits change as a function of subject aging and behavioral test replications. Portions of the neonatal rat cerebral hemispheres were X-irradiated in order to selectively damage the granule cells of the dentate gyrus. The brains of experimental animals received a fractionated dose of X-rays (13 Gy total) over postnatal days 1 to 16 and control animals were sham-irradiated. Rats between the ages of 71–462 days were tested 3 separate times on each of the following 3 behavioral tests: 1) spontaneous locomotion, 2) passive avoidance acquisition, and 3) spontaneous circling in a large plastic hemisphere. Rats with radiation-induced damage to the fascia dentata exhibited long bouts of slow turns without reversals. Once they began, irradiated subjects perseverated in turning to an extent significantly greater than sham-irradiated control subjects. This irradiation effect was significant during all test series. Moreover, in time, spontaneous perseverative turning was significantly potentiated in rats with hippocampal damage but increased only slightly in controls. Early radiation exposure produced locomotor hyperactivity in young rats. While activity levels of controls remained fairly stable throughout the course of the experiment, the hyperactivity of the irradiated animals decreased significantly as they matured. The total distance traveled by irradiated rats was significantly above sham-irradiated controls at the beginning of the study but was significantly lower than controls at the conclusion. Young, irradiated rats learned a passive avoidance task more slowly than controls. They not only took more trials to meet criterion but their latency to move into an area in which they had just been shocked was significantly shorter than sham-irradiated rats. Passive avoidance learning improved in mature animals. These data suggest that radiation-induced damage to the fascia dentata produces task-dependent behavioral deficits that change as a function of subject age and/or behavioral testing.

ionizing radiation Brain damage Spontaneous locomotion Rotation Hippocampus Fascia dentata Passive avoidance Rats Longitudinal

The central nervous system (CNS) is most sensitive to structural damage from ionizing radiation exposure during critical periods of morphogenesis that occur prenatally and soon after birth. The consequences of radiation-induced malformations of the CNS are manifested in the function of the nervous system and in subsequent behavioral aberrations as well [for reviews see (20, 35)].

Through methods that utilize precise timing of brain irradiation coupled with selective shielding techniques it has been possible to determine the behavioral changes associated with early radiation-induced damage to particular brain nuclei. Bayer and Peters have utilized these techniques to study the behavioral consequences of radiation-induced hypoplasia of the granule cells in the hippocampal dentate gyrus (fascia dentata). Since lesions of the granule cells effectively remove a major input to the hippocampus through the perforant path from entorhinal cortex they often produce behavioral consequences similar to total hippocampectomy (2, 36). Bayer, Brunner, Hine and Altman (6) described locomotor hyperactivity, reduced spontaneous alternation in a T maze and retarded acquisition of a passive avoidance task in rats with early radiation-induced hippocampal damage. More recently, we (26) have replicated and extended this work by revealing that rats with hypoplasia of the fascia dentata granule cells exhibit perseverative spontaneous turning, with few reversals, in a bowl-shaped apparatus. These data illustrate behavioral effects of early radiation

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exposure and suggest a role for hippocampal granule cells in working memory (30), response inhibition (14, 18, 19), and perhaps spatial mapping (29).

Most previous studies have described behavioral effects of radiation-induced damage to the neonatal dentate gyrus by measuring performance deficits at a single point in time. These analyses are typically done in young-adult subjects (6, 16, 36, 37). The purpose of the present experiments was to trace the consequences of early brain damage longitudinally. The same rat subjects were assessed on a battery of tests during 3 separate periods in their maturation. Here we report that radiation-induced behavioral deficits are not always stable throughout maturation. Instead, the progress of behavioral decrements following damage to the fascia dentata is task-dependent and performance may get progressively more, or less, severe as the brain-damaged animal matures.

METHODOLOGY

Subjects

Pregnant rats [Crl: CDiSD1BR] obtained from Charles River Laboratories, Kingston, NY, and screened for evidence of disease were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Temperature and relative humidity in the animal rooms were held at 19-21°C and 50% - 70%, respectively, with 10 air changes hour. Full-spectrum lighting was cycled at 12 hour intervals (lights on at 0600) with no twilight. The rats used in these experiments came from a total of 19 different litters. On the day of birth (day 1 litters were culled and only 4-8 males litter were reared together. Based on a random selection process, from 1-5 of these rats in each litter were actually used in the experiments reported here. All rats were weaned at the same time (between 23-28 days after birth) and then individually housed in microisolator, polycarbonate cages on hardwood chip contact-bedding. Rats were given ad lib Wayne Rodent Blox and acidified water (HC), pH 2.5 to prevent the spread of Pseudomonas. Palatability studies indicate that animals do not prefer tap water to acidified water and that there are no deleterious effects of this water treatment over the lifetime of the subject [for review, see (38)].

Irradiated rats weighed less than sham-irradiated controls throughout most of the experiment (see Fig. 1). This weight difference was noted before weaning on postnatal day 21 [irradiated = 51.1 ± 0.8 g (SEM); sham-irradiated = 63.7 ± 1.1 g (SEM); t(52) = 9.08, p < 0.001] and persisted in 71-day-old adolescents [irradiated = 356.2 ± 5.7 g (SEM); sham-irradiated = 434.2 ± 8.4 g (SEM); t(46) = 5.79, p < 0.001] as well as mature (200-day-old) subjects [irradiated = 527.9 ± 10.4 g (SEM); sham-irradiated = 640.8 ± 11.2 g (SEM); t(58) = 7.36, p < 0.001]. However, absolute weights for both irradiated and control rats were initially higher than published norms for this species (21, 32). This may be due to the fact that litters were quite small after they were culled to contain only males. Rats raised in small litters tend to be significantly larger than are those reared in standard-sized litters (27).

The rats in the current study were sacrificed at the end of the experiment to allow histological analysis of the brain (see below). However, in another set of animals, treated identically, we found partial head irradiation did not significantly alter the life span of the animals (median = 589 days; range = 127-769; N = 40) as compared to sham-irradiated controls (median = 640 days; range = 180-782; N = 32).

See Table 1 for the number of subjects and their ages at the beginning of behavioral testing. Although their treatment was otherwise identical, some of the rats in this experiment served as 'sham-surgical' controls for another study. In this context 15 of the sham-irradiated rats and 11 of the irradiated animals underwent a surgical procedure after the first behavioral test series (mean age at time of surgery = 182 ± 4 (SEM) days). The animals were injected with atropine sulfate (0.4 mg/kg, IP) and then anesthetized with sodium pentobarbital (50 mg/kg, IP). Using aseptic techniques, the scalp was opened, a hole was drilled on both sides of the cranium adjacent to the midline, and the dura mater was broken with a probe. Following this procedure the scalp was sutured and subjects were placed back into their home cages. Full recovery was allowed since the next behavioral test series did not commence until almost 100 days after this surgery. Within each statistical analysis we compared the data derived from these animals to the data from rats not having the surgery (see Statistical Analysis sections) and found them not to differ significantly.

FIG. 1. Growth of irradiated and sham-irradiated rats throughout the course of this experiment. The weights presented here were recorded when subjects were being tested on the rotation measure. There are 3 data points per subject reflecting each of the 3 test series. See statistical comparisons of irradiated and sham-irradiated rat weights in the Method section of the text. Also presented for comparison are normative weights for this strain (21, 32). Lines were fit using the least squares method (39).
PROGRESSIVE BEHAVIORAL CHANGES

TABLE 1

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<td>N</td>
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<td>N</td>
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*All rats were tested on these 3 behavioral measures. Variations in N between different tests are due to slight procedural problems or equipment malfunction that disqualified a particular subject’s data from inclusion. Mean ± SEM.

Irradiation

Subjects from each litter were randomly assigned to either the X-irradiated or the sham-irradiated (control) group. Irradiated rats received collimated X-rays (Phillips Industrial 300 kvp X-ray machine, Phillips, Inc., Mahwah, NJ; configured with 1.5 mm of copper filtration, with a half-value layer of 2.5 mm copper) delivered dorsally, in the coronal plane, through a narrow slot in a lobe-fitting whole-body lead shield. X-rays were confined to that area of the head previously determined to contain the hippocampus. Determination of the location of the hippocampal formation relative to external landmarks (e.g., snout, eye, ears) was made during preliminary dissections of other neonatal rats. These external landmarks were subsequently used to set the position of the slot in the lead shield during our irradiation procedure. The measurements and anatomical landmarks we used for shield placement corresponded closely to those previously reported by Bayer and Peters (7). The slot in the shield was the opening between 2 movable lead strips (22.8 × 6.8 × 2 cm) suspended just above the heads of the rats in the radiation exposure array. The opening extended laterally beyond the full width of each rat head and varied between 5-10 mm in the anterior-posterior plane in order to accommodate the growth of the head brain over the course of the radiation treatment [see (7) for a complete explanation of this procedure]. Irradiated rats were exposed to 3.0 Gray (Gy) of postnatal days 1 and 2 (day of birth - postnatal day 1), and to 1.5 Gy on postnatal days 5, 7, 9, 12, 14, and 16 for a total partial-head-only dose of 13 Gy (1 Gy = 100 rads). Doses were determined by using Exradin 0.05 cc tissue-equivalent ion chambers with calibration traceable to the National Institute of Standards and Technology. X-rays were delivered at a rate of 0.49 Gy minute (total irradiation time = 3.5-4.0 min) at a depth of 2.0 mm in tissue. The sham-irradiated control rats were restrained for the same time period as the irradiated rats but were not exposed to X-rays.

The entire anterior-posterior extent of the hippocampal formation was irradiated as were brain areas dorsal and ventral to this structure [see (13) for a listing of these other brain areas]. Brain structures anterior and posterior to the slot in the lead were shielded. At the time of our postnatal radiation exposures the rat brain contained three remaining populations of dividing (and therefore radiosensitive) cells: neuronal precursors of granule cells in the hippocampus, cerebellum and olfactory bulbs (5, 6). Two of these major neuronal precursor populations (in the cerebellum and olfactory bulbs) were covered by the radio-opaque shielding. Unshielded were the mitotic (radiosensitive) granule cells of the dentate gyrus and the mature neurons in other brain structures residing in the same coronal plane as the hippocampus. This procedure produces selective hypoplasia of granule cells in the dentate gyrus (7, 17) while sparing the radioreistant (11, 18) mature neurons of other brain structures. The technique has been validated through a variety of neuroanatomical methods (5, 17, 40).

Apparatus and Procedures

Following irradiation, rats were allowed to mature for approximately 4 months (see Table 1) before behavioral testing began. At this time, and during 2 subsequent time periods (approximately 9 and 12 months after birth) we recorded performance on 3 behavioral measures (see below). There was no systematic order in the conduct of these behavioral tests within each test series.

Spontaneous rotation: Rotation was measured in one of two opaque, sound-insulated, 60-cm diameter plastic hemispheres (bowls). Rotation within the hemisphere was inevitable since this was the primary gross movement permitted by the shape of the apparatus. Circling was measured through a projector-drive cable clipped to a wide rubber band around the rat's thorax. Following the design of Greenstein and Glick (15), the cable turned a slotted illuminated disk uncovering one of four phototransistors. The time and direction of each quarter turn in each of six 30-minute sessions was recorded on a microcomputer.

Rats were tested at approximately the same time during the light portion of the day for six days. After each rat was put into the hemisphere and the cable clipped to its head harness, the hemisphere's sound-attenuating lid was lowered, leaving a 0.5-mm gap. Spontaneous turning was recorded for 0.5 hr. Bowls were thoroughly washed between runs. In order to control for an apparatus bias, daily runs were alternated between two test hemispheres.

Locomotor activity: Locomotor behaviors were automatically recorded using Digiscan Animal Activity Monitors, Model DCM-16 (Omnitech Electronics, Columbus, OH). Monitors consisted of a square acrylic activity arena (40 × 40 × 30 cm) and an array of infrared horizontal and vertical sensors 1 inch (2.54 cm) apart. Horizontal and vertical sensors were positioned 1.3 and 11.0 cm, respectively, above the floor of the cage. Data were processed and recorded by a Digiscan Analyzer (Omnitech, Model DCM) and an IBM-PC/XT computer.

Measurements were made in a Digiscan activity monitor for at least two 1-hour periods separated by 1 week or more. The following activity parameters were recorded during the lighted portion of the dark-light cycle: 1) Total distance traveled a measure of horizontal locomotion that goes beyond inferred beam break counts, takes into account diagonal movements, and computes actual distance traversed; 2) Vertical activity, total number of beam interruptions that occurred in the vertical sensor (this parameter included, but was not limited to: rearing, high sniffing movements were also recorded by vertical-sensor beam breaks; 3) Stereotypic movements—number of occurrences of quick (< 1 sec) repetitive breaks of the same horizontal beam.

Passive avoidance: Passive avoidance was measured within a shuttle box comprised of 2 compartments each 22 × 22 × 22 cm separated by a black plastic guillotine door. Each compartment was made of black plastic except for the top and the front walls which were clear. The goal compartment was identical to the
start box except for a small nonoperative light on the wall at the far end and a plastic food tray on this wall 2 cm off the floor. The floor was made of metal rods (0.5 cm diameter, 1.7 cm apart). Leads from a Coulbourn Instrument’s (Lehigh Valley, PA) constant-current shaker (Model E13-04) were connected to the floor bars in the goal compartment but not to the bars in the start compartment.

Three to 7 days before training, rats were given daily rations of 5 g of Wayne Rodent Blox and 1-5 g of highly palatable breakfast cereal (Froot Loops®; Kellogg Company, Battle Creek, MI). For 2 to 4 days before training, each rat was allowed to fully explore the shuttle box (with cereal in the food-tray) for 15 to 45 minutes. Body weight on the first day of training averaged 87% of weight for the week prior to food reduction. During training (15 trials each day) the rat was placed in the start compartment with the divider door closed. After 5 seconds the door was raised and a timer started. The trial ended either when the rat grasped a Froot Loop® or in 2 minutes. Between training trials, the rat was returned to its home cage for 30 seconds. Avoidance testing was started when a rat both averaged less than 10 sec per trial to grasp the food during a training session and, on the first 5 trials of the following day, had a median latency to grasp the food of 10 sec or less.

Avoidance training-testing consisted of initiating a regular training trial as usual. However, when the rat’s four feet were in the goal box, the experimenter administered a 0.25 mA scrambled footshock until the rat retreated to the safe side. The rat was then returned to its home cage for 60 seconds. This procedure was repeated on subsequent trials except that the floor on the food side of the chamber was continuously electrified. The critical latency measured was the time for each rat to cross onto the shock grid. A trial was terminated if the rat either crossed onto the food/shock side of the chamber (registering a decrease in resistance across the rods) or stayed on the safe side for 120 seconds. The session (and test) was ended when the rat remained in the safe area for 120 seconds on three consecutive trials or after 20 test trials. After each session the apparatus was thoroughly cleaned and paper under the rod floor was replaced.

**Histology**

After behavioral testing, our rats were anesthetized and perfused with heparinized saline followed by 10% buffered formalin. Brains were embedded in paraffin, serially sectioned (6 µm) (in either the coronal or sagittal plane) and then stained with cresyl violet and luxol fast blue (23). All brains received a preliminary review to confirm radiation-induced damage to the dentate gyrus. In addition, brains sliced in the sagittal section (irradiated, N = 20; sham-irradiated, N = 20) were analyzed in more detail. A single section (approximately 1.9 mm lateral to the midline (31)) was used for this analysis to 1) estimate the degree of fascia dentata injury, and 2) survey the other brain areas (olfactory bulb and cerebellum) that, although shielded from irradiation, are known to contain granule cells mitotic at the time of radiation treatment. We counted the total number of granule cells that could be visualized in the single section of the dentate gyrus used in this analysis. Cell counts were accomplished under 250 x total magnification by a single observer. Nuclear cell counts were used in order to avoid the error caused by double or triple nuclei. The size, cytoplasmic staining and nuclear structure of granule cells usually makes them distinguishable from glial cells (3.33). However, the possibility cannot be ruled out that some of the astroglial cells may have also been counted. The impact of this possible error is reduced by the fact that the number of glial cells in the fascia dentata is extremely low (22). In addition, after neonatal irradiation similar to those described here, Bayer and Altman (4) reported that the granule cell population remains significantly reduced into adulthood while the glia show an initial reduction in number followed by a complete regeneration to normal levels within 60-90 days (5). Thus our cell counts in the fascia dentata of the 1-year-old adult rat would presumably not reflect a radiation-induced alteration in glial population. Using an imaging system (Bioquant System IV, R and M Biometrics, Inc., Nashville, TN) we also derived the area of the dentate gyrus, computed the cellular density of the structure and the thickness of the granule cell layer. In order to confirm that the shielding of other brain areas was sufficient, we also counted granule cells in a 0.004 mm² area in the cerebellum and olfactory bulb. Further, we evaluated the sparing of other more mature, and therefore less radiosensitive, hippocampal structures by counting the thickness of the CA1 pyramidal cell layer that was dorsal to the dentate and directly in the path of the X radiation.

Using irradiation procedures similar to ours, Bayer and Peters (17) have previously determined that X irradiation destroys approximately 85% of the granule cells in the dentate gyrus of the hippocampus. This procedure spares 1) hippocampal pyramidal cells and adjacent brain nuclei (e.g., caudate) in the path of the X-rays and 2) shielded neurons anterior and posterior to the hippocampus (6,26). A foreshortening of cranial bone growth (28) and secondary brain changes in reaction to granule cell hypoplasia (40-42) may explain the slight shortening of hippocampus (24-26) and other brain structures (26) that have been previously reported.

**Statistical Analyses**

The effects of early radiation exposure on our behavioral parameters were evaluated through the use of analyses of covariance (ANCOVAs) with “subject age” as the covariate and “test series” (initial test and retests 2 and 3) as a within-subjects repeated measure. In one analysis of locomotor activity body weight was used as a covariate instead of “age.” Initially, these analyses also included “surgery” as a factor in order to determine if the mild surgical treatment received by some of the subjects (see the Method section) significantly influenced the behavioral measures. Since no effect of surgery was revealed by these analyses, this factor was dropped in subsequent treatments of the data and the surgical groups were combined. Our analyses were conducted using the logarithms of the data in order to meet the ANCOVA assumptions of homogeneity of variance and normality of distribution (39).

In the analyses of the rotation data, the mean turning bout length for each of the 6 daily test sessions was computed by dividing the total quarter turns in the dominant direction by the number of bouts of quarter turns (without reversal of direction) in that session. Statistical comparisons were computed using each rat’s average bout length score. This was the mean of the daily bout lengths for the 6 sessions in the test series.

**RESULTS**

In general, the data reveal that early radiation-induced granule cell hypoplasia of the fascia dentata causes 1) perseverative turning in a bowl apparatus (long turning bouts in the same direction, without reversal), 2) locomotor hyperactivity, and 3) deficits in the acquisition of a passive avoidance task. These behavioral changes were identified in young rats during the first performance tests but changed over time. Radiation-induced perseverative turning became more intense through the course of the experiment while passive avoidance acquisition improved.
Progressive Behavioral Changes

Table 2

Histological Data Derived from Analysis of Sagittal Sections of Rat Hippocampus

<table>
<thead>
<tr>
<th>Anatomical Parameter</th>
<th>Irradiated (N = 20)</th>
<th>Sham-Irradiated (N = 20)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Dentate Granule Cells</td>
<td>177.7 (9.3)*</td>
<td>177.1 (9.7)</td>
<td>100%</td>
</tr>
<tr>
<td>Dentate Area [sq mm]</td>
<td>0.5 (0.02)*</td>
<td>2.2 (0.15)</td>
<td>24%</td>
</tr>
<tr>
<td>Density of Dentate Granule Cells</td>
<td>349.1 (28.0)*</td>
<td>824.2 (69.9)</td>
<td>42%</td>
</tr>
<tr>
<td>Thickness of Dentate Granule Cell Layer</td>
<td>3.4 (0.2)*</td>
<td>9.3 (0.4)</td>
<td>36%</td>
</tr>
<tr>
<td>Thickness of CA I</td>
<td>2.6 (0.1)</td>
<td>2.7 (0.1)</td>
<td>96%</td>
</tr>
</tbody>
</table>

*Significantly different t-tests from sham-irradiated, p < 0.001; Numbers are means and (in parentheses) SEMs.

Spontaneous locomotor hyperactivity was progressively reduced as subjects matured.

Histology

Exposure of a portion of the neonatal cerebral hemispheres to early, fractionated doses of ionizing radiation produced a selective reduction of granule cells of the hippocampal dentate gyrus while sparing other brain areas. Specifically, exposure of the neonatal rat hippocampus to ionizing radiation produced a significant, F(2, 28) = 17.5, p < 0.001, depletion of dentate granule cells (see Table 2). Similarly, the thickness of the dentate gyrus and the length of the turning bout in the bowl apparatus, r(18) = -0.48, p < 0.05. These data suggest that the shielding of the olfactory bulb and cerebellum during the irradiation treatment was effective.

We also calculated correlations between anatomical data and the results of the behavioral tests of the third test series (i.e., those conducted just before the animal was sacrificed). Statistically significant negative correlations existed between, for example, the number of cells in the irradiated dentate gyrus and the length of the turning bout in the bowl apparatus, r(18) = -0.48, p < 0.05. Similarly, the area of the dentate gyrus of irradiated rats was negatively correlated with horizontal activity counts, r(18) = -0.44, p < 0.05, and measures of locomotor stereotypes, r(28) = -0.39, p < 0.05. These data suggest that radiation-induced damage to the granule cells of the dentate gyrus predict locomotor hyperactivity and perseverative turning in a plastic hemisphere. We did not find similar correlations between our behavioral parameters and anatomical measures derived from the cerebellum or olfactory bulb.

Spontaneous Rotation

Radiation-induced hippocampal damage caused rats to make long bouts of turns in a plastic hemisphere without reversal of direction (see Fig. 2). Once they began moving in either direction, irradiated rats perseverated in that turning to an extent significantly greater than the sham-irradiated subjects, F(3, 28) = 17.09, p < 0.001.

The ANCOVA also revealed that there was a statistically
significant interaction between the effect of the radiation treatment and the test series, \( F(2,79) = 4.17, p = 0.01 \). These data suggest that, during the course of the experiment, perseverative turning became progressively more pronounced in irradiated animals than it did in controls. t-Tests comparing irradiated and sham-irradiated subjects at each test series confirmed this finding [Test series 1: \( \pi(79) = 2.99, p = 0.01 \); Test series 2: \( \pi(79) = -5.72, p = 0.001 \); Test series 3: \( \pi(79) = 7.02, p = 0.001 \)].

The total number of quarter turns is a reflection of spontaneous activity that took place in the bowl apparatus. There was no difference between the total quarter turns exhibited by irradiated and sham-irradiated rats. However, the ANCOVA did reveal a significant decrease in this activity parameter as subjects matured [effect of the covariate of "age": \( F(1,79) = 6.94, p = 0.01 \)]. It is of interest that, in mature adult animals that move less in the bowl apparatus, perseverative spontaneous turning becomes most prominent. These data suggest that increases in spontaneous perseverative turning in older subjects is not merely an outcome of enhanced frequency of turns.

**Passive Avoidance**

Rats with radiation-induced damage to the fascia dentata took more trials to learn the passive avoidance task, i.e., to meet a criterion of staying on the safe side for 120 seconds, \( F(1,38) = 4.4, p = 0.04 \). Irradiated rats took an average of 6.8 ± 0.7, SEM, trials (over all test series) to meet this criterion while sham-irradiated rats learned the task in only 4.4 ± 0.3, SEM, trials. Although the ANCOVA revealed no effect of age, there was a tendency for the data (mean trials to criterion of the irradiated and sham-irradiated subjects to converge during the final test series (see Fig. 3). During the first 2 behavioral tests, irradiated rats took more trials than did the sham-irradiated rats to meet the performance criterion [Test series 1: \( \pi(39) = 1.80, p = 0.05 \); Test series 2: \( \pi(39) = 2.72, p = 0.01 \)]. During the final test series impaired reacquisition of the passive avoidance task was not observed in the rats with granule cell hypoplasia.

Irradiated rats also spent less time on the safe side of the passive avoidance chamber during the second trial (the one immediately following the first foot shock) on the test day, \( F(1,38) = 5.3, p = 0.03 \). Overall the test series, rats with radiation-induced hippocampal damage had an average latency to move onto the shock grid of only 10.8 ± 2.7, SEM, while sham-irradiated rats waited over twice as long (24.9 ± 4.5, SEM). According to the ANCOVA, these differences between irradiated and sham-irradiated rats did not change significantly with animal age. As Fig. 4 illustrates, however, the difference between the latencies of irradiated and sham-irradiated rats is most dramatic during the first 2 behavioral tests [Test series 1: \( \pi(39) = 1.91, p = 0.05 \); Test series 2: \( \pi(39) = 2.54, p = 0.01 \); Test series 3: not a significant difference between groups].

Learning deficits in irradiated rats with fascia dentata damage seem to be specific to passive avoidance acquisition. These animals readily learned to run to the adjacent compartment for food reward during the initial stages of training. Further, long-term retention of the learned response of running for food reward was illustrated by the fewer and fewer training trials required by the irradiated animals during the second and third behavioral test series. Thus, irradiated and sham-irradiated rats were similar in their learning and retention of this skill of shuttling from one compartment of the box to another.

**Spontaneous Locomotion**

Locomotor activity parameters produced by radiation-induced hippocampal damage were evident at the time of the first performance tests but changed significantly over time. After adjusting for test series in the ANCOVA, subject age seemed to affect all locomotor parameters measured: vertical activity, \( F(1,77) = 6.26, p = 0.015 \); stereotypy, \( F(1,77) = 14.84, p = 0.0002 \); and total distance traveled, \( F(1,77) = 20.38, p = 0.0001 \). Changes in spontaneous locomotion were most evident in aging subjects with radiation-induced brain damage while remaining fairly stable over time in sham-irradiated control rats (see Fig. 5). Radiation-induced changes in locomotion were dependent on the test replication within which these measures were recorded [radiation treatment × test series interactions: \( F(1,7) = 3.8, p = 0.052 \); \( F(1,7) = 3.6, p = 0.052 \)]. Subsequent t-tests revealed that measures of stereotypy and total distance traveled were initially higher in irradiated rats with hippocampal damage than in sham-irradiated animals (stereotypy,

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**FIG. 3.** Mean number of trials to achieve criterion (staying in the safe area for at least 120 seconds) on the passive avoidance task during each of the 3 behavioral test series. Irradiated rats and fascia dentata damage take longer to learn this task than do sham-irradiated controls. Lines are fit using the least squares method. Vertical bars represent the SEM. *Different from sham-irradiated controls, \( p = 0.05 \).
PROGRESSIVE BEHAVIORAL CHANGES

FIG. 4. Mean time (seconds) spent in the safe compartment of the passive avoidance apparatus on test trial 2 (following the initial shock in the adjacent compartment). Latencies are illustrated for each of the 3 test series. Irradiated rats with fascia dentata damage more quickly re-enter the chamber in which they have just been shocked than do sham-irradiated controls. Lines are fit using the least squares method (39). Vertical bars represent the SEMs. *Different from sham-irradiated controls *p<0.05.

\[ t(77) = -3.87, \ p<0.001 \] total distance: \[ t(77) = -3.41, \ p<0.01 \]

This hyperactivity diminished and, in some cases (e.g., total distance traveled) became depressed during subsequent testing [Test series 3: \( t(77) = 2.96, \ p<0.01 \)]. Similarly, vertical activity, which was initially as prevalent in irradiated as in control animals, was later significantly reduced in irradiated subjects [Test series 2: \( t(77) = 2.91, \ p<0.01 \); Test series 3: \( t(77) = 4.50, \ p<0.001 \)].

Throughout most of this experiment, weight gains were observed both in control animals and (to a lesser extent) in irradiated subjects (see Fig. 1 and "Subject" data in the Method section). ANCOVAs used to determine the contribution of weight to the decline of stereotypy and vertical activity revealed that body weight was not a significant covariate. However, weight was a significant covariate within an analysis of total distance traveled, \( F(1,77) = 6.83, \ p = 0.01 \). Although enhanced weight may be thought to explain the reductions of spontaneous locomotion reported here, the variability of the effect of body weight on these locomotor parameters suggests that weight may actually play a more limited role in producing these behavioral declines. In this study, age seems to be a more consistent covariate of locomotor decline than is weight (see above). Further, the fact that locomotion is reduced in predominantly the irradiated rats, (subjects that

FIG. 5. Spontaneous locomotion of irradiated rats with fascia dentata damage and their sham-irradiated controls during 3 separate behavioral test series. Means for each of the following measures are illustrated: (A) Total distance traveled, (B) Stereotypy, and (C) Vertical activity (see text for definitions of these parameters). Lines are fit using the least squares method (39). Vertical bars represent the SEMs. *Different from sham-irradiated controls *p<0.05.
weight less than sham-irradiated animals suggests that weight may not be a significant predictor of locomotor hypoactivity in the mature adult rats of this study.

**DISCUSSION**

The data reported here suggest that behavioral decrements following radiation-induced damage to the neonatal fascia dentata are task-specific and dynamic. Behavioral deficits may become more severe (e.g., spontaneous perseverative turning without reversals) or less severe (e.g., spontaneous locomotor activity) as the brain-damaged rat is tested during maturation. The progressive and task-dependent nature of these behavioral alterations highlights the need for multiple behavioral assessments throughout the development of the subject in order to fully characterize certain neurotoxic effects.

The results of the studies reported here are, in many ways, similar to those published by Brunner and collaborators (9, 10). Despite the use of different experimental designs, behavioral techniques and apparatus, both of our laboratories describe a decline in spontaneous locomotion in maturing rats with radiation-induced damage to the hippocampal granule cells. This effect was marked compared to the relatively stable activity of sham-irradiated rats.

Brunner (10) also reported either 1) "persistent" deficits in the spontaneous alternation in a T maze of 6-month-old rats that were irradiated as neonates, or 2) a continuing "deterioration" in the spontaneous alternation of slightly older irradiated subjects (6-8 months) (9). In an important way the bowl apparatus, in which our rats spontaneously turn, is like a T maze. In a T maze there is one choice point, while the bowl gives a circular continuum of choice points. In a T maze a spontaneously alternating rat reverses direction on each discrete trial. In the bowl there is no discrete choice point. When a rat has made a full circle in the plastic hemisphere it begins to retrace the path most recently traversed. This presents an opportunity to either continue or reverse direction. The mean turning bout length is essentially a measure of alternation frequency corrected for the total number of quarter turns made. Therefore mean bout length may be considered a continuous (nondiscrete-trial) measure of spontaneous alternation. During the first behavioral test series sham-irradiated rats tended to stop and reverse direction after making almost full circle (mean of 3.6 quarter turns) while rats with damage to hippocampal dentate granule cells continued beyond this point to a mean of 5.9 quarter turns before reversing direction. Our mature rats were on average 4 months older than Brunner and Allman's (10) oldest subject and therefore a further "deterioration" of spontaneous alternation might be expected. Indeed, our mature adult animals showed a clear lengthening of their mean circular bout and made over 10 quarter turns before alternating their direction of movement. When these data are taken in conjunction with those of Brunner (10) who used a design that did not call for repetitive testing, we may conclude that maturation plays a dominant role in producing enhanced perseverative responding in mature rats with fascia dentata damage.

Because our rats were irradiated-sham-irradiated as neonates and then tested 3 times on 3 different behavioral measures, they received a significant amount of handling. In fact, the subjects quickly became quite tame and remained so throughout the experiment. Others have reported that neonatal handling can prevent behavioral deficits and hippocampal damage normally seen in aged animals (25). Further, Tiber and collaborators (24) suggest that handling can eliminate the rat's hypersensitivity normally observed after colchicine-induced damage to the adult dentate gyrus. These behavioral effects of handling were observed in spite of significant decreases in both granule cell number and volume of hippocampal mossy fibers. Here we report that: 1) the initial horizontal locomotor hyperactivity of our mature irradiated handled rats decreased to a level similar to, or below, that of the controls (also handled); and 2) handled animals with neonatally damaged hippocampi exhibited several behavioral deficits some of which (e.g., perseverative turning) became potentiated in the mature adult. These data may suggest some task-specific relationship between handling, early hippocampal damage and maturational effects that requires further study.

The neuronal substrate of the progressive behavioral changes that we report remains obscure. The mean percent reduction in the number of hippocampal granule cells in our rats (as compared to controls) was 90% when the subjects were sacrificed at over 1 year of age. This damage was roughly comparable to the radiation-induced hypoplasia previously reported in rats 30-125 days old (6,24). Thus the percentage of cells remaining in the damaged dentate gyrus after radiation exposure is quite stable over time and does not change in synchrony with our behavioral alterations. We know, however, that the total number of granule cells in the rat dentate gyrus is not static even in the adult. Bayer (15) has reported a 38-43% linear increase in the number of dentate granule cells between 1 month and 1 year. Others have observed significant changes in the mature hippocampus that they attribute to the development of axons and dendrites (12) or astroglia (24) rather than perikaryon. Perhaps most relevant to the present study are the findings of Zimmer and his colleagues (14-14) who have shown that the brain may compensate for early radiation-induced damage to the hippocampal granule cells by stimulating dendritic growth. Their results demonstrate that a reduction of a specific neuronal population can induce: 1) a compensatory increase in the neuritic layers containing the dendrites of the remaining neurons, 2) a corresponding relative increase in their axonal projections, and 3) a shift and expansion ofafferent projections to an adjacent neuronal population. Thus, although our hippocampal radiation produces damage specific to the granule cells, subsequent brain changes, in reaction to this initial damage, may produce more pervasive changes in neuroanatomy. These, or other changes in neuroanatomy, may contribute to the progressive behavioral changes that we observed in mature adult rats that were partially head irradiated as neonates.

Our longitudinal experiments have followed changes in the behavioral responses of rats with early radiation-induced hypoplasia of the dentate gyrus granule cells. Behaviors observed a year or more after the early brain damage are, in many ways, different from the acute behavioral response. These progressive behavioral changes may be mediated by normal developmental processes, the brain's natural compensatory reactions to early damage, experimental variables (e.g., handling) or other mechanisms that have yet to be fully elucidated.

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REFERENCES

1. Altman, J., Brunner, R. L.; Bayer, S. A. The hippocampus and

2. Altman, J. An animal model of minimal brain dysfunction. In Lewis,
M., ed. Learning disabilities and prenatal risk. Urbana and Chicago:


4. Bayer, S. A., Altman, J. Hippocampal development in the rat
Cyto genesis and morphogenesis examined with autoradiography and

5. Bayer, S. A., Altman, J. Radiation-induced interference with postna
tal hippocampal cyto genesis in rats and its long-term effects on the

6. Bayer, S. A.; Brunner, R. L.; Hine, R., Altman, J. Behavioral effects of
interference with the postnatal acquisition of hippocampal granule


8. Bayer, S. A. Changes in the total number of dentate granule cells in
juvenile and adult rats. A correlated volumetric and 'H-thymidine

9. Brunner, R. L., Altman, J. The effects of interference with the maturation
of the cerebellum and hippocampus on the development of adult
behavior. In Stein, D. G., Rosen, J. J., Butters, N., eds. Plasticity and
recovery of function in the central nervous system. New

10. Brunner, R. L. A cross-sectional study of behavior at three ages after
neonatal X-irradiation of the hippocampus. Behav. Biol. 22:211-218,
1978.


12. Coleman, P. D., Flood, D. G., West, M. J. Volumes of the compo
ments of the hippocampus in the aging F344 rat. J. Comp.


14. Grazzara, R. A.; Altman, J. Early postnatal X-irradiation of the
hippocampus and discrimination learning in adult rats. J. Comp.
Physiol Psychol 95:484-495, 1981.

15. Greenstein, S., Gluck, S. D. Improved automated apparatus for
recording rotational testing behavior in rats or mice. Pharmacol.
Biochem Behav. 3:491-500, 1975.

granule cell agenesis on acquisition of escape from fear and
one-way active avoidance responses. J. Comp. Physiol.

17. Hicks, S. P. Radiation as an experimental tool in mammalian

18. Hicks, S. P., D'Amauro, C. J. Effects of ionizing radiation on
mammalian development. In Woollam, D. H. M., ed. Advances in


20. Kuncel, D. J., Hunt, E. I. Ionizing radiation: Neural function and

in long-term toxicity studies. In The laboratory animal in drug testing.
Hanover: 9th ICFA Symposium, 1972.

22. Lauter, R. H., Cowan, W. M. Electron microscope studies of the
dentate gyri of the rat I. Normal structure with special reference to

23. LaBossiere, E. Histological processing for the neural sciences. Spring

24. Landfield, P. W., Rose, G., Sandler, L., Wolfsthaler, T. C., Lynch,
G. S. Patterns of astrogial hypertrophy and neuronal degeneration

Sapolsky, R. M. Effect of neonatal handling on age-related impair

Alder, C. E. Spontaneous perseverative turning in rats with radia

27. Moore, B. J., Stern, J. S., Horwitz, B. A. Brown fat mediates
increased energy expenditure of cold-exposed neonatal rats.

28. Mosier, D. J., Janowsky, R. A. Effect of X-irradiation of selected areas
of the head of the newborn rat on growth. Radiat. Res. 42:92-104,
1970.


30. Olson, D. S. Memory functions and the hippocampus. In Scantt, W.
ed Neurobiology of the hippocampus. London: Academic Press,


32. Petley, J. A. Growth tables for strains and stocks of laboratory

33. Seres, L., Pokorny, J. Structure of the granular layer of the rat

34. Tolson, H. A., Hart, G. J., Sanya, K., Rogers, B. C., Peterson, N.
J., Jensen, K. D. P. Experimental factors in the expression of
hippocampal hyper trophy produced by intradendritic colchicine. Lack
of effect of GMI ganglioside on colchicine-induced loss of granule cells

35. Van Cleve, C. D. Irradiation and the nervous system. New

36. Wallace, R. B., Kaplan, N. F., Werbott, J. Behavioral correlates of
hippocampal X-irradiation in rats. Exp. Brain Res. 24:343-349,
1976.

37. Wallace, R. B., Graznadel, R., Werbott, J. Behavioral correlates of
local hippocampal X-irradiation in rats II. Exp. Brain Res.

38. Wersboth, N. H. Bacterial and mycotic diseases. In Baker, H. L.,
Lindsey, J. R., Wersboth, N. H., eds. The laboratory rat: vol 1

39. Winer, B. J. Statistical principles in experimental design. New

40. Zimmer, J., Sunde, D., Svin, R. T. Reorganization and re-formation of
central nervous connections after injury: An lesion and transplant
study of the rat hippocampus. In Will, B. F., Schmitt, P., Darius,
Aldorf, J. C., eds. Brain plasticity: learning and memory. London:

41. Zimmer, J., Laberge, S., Sunde, N. Non-cholinergic factors in the
development of the cholinergic septo-hippocampal projection. A study of
the AChE staining patterns of the tecto-dentata and hippocampus after
lesions, irradiation, and intracerebral grafting. Exp. Brain Res. 64:158-178,
1989.

42. Zimmer, J., Poulsen, P. H., Blackstad, T. W., West, M. J. Radiation-
induced reduction of the rat dentate gyrus. Compensatory cellular and
What's your Diagnosis?

Stomach Nodules in Pigeons

By

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Sixty male white Carneau pigeons, 6 to 12 months old, were procured from a reputable supplier for subsequent use in behavioral studies. Upon receipt, the pigeons were housed individually in stainless steel cages and acclimated to the animal room environment for a period of 3 weeks. During this time the pigeons were provided grit, mixed grains, and water ad libitum.

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The animal room was monitored at least twice daily and kept at 70°±2° F temperature and 50%±5% humidity. There were between 10 and 15 complete room air changes per hour. Following the acclimation period, the pigeons were maintained at specified weights (70%, 80%, or 100% of free-feeding weight) through individualized feeding of mixed grain. Water and grit were continued ad libitum. The animals were then started on behavioral training protocols.

Clinically and behaviorally the pigeon colony remained healthy. Pigeons were periodically submitted to necropsy according to protocol or at the termination of experiments. Most observed lesions were attributable to specific experimental manipulation. However, about 30 to 35% of all the pigeons (both control and treated), demonstrated a similar, distinctive gross necropsy finding: The serosal surface of the proventriculus contained multiple slightly raised black nodules of 1 to 2 mm in diameter (see Figure 1). The degree of involvement ranged from a half dozen nodules to total coverage of the organ. The mucosal surface was thickened and contained multiple .5 mm craterform structures with central open pores. On cut section the mucosa contained multiple round to oval cystic spaces filled with a black granular material, which, if squeezed, popped out, leaving a clear, mucin-filled space (see Figure 2). Tissue samples were taken for histology.

What's your diagnosis? How common is this problem? Why are there no clinical symptoms? Can it be treated?

Figure 1. Proventriculus, gizzard, and duodenum showing multiple 1 to 2 mm dark nodules in the wall of the proventriculus.

Figure 2. Proventriculus showing craterform thickening of the mucosa and cross sections of black nodules in the wall.
THE BLACK TO DARK RED nodules embedded in the mucosa of the proventriculus were gravid female nematode parasites. *Tetrameres* sp. The histologic cross sections of the proventriculus demonstrated multiple dilated gastric glands containing large, globular parasites (see Figure 3). One gravid female usually occupied each gland unless a much smaller male parasite was also present (see Figures 3 and 4). The female parasite is composed predominantly of uteri containing numerous thick-shelled embryonated and unembryonated eggs and an intestinal tract lined by uniciliated, cuboidal, epithelial cells with microvilli. *Tetrameres* sp. have a thick cuticle with bilateral alae, often prominent lateral cords, and coelomyarian-polymyarian musculature (1). (Body wall structure of the parasite is more easily characterized in the male than in the expanded gravid female.)

Histopathological changes in the mucosa were mild. They included compression and flattening of the secretory epithelium in affected glands and a multifocal, mild, interstitial infiltrate of lymphocytes and plasma cells. Preexisting lymphoid nodules within the mucosa often appeared enlarged with increased numbers of surrounding plasma cells. Although the affected pigeons in this report were asymptomatic, previous reports of tetrameriasis in pigeons have described clinical illness in some birds (2-4). Affected birds may show weight loss, anemia, and generalized weakness, especially after flight. Some birds may also develop diarrhea. Oral treatment with levamisole at 33 mg/kg has been the recommended method for controlling the infestation (2).

*Tetrameres* sp. are spirurid nematodes in the order Spiruridae and suborder Spirurina. *Tetrameres americana* and *T. columbicola* are the species most described in pigeons (2-4). These spirurids have an indirect life cycle and require an intermediate host (5), such as grasshoppers, sowbugs, cockroaches, pillbugs, and earthworms. Thus, control of these vectors may help reduce the incidence of infestation.

**References**


Contributions to “What’s Your Diagnosis?” by readers of *LAB ANIMAL* are welcomed. Selections will be made on the basis of relevancy and interest to readers. Submission of black and white illustrations or photos is encouraged. Please address contributions to Thomas M. Donnelly, *LAB ANIMAL*, 65 Bleecker St., New York, NY 10012.
An Assessment of the Behavioral Toxicity of High-Energy Iron
Particles Compared to Other Qualities of Radiation

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Conditioned taste aversion was used to evaluate the behavioral toxicity of exposure to high-energy iron particles (7Fe, 600 MeV/amu) in comparison to that of gamma photons (60Co). High-energy electrons or fission neutrons. Exposure to high-energy iron particles (50-500 eGy) produced a dose-dependent taste aversion with a maximal effect achieved with a dose of 30 eGy. Gamma photons and electrons were the least effective stimuli for producing a conditioned taste aversion, with a maximal aversion obtained only after exposure to 500 eGy, while the effectiveness of fission neutrons was intermediate to that of photons and iron particles, and a maximal aversion was obtained with a dose of 100 eGy. In the second experiment, rats with lesions of the area postrema were exposed to iron particles (30 eGy), but failed to acquire a taste aversion. The results indicate that (1) high-energy iron particles are more toxic than other qualities of radiation and (2) similar mechanisms mediate the behavioral toxicity of gamma photons and high-energy iron particles.

INTRODUCTION

As manned exploration of the solar system increases in the coming decades, astronauts will be leaving the protection provided by the earth's magnetic field. As a result, they will be exposed to radiation qualities and doses that will differ significantly from those experienced in earth orbit, largely from intergalactic cosmic rays. Intergalactic cosmic rays are composed of protons, alpha particles, and heavy particles with high energy and charge (HZE). The toxic effects of such exposures, particularly nausea and emesis, may interfere with the ability of astronauts to successfully complete their assigned missions. Protection against the effects of these exposures will require an understanding of the toxicity of high-energy particles and their relationship to the
toxicity of other qualities of radiation and of the mechanisms by which such exposures can produce effects on behavior.

Although there has not been much research evaluating the effects of different qualities of radiation on behavioral tasks, the research that is available suggests that the toxic effects of exposure may be specific to the quality of the radiation (1, 2). As such, in the absence of specific data, attempts to generalize to the behavioral effects of exposure to high-energy particles on behavior based upon the effects produced by other qualities of radiation may not provide an accurate guide to the potential effects of such exposures. It may not be possible, therefore, to predict the effects of exposure to high-energy particles on behavior based upon research using other qualities of radiation. The present experiments were designed specifically to provide an initial assessment of the behavioral toxicity of high-energy iron particles in relation to other qualities of radiation and of the mechanisms by which they can affect behavior.

The behavioral task utilized for these experiments was the conditioned taste aversion. A conditioned taste aversion is acquired when a normally preferred novel tasting saccharin or sucrose solution is paired with a toxic unconditioned stimulus, such as lithium chloride or gamma or x radiation, such that the organism will avoid ingestion of that solution at a subsequent presentation. This avoidance behavior is typically acquired in a single pairing of the solution and toxin and may be observed at dose (or exposure) levels that produce no obvious signs of illness in the organism (3). Conditioned taste aversion is functionally related to emesis in that both responses serve to limit the intake and/or absorption of toxic substances (4, 5). As such the conditioned taste aversion is a standard paradigm for assessing the potential toxicity of a wide range of stimuli (6).

EXPERIMENT I

The first experiment was designed to establish the dose–response relationships between exposure to high-energy iron particles and the acquisition of a conditioned taste aversion in comparison to exposure to other qualities of radiation.

Methods

The subjects were 248 male Crl:CD BR ICR Plus rats (Rattus norvegicus) weighing 300–400 g at the start of the experiment. Rats were quarantined on arrival and screened for evidence of disease before being released from quarantine. They were maintained in AAALAC-accredited facilities in plastic Macrolon isolator cages on hardwood chip contact bedding and provided commercial rodent chow and acidified water. Animal holding rooms were maintained at 21 ± 1°C with 50 ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. The rats were maintained on a 12:12 light dark full-spectrum lighting cycle with no twilight.

Procedure

The behavioral procedures have been detailed previously (7). Briefly, all rats were adapted to a water deprivation schedule for 5–7 days, during which they received water for 30 min each day. On the conditioning day, the rats were presented with a single calibrated drinking tube which contained a 10% sucrose solution in place of the water, and the intake was recorded. Immediately following the drinking period, the experimental animals were irradiated with one of the four qualities of radiation. The control animals were taken to the radiation source, but not exposed, to provide controls for the handling required to bring the
animals to the various sources. On the test day, 24 h later, the rats were again presented with the single drinking tube containing the 10% sucrose solution, and their intake was measured. Statistical analysis of the data was done using two-way analyses of variance.

Irradiation with heavy particles was done using the BEVALAC at the Lawrence Berkeley Laboratory (LBL). Groups of rats (9-12/group) were exposed to doses of 5, 10, 20, 30, 40, 50, 100, or 500 cGy of $^{56}$Fe particles at an average dose rate of 10-50 cGy/min. The energy of the particles was 600 MeV/amu to take advantage of the plateau of the Bragg curve. Dosimetry was provided by the staff of the BEVALAC facility using standard procedures that have been described previously (8, 9).

All other irradiations were done using the sources at the Armed Forces Radiobiology Research Institute. Irradiation with fission neutrons was performed using the TRIGA reactor, which was set to deliver a neutron-gamma ratio of 20:1. The tested doses were 5, 10, 20, 30, 40, 50, or 100 cGy delivered at a dose rate of 5 cGy/min. Exposure to electrons was performed using a linear accelerator which provided 4-μs pulses (15 pulses/s) of 18.5 MeV electrons. Gamma irradiation was provided by a $^{60}$Co source. For both electron and gamma irradiation, the tested doses were 10, 50, 100, or 500 cGy, at a dose rate of 130 cGy/min for electrons and 40 cGy/min for gamma rays. Radiation dosimetry was performed using paired 50-ml ion chambers. Dose was calculated using a tissue-equivalent ion chamber placed inside an acrylic rat phantom and expressed as the ratio of the dose measured in the phantom to that measured free in air ($^\circ$).

**Results**

The results are summarized in Fig. 1, which presents actual test day sucrose intake, and Fig. 2, which presents test day sucrose intake as the percentage of conditioning day intake. These figures show that the acquisition of a conditioned taste aversion following irradiation is a function of both the quality and the dose of the radiation. Both the threshold dose and the slope of the dose–response curve vary as a function of the quality of radiation.

Statistical comparisons indicated that the effects of exposure to $^{60}$Co photons on the acquisition of a conditioned taste aversion were identical to those obtained following exposure to high-energy electrons. There were no differences in either the threshold at which a significant response was first observed, 50 cGy, or the dose at which the maximum taste aversion was observed.

In contrast, the behavior of both the gamma- and electron-irradiated animals differed from those exposed to fission neutrons. The threshold for observing a condi-
tioned taste aversion between 10 and 20 cGy was considerably lower than that following exposure to gamma photons and electrons. In addition, as shown by the significant quality-by-dose interaction ($F(3,61) = 5.57$, $P < 0.01$), the dose–response curve following exposure to fission neutrons was steeper than that obtained with these other two qualities of radiation.

The effects of exposure to high-energy iron particles on the acquisition of a conditioned taste aversion were, in turn, significantly greater than those observed following exposure to fission neutrons ($F(1,134) = 15.59$, $P < 0.001$). Although the threshold at which a taste aversion was first observed was similar, exposure to 20 cGy produced a much greater avoidance of the sucrose following exposure to the iron particles than following exposure to neutrons. In addition, a nearly total avoidance of the conditioned stimulus was observed following exposure to only 30–40 cGy of high-energy iron particles which showed no further changes despite increasing the dose to 500 cGy. In contrast, exposure to neutrons did not produce an equivalent effect until a dose of 100 cGy had been utilized. The significant quality-by-dose interaction ($F(7,143) = 2.52$, $P < 0.05$) confirms that the pattern of responding as a function of dose was not identical between the two groups, with the animals exposed to $^{56}$Fe showing a maximal aversion at a much lower dose than the animals exposed to the fission neutrons.

Discussion

These results indicate that different qualities of radiation differ in terms of their capacity to lead to the acquisition of a conditioned taste aversion. Since the taste aversion is a measure of the toxicity of a stimulus (6), the results show that these different qualities of radiation differ in terms of their toxic effect on behavior. At the lowest level of toxicity are electrons and gamma photons which show identical profiles in terms of their capacity to induce a conditioned taste aversion. The most toxic radiation stimulus is produced by exposure to high-energy iron particles, which showed the lowest threshold and steepest dose–response curve. Exposure to fission
neutrons produced intermediate levels of toxicity, both in terms of thresholds and in terms of the slope of the dose–response curve.

Previous research on a variety of physiological endpoints indicates that the relative biological effectiveness for lethality of neutron irradiation is greater than that of gamma photons (10, 11). Similarly, studies looking at physiological and morphological endpoints of exposure to $^{56}$Fe suggest that exposure to these high-energy particles produces more damage than exposure to equivalent doses of lower linear energy transfer (LET) radiation (12–14). The present results are therefore consistent with these previous studies in showing increasing effects of irradiation on the acquisition of conditioned taste aversion with increasing LET.

Although there are fewer studies available examining the effects of variations in radiation quality on behavioral performance, these studies also indicate that the effect of irradiation on behavior may vary as a function of the quality of the radiation. Thus it has been reported that the frequency of vomiting is greater following exposure to a radiation field containing a higher proportion of fission neutrons to gamma photons (15). Similarly, studies of maze learning following irradiation of the hippocampus with $^{56}$Fe particles showed deficits in performance following exposure to doses of 50 cGy (16). In contrast, using a different behavioral task, the accelerom which measures a motor performance decrement following high-dose irradiation, exposure to a given dose of fission neutrons or gamma photons produces the smallest decrease in performance while exposure to electrons causes the greatest decrement (2). The present results, showing that neutron irradiation has a greater effect on the acquisition of a conditioned taste aversion than does gamma irradiation, are consistent with the data on emesis following irradiation. This agreement between the data for taste aversion and emesis may be a reflection of the fact that taste aversion, unlike the accelerom which measured motor activity, is a behavioral measure of stimulus toxicity which is functionally related to emesis (5). Similarly, the present data concerning the toxicity of $^{56}$Fe particles are consistent with the observations of $^{56}$Fe-induced deficits in maze learning by showing that exposure to low doses of high-energy particles can produce severe effects on behavior (16). These data therefore suggest that the effects of radiation on behavior are a function of both the quality of the radiation and of the nature of the behavioral task.

**EXPERIMENT 2**

The finding that exposure to $^{56}$Fe produces a conditioned taste aversion at lower doses and with a significantly steeper dose–response curve than the other qualities of radiation raises the question of whether this represents quantitative differences between the different radiation qualities, or whether it reflects the operation of fundamentally different mechanisms leading to changes in behavior.

Studies with gamma photons have shown that conditioned taste aversion following exposure to $^{60}$Co requires the mediation of the area postrema (17, 18), the brainstem chemoreceptive trigger zone for emesis (19). This finding has been interpreted as indicating that the acquisition of a conditioned taste aversion following exposure to gamma radiation is a peripheral effect of the exposure: that irradiation causes the release of some humoral factor which circulates in the blood and/or cerebrospinal
fluid affecting area postrema activity which, in turn, leads to the behavioral response
(5). In contrast, most research on the mechanisms by which exposure to high-energy
particles affect the organism has focused on the concept of a "microlesion" (20, 21).
This is the concept that irradiation with these particles produces a series of discrete
microscopic tracks or "lesions" in the tissue, which are responsible for both the mor-
phological and behavioral effects of exposure. Since head-only exposure to higher
dose gamma photons (300 cGy) can produce a conditioned taste aversion which is
partially independent of the area postrema (22), there is the possibility that the taste
aversion seen following exposure to 56Fe particles may result from direct effects
on neural tissue and not from the release of a humoral mediator, as with gamma
photons.

The present experiment was designed to evaluate the role of the area postrema in
the acquisition of a conditioned taste aversion following exposure to high-energy iron
particles to determine whether or not mechanisms similar to those observed with
gamma irradiation are involved in its acquisition.

Procedure

The subjects were 57 male albino rats weighing 250-275 g at the time of
surgery. Histologically verified lesions were made in the area postrema of 37 rats at AFRR1
using techniques that have been detailed previously (17). Briefly, the rats were anesthetized with pentobarbital
sodium (35 mg/kg, ip), the brainstem was exposed, and the area postrema was cauterized under direct visual control. The wound was closed and
the animals were given a prophylactic injection of Bicillin (100,000 units). The 20 sham-operated controls
were treated identically, except that the area postrema was not cauterized. The animals were then allowed
a recovery period of 3-4 weeks before being shipped to the LBI for the remainder of the experiment.

The behavioral procedures were begun 2 weeks after the animals were shipped to LBI and were identical
to those detailed above, except that only a single dose of iron particles was utilized. The rats with area
postrema lesions and the sham-operated controls were each divided into two groups. The experimental
subjects were 21 rats with area postrema lesions and 10 sham-operated controls which were exposed to 30
cGy 56Fe particles at a nominal dose rate of 10 cGy/min. Sixteen rats with area postrema lesions and 10
sham-operated rats were treated identically, except that they were not exposed in order to serve as controls
for the shipping and handling procedures.

At the conclusion of the experiment, all rats were sacrificed with an overdose of pentobarbital (50 mg),
perfused with isotonic saline and 10% formalin saline, and the brains were removed for histological exami-
nation. Frozen sections were cut through the brainstem at the level of the area postrema at 50 μm and
stained with thionin. Sample photomicrographs of the area postrema and a representative lesion are pre-
sented in Fig. 3.

Results

As shown in Fig. 4, lesions of the area postrema completely disrupted the acquisi-
tion of a conditioned taste aversion following exposure to high-energy iron particles.
In contrast to the area postrema-operated animals, the sham-operated controls
showed a significant reduction in test day sucrose intake (F(1, 29) = 22.87, P < 0.001). There were no differences between the area postrema- and sham-operated groups
(F(1, 24) = 1.14, P > 0.05) which served as shipping and handling controls and which
were not irradiated.

Histological examination of the brains of the rats indicated that, for the most part,
the lesions were restricted to the area of the area postrema, although they did, in some
cases, infringe upon the dorsal parts of the nucleus of the solitary tract or dorsal motor
nucleus. However, given destruction of the area postrema, there was no apparent relationship between this additional tissue destruction and the lesion effects on the acquisition of a conditioned taste aversion.

Discussion

The observation that lesions of the area postrema disrupt the acquisition of conditioned taste aversion induced by \(^{56}\)Fe indicates that the mechanisms by which exposure to these high-energy particles exert their toxic effect on behavior are the same as
those involved in the acquisition of a conditioned taste aversion following exposure to gamma photons, which also depends upon the integrity of the area postrema (17, 18). These data therefore suggest that the differences in toxicity obtained in the first experiment of this series are the result of quantitative and not qualitative differences between these two forms of radiation: that $^{56}$Fe is a much more potent toxin than is $^{60}$Co, but that both produce their effects on behavior through the operation of similar area postrema-dependent mechanisms. While these data do not directly deal with the microlesion concept, the observation that the conditioned taste aversion induced by $^{56}$Fe is also dependent upon the integrity of the area postrema would be consistent with the hypothesis that this response, at this dose (30 cGy), is a peripheral effect of the exposure and does not result from the direct action of the particles on the central nervous system, as has been reported for other behavioral effects of $^{56}$Fe exposure (16).

**GENERAL DISCUSSION**

The present results support the general conclusion that these different radiation qualities differ in terms of behavioral toxicity: $^{56}$Fe is the most toxic, followed by fission neutrons, while $^{60}$Co and electrons produce the least toxic, but identical, effects on behavior. The factors that might be responsible for this ranking are not completely clear. While it might be possible that these behavioral differences reflect differential damage to taste receptor units caused by the different qualities of radiation, resulting in a differential responsiveness to the sucrose conditioned stimulus, this does not seem likely because exposure to gamma photons and cyclotron fast neutrons produce equivalent changes in taste acuity and in detection and recognition thresholds (23). Another possibility is that the toxicity of radiation may be related to L.E.T. Since the intensity of the taste aversion produced by the various qualities of radiation paralleled the L.E.T of those radiation qualities, it may be that the behavioral toxicity of the different radiation qualities depends upon their L.E.T. However, the basis for these differences in behavioral toxicity must await further research utilizing other high-energy heavy particles. Nonetheless, these data, in combination with previous research about the effects of different qualities of radiation, clearly suggest that the
Effects of exposure on behavior are a joint function of both the specific quality of the radiation and the specific nature of the behavioral task. As a result, our ability to generalize about the potential effects of exposure to one radiation quality based upon data from another and from one endpoint to another may be very limited.

With regard to the specific effects of exposure to high-energy heavy particles on behavior, the present results are consistent with the hypothesis that these differences result from a greater effectiveness of these particles in producing the toxic reactions measured by conditioned taste aversion. These results are, in general, consistent with other research using a variety of physiological and behavioral endpoints; this indicates the significantly greater relative biological effectiveness of exposure to high-energy heavy particles (13, 16).

Although this research presents only an initial assessment of the behavioral toxicity of exposure to heavy particles, it does have some implications for manned flights outside the magnetic field of the earth. The relative biological effectiveness of high-energy iron particles is greater than that of gamma photons by a factor of ten, meaning that behavioral effects can be observed following exposure to doses much less than might be expected based upon the data derived from the use of gamma irradiation. As such, these results suggest that estimates of mission failure due to exposure to heavy particles may have to be revised upward.

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REFERENCES

L-LEUCYL-L-LEUCINE METHYL ESTER TREATMENT OF CANINE MARROW AND PERIPHERAL BLOOD CELLS

INHIBITION OF PROLIFERATIVE RESPONSES WITH MAINTENANCE OF THE CAPACITY FOR AUTOGLOGOUS MARROW ENGRAFTMENT

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Incubation of canine marrow and peripheral blood mononuclear cells with L-leucyl-L-leucine methyl ester resulted in the inhibition of mitogen- and alloantigen-induced blastogenesis, the elimination of allosensitized CTL and NK activity, and prevented the development of CTL from pCTL. The effects of these incubations were similar to those described in mice and humans. Additionally, in vitro CFU-GM growth from treated canine marrow was reduced, but could be regained when the Leu-Leu-OMe-treated treated marrow was cocultured with either untreated autologous peripheral blood mononuclear cells or monocyte-richenched PBMC but not with untreated monocytedepleted PBMC. Six of seven dogs conditioned with 920 cGy total-body irradiation engrafted successfully after receiving autologous marrow that was incubated with Leu-Leu-OMe prior to infusion. These cumulative results indicate that incubation with Leu-Leu-OMe is a feasible method to deplete canine marrows of alloreactive and cytotoxic T cells prior to transplantation.

The success of allogeneic marrow transplantation as treatment for malignant and nonmalignant hematopoietic diseases has been restricted by the serious complications of graft-vs.-host disease (1, 2). Experiments in a variety of mammalian marrow transplant model, have shown that removal of mature T cells from donor marrow permits engraftment without the development of GVHD (3–6). Based on these and similar observations, studies have been carried out to evaluate the effects of T cell depletion prior to allogeneic marrow transplantation in humans. Most studies have employed marrow treatments with anti-T cell monoclonal antibodies plus complement or with soybean agglutinin followed by E rosette formation and density gradient centrifugation (7–9). In general, removal of T cells has been associated with a marked decrease in both the incidence and severity of GVHD. However, the use of T cell-depleted marrow has also been associated with an increased incidence of marrow graft rejection (10).

Recently, Thiele and Lipsky have described a dipeptide methyl ester, L-leucyl-L-leucine-methyl ester (Leu-Leu-OMe) that can eliminate natural killer cells (NK), monocytes (Mo), and precursors of alloantigen-specific cytotoxic T cells (pCTL) from mouse spleen cell suspensions and from both mouse and human peripheral blood. This treatment leaves intact B cells, helper T cells, and murine erythrocyte and hematopoietic stem cells (11–14). In a murine histoincompatible marrow transplant model (C57BL/6J→C57BL/6×DBA/2F1), treatment of donor marrow and spleen cells with Leu-Leu-OMe resulted in successful donor marrow engraftment and the development of stable long-term hematopoietic chimerism without GVHD (14–16). The use of Leu-Leu-OMe to treat marrow may have advantages over currently used methods. The use of Leu-Leu-OMe is very simple, requiring but a single 15-min incubation. In addition, it appears that marrow incubation with Leu-Leu-OMe results in the elimination of the cells responsible for acute GVHD while at the same time preserving hematopoietic stem cells needed for engraftment and the cells required for immune reconstitution (15, 16).

We and others have used dogs as a large, outbred animal model for us in experimental marrow transplantation (17, 18). The present studies were undertaken to determine whether the incubation of canine marrow and peripheral blood cells with Leu-Leu-OMe would yield alterations of intracellular immune function comparable to those described in human and murine cells and to investigate the effects of marrow incubation with Leu-Leu-OMe on early hematopoietic progenitors and stem cells assayed both in vitro and in vivo function.

MATERIALS AND METHODS

Dogs Beagles, hounds, and mixed breed dogs, obtained from commercial vendors in Washington and Virginia or raised at the Fred Hutchinson Cancer Research Center (FHCR), were dewormed and vaccinated against distemper, hepatitis, leptospirosis, and parvovirus before use in this study. All dogs were at least 6 months of age and were maintained at the FHCR canine kennel facilities, which guidelines stipulated by the National Academy of Sciences. National Research Council. The research protocol was approved by the Internal Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

Medium Waymouth's MB 752-1 medium (FHCR media preparation facility, supplemented with 0.1 mM nonessential amino acids and 100 µl penicillin and 100 µl streptomycin (4)/ml cl (4).

* Abbreviations—Leu Leu OMe, L-leucyl-L-leucine methyl ester; Mo, monocytes; BML, bulk ML; NWNA, nucleus nonadherent; CFU-C, colony forming unit in culture; PDL, pokeweed mitogen; CTX, canine thyroid adenoma cell line.
was used for the dilution of heparinized whole blood and marrow for the cell separation procedures. Weymouth's medium supplemented as above with the addition of $10^5$ to $20^6$, heat inactivated (56 C) normal pooled dog serum (M:NPS) was used for the mixed leukocyte culture microassays, bulk MLA (H MLA), cell mediated lympholysis, and NK assays.

**Cell preparation.** Peripheral blood mononuclear cells were obtained by the centrifugation of heparinized venous whole blood diluted 1:2 with medium over Ficoll-Hypaque density gradients (Sp. dens. 1.074) as previously described (4). Bone marrow cells (BMC) for in vitro assays were obtained by syringe aspiration from the humeral head of an anesthetized dog. The marrow was diluted 1:2 with medium and overlayed onto Ficoll-Hypaque density gradients for centrifugation (1000 x g), following which the interface cells were washed once with heparinized buffer and twice with medium. The PBMC and marrow cells were resuspended into medium for cell counts and viability assessment using the trypan blue exclusion technique.

Monocyte enriched cells were obtained by treating PBMC with the antiratine murine monoclonal antibody (Dan 6) (20) as follows: 1000 x 10^6 MBMC were incubated for 30 min at room temperature in 30 ml of 1:10 diluted Dan 6 (containing antibody), and then an equal volume of 1:1 diluted rabbit serum complement (Pel Freeze, Rogers, AR) was added for an additional 60 min. The cells were washed once with medium, resuspended in 30 ml of 1:1 diluted rabbit serum complement, and incubated again for 60 min. After washing twice in medium, these cell suspensions contained 61.8% (SEMF) viable monocytes, 16.7% lymphocytes, and 21.5% granulocytes, primarily eosinophils.

Monocyte-depleted PBMC were obtained by first passing PBMC over nylon wool columns as previously described (21) and then transferring 20x10^5 nylon wool nonadherent (NWNA) cells in 10 ml of medium, containing 5% fetal calf serum, into plastic petri dishes (Falcon No. 3000, Lincoln Park, NJ) for 2 hr incubation at 37 C, 7% CO.

This depletion technique yielded approximately 95.1% lymphoid cells with greater than 90% viability and less than 3% monocytes as determined by morphologic assessment of Wright stained extospin preparations.

**Preparation of Leu-Leu OMe.** The Leu-Leu OMe was synthesized from L-leucyl-L-leucine (Sigma Chemical Co., St. Louis, MO) as previously described (11). Qualitative assessment of Leu-Leu OMe purity was obtained by thin layer chromatography (TLC) (22). Briefly, 5 x 5 cm plates of silica gel (Faster, Lincoln Park, NJ) were prepared and the plate was developed and run in a solvent system of chloroform/methanol/20% ammonium hydroxide, 8:2:1, v/v/v. The migration of the four compounds was visualized by using an aerosol spray of 0.2% ninhydrin in ethanol and then placing the plates at 60 C oven for 30 min. R values and the ratio of the distance the compound travels to the distance the solvent front travels were calculated, in order to assess the resultant migration patterns, according to the formula (22):

$$R = \frac{distance \ traveled \ by \ compound}{distance \ traveled \ by \ solvent \ front}$$

Leu-Leu OMe was stored at -20 C in absolute methanol and, based on repeated TLC analysis, was stable for at least three months.

**Incubation of PBMC or marrow cells with Leu-Leu OMe.** Equal volumes of PBMC or marrow cell suspensions and Leu-Leu OMe at the indicated final concentrations were incubated for 15 min at room temperature. Cells for in vitro studies were washed twice and resuspended in medium. Marrow cells used for autologous infusion were incubated at cell concentrations of 2x10^7/ml. Leu-Leu OMe solutions that contained 0.1 U/ml DNAse (Worthington, Enzymes and Biochemicals, Freehold N J). After incubation, these cells were washed, counted, and resuspended within 1-3 hr.

**Mixed leukocyte culture and mitogen assays.** MLRs were established, labeled with [3H]thymidine, harvested, and prepared for liquid scintillation counting as previously described (20) with minor modifications. The 10 Leu-Leu OMe treated or untreated responder and 10 irradiated (2500 rad) untreated, stimulator PBMC were cocultured in a final volume of 200 ml M NPS/20% per cell. Mitogen stimulation was assessed by adding either 0.75 mg/ml PHA (DIFCO, Detroit MI), 200 pg/ml Con A (Calbiochem, San Diego CA), or 200 pg/ml PWM (GIBCO, Grand Island, NY) to 10 treated or untreated responder cells in a final volume of 200 ml M NPS/20%. All cultures were initiated in triplicate in multiwell plates (Costar No. 3596, Cambridge MA) for 7 days at 37 C, 7% CO 2 in a humidified incubator.

**Bulk MLA (H MLA) and cell mediated cytolysis assays.** Bulk MLA were established using either untreated or Leu-Leu OMe treated PBMC or marrow cells as responders, and untreated, irradiated PBMC as stimulators, to generate CTL for CMI assays, as previously described (21). With modifications, CTL were derived from these cultures to form two CMI assay groups: 1) responder PBMC or marrow cells treated with Leu-Leu OMe or MeOH on day 0 prior to mixing with irradiated stimulator PBMC in H MLA (day 9) and 2) Leu-Leu OMe or MeOH treatment of 7-day MLA generated CTL, used in CMI assays. CTLs were mixed at a 5:1 effectortarget ratio with 3L labeled Na Cr, 1000 500 cpm/ml, Nitro, and stimulated with Con A on day 4 of culture. The 4-h 3CI release assay was performed as previously described (11). The mean spontaneous release was 10.1% of total release. The 10% release was 30.2%, for targets used in these studies of CMI assays.

**Proliferation of treated or untreated responder PBMC or marrow cells.** 7-day MLA was measured by counting 200 ul aliquots from each H MLA flask into triplicate microculture wells and labeling for 5 hr with 10 H]thymidine. Cell harvest and liquid scintillation counting were performed as described for MLA (20).

**Natural killer (NK) assays.** Leu-Leu OMe or MeOH treated or untreated PBMC and marrow cells were assayed for NK activity against a C1r labeled canine thymic adenocarcinoma cell line (CTAC) in a 4-hr assay with the percentage of 3CI release calculated as previously described (21).

**In vitro marrow cultures for CFU-GM growth.** Leu-Leu OMe and MeOH treated and untreated marrow cells were tested for in vitro hematopoietic progenitor cell growth using an agar based colony formation assay that utilizes post osteodox serum (PESO) as the source of colony stimulating factor (24). Growth of granulocyte-macrophage colonies (CFUGM) was assayed in cultures containing 10 or 15 x 10^6 treated or untreated marrow cells only, and in cultures with marrow cells and freshly isolated PBMC at both 10 and 15x10^6 cells/ml of PBMC. Leu-Leu OMe treated PBMC depleted with 1000 x 10^6 Leu-Leu OMe treated PBMC were incubated with 1000 x 10^6 Leu-Leu OMe treated PBMC. Cultures were incubated in triplicate for 10 days at 37 C, 7% CO humidified incubator. CFU-GM colonies were enumerated as previously described (24).

**Autologous marrow transplantation.** Marrow for autologous transplantation was obtained by a vacuum pump aspiration procedure (26), processed over a Ficoll Hypaque density gradient and incubated with Leu-Leu OMe. All recipients were conditioned with 250 x 65, total body irradiation delivered as a single exposure to two opposing Co sources at 70 cGy/mm (27). The recipients were infused with treated autologous marrow within 3 hr of THI. Supportive care pre- and posttransplantation with antibiotics, fluids, and whole blood transfusions, was given as previously described (27). In addition, recipients were given oral antibiotics (Neomycin sulfate and polymyxin B sulfate) daily for five days before THI and posttransplant until the granulocyte count reached 500/mm.
RESULTS

Thin-layer chromatographic analysis of synthesized Leu-Leu-OMe. Three batches of Leu-Leu-Ome were synthesized for use in the in vivo studies and autologous transplant experiments described. Consistent R values were obtained for each batch of Leu-Leu-OMe and for the three control compounds tested. Within the Leu-Leu-Ome, there was a secondary spot that migrated with a R value equal to that observed for the Leu-Leu-L-leucine. The consistency of the R values indicated that constant yield and purity was achieved with minimal batch-to-batch variations.

Viability of PBMC and marrow cells after incubation with Leu-Leu-Ome. The viability of treated PBMC and marrow cells was assessed within 1 hr after incubation with Leu-Leu-Ome. There was no significant difference in viabilities observed in cells treated with either Leu-Leu-Ome (up to 1000 μM, 0.5% MeOH in PBS, or PBS only. No time-course studies were done to assess the viability of PBMC and marrow cells more than 1 hr after incubation with Leu-Leu-Ome.

Elimination of allogeneic responses and antigen-induced Epstein-Barr virus blastogenesis by incubation of PBMC with Leu-Leu-Ome. PBMC were treated at either 20 μM or 200 μM with Leu-Leu-Ome and tested in mitogen MLTC and blastogenesis assays (Fig. 1). In all assays there was a Leu-Leu-Ome dose-dependent reduction in the proliferative response such that virtually no blastogenesis was observed after incubation with 1000 μM Leu-Leu-Ome.

Treatment of 200 μM responder PBMC or marrow cells before bulk MLTC resulted in similar Leu-Leu-Ome dose-dependent reductions in proliferative response (data not shown). The marrow cells gave a lower baseline level of [3H]thymidine incorporation and were more sensitive than the PBMC to treatment with Leu-Leu-Ome (data not shown). Treatment of PBMC marrow cells with 0.5% MeOH had no effect on the alloproliferative response in MLTC (data not shown).

Elimination of the generation of antigen-specific cytotoxic T cells in vitro to an antigen of PBMC and marrow cells with Leu-Leu-Ome. The effect of Leu-Leu-Ome on the generation of antigen-specific cytotoxic T cells was measured by incubating responder PBMC and marrow cells with Leu-Leu-Ome (day 0 treatment), and then testing these cells in an in vitro assay after 5 days of culture in RMLTC. Treatment with 1000 μM Leu-Leu-Ome eliminated the generation of cytotoxic activity against 3H-labeled allogeneic-specific Con A-stimulated PBMC (Fig. 2). These results indicated that the generation of CTL from precursors is sensitive to incubation with Leu-Leu-Ome. The effects of Leu-Leu-Ome incubation on CTL already generated in 5 day bulk MLTC (day 5 treatments) were also evaluated. This treatment eliminated antigen-specific cytotoxic activity (Fig. 2). Treatment with 0.5% MeOH, either on day 0 or day 5, did not interfere with either the development of CTL, or the specific lysis of targets assayed on day 5 (data not shown).

Elimination of NK activity after incubation of PBMC and marrow cells with Leu-Leu-Ome. Marked elimination of NK activity, in a dose-dependent manner was seen after incubation of PBMC or marrow cells with Leu-Leu-Ome. NK activity was essentially eliminated after treatment with 1000 μM Leu-Leu-Ome (Fig. 3).

In vitro CTL assay: day 5 culture. PBMC were cultured with Leu-Leu-Ome.
treatment of marrow cells. There was a marked reduction in 
CFU-GM colony growth obtained from marrow cells incubated 
with varying concentrations of Leu-Leu-OMe (Table 1). Minim 
al CFU-GM growth was observed with 10\(^{-6}\) Leu-Leu-OMe 
treated marrow cells per plate were cultured. There was some 
recovery of CFU-GM growth when the cell number was 
increased to 3x10\(^{4}\) treated marrow cells per plate, but only to 
a level that was approximately 25% of that observed with 3x10\(^{4}\) 
untreated marrow cells. Addition of either untreated, autol 
ologous PBMC or M\(^e\)-enriched autologous PBMC increased 
CFU-GM growth, but not to the levels observed with untreated 
marrow cells in similar cocultures. The addition of M\(^e\)-depleted 
or Leu-Leu-OMe treated autologous PBMC to treated marrow 
cells did not augment growth.

The effect of incubating marrow cells with Leu-Leu-OMe on 
autologous marrow engraftment. Six of the seven dogs given 
Leu-Leu-OMe treated autologous marrow engrafted and did so 
with kinetics similar to those seen in recipients of untreated 
autologous marrow (Table 2 and Fig. 4). Platelet counts re 
turned to normal levels between 20 to 30 days posttransplant. 
Dog C521 did not survive past day 20 posttransplant. After 
Leu-Leu-OMe incubation, the marrow cells from this dog were 
clumped and had only 20% viability, resulting in the infusion 
of a very low marrow cell dose. The marrow from C580 was 
incubated with the same concentration of Leu-Leu-OMe as 
C521, but the treated marrow cells of C580 did not clump and 
this dog survived with rapid engraftment. Dog BB8701 had to 
be euthanized on day 19 posttransplant due to an accidental 
sepsis. They further showed that incubation of mix 
tures of murine marrow and spleen cells with Leu-Leu-OMe 
did not interfere with engraftment and could, in certain circum 
stances, prevent GVHD (14-16). In the studies presented here, 
we found that canine peripheral blood and marrow cells behave 
similarly following exposure to Leu-Leu-OMe with the elimi 
nation of functional monocytes, NK cells, and alloantigen 
sensitized CTL, and inhibited the development of CTL from 
pCTL. Further, we found that incubation of marrow cells with 
Leu-Leu-OMe (even at very high doses) did not inhibit autol 
ologous engraftment in recipients conditioned with 920 cGy TBI. 
These studies also demonstrated that the treatment of canine

### Table 1. In vitro CFU-GM colony growth from marrow cells incubated with varying concentrations of Leu-Leu-OMe

<table>
<thead>
<tr>
<th>Leu-Leu-OMe (cM)</th>
<th>10(^{-6}) BMC cultures</th>
<th>10(^{-5}) BMC cultures</th>
<th>10-4 BMC cultures</th>
<th>10-3 BMC cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 No. coculture</td>
<td>10 M(^e) coculture</td>
<td>10 M(^e)-enriched coculture</td>
<td>10 M(^e) coculture</td>
</tr>
<tr>
<td>Nil</td>
<td>66±14 84±29</td>
<td>96±31 76±23</td>
<td>78±13</td>
<td>207±45 255±18</td>
</tr>
<tr>
<td>250</td>
<td>0.4±0.2 64±32</td>
<td>14±5 14±5</td>
<td>12±4</td>
<td>3±2 5±24</td>
</tr>
<tr>
<td>500</td>
<td>0 14±5</td>
<td>14±5 14±5</td>
<td>12±4</td>
<td>3±2 5±24</td>
</tr>
<tr>
<td>1000</td>
<td>1.2±0.5 10±3</td>
<td>29±1 24±6</td>
<td>2±0.6</td>
<td>8±3 42±3 74±29 0.3±0.3 3±1</td>
</tr>
<tr>
<td>2000</td>
<td>2±1 5±2</td>
<td>2±0.8 13±5</td>
<td>1±1</td>
<td>0±0.5 16±29</td>
</tr>
<tr>
<td>4000</td>
<td>0.7±0.6 11±5</td>
<td>0±0.5 16±29</td>
<td>1±1</td>
<td>0±0.5 16±29</td>
</tr>
</tbody>
</table>

Represented are mean values ± SEM, obtained from multiple experiments in which triplicate CFU-GM cultures were established for each parameter tested.

1 All cocultures employed autologous cells. The limited yield of M\(^e\) prevented coculture with all concentrations of Leu-Leu OMe tested. There was no CFU-GM growth when untreated PBMC (between 10\(^{-6}\) to 6x10\(^{4}\) /ml) were cultured without marrow cells.

2 The concentration of marrow cells treated was constant at 2x10\(^{5}\) /ml.

### Table 2. Recipients conditioned with 920 cGy TBI and receiving Leu-Leu OMe treated autologous marrow

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>Leu-Leu OMe (cM)</th>
<th>No viable treated BMC (x 10(^{4}) /kg)</th>
<th>Survival days post RMT</th>
<th>Cause of death</th>
<th>Marrow cellularity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C563</td>
<td>1.000</td>
<td>1.4</td>
<td>172</td>
<td>Sodium pentothal^2</td>
<td>Normocellular</td>
</tr>
<tr>
<td>BB8701</td>
<td>1000</td>
<td>0.7</td>
<td>19</td>
<td>Sodium pentothal^3</td>
<td>7% of Normocellular</td>
</tr>
<tr>
<td>C622</td>
<td>1000</td>
<td>1.41</td>
<td>&gt;124</td>
<td>Still living</td>
<td>Normocellular</td>
</tr>
<tr>
<td>C521</td>
<td>2000</td>
<td>0.052</td>
<td>20</td>
<td>Pneumonia, sepsis</td>
<td>Focal hematopoeisis</td>
</tr>
<tr>
<td>C580</td>
<td>2000</td>
<td>1.67</td>
<td>174</td>
<td>Sodium pentothal^4</td>
<td>Normocellular</td>
</tr>
<tr>
<td>B920</td>
<td>4000</td>
<td>1.3</td>
<td>114</td>
<td>Sodium pentothal^5</td>
<td>Normocellular</td>
</tr>
<tr>
<td>B622</td>
<td>4000</td>
<td>1.5</td>
<td>112</td>
<td>Sodium pentothal^6</td>
<td>Normocellular</td>
</tr>
</tbody>
</table>

^2 Autologous marrow aspiration, BMC treatment, and infusion on same day as 920 cGy TBI to recipient (RMT: bone marrow transplant)

^3 2x10\(^{5}\) BMC/ml treated with Leu-Leu OMe.

^4 Viability of bone marrow cells infused determined by trypan blue stain exclusion technique

^5 Sodium pentothal injection for euthanasia at end of study.

^6 The Leu-Leu OMe treated autologous marrow was frozen and stored at -80° C for one week prior to reinfusion. The procedure for marrow cryopreservation was as described (26).
marrow cells with Leu-Leu-OMe, in a concentration-dependent fashion, could reduce or eliminate in vitro CFU-GM growth. This reduction could be partially reversed with the addition of unfraccionated autologous PBMC or Mφ-enriched PBMC but not with Mφ-depleted PBMC or autologous PBMC treated in vitro with Leu-Leu-OMe. Our assay employs postendotoxin-treated dog serum as a source of colony-stimulating factor. Reversal of the effects of Leu-Leu-OMe on CFU-GM growth by monocytes demonstrated the monocyte dependence of this CFU-GM assay system and suggests that PDSs does not act directly as a single factor on the granulocyte/macrophage progenitors.

Marrow were treated at two different cell concentrations in anticipation that this procedure might be useful for allogeneic marrow transplantation in large animals and possibly in man. Reduction or elimination of various cells involved either in response to alloantigen stimulation (MLC, pCTL, CTL) or NK function could be accomplished by treating PBMC or marrow cells at 2x10^6/ml with 1000 μM Leu-Leu-OMe. The murine models using H2-disparate P→F marrow donor/recipient pairings showed that Leu-Leu-OMe treatment could prevent acute GVHD. The resultant chimeras developed normal cellular immune responses and showed donor- and host-specific immunologic tolerance (176). The cell concentration treated in those studies was 2x10^6/ml, and the highest concentration of Leu-Leu-OMe used was 250 μM. Cell concentration directly influenced the concentration of Leu-Leu-OMe required to achieve a given effect. Thus, in the present study where the cell concentration was increased tenfold, the concentration of Leu-Leu-OMe had to be increased approximately fourfold to achieve the same inhibition of cellular immune functions. The potential problem of larger cell numbers and expanded marrow volumes needed for the transplantation of larger animals can be circumvented by increasing the concentration of Leu-Leu-OMe.

A diagram showing hematologic recovery in recipients conditioned with 920 cGy TBI and infused with Leu-Leu-OMe-treated autologous marrow. The ordinate indicates the absolute granulocyte counts obtained daily post-transplant until death or recovery to the normal range. The shaded area represents the normal range (mean ± SD) of recovery observed in 16 dogs conditioned with 920 cGy TBI and given untreated autologous marrow (176).


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Rapid Communication

Localization of Cyclo-oxygenase and Prostaglandin E\(_2\) in the Secretory Granule of the Mast Cell

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The application of anti-cyclo-oxygenase and anti-prostaglandin E\(_2\) immunoglobulins to A23187-stimulated rat connective tissue mast cells has permitted the localization of cyclo-oxygenase activity (prostaglandin H\(_2\) synthetase) and the site of prostaglandin E\(_2\) (PGE\(_2\)) formation in the secretory granules. Because binding was carried out after stimulation but before dehydration and embedding, we have limited the loss of these antigens due to normal degradation and to aqueous and solvent washes. As this method permits labeling of exposed cell surfaces, only granules that have been exteriorized can be labeled. Contrary to what might have been expected, no labeling was associated with plasma membranes or with any portion of damaged cells. Antibodies to PGE\(_2\) were bound evenly over the surface of the granule matrix, whereas antibodies to cyclo-oxygenase appeared to be bound to strands of proteo-heparin projecting from the surface of the granule matrix. Where granule matrix had become unraveled and dispersed, label appeared to adhere throughout the ribbon-like proteo-heparin strands. These results support our previous conclusion that the secretory granule is the site of the arachidonic acid cascade during exocytosis. (J Histochem Cytochem 37:1319-1328, 1989)

KEY WORDS: Cyclo-oxygenase, Prostaglandin E\(_2\), Secretory granules, Mast cell, Eicosanoids, Lipid mediators, Inflammation, Arachidonic acid, Eicosanoids, Immunocytochemistry.

Introduction

It has long been assumed that the cell membrane is the source of phospholipid which provides the arachidonic acid required for synthesis of prostaglandins and other eicosanoids (1-3). Eicosanoid release has also been assumed to occur as a result of cell and membrane damage (4-6). However, membrane damage cannot be the sole source for the large pool of free arachidonic acid required for eicosanoid synthesis, as calcium ionophore-stimulated release of eicosanoids from neutrophils can occur without damage to membrane or loss of cell function (6). In addition, macrophages that produce large amounts of eicosanoids are known to contain no stored free arachidonic acid (7). These contradictions are compounded when one considers that phospholipase A\(_2\), the enzyme necessary for arachidonic acid release from phospholipid, requires millimolar calcium concentrations for its activity (4,8). This calcium requirement cannot be met by a membrane bilayer source.

Recently, we found that the secretory granules of the mast cell contain a large non-bilayer phospholipid store (9). During granule activation, a portion of this phospholipid spontaneously assembles into vesicles as a result of a water influx from the cytoplasm into the granule (9-11). Fusion of these newly assembled membrane vesicles with the perigranular membrane enables the activated granule to enlarge and its perigranular membrane to lift from the surface of the granule matrix. The contact and fusion of the expanding perigranular membrane with the plasma membrane culminates in exocytosis (10-13). Like many other secretory systems, the mast cell also produces a variety of lipid-derived mediators, such as prostaglandins and leukotrienes, during histamine release (14). Because mast cell phospholipid contains a high concentration of arachidonic acid (15), the remaining matrix-bound phospholipid would be a convenient arachidonic acid source for eicosanoid synthesis if the enzymes of the arachidonic acid cascade are also found in the granule. To this end, we have demonstrated that the mast cell granule not only contains the substrate for eicosanoid synthesis but also contains the machinery for rapid production of eicosanoids during granule activation (16). In this communication, we further establish that the granule is the site as well as the source of eicosanoid production, by localizing the presence of cyclo-oxygenase and its product, prostaglandin E\(_2\), (PGE\(_2\)) to the granule matrix using immunocytochemical techniques.

Materials and Methods

Rat serosal mast cells were obtained by peritoneal lavage according to a pub-
Figure 1. Cyclo-oxygenase localization in mast cells stimulated with A23187. The cyclo-oxygenase activity was localized by using monoclonal anti-cyclo-oxygenase antibodies and the resulted antigen–antibody complex was visualized ultrastructurally by binding of ferritin-conjugated antibodies. The ferritin label was confined to the strands of proteo-heparin projecting from the surface of the secreted granules. Eosinophils (Es) showed no label. Membrane vesicles were often seen in association with secreted granules (arrow). (A–D) High-magnification images of areas marked a through d. Original magnification × 10,120, A–D × 36,550. Bars = 0.5 μm.
Figure 2 Association of cyclo-oxygenase with vesicles assembled from granule matrix phospholipid. The presence of cyclo-oxygenase activity, as visualized by the ferritin label, is seen to decorate the secreted membrane vesicles (arrow) (A-D). High-magnification images of serial sections of the cluster of vesicles. Note that the damaged mast cell (*+) shows no ferritin label. Original magnification = 11,302, A–D = 48,375. Bars = 0.5 μm.
established procedure (9), except that plain normal Hanks' balanced salt solution (HBSS) was used as a lavage buffer. After one wash with HBSS and centrifugation at 30 x g for 10 min, mast cells were activated by suspension in HBSS containing 1 mg/ml A23187 for 5 min at 20°C. The reaction was stopped and cells were fixed by the addition of an equal volume of fixative containing 3% glutaraldehyde, 100 mM cacodylate, and 4 mM MgCl₂. After a 30-min incubation at 20°C, the cells were washed three times with HEPES-buffered saline (0.15 M NaCl and 20 mM HEPES, pH 6.8). Each wash was for 15 min with gentle agitation and was followed by a 2-min 1500-rpm centrifugation in a Beckman microfuge. During the third wash, the cell suspension was divided into aliquots for the various intended experiments. After centrifugation, the cell pellets were re-suspended in the respective antibodies or in pre-immune serum for the corresponding control experiments.

For localization of cyclo-oxygenase, the cells were re-suspended and incubated for 75 min with monoclonal mouse anti-cyclo-oxygenase immunoglobulin G₁ (Cayman Chemical; Ann Arbor, MI) diluted to an approximate concentration of 50 μg/ml in HEPES-buffered saline containing 20 μM digitonin (Fluka, Buchs, Switzerland) and 10 mM EDTA. A cell sample for the corresponding control experiment was incubated in pre-immune mouse serum diluted to 50 μg/ml in the same buffer.

For localization of PGE₂, the cells were incubated for 75 min with polyclonal rabbit anti-PGE₂ immunoglobulin G (Cayman Chemical) diluted to about 50 μg/ml as for the anti-cyclo-oxygenase antibody. The corresponding control was incubated with pre-immune rabbit serum at about 50 μg/ml.

After incubation, the cells were treated with three 5-min washes in HEPES-buffered saline to remove unbound antibodies. To visualize the immunogenetic activity of the cyclo-oxygenase, both the control and the experimental samples were incubated with about 1 mg/ml ferritin-conjugated goat anti-mouse IgG antiserum (Cappel, West Chester, PA) for 60 min, followed by three 5-min washes with HEPES-buffered saline to remove unbound ferritinated antiserum. To visualize the PGE₂, the control and the experimental samples were incubated with diluted ferritin-conjugated goat anti-rabbit IgG antisera (Sigma, St Louis, MO) for 60 min, followed by washes to rid of excess unbound antibodies. Similarly, gold-conjugated antiserum (Sigma) was also used in place of the ferritin-conjugated antibodies for ultrastructural localization of PGE₂.

After a last wash to rid the cells of the unbound heavy metal-conjugated antiserum, the specimens were osmicated for 20 min in 1% osmium tetroxide, followed by routine dehydration and embedding in Epon 812 according to established procedures (12). Unstained thin sections were examined with a Philips 400 transmission electron microscope.

Results

Cyclo-oxygenase Localization

The application of monoclonal anti-cyclo-oxygenase antibodies to A23187-stimulated peritoneal lavage cells permitted the ultrastruc-
Figure 4 Localization of the secretory granule as the site of PGE$_2$ production. Mast cells were stimulated with A23187 and PGE$_2$ was localized by using a polyclonal anti-PGE$_2$ antibody. The resulting antigen–antibody complex was visualized ultrastructurally by binding of ferritin-conjugated IgG. PGE$_2$ is confined to the exposed surface of the secreted granules (A-E). High-magnification images of areas labelled a through e. Original magnification × 11,730, A–E × 34,400. Bars = 0.5 μm.

Cyclo-oxygenase localization to the secretory granule of the mast cell. The association of cyclo-oxygenase with secreted mast cell granule matrix was readily visualized (Figure 1). Antibody binding was prominently localized to the proteo-heparin strands projecting from the surfaces of the secreted granules. All secreted mast cell granules were labeled. Binding was not seen in the interior of the granules. This may indicate the inaccessibility of the condensed granule matrix to the antibodies. Dispersed granules which re-
Figure 5. PGE₂ localization on unraveled secreted granule. Complete unraveling of a secreted granule has permitted visualization of PGE₂ binding on the ribbon-like proteo-heparin strand. Bar at the lower right-hand corner = 2 μm. Insets A, B, and C correspond to areas a, b, and c on the strand. Even distribution of ferritin label on the matrix proteo-heparin strands can be observed. Original magnification × 5698, A-C × 33,325. Inset bar = 0.5 μm.
Figure 6. Control experiment for PGE\textsubscript{2} localization. Parallel experiment in which pre-immune serum is used in place of the polyclonal anti-PGE\textsubscript{2} antiserum (inset) High-magnification of the secreted granule (arrow). Original magnification × 13,570. Inset × 24,725. Bars = 0.5 μm.

maintained within the confines of the cell but which were in communication with the outside via a pore often had little or no label. No cyclo-oxygenase was localized to the plasma membranes of any cell type present in the lavage.

The lipid nature of the granule contents could be seen, to a limited extent, with the appearance of a single vesicle adjacent to a secreted granule (Figure 1A). It became more apparent in Figure 2, where a large cluster of vesicles was associated with a partially secreted mast cell. This mass of vesicles was prominently labeled for cyclo-oxygenase, especially in the intervesicular regions of the mass.

A control cell which received pre-immune serum rather than anti-cyclo-oxygenase antibodies is shown in Figure 3. The absence of label on the secreted granules of the control cells illustrated the specificity of the antigen antibody reaction.

**Prostaglandin E\textsubscript{2} Localization**

The application of polyclonal anti-PGE\textsubscript{2} antibodies to A23187-stimulated peritoneal lavage cells has established the mast cell granule as the site of PGE\textsubscript{2} synthesis during histamine release (Figure 4). All secreted mast cell granules were labeled. Although other granule-containing cell types were present at the lavage, including eosinophils and neutrophils, these cells did not appear to have undergone secretion and were generally not associated with PGE\textsubscript{2} label. Antibody binding was predominantly localized to the peripheries of the secreted granules. Unlike the cyclo-oxygenase label which was apparent on the proteo-heparin projections from the granule matrix, the PGE\textsubscript{2} label was distributed evenly and in a more continuous fashion on the surface of the matrix. This suggests a strong hydrophobic interaction between the PGE\textsubscript{2} and the hydrophobic components of the matrix.

It is not uncommon to find extruded granules that have become so dispersed and unraveled that they reveal their proteo-heparin cores as being made up of long ribbon-like strands (17). A portion of an extruded granule can be seen in Figures 5A–5C. The adherence of the label throughout the strand indicates that much of the newly synthesized prostaglandin remains bound to the granule matrix long after the extrusion of the granule. We never observed any gradient of label in association with the granule matrix. The label was always tightly associated with the matrix protein and did not appear to diffuse from this bound state.

Results of the control experiment in which cells received pre-immune serum rather than PGE\textsubscript{2} antibodies can be seen in Figure 6. The absence of label in the control sample lends credence to the results. The localization of both the cyclo-oxygenase and its
also gave no evidence of cyclo-oxygenase activity or PGE₂ localization, even though the antibodies often had access to internal cytosolic locations (Figure 2).

Prostaglandin E₂ appeared to be bound to the granule matrix even when the granule was unraveled (Figure 5). Since PGE₂ is very hydrophobic, as evidenced by its low solubility in water, much of it may have remained bound to the hydrophobic matrix after secretion. Many cells of the immune system, such as neutrophils and macrophages, are rapidly attracted to secreted mast cell granules because their secretion is associated with the production of the so-called "slow-reacting substances of anaphylaxis" (SRSA). These SRSA, which are primarily leukotrienes, are potent leukocyte chemotacticants. In Figure 8, a granulocyte appears to be phagocytosing a labeled mast cell granule. Since the eicosanoids, as represented by PGE₂, remain bound to the granule matrix via hydrophobic interaction, it seems possible that these bound lipid mediators may confit a signal to the phagocytosing cells by activating their cell surface receptors at the points of contact. The phagocytosis of the granule matrix would result in formation of a lysosome which contains lipid mediators. However, it is also conceivable that mediator-containing vesicles, such as some of those shown in Figure 2, may fuse directly with a target cell on contact. Their fusion would result in the direct release of lipid mediators into the cytoplasm of the

Discussion

In agreement with our previous biochemical evidence, our current ultrastructural results also confirm the fact that the secretory granule of the mast cell is the site of cyclo-oxygenase activity and prostaglandins synthesis during exocytosis. Because we applied antibodies to secreting whole cells, the labeling was limited to exposed surfaces. The fact that only the matrices of the secreted granules were labeled (Figures 1, 4, 5, and 7) implies the presence of an eicosanoid-synthetic machinery in the secretory granule.

Contrary to what might have been expected, cell plasma membranes were never labeled with either cyclo-oxygenase or PGE₂ activity. Disrupted cells, broken or damaged as a result of handling,
target cell. The internalization of non-membrane-delimited mediators opens up the possibility of some yet unknown intracellular functions for the lipid mediators. A role of arachidonic acid metabolites as intracellular modulators of K+ channel has been recently suggested (18).

Previously, eicosanoid synthesis was considered to originate from membrane phospholipid (1–5), with damage to these membranes as the event that made phospholipid available for eicosanoid synthesis. Our results indicate that prostanoid formation may actually be the result of receptor-mediated granule exocytosis. The coupling of granule activation to triggering of the arachidonic acid cascade explains why, for the mast cell, the initial time course of histamine release closely parallels the time course for prostanoid production (12,13). This explanation is also consistent with the coincident production of eicosanoids with the process of secretion in other secretory systems (20,21).

In the eicosanoid biosynthetic cascade, the first requirement for the synthesis of prostanoids is availability of phospholipid. In the mast cell granule, this is easily met by the existence of a large non-bilayer phospholipid store bound to the granule matrix (9). The second requirement is a phospholipase to liberate the arachidonic acid from the phospholipid. Since arachidonic acid is usually esterified in the sn-2 position of phospholipid (7), the presence of a phospholipase A2 is implied. The existence of a phospholipase A2 in the mast cell granule has now been verified in our laboratory (submitted for publication). In fact, the formation of PGE2 from endogenous granule phospholipid detected here by using a specific antibody to PGE2 is in itself strong evidence for the presence of a phospholipase A2 in the granule. This phospholipase A2, like that found in the pancreas and in the macrophage, requires high calcium concentrations for its activity (4,8). This requirement can be easily met in the granule (22), but not by a membrane bilayer source. The phospholipase A2 of macrophage granules was assumed to be bound to the granule membrane (4). In the mast cell, our studies suggest that it is bound to the granule matrix instead. The third requirement in the pathway is the presence of a cyclo-oxygenase, the topic of this study, to catalyze the conversion of arachidonic acid to PGE2. Current theory has also placed this enzyme, along with other key enzymes of the cascade, in or on the plasma membrane. However, it should be pointed out here that fragmented granule matrix components can be easily sedimented with the microsomal fraction at 100,000 × g. Therefore, it is possible to confuse granule matrix components with membranous components. This confusion becomes more obvious in experiments involving the use of fragmented cells or tissues as starting materials. Our observations indicate that cyclo-oxygenase is tightly bound to the matrix of the secretory granule, where a large amount of phospholipid is stored. All these points, taken together, suggest that the secretory granule contains all the necessary ingredients to function as an eicosanoid-producing entity during granule exocytosis.

There are many inconsistencies surrounding the current dogma which places the prostanoid-synthesizing machinery on the plasma membrane. Aside from the difficulty of coping with the consequence of placing many membrane-destructive enzymes on the plasma membrane, this concept also provides no credible explanation of how hydrophobic products such as prostanoids can be released from their hydrophobic membrane environment during exocytosis. More serious challenge to this dogma ensues after the identification of a key enzyme in the cascade, prostanoid D-isomerase, being a cytosolic enzyme instead of an expected membrane enzyme (23). This difficulty of placing the cyclo-oxygenase complex on the plasma membrane while the D-isomerase/synthetase is in the cytosol, has been pointed out by Giles and Leff (24). Furthermore, the use of immunocytotoxic chemical technique also failed to demonstrate the presence of cyclo-oxygenase on the plasma membrane (25). Our present finding of localizing both the substrate and the necessary enzymes of the cascade to the secretory granules not only can overcome the difficulties facing the current dogma but also can provide the physical basis for linking of granule activation to the production of eicosanoids during exocytosis (16).

We now understand that stimuli in the form of trauma, infection, or radiation cause certain cells to secrete and thus produce various lipid mediators which can initiate an inflammatory response. Many of these mediators are also potent chemotactic factors. The involvement and the recruitment of the cells of the immune system into the site of inflammation usually results in tissue destruction. Under physiological conditions, the process of inflammation is self-contained in the sense that the release of a deleterious mediator eventually elicits the release of opposing factors via a feedback mechanism. This line of thinking is consistent with the concept of Chandler and Fulmer who suggested that the inflammation-promoting eicosanoids are released to initiate an inflammatory response and that this inflammatory state is later terminated by the release of immunosuppressive eicosanoids (26). Many studies on the roles of eicosanoids in the mechanism of inflammation and how they interact with the various lymphokines, helper cells, and suppressor cells to elicit a specific immune response have been published (27–30). The localization of the site of eicosanoid production to the secretory granule and the linking of their production to the process of secretion will lead to a better understanding of the mechanism of inflammation and open the way to specific manipulation of the immune system.

Acknowledgments

We wish to thank Mr. Joe L. Parker and Christine S. Cho for technical assistance.

Animal usage was in compliance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Literature Cited


Ishizaka T, Ishizaka K. Activation of mast cells for mediator release through IgE receptors. Prog Allergy 1984;44:188


Parker BM, Parker DM. Metabolism of arachidonic acid in tumour necrosis factor-α-stimulated macrophages. J Cell Physiol 1990;140:255


Radioprotection of Mice with Interleukin-1: Relationship to the Number of Spleen Colony-Forming Units

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Compared to saline-injected mice 9 days after 6.5 Gy irradiation, there were twofold more Day 8 spleen colony-forming units (CFU-S) per femur and per spleen from B6D2F1 mice administered a radioprotective dose of human recombinant interleukin-1-α (rIL-1) 20 h prior to their irradiation. Studies in the present report compared the numbers of CFU-S in nonirradiated mice 20 h after saline or rIL-1 injection. Prior to irradiation, the number of Day 8 CFU-S was not significantly different in the bone marrow or spleens from saline-injected mice and rIL-1-injected mice. Also, in the bone marrow, the number of Day 12 CFU-S was similar for both groups of mice. Similar seeding efficiencies for CFU-S and percentage of CFU-S in S phase of the cell cycle provided further evidence that rIL-1 injection did not increase the number of CFU-S prior to irradiation. In a marrow repopulation assay, cellularity as well as the number of erythroid colony-forming units, erythroid burst-forming units, and granulocyte-macrophage colony-forming cells per femur of lethally irradiated mice were not increased in recipient mice of donor cells from rIL-1-injected mice. These results demonstrated that a twofold increase in the number of CFU-S at the time of irradiation was not necessary for the earlier recovery of CFU-S observed in mice irradiated with sublethal doses of radiation 20 h after rIL-1 injection.

INTRODUCTION

Previous studies demonstrated that more mice survived after exposure to otherwise lethal doses of γ radiation or cytoxan if they had been administered a single injection of recombinant interleukin-1 (rIL-1) 18-24 h before these hematopoietic suppressive treatments (1-5). Associated with the increased survival, there was an earlier recovery of mature cells in the blood and hematopoietic colony-forming cells in the bone marrow (2-5). Similarly, an earlier recovery of CFC was observed in mice administered rIL-1 prior to sublethal doses of radiation (3, 4). The physiological mechanisms pro-
moting the earlier recovery of CFC after irradiation of mice pretreated with rIL-1 are not well understood.

In mice, assays for spleen colony-forming units (CFU-S) have provided useful and sensitive measurements of damage and recovery of bone marrow cells after irradiation (6, 7). However, CFU-S represent a heterogeneous population of cells that varies in time of appearance (8), cell cycling characteristics (7, 9, 10), and potential for self-renewal (7, 11, 12). These subpopulations of CFU-S have been shown to differ in their tissue distribution (7, 11-13), radioprotective ability after transplantation (11, 14), and sensitivity to irradiation (9, 10, 13, 15-17). Neta et al. (18) demonstrated that injection of murine rIL-1 induced an increase in the number of endogenous CFU-S. However, they reported that the increase in endogenous CFU-S observed after rIL-1 injection was not necessarily predictive of radioprotection (18). Others demonstrated that an increase in the number of endogenous CFU-S is not always correlated with earlier bone marrow recovery or increased survival (19-21). Determinations of the effect of rIL-1 injection on the number and cell cycle status of other populations of CFU-S may be useful in further delineating the mechanisms responsible for the earlier hematopoietic recovery that occurs after irradiation of rIL-1-injected mice.

The studies in the present report were done to investigate the changes in CFU-S number and the proportion of CFU-S in S phase of the cell cycle in tissues of normal mice that had occurred by 20 h after a single injection of a radioprotective dose of rIL-1. Data in an earlier report demonstrated that 150 ng rIL-1 from the same stock solutions as used in the present studies increased the number of B6D2F1 mice that survived after 10.5 Gy irradiation from $7 \pm 1.1$ to $85 \pm 7.1$% (4). Also, compared to saline-injected mice, there was an earlier recovery of erythroid and granulocyte-macrophage hematopoietic colony-forming cells in the bone marrow of rIL-1-injected mice 8 days after 6.5 Gy irradiation (4). In the present report, compared to saline-injected mice, there were twofold more Day 8 CFU-S in both bone marrow and spleens from rIL-1-injected mice 9 days after 6.5 Gy irradiation. At the time of irradiation, the number and seeding efficiency of Day 8 CFU-S and Day 12 CFU-S were compared for rIL-1-injected mice and saline-injected mice. The decrease in CFU-S after hydroxyurea injection was used to measure the proportion of CFU-S in S phase of the cell cycle. Also, bone marrow cells from rIL-1-injected and saline-injected mice were assayed for their ability to repopulate the bone marrow and spleens of lethally irradiated mice.

**MATERIALS AND METHODS**

**Mice**

B6D2F1 or (C57Bl/6J × DBA/2)F1, female mice were purchased from Jackson Laboratories (Bar Harbor, ME). Upon arrival, mice were maintained in an AAALAC-accredited facility. They were housed 10 per cage in plastic microisolator cages on hardwood-chip contact bedding and were allowed food (Wayne Rodent Blox) and HCl acidified water (pH 2.4) *ad libitum*. Animal holding rooms were maintained at 70 ± 2°F and 50 ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air and exposed to full-spectrum light from 6:00 AM to 6:00 PM. Upon arrival, mice were tested for *Pseudomonas* contamination and quarantined until test results were obtained. On healthy mice were released for experimentation. Twelve- to 16-week-old mice were used for these studies. Research was conducted ac-
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According to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animals Resources, National Research Council.

Irradiation

Mice were exposed bilaterally to γ radiation at a dose rate of 0.40 Gy/min from a 137Cs radiation source. Mice received total-body doses of 6.5 Gy for sublethal irradiations studies and 10.5 Gy for CFU-S and bone marrow and spleen repopulation studies.

Treatment with Interleukin-1 (IL-1)

Purified human recombinant IL-1α, a generous gift from Dr. S. Gillis of Immunex (Seattle, WA), was used in these studies. The IL-1α (three different lots) was supplied in a solution of phosphate-buffered saline at pH 7.2 with a specific activity of 7.5 × 10⁶ U/IL-1/mg protein, and aliquots were maintained at −70°C. Immediately before use, stock solutions of IL-1α were diluted with pyrogen-free saline (McGaw). In a volume of 0.5 ml 75, 150, or 200 ng was administered to normal mice by intraperitoneal injection. The mean body weight of mice used in these studies was 27 ± 2.5 g so that the average dose of IL-1α was approximately 5.6 μg/kg body wt. Control animals were administered 0.5 ml saline at the same time. Endotoxin (LPS) contamination in IL-1 stock solutions was measured by the Limulus lysate assay. On the basis of these results less than 0.2 ng of LPS was administered per injection.

Preparation of Cell Suspensions

Mice were sacrificed by cervical dislocation, and the femurs and spleens were excised. Cells were flushed from the bone marrow with Hanks' balanced salt solution without Ca²⁺ or Mg²⁺ (HBSS; Grand Island Biological Co., Grand Island, NY) using a syringe and 25 gauge needle. Spleen cells were obtained by using a syringe plunger to disperse spleen cells through a wire screen (Millipore). Bone marrow and spleen cells were then dispersed through a 25 gauge needle until a single cell suspension was obtained. All cell concentrations were determined by hemacytometer counts.

Bone Marrow and Repopulation Assay

Bone marrow cells from mice 20 h after saline or rIL-1 injection were diluted, and 2 × 10⁴, 2 × 10⁵, or 2 × 10⁶ cells were infused into a caudal vein of individual mice 24 h after 10 Gy irradiation. Four and 12 days later, bone marrow and spleen cells were assayed for cellularity and hematopoietic colony-forming cell content.

Assays for In Vitro Colony-Forming Cells

Granulocyte-macrophage colony-forming cells (GM-CFC) 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% tryptose soy broth, 0.02 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 0.3% agar (Bactoagar, Difco) containing 10% (vol/vol) L-929 cell conditioned medium as a source of colony-stimulating activity. The top layer contained 1 ml of a 1:1 mixture of culture medium and 0.66% agar containing bone marrow or spleen cells. Cultures were incubated at 37°C in 5% humidified CO₂ in air. After 10 days of culture, colonies greater than 50 cells were scored as GM-CFC.

Determinations of erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E) were made using a plasma clot culture system basically as described by Weinberg et al. (23). Iscove's modified Dulbecco's medium (Grand Island Biological Co.) was substituted for medium. Cells were plated with 0.25 ml/ml for BFU-E or 3.0 U/ml for BFU-E anemic sheep plasma [step III erythropoietin (Connaught Laboratories, Inc., Lot No. 3092-2)] in 0.4-ml plasma clot in four-well Nuncclone culture dishes (Nunc). CFU-E and BFU-E cultures were placed into a humidified 37°C incubator with 5% CO₂ for 2.5 and 8 days, respectively. Cultures were then harvested, fixed, stained, and evaluated as described by McLeod et al. (24).
Determination of CFU-S Seeding Efficiencies

Twenty-four-hour seeding efficiencies were determined for both Day 8 and Day 12 CFU-S in bone marrow from saline- or IL-1-injected mice (25, 26). In these studies, Day 8 and Day 12 CFU-S per femur were determined for saline- or IL-1-injected mice. Bone marrow cells ($5 \times 10^7$) from the same suspensions were also transplanted into another group of 6-10 mice after their exposure to 10.5 Gy radiation (primary recipients). After 24 h, 7.5 $\times 10^7$ (for Day 8 CFU-S) or $3.7 \times 10^7$ (for Day 12 CFU-S) splenic cells from the primary recipients were slowly infused into a caudal vein of mice exposed to 10.5 Gy radiation 24 h earlier (secondary recipients). The number of colonies on spleens from secondary recipients was counted on Day 8 or Day 12. This number was used to calculate the number of CFU-S per spleen from primary recipients. The percentage of bone marrow derived CFU-S that had seeded into the spleens of primary recipients by 24 h was calculated by dividing CFU-S recovered/spleen of primary recipient mice by the number of CFU-S in bone marrow cells from nonirradiated saline- or IL-1-injected mice.

Determination of CFU-S in S Phase of the Cell Cycle

The percentage of CFU-S in S phase of the cell cycle was determined basically as described by Rickard et al (12, 19). Mice were administered 900 mg/kg body wt hydroxyurea (Sigma) in Dulbecco's phosphate-buffered saline (PBS; Grand Island Biological Co.) by intraperitoneal injection. Control groups of mice were administered PBS without hydroxyurea at the same time. Tissues were assayed for surviving CFU-S 2.5 to 3 h later. The number of CFU-S in hydroxyurea-injected mice was compared to the number in PBS-injected mice, and the percentage decrease in CFU-S after hydroxyurea was calculated as the percentage of CFU-S in S phase of the cell cycle for IL-1-injected mice and saline-treated mice.

Statistics

The two-tailed Student's t test was used to test for significant differences in cellularity and colony-forming cells per tissue between groups of mice.

RESULTS

Effect of IL-1 on Early Recovery of CFU-S after Sublethal Irradiation

The number of Day 8 CFU-S per femur was determined for mice exposed to a radiation dose of 6.5 Gy 20 h after saline or IL-1 injection. Five, 9, and 12 days after irradiation, the number of CFU-S was twofold greater in bone marrow from IL-1-injected mice than in bone marrow from saline-injected mice. The values were similar to Day 12 CFU-S values previously reported (4). Also, 9 days after exposure to 6.5 Gy radiation, the number of Day 8 CFU-S/spleen was twofold greater ($P < 0.01$) for IL-1-injected mice ($111 \pm 23.5$) than for saline-injected mice ($55 \pm 17.0$). These results demonstrate that IL-1 injection induced an earlier recovery of CFU-S in both...
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bone marrow and spleens of mice injected with 150 or 200 ng rIL-1 20 h prior to irradiation.

**Number of Spleen Colony-Forming Units after rIL-1 Injection**

The content of CFU-S was measured in bone marrow and spleens from nonirradiated mice 20 h after saline or rIL-1 injection (Table I). The number of Day 8 CFU-S/femur was not significantly different (P > 0.05) for mice 20 h after injection with saline or 75-200 ng rIL-1. Also, the number of Day 12 CFU-S/femur and Day 8 CFU-S/spleen was similar for mice 20 h after saline injection or rIL-1 injection. Seeding efficiencies for both Day 8 and Day 12 CFU-S from the bone marrow of saline-injected mice were not significantly different from seeding efficiencies determined for CFU-S in bone marrow from rIL-1-injected mice (Table II A). Thus the number of CFU-S/femur after correction for seeding efficiencies was also similar for saline-injected mice and rIL-1-injected mice. These results demonstrate that rIL-1 injection did not induce a change in the number or seeding efficiencies of Day 8 and Day 12 CFU-S in mice prior to irradiation.

**Effect of rIL-1 on Sensitivity of CFU-S to Hydroxyurea**

The decrease in CFU-S after hydroxyurea injection was used to determine the percentage of CFU-S that were in S phase of the cell cycle 20 h after rIL-1 injection. The number and percentage of Day 8 CFU-S surviving after treatment with hydroxyurea was not significantly different for saline-injected mice and rIL-1-injected mice (Table II B). In one study, the number of Day 12 CFU-S sensitive to hydroxyurea was also similar for mice 20 h after saline or rIL-1 injection. These results demonstrate that a twofold increase in the number of CFU-S/femur in S-phase of the cell cycle 20 h after rIL-1 injection was not necessary for the earlier recovery of CFU-S observed in rIL-1-injected mice after 6.5 Gy.

**Recovery of Colony-Forming Cells after Bone Marrow Transplantation**

In a bone marrow repopulation assay, the recovery of CFU-E, BFU-E, and GM-CFC was determined for lethally irradiated mice receiving bone marrow cells from saline-injected mice or rIL-1-injected mice (Table III). Similar numbers of cells and Day 8 CFU-S from saline-injected or rIL-1-injected donor mice were transfused into lethally irradiated mice. Four and 12 days later, bone marrow cellularity and content of colony-forming cells were not significantly different for either group of recipient mice. Fewer GM-CFC per spleen were observed in recipients of bone marrow cells from rIL-1-injected donor mice. These studies demonstrate that at 4 and 12 days, there was no evidence that bone marrow cells from rIL-1-injected mice accelerated hematopoietic recovery of lethally irradiated recipient mice.

**DISCUSSION**

Data in this report demonstrated that a single injection of rIL-1 administered to B6D2F1 mice 20 h prior to sublethal irradiation promoted an earlier recovery of Day 8 CFU-S in the bone marrow and spleen. Data in this report and a previous report...


<table>
<thead>
<tr>
<th>Strain</th>
<th>Saline (mL)</th>
<th>rHL-1 (mL)</th>
<th>Saline (n°)</th>
<th>rHL-1 (n°)</th>
<th>Saline (n°)</th>
<th>rHL-1 (n°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 75 ng rHL-1</td>
<td>2.3 ± 0.2 (7)</td>
<td>2.2 ± 0.6 (7)</td>
<td>2.0 ± 0.4 (7)</td>
<td>1.5 ± 0.6 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. 150 ng rHL-1</td>
<td>4.5 ± 0.6 (5)</td>
<td>4.0 ± 1.0 (5)</td>
<td>1.0 ± 0.2 (4)</td>
<td>1.2 ± 0.3 (5)</td>
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<td></td>
</tr>
<tr>
<td>2°</td>
<td>3.8 ± 0.4 (5)</td>
<td>4.1 ± 0.5 (5)</td>
<td>3.7 ± 0.9 (4)</td>
<td>1.1 ± 0.3 (6)</td>
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</tr>
<tr>
<td>4°</td>
<td>4.3 ± 0.8 (5)</td>
<td>3.3 ± 0.6 (6)</td>
<td>0.8 ± 0.3 (6)</td>
<td>0.9 ± 0.4 (6)</td>
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<tr>
<td>5°</td>
<td>4.6 ± 0.9 (6)</td>
<td>3.5 ± 0.9 (6)</td>
<td>2.0 ± 0.5 (5)</td>
<td>2.4 ± 0.6 (5)</td>
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<tr>
<td>6°</td>
<td>4.5 ± 0.2 (5)</td>
<td>4.1 ± 0.5 (5)</td>
<td>4.5 ± 1.0 (4)</td>
<td>3.0 ± 1.0 (3)</td>
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</tr>
<tr>
<td>7°</td>
<td>3.9 ± 0.5 (4)</td>
<td>3.4 ± 0.5 (4)</td>
<td>3.9 ± 0.5 (4)</td>
<td>3.4 ± 0.6 (5)</td>
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<td></td>
</tr>
<tr>
<td>C. 200 ng rHL-1</td>
<td>4.5 ± 0.9 (5)</td>
<td>3.4 ± 0.5 (5)</td>
<td>5.8 ± 1.3 (3)</td>
<td>3.5 ± 0.9 (5)</td>
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<tr>
<td>8°</td>
<td>3.7 ± 0.5 (5)</td>
<td>3.8 ± 0.1 (5)</td>
<td>2.6 ± 1.1 (5)</td>
<td>2.6 ± 0.0 (5)</td>
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<tr>
<td>9°</td>
<td>4.0 ± 1.6 (4)</td>
<td>4.1 ± 0.2 (4)</td>
<td>1.8 ± 0.5 (5)</td>
<td>1.0 ± 0.3 (4)</td>
<td></td>
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</tr>
</tbody>
</table>

*Female B6D2F1 mice, 12–16 weeks old, were administered 0.5 mL saline or 75, 150, or 200 ng rHL-1 20 h prior to assays.

*Cells were pooled from both femurs and spleens from three mice per group in each study. Values represent the means ± SD of mean colony values in individual studies.

*Number of mice.

*Groups of mice were also evaluated for survival or bone marrow recovery after 6.5 Gy irradiation.
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TABLE II

Effect of rIL-1 Injection on Bone Marrow CFU-S Seeding Efficiencies and Survival after Hydroxyurea Injection of Saline or rIL-1 Injected Mice

<table>
<thead>
<tr>
<th>Treatmentb</th>
<th>Saline injection (n)a</th>
<th>rIL-1 injection (n)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Seeding efficiencies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8 CFU-S</td>
<td>2.4 ± 0.4 (3)</td>
<td>2.7 ± 0.7 (3)</td>
</tr>
<tr>
<td>Day 12 CFU-S</td>
<td>3.1 ± 0.1 (2)</td>
<td>4.4 ± 0.2 (2)</td>
</tr>
<tr>
<td>B. Survival of Day 8 CFU-S after hydroxyurea injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number/Femur (x 10³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninjected mice</td>
<td>4.0 ± 0.35 (3)</td>
<td>3.6 ± 0.42 (3)</td>
</tr>
<tr>
<td>Injected mice</td>
<td>3.5 ± 0.65 (3)</td>
<td>3.0 ± 0.36 (3)</td>
</tr>
<tr>
<td>% Surviving after injection</td>
<td>88 ± 9.1</td>
<td>84 ± 17.1</td>
</tr>
</tbody>
</table>

a B6D2F1 female mice, 12-16 weeks old.
b Mice were administered 0.5 ml saline or 150 ng rIL-1 20 by ip injection 20 h prior to administration of hydroxyurea (900 mg/kg body wt) or saline.

Number of studies demonstrated that, compared to saline-injected mice, there were twofold more Day 8 and Day 12 CFU-S per femur of rIL-1-injected mice 5-12 days after 6.5 Gy irradiation. Results in this report further demonstrate that the earlier recovery of CFU-S observed in rIL-1-injected mice was not due to a twofold increase in numbers of CFU-S at Day 8 or Day 12 or proportion of CFU-S in S phase of the cell cycle at the time of irradiation. Also, in a marrow repopulation assay, the recovery of erythroid and granulocyte-macrophage colony-forming cells in bone marrow of lethally irradiated recipients of bone marrow cells from saline-injected mice was similar to the recovery in recipients of bone marrow cells from rIL-1-injected mice.

In some studies, the fraction of CFU-S in injected bone marrow cells that lodged in the spleen and proliferated to form colonies was decreased for populations of CFU-S in S phase of the cell cycle. For example, 24 h after endotoxin injection the number of CFU-S per femur was decreased by 50%, but when corrected for a lower seeding efficiency in the spleen (i.e., f-fraction) there was no difference in the number of CFU-S from control and treated mice. In the present studies, there was no significant difference in the 24-h seeding efficiency of Day 8 and Day 12 CFU-S in bone marrow from saline-injected mice and rIL-1-injected mice. In general, the ratio of 2-h seeding efficiency:24-h seeding efficiency has been reported to be approximately 3:1. In the present studies, the 24-h seeding efficiency for Day 8 CFU-S was lower than expected from the 13.4% 2-h seeding efficiency previously reported for similarly aged female B6D2F1 mice. This lower value was observed for both saline-injected mice and rIL-1-injected mice. Saline injection alone may have stimulated some CFU-S into cell cycle. In one study, the percentage of mice surviving >30
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TABLE III
Bone Marrow Recovery in Irradiated Recipients of Bone Marrow Cells from Mice 20 h after Saline or rIL-1 Injection

<table>
<thead>
<tr>
<th>Assay</th>
<th>Time after transplantation and source of donor cells*</th>
<th>Day 4</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saline donors</td>
<td>rIL-1 donors</td>
</tr>
<tr>
<td></td>
<td>(% of normal values)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>3.4 ± 0.1</td>
<td>3.3 ± 0.2</td>
<td>69 ± 25</td>
</tr>
<tr>
<td>BFU-E</td>
<td>3.8 ± 1.1</td>
<td>4.2 ± 2.4</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>GM-CFC</td>
<td>0.3 ± 0.5</td>
<td>0.5 ± 0.7</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>1.7 ± 0.7</td>
<td>1.8 ± 0.5</td>
<td>43 ± 12</td>
</tr>
<tr>
<td>GM-CFC</td>
<td>6.1 ± 0.6</td>
<td>5.7 ± 0.2</td>
<td>94 ± 7.8</td>
</tr>
<tr>
<td>CFU-E</td>
<td>8.0 ± 6.9</td>
<td>5.6 ± 3.0</td>
<td>605 ± 61</td>
</tr>
<tr>
<td>Splen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>56 ± 9.6</td>
<td>19.3 ± 6.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* B6D2F1 female mice, 12-16 weeks old, were administered 0.5 ml saline or 150 ng rIL-1. Bone marrow cells (2 × 10^6) from mice 20 h after saline or rIL-1 injection were injected into B6D2F1 mice that had received a radiation dose of 10.5 Gy. CFU-S values in 2 × 10^6 cells from saline-injected mice were 620 ± 84 and 620 ± 78; CFU-S values for 2 × 10^6 cells from rIL-1-injected mice were 660 ± 262 and 600 ± 156.

b Values are the means ± SD of mean values from two studies.

Significantly different from values for recipients of cells from saline-injected mice (P < 0.05)

days after 10.5 Gy irradiation was similar for noninjected mice (1/10), saline-injected mice (1/10), and mice injected with heat-inactivated rIL-1 (1/10). That study demonstrated that the possible stimulation of CFU-S into cell cycle after saline injection did not result in an increase in the number of mice surviving after irradiation. Similarities in CFU-S numbers and seeding efficiencies further demonstrated that a twofold increase in the number of CFU-S at the time of irradiation was not necessary for the earlier recovery of CFU-S observed in mice after 6.5 Gy irradiation.

Neto et al. (31) suggested that one possible mechanism for the radioprotective effects of rIL-1 might be that rIL-1 injection induces bone marrow cells into a radioreistant late S phase of the cell cycle. Reports on differences in the radiosensitivity of cycling and noncycling populations of CFU-S are not definitive (9, 10, 13, 15-17). This may be due to the heterogeneity of CFU-S, as well as to the difficulty in establishing and maintaining a synchronized population of CFU-S in vivo. Several studies indicated that some subpopulations of CFU-S in S phase of the cell cycle were more resistant to radiation injury than noncycling CFU-S (9, 10, 15). Dumenil et al. (32) demonstrated that a decrease in seeding efficiency was not always observed when there was an increase in the proportion of CFU-S in S phase of the cell cycle. Therefore, in the present studies, the decrease in the number of CFU-S after hydroxyurea injection was used to determine the percentage of CFU-S in S phase of the cell cycle. The decrease in the number of Day 8 and Day 12 CFU-S in the bone marrow after hydroxyurea injection was similar for saline-injected mice and rIL-1-injected mice.
Thus, within the limits of these assays, it does not appear that the twofold increase in the number of CFU-S after 6.5 Gy irradiation of rIL-1-injected mice can be accounted for by a similar increase in number of CFU-S in S-phase of the cell cycle. However, the effect of rIL-1 injection on the number of endogenous CFU-S in S phase of the cell cycle was not investigated in studies for this report. Boggs and Boggs (10) suggested that the earlier hematopoietic recovery observed after irradiation of endotoxin-injected mice was a result of an increase in the proportion of endogenous CFU-S in S phase of the cell cycle. Further studies of the effects of rIL-1 injection on cell cycle kinetics of subpopulations of CFU-S will be important to further delineate the mechanisms for the earlier hematopoietic recovery after irradiation of rIL-1-injected mice.

Others demonstrated that CFU-S in the bone marrow were induced into cell cycle within 24 h after endotoxin injection (29). The increase in the number of CFU-S in S phase of the cell cycle after endotoxin injection might be related to the replacement of CFU-S and other hematopoietic colony-forming cells lost due to mobilization of CFU-S from the bone marrow to the blood and the spleen early after endotoxin injection (29). In the present studies, 20 h after rIL-1 injection, there was no increase in the number of CFU-S in the spleen or in the number of CFU-S decreased after hydroxyurea injection. Also, the numbers of endogenous CFU-S reported for mice administered similar doses of rIL-1 used in the present studies were low (18) compared to the numbers reported for mice after injection of glucan (33) or endotoxin (19). These results suggest that mobilization of CFU-S from the bone marrow to the spleen early after the injection of the doses of rIL-1 used in the present studies was minimal. This may be one reason for the observations in the present studies in which the number of CFU-S in S phase of the cell cycle in the bone marrow was not increased in mice 20 h after rIL-1 injection.

A previous report demonstrated that rIL-1 injection did not prevent the expression of radiation-induced long-term decreases in the number of CFU-S (4). Also, the number of Day 8 and Day 12 CFU-S per femur was similar for saline-injected mice and rIL-1-injected mice 2 and 24 h after their irradiation (4). Those studies demonstrated that rIL-1 injection did not protect CFU-S from radiation injury or decrease their radiosensitivity (4). However, rIL-1 injection may induce an increase in a subpopulation of CFU-S with a greater capacity for repair of radiation damage. Studies by Till and McCulloch (34) suggested that endogenous CFU-S have a greater capacity for repair of radiation damage than CFU-S (10). Neta et al. (18) demonstrated that rIL-1 injection induced an increase in the number of endogenous CFU-S. However, an increase in the number of endogenous CFU-S has not always been associated with earlier bone marrow recovery or increased survival (19–21). Another possible mechanism for the earlier recovery of CFU-S after irradiation of rIL-1-injected mice is that rIL-1 injection may induce an earlier recruitment of surviving CFU-S into cell cycle after irradiation, as proposed by Smith et al. (35) for the radioprotective effects of endotoxin.

Manori et al. (36, 37) demonstrated that IL-1 containing supernatants were radioprotective when added to T cells within less than 24 h after their irradiation. This effect was related to the induction of IL-2 by IL-1 (37). In vitro studies demonstrated that IL-1 induced a manyfold increase in the production of colony-stimulating activ-
ity or factors for erythroid, granulocyte, macrophage, and multipotential colony-forming cells from a variety of cell types that include cells of the hematopoietic microenvironment (38-42). For example, IL-1 has been shown to induce the expression of genes for granulocyte-macrophage colony-stimulating factors, granulocyte colony-stimulating factors, and IL-6 from cell lines derived from endothelial, fibroblast, and bone marrow stromal cells (38-41). The maximum amounts of colony-stimulating factors occurred 8-48 h after addition of rIL-1 to cell lines (40, 42). In vitro studies demonstrated that hematopoietic functions of bone marrow stromal cells, thought to make up part of the hematopoietic microenvironment, are relatively radioresistant (43, 44). After radiation doses of 13-500 Gy, stromal cells still supported prolonged production of GM-CFC or produced granulocyte-macrophage colony-stimulating factors. Also, after exposure to 500 Gy radiation, murine bone marrow stromal cells still were able to respond to endotoxin by increased production of colony-stimulating factors (43). The production and release of colony-stimulating factors from accessory cells that make up part of the hematopoietic microenvironment may be one mechanism for the earlier bone marrow recovery observed in mice irradiated after rIL-1 injection.

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REFERENCES

Survival after Total-Body Irradiation. I. Effects of Partial Small Bowel Shielding

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The small intestine of the rat was shielded during total-body irradiation (TBI) to evaluate the effects of radiation dose and length of intestine shielded on survival. Sprague-Dawley rats were anesthetized in groups of 10. Using aseptic surgical procedures, 30, 40, 50, or 60 cm of the proximal or distal small intestine were temporarily exteriorized and shielded during irradiation with photons from an 18 MeV linear accelerator. Less than 1% of the dose was delivered to the shielded intestines. In unshielded animals, deaths occurred from Days 4 to 6 with 13, 15, or 17 Gy and from Days 8 to 10 with 9, 11, and 12 Gy. However, in all animals exposed to 15 Gy, with all or part of the small intestine shielded, survival was increased to between 5 and 9 days. Shielding of the distal small intestine was more effective in prolonging survival than shielding of the proximal small intestine. The previously identified target of radiation damage in the small intestine is the crypt stem cell. In this study, the analysis of histological specimens of shielded and irradiated small intestine suggested that humoral factors also influence intestinal histology and survival after irradiation. These humoral factors are thought to originate from the irradiated body tissues, the shielded proximal intestine, and the shielded distal intestine. Further studies are required to identify these factors and to determine their mode of action and their therapeutic potential after radiation damage to the small intestine.

INTRODUCTION

Total-body irradiation (TBI) of experimental animals has been studied to explore treatment options for victims of accidental radiation exposures or for patients in need of a bone marrow transplant (1). Dose-effect studies of TBI have been reported in a number of animal species since 1945 (2, 3). There is a threshold dose for lethality with total-body irradiation which varies between species. After a TBI dose just above the threshold dose, animals begin to die during the second week postirradiation from

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the sequelae of bone marrow aplasia, i.e., hemorrhage and/or infection. A further increase in TBI dose decreases survival to between 4 and 6 days. At these higher doses, death is caused by intestinal damage that leads to fluid and electrolyte loss (3). After very high doses (> 100 Gy) of TBI, death will ensue within hours due to respiratory and cardiac arrest caused by radiation damage in the central nervous system. For low-LET TBI, the relationship of dose to survival is different for each species. In a given species, these patterns occur in clearly separate dose ranges (4). Bone marrow transplantation can protect an irradiated individual after low-dose TBI (e.g., a single dose of < 10 Gy) (5, 6). With an appropriate bone marrow transplantation protocol, survival will occur at twice the dose that is lethal in animals without transplantation. When TBI is combined with bone marrow transplantation, the intestinal tract becomes the dose-limiting tissue for acute side effects of radiation. Fluid and electrolyte replacement, as well as total microbiologic gastrointestinal decontamination, have proven to be useful in decreasing radiation-induced intestinal toxicity. However, the TBI dose is increased by only 1 to 2 Gy (i.e., < 12.5 Gy) (7-10). The crypt stem cell of the small intestine has been identified as a critical target for radiation injury of the intestine (2, 3). The number or function of crypt stem cells that survive radiation is not readily influenced by currently available therapeutic agents. Other studies with intestinal shielding during TBI appear to indicate that factors other than crypt stem cells also influence survival (11-14). The identification of these factors might be useful for the treatment of intestinal radiation injury.

The present study was initiated in rats to explore new pathophysiological mechanisms in acute intestinal radiation damage.

MATERIALS AND METHODS

Animals

Purpose-bred Sprague-Dawley rats 6 to 8 weeks old and weighing 200-300 g were quarantined on arrival and screened for evidence of disease and pseudomonas before being released. They were maintained in an AAALAC-accredited facility caged in pairs and provided commercial rat food pellets and acidified tap water (pH 2.5) ad libitum. Animal holding rooms were maintained at 21 ± 2°C with 50 ± 10% relative humidity using at least 10 air changes per hours of 100% conditioned fresh air. They were on a 12-h light-dark full-spectrum light cycle with no twilight.

Surgery and Anaesthesia

General anaesthesia was maintained during surgical procedures using ketamine HCl hydrochloride (50 mg/ kg) in combination with xylazine (10 mg/kg) given intramuscularly and supplemented as needed. Aseptic procedures were used. A midline abdominal incision was made, and the portion of the small intestine to be shielded was exteriorized. Hemorrhage was not normally a problem in this surgical procedure; however, if it occurred, 4/0 chromic gut suture material was used to ligate bleeding vessels. The exteriorized portion of the intestine was tagged with colored nonabsorbable suture material. The animal was irradiated as described below. After irradiation, the small bowel was repositioned in the abdominal cavity, and the abdominal wall was closed using single interrupted 4/0 monofilament nylon sutures. The skin was closed with clamps. Anaesthesia was maintained during this entire time (approximately 40 min). Pedal reflex was monitored to assure maintenance of adequate anaesthesia depth. Animals were allowed to regain consciousness in individual cages after completion of the radiation exposure and surgical closure. The influence of anaesthesia and surgery on survival after TBI was analyzed by irradiating animals in the same restraint.
An 18 MeV Linac Accelerator was used at a dose rate of 10 Gy/min. A bremsstrahlung beam with an average energy of 6 MeV was produced by electrons impinging on a 4-mm water-cooled tantalum target. The dose per pulse was determined prior to irradiation of each group of animals. The restraint device allowed for shielding of the exteriorized portion of the small intestine with 18 cm of lead during irradiation. Figure 1 is a diagram of the physical setup. Ten centimeters of the small intestine or multiples thereof was shielded while the remainder of the rat's body was exposed. The following intestinal segments were shielded: the first 10, 20, or 40 cm of the proximal small intestine; the last 10, 20, or 40 cm of the distal small intestine; the middle 40 cm of the small intestine; or the entire 80 cm of the small intestine. Every radiation exposure was monitored by two ionization chambers in the field that were calibrated to the midline dose in a rat phantom for each irradiation day. The actual dose delivered was always within ±5% of the prescribed dose.

Dose measurements were made with ionization chambers and thermoluminescent dosimeters (TLDs) placed in an acrylic rat phantom. Ionization chambers were used to determine the dose rate according to the protocol established by Task Group 21 of the American Association of Physicists in Medicine (14). The TLDs were used to determine dose uniformity and the effectiveness of the shielding.

**Animal Care after Irradiation**

Rats remained in individual cages after the irradiation. They were evaluated for appetite, hydration, health status, and diarrhea twice a day during the normal work week and once daily during the weekends. Moribund animals were euthanized by CO₂ inhalation.

**Morphometric Analysis**

Computerized morphometric studies (Bioquant System IV, R & M Biometrics, Inc., Nashville, TN (1985)) were performed on tissues obtained from moribund animals. The small intestine samples were taken at 5, 10, 20, 40, 60, and 80 cm of an average 80 cm total length. The samples at 20 (proximal) and 60 cm (distal) were selected for complete histological and statistical analysis. The tissues were fixed in 10% buffered formalin. Transverse sections were embedded in paraffin. Five micron sections were cut, placed on glass slides, and stained with hematoxylin and eosin. At least five slides per animal were analyzed for...
Fig. 2. Relative dose distribution in plastic rat phantom in percentage of dose in midphantom using intestinal shielding. The same orientation is used for A and B.

**Results**

**Dosimetry**

Dosimetry results are summarized in Fig. 2. The rat’s body received a homogeneous dose (maximum/minimum ratio 1.08). The shielded intestine positioned more than 5 mm from the edge of the shield received less than 17% of the midline body dose.

**Dose-Effect Curve**

The LD_{50/60} was 10.0 Gy while the LD_{50} was 12.5 Gy. Anesthesia increased both of these LD_{50}'s by approximately 1.0 Gy (Fig. 3). The addition of surgery decreased the LD_{50} for the intestinal syndrome death by approximately 0.5 Gy.
Radiation and Small Bowel Shielding

Fig. 3. Lethality after total-body irradiation in anesthetized and unanesthetized rats. Ten to 20 rats per point. Solid circles indicate death before Day 6 (intestinal syndrome). Open circles indicate death before Day 30 (hematopoietic syndrome). The diamond symbol indicates animals that died before Day 6 that received anesthesia but no surgery. The broken lines connect results in rats that received anesthesia and surgery. The drawn lines connect results in rats that did not receive anesthesia or surgery.

Shielding of the Small Intestine

Shielding of as little as 10 cm of the small intestine increased survival time significantly \((P < 0.05)\). Shielded animals died between days 5 and 9 after TBI (see Fig. 4).

![Fig. 4. Mortality of irradiated and shielded rats after total-body irradiation. Ten to 20 rats per point. Number of deaths per day is circled. Day of death of last animal is indicated by vertical mark. Continuation of survival is indicated by > mark and the number of survivors to Day 14 is indicated.](image-url)
Survival Time of Rats after 15.0 Gy Irradiation

<table>
<thead>
<tr>
<th>Small intestine sub-system</th>
<th>Shielding (cm)</th>
<th>Group</th>
<th>N</th>
<th>Mean survival (days ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fore</td>
<td>0</td>
<td>27</td>
<td>4.6 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>10</td>
<td>9</td>
<td>6.4 ± 0.18*</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>20</td>
<td>10</td>
<td>5.6 ± 0.26**</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>40</td>
<td>12</td>
<td>6.2 ± 0.17**</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>60</td>
<td>10</td>
<td>6.3 ± 0.23**</td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>80</td>
<td>9</td>
<td>7.1 ± 0.20**</td>
<td></td>
</tr>
<tr>
<td>Middle</td>
<td>40</td>
<td>18</td>
<td>6.8 ± 0.18**</td>
<td></td>
</tr>
<tr>
<td>Entire</td>
<td>80</td>
<td>9</td>
<td>9.2 ± 0.99**</td>
<td></td>
</tr>
</tbody>
</table>

* (P < 0.05) significant difference from 0 cm (no shielding).
** (P < 0.05) significant difference between shielding groups.

and Table 1). Shielding of the proximal small intestine was less effective than distal shielding. A gradual increase in survival time of shielded animals was noted with increasing length of intestine shielded. In contrast, the dose-effect TBI studies in unshielded animals showed a discontinuous distribution of survival times, i.e., < Day 5 for intestinal syndrome death and ≥ Day 9 for hematopoietic syndrome death. The survival times of animals in different groups are shown in Table I and were compared using a t test.

**Histopathology of Shielded and Irradiated Small Intestine**

The highest number of crypts was found in the unirradiated proximal small intestine. Shielding protected the crypts in the distal small intestine completely (100%) but was less effective in the proximal small intestine (75%). Proximal shielding increased survival of crypts in the irradiated distal small intestine by 35% (Fig. 5, see ascending arrow). However, distal shielding did not increase the number of crypts per millimeter in the proximal and irradiated small intestine (Fig. 5, see descending arrow).

Proximal shielding did not change crypt depth in the irradiated distal intestine. In contrast, shielding the distal small intestine increased the crypt depth in the irradiated proximal small intestine (Fig. 6, see arrows). Crypt depth in the shielded distal small intestine was significantly increased compared to the unirradiated control on Day 6 (P < 0.01). However, this effect was transient since it was no longer observed on Day 7.

Villus height was greater in the unirradiated proximal small intestine than in the unirradiated distal small intestine (P ≤ 0.01). Villus height in the proximal small intestine was decreased by irradiation and conserved by shielding (Fig. 7). Distal shielding did not alter villus height in the irradiated proximal small intestine. However, with proximal shielding, the villus height in the irradiated distal small intestine was significantly (P ≤ 0.01) greater than the unirradiated control (Fig. 7, ascending
With distal shielding, the villus height in the distal-shielded small intestine was increased to 200% of the unirradiated controls. This effect appeared to be sustained until at least Day 7 (Fig. 7, descending arrow).

Results were best explained by the release of humoral factors (see Discussion). Humoral factors produced in shielded segments of the small intestine appeared to induce

Fig. 5. Crypts per millimeter length after shielding rat small intestines from 15 Gy total-body irradiation.

Fig. 6. Crypt depth in millimeters after shielding rat small intestines from 15 Gy total-body irradiation.
cell replication in the irradiated small intestine (Fig. 8, see arrows). Humoral factors released by the tissues that were irradiated influenced the shielded distal small intestine. The cell number in shielded distal small intestine was decreased compared to unirradiated controls (Fig. 8), whereas the villus-crypt length in the same tissue was

![Graph showing villus height in millimeters after shielding rat small intestines from 15 Gy total-body irradiation.](image)

Fig. 7. Villus height in millimeters after shielding rat small intestines from 15 Gy total-body irradiation.

![Graph showing number of cells per villus to crypt length after shielding rat small intestines from 15 Gy total-body irradiation.](image)

Fig. 8. Number of cells per villus to crypt length after shielding rat small intestines from 15 Gy total-body irradiation. P values apply to labeled bars in the same anatomical location only.
increased compared to unirradiated controls (Fig. 9, see arrow), indicating that cell size had increased.

In summary, the supportive evidence that the shielded proximal small intestine influenced the irradiated distal small intestine, probably by release of a humoral factor was (a) accelerated regeneration of crypts (Fig. 5); (b) increased villus height (Fig. 7); and (c) increased cell number (Fig. 8). Similarly, that the shielded distal small intestine influenced the irradiated proximal small intestine was indicated by (a) increased crypt depth (Fig. 6) and (b) increased cell number (Fig. 8). Humoral factors released by irradiated tissues appeared to increase cell size in the shielded distal small intestine only (Figs. 8 and 9).

**DISCUSSION**

Shielding the small intestine from radiation improved survival and resulted in changes in the intestinal mucosa. These studies required the use of anesthesia (Ketamine and Xylazine) which introduced a dose-modifying factor of approximately 1.15. The LD_{50/10} and the LD_{50/20} for mice restrained during TBI without anesthesia are also higher than for unrestrained animals. The radioprotective effect of anesthesia or restraint has been attributed to various mechanisms which include: (i) modulation of the central nervous system; (ii) the induction of hypoxia in cells; and (iii) a direct radioprotective effect (15, 16). In contrast, radiosensitization by anesthetic

agents has also been observed in normal and malignant tissues in mice (17–19). There is a need for further clarification of the interactions between anesthesia and radiation.

Shielding the small intestine from radiation increases the survival time. Data in Fig. 4 indicate that intestinal shielding changed the time of death of rats given 15 Gy TBI to a period midway between the classic intestinal syndrome (4–6 days) and the classic hematopoietic syndrome (9–30 days). If the intestinal crypt stem cells were the only determinant of survival after TBI, shielding of the small intestine should increase survival into the range associated with hematopoietic syndrome (≥9 days) as soon as enough crypt stem cells are shielded. However, since this was not observed, factors other than the intestinal crypt stem cells appear to influence survival. The survival times obtained in shielded animals in our experiments are similar to those observed by previous investigators (11–13). However, we were able to quantify the effects of shielding the small intestine more precisely by: (a) using higher energy photons that allowed for more precise shielding; (b) using a more detailed dosimetry; (c) comparing our results to a TBI dose–effect curve for unshielded animals; and (d) making a quantitative morphometric analysis of shielded and irradiated small intestine histology after irradiation. The strip of shielded intestine that received a higher dose than the rest of the shielded intestine is so small that it is not considered to influence our results (see Fig. 2).

The protective effects of shielding from radiation can be explained in several ways. One possibility is that cells may migrate from shielded to irradiated sites. The classic example is the protective effect of spleen or leg shielding in the lower lethal TBI dose range (20). It has been shown that hematopoietic recovery results from migration of stem cells from the shielded body areas (21–23). Recovery from intestinal radiation damage is unlikely to occur by migration of stem cells from shielded small bowel to irradiated small bowel. Instead, we postulate that humoral substances contribute to recovery from intestinal radiation injury. Humoral substances are released after irradiation and may influence the intestinal mucosa at shielded or irradiated sites by stimulating cell proliferation, cell traffic, or cell function after TBI. Prior studies of intestinal growth control mechanisms have indicated that intraluminal as well as circulatory factors are involved (24). The observation that shielding of the distal small intestine influenced the mucosal histology of the irradiated proximal small intestine supports the idea of a systemic transport of such a humoral factor and not intraintestinal lumen transport of that particular factor.

Humoral factors released by irradiated tissues would appear to differ from those released by shielded tissues. The former produce changes in intestinal cell size, the latter in intestinal cell proliferation (see Figs. 8 and 9). Irradiated tissues might release factors from cells damaged by radiation. In contrast, shielded tissues can produce factors actively as part of a physiological response to damaged intestinal mucosa. Known factors that should be considered include (i) pleiotropic growth regulators (hormones) such as gastrin, glucagon, thyroid hormone, or others (24); (ii) chalone that specifically suppress intestinal growth and change in concentration after irradiation (25); and (iii) growth factors which are not identified yet that are specific for the intestinal mucosa renewal system and comparable to factors identified for the hematopoietic system (26). The nature and number of humoral factors involved in
regeneration of intestinal tissues after irradiation can be determined only by biochemical identification, separation, purification, and testing of such factors by bioassay systems. The findings in this communication provide a good start for such assays.

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REFERENCES

Laboratory x-ray irradiator for cellular radiobiology research studies: Dosimetry report

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G. H. Zeman
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Dosimetry measurements were performed on a 50 kVp laboratory x-ray irradiator for cellular radiobiology research. The measurements were done with a parallel-plate ionization chamber, which had a thin aluminized-mylar window, and an extrapolation chamber. Intercomparison of dosimetry data from the two independent methods showed excellent agreement. Results are presented for radiation dose rate, uniformity, and beam quality for operation of the tungsten target, beryllium window x-ray tube at 50 kVp, 20 mA, and with filters either 0.012 mm, 0.180 mm, or 0.633 mm thick, for arrays positioned at a range of distances from the x-ray tube. The data in this report establish a dosimetric link between the cellular radiobiology research to be performed at AFRI with the 50 kVp laboratory x-ray irradiator and the landmark work performed by Elkind and Sutton (1960) with a similar device.
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In 1985, the Armed Forces Radiobiology Research Institute (AFRRI) acquired a 60 kVp x-ray irradiator (figure 1) from Argonne National Laboratory, Argonne, IL, for cellular radiobiology experiments. This irradiator is similar to one used by Elkind and Sutton (1) for their landmark investigations. The shielding, space, and logistical requirements for the irradiator are minimal, allowing it to be conveniently located in a typical laboratory. Because of its simplicity of operation and inherent safety mechanisms, an investigator or technician can qualify easily to use the radiation source independently.

This report describes the dosimetry methods used to calibrate the beams planned for cell irradiations. Measurements of exposure rate, beam quality, and field uniformity were made with a thin-window, parallel-plate ionization chamber. Elkind and Sutton (1) used a variable-volume extrapolation chamber to provide dosimetry for their irradiator because of its excellent capability of measuring low-energy photons. We had the good fortune to use Elkind's variable-volume extrapolation chamber for comparison with the parallel-plate chamber. Comparison of the dosimetry data showed very good agreement between results obtained by the two methods.
MATERIALS AND METHODS

Irradiator

The x-ray irradiator consists of a x-ray machine (Model No. DXE-225, General Electric Company, Milwaukee, WI), a x-ray tube (Model No. OEG-60, Machlett Laboratories Inc, Stamford, CT), and a shielded cabinet. The x-ray machine operated at peak voltages and currents of up to 60 kVp and 25 mA, respectively. The tube head is water-cooled for continuous operation. The tungsten target angle is 45 degrees to minimize anode heel effects. The tube window has an inherent filtration of 1.5 mm of beryllium (Be), which allows passage of a significant portion of the low-energy x rays. The irradiation time is controlled by a microprocessor-based solid-state timer (Model CX300, Eagle Signal Industrial Controls, Davenport, WI).

The irradiation cabinet has interior dimensions of 56 cm by 42 cm by 45 cm. The inner walls (including the door, top, and bottom) are lined with 2 mm of lead for effective personnel protection. A plastic shelf inside the chamber provides a stable, highly repeatable platform for experimental dosimetry devices and cell samples. The shelf is movable in the vertical (Z) axis by increments of 1 cm. The Z axis is at the center of crossed lines etched into the plastic shelf.

Radiation surveys at the exterior surface of the irradiator during operation at maximum power have shown no radiologic hazard due to beam leakage, thereby permitting experimental preparations to proceed in the lab during irradiation. The only detectable response was 0.5 mrem/hour on contact with the x-ray tube. At 10 cm from the tube, this response was negligible. As a precaution, two safety lights (and an audible alarm, if activated) alert occupants in the laboratory when x rays are being generated. Also, an interlock mechanism prevents the machine from working when the cabinet door is open and automatically turns the irradiator off when the door is opened during operation.

Beam Filtration

Aluminum (Al) filters were added at the exit port of the Be window to increase the effective energy of the x-ray beam by removing many of the low-energy x rays (2). Three thicknesses of Al (that is, 0.633 mm, 0.180 mm, and 0.012 mm) were used for all dosimetric measurements. The tube voltage and current used were 50 kVp and 20 mA, respectively. The 0.633-mm Al filter was used for intercomparison measurements because it simulates a field at the National Institute of Standards and Technology (NIST), Gaithersburg, Maryland, and was used to calibrate the parallel-plate ion chamber. The 0.180-mm Al filter is comparable to that used by Elkind (1). The 0.012-mm Al filter provides negligible filtration of low-energy x rays, hence, it offers a higher dose rate.
Culture Dish Arrays

Typically, cells are maintained in tissue culture petri dishes under monolayer growth conditions bathed in nutrient media to a depth of about 3 to 5 mm (approximately 5 ml per 60-mm-diameter dish or 20 ml per 100-mm-diameter dish). The petri dish is capped with a plastic cover approximately 1 mm thick, which assists both to maintain sterility and to decrease evaporative loss of media.

The x-ray energy spectrum produced at a peak voltage of 50 kV and with added Al filters readily undergoes attenuation by the plastic tissue-culture petri dish covers or the culture media. For example, using a beam hardened with 0.180 mm of Al the attenuation due to the medium can be as high as 60%, and the plastic cover will reduce the beam an additional 15%. Further, inconsistencies in the thickness of medium above the cells can result in large changes in the x-ray energy spectra reaching the cells. To avoid these problems, Elkind and Sutton (1) irradiated cells with a minimum of absorbing material between the radiation source and biological target. An ultra-thin cover of plastic wrap (Saran) secured in a plastic ring with brass screws substitutes for the petri dish cover during irradiations and decreases the possibility of contamination without severely diminishing the photon fluence. Further, all excess culture media was aspirated from the petri dish, leaving the cells without a significant media-induced shielding during irradiation.

Reproducibility of the positioning of the biological target material relative to the radiation source was accomplished in the following manner. Either 60-mm or 100 mm-diameter tissue-culture petri dishes were placed on the plastic tray in custom acrylic holders to provide several arrays for irradiation (figure 2). The petri dish array was centered on the Z axis of the irradiator by a centering plate. Cell cultures can be irradiated at room temperature or at the temperature of ice water. For ice water irradiation, an adaptor raises the large petri dish by 1 cm for ice water to be placed beneath the cell plate, thereby facilitating a common vertical scale (3).
Figure 2. Culture dish arrays: a. 100-mm diameter; b. 60-mm diameter; c. 60-mm diameter in three dish array.
Radiation Detectors

Parallel-Plate Ionization Chamber

A parallel-plate ionization chamber made by Capintec, Inc. (Model No. PS-033, Pittsburg, PA) was used to measure the exposure rate. Its thin window has a thickness of 0.5 mg/cm$^2$ of aluminized mylar, and its sensitive volume is 0.5 cm$^3$. The detector body is made of polystyrene. The chamber was calibrated at NIST with two x-ray fields (Table 1) (4). Appendix A shows the NIST energy spectrum for the unshielded, 20 kVp calibration field and an energy spectrum with irradiation parameters similar to the NIST L50 x-ray field (5). The difference between the calibration factors for the two beams was 2.4%. This indicated that the chamber response changed minimally over a range of low energies. Charge accumulation was made with a Keithley Model 616 digital electrometer (Cleveland, OH) for the duration of exposure. Collecting charge was the appropriate method of ionization measurement because this is the mode used by NIST to calibrate the chamber. The half-value layer (HVL) is the thickness of material required to reduce an x-ray beam to half its original intensity. The homogeneity coefficient is simply the ratio of the first and second HVL's. Appendix B shows the method of calculation for exposure and dose rates.

Preliminary measurements on the x-ray irradiator indicated that the parallel-plate chamber showed negligible ion recombination characteristics and polarity differences. Readings taken over several days at the same position showed a precision of ±1.5%.

Table 1. NIST Calibration Parameters of Parallel-Plate Chamber

<table>
<thead>
<tr>
<th>NIST field</th>
<th>kVp</th>
<th>Added filter (mm Al)</th>
<th>HVL (mm Al)</th>
<th>Homogeneity coefficient</th>
<th>Calibration factor (R/nC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L20</td>
<td>20</td>
<td>None</td>
<td>.071</td>
<td>.76</td>
<td>6.349</td>
</tr>
<tr>
<td>L50</td>
<td>50</td>
<td>.639</td>
<td>.75</td>
<td>.58</td>
<td>6.502</td>
</tr>
</tbody>
</table>
Elkind Variable-Volume Extrapolation Chamber

An extrapolation chamber is an ionization chamber in which the sensitive volume may be accurately changed by varying the separation distance between the window and collecting plate. It is a fundamental method of measuring exposure rate so that instrument calibration is unnecessary (6). Appendix C shows the derivation of the equations used to compute exposure rate from extrapolation chamber measurements. The extrapolation chamber (figure 3) used for comparison with the parallel-plate chamber is a custom device provided by Dr. M. M. Elkind. The chamber has a 50-μm-thick Be window (1) and a dual anode with collecting surface diameters of 1 cm and 4 cm. The potential difference applied to the collecting plate was -1,500 volts per centimeter with respect to the Be window. The collected current was read on the same electrometer as used with the parallel-plate chamber. (The difference between the electrometer current (nA) and charge (nC/sec) modes was measured to be 1.6% with a Keithley 261 Picoampere source in conjunction with a Hewlett-Packard 85 computer and 3421A Data Acquisition/Control Unit (Corvallis, OR.).

Figure 3. Elkind's extrapolation chamber with collecting plate.
RESULTS

Tube Potential and Timer Accuracy

Peak tube potential and quality assurance measurements for timer accuracy were made with a Mini-X kVp/Timer Meter (RTI Electronics Lab, AB, Sweden). The Mini-X was capable of accurately measuring x-ray tube voltage down to 44 kVp and timer accuracy for times up to 20 seconds. The measured value of kVp from the Mini-X agreed with the x-ray generator settings of 50 kVp and 55 kVp to within +/- 1% and at 45 kVp to within 3.5%. The x-ray timer setting was consistently 2% higher than the time measured by the Mini-X. This difference is due partly to the fact that the x-ray timer indicated the full exposure time while the Mini-X displayed the time that the exposure rate exceeded 75% of the maximum. To further evaluate the timer accuracy, charge was collected by the parallel-plate chamber over a range of exposure times for each beam quality (figure 4). The slopes of the fitted values have units of nC/sec. Comparison of each slope with the measured current shows an overresponse of the charge readings relative to the current readings by an average of 1.5%. This agrees with measurements taken with the picoampere source. The intersection of each line with the time axis shows negligible offset from the origin.

Figure 4. X-ray timer evaluation.
Focal Spot Measurements

The focal spot is the area on the target that accelerated electrons strike to produce x rays, and it correlates to the effective source size. The focal spot image of the irradiator was recorded on radiographic film through a pinhole aperture of 0.2 mm in a sheet of 1.6-mm lead placed midway between the source and the film. The size of the effective focal spot was calculated to be 6 mm by 7 mm using a similar triangles technique (7). Figure 5 shows an enlargement of the focal spot radiographic image.

![Focal Spot Image](image)

Figure 5. Enlargement of focal spot image.

Beam Quality

When an energy spectrum of an x-ray beam is not available, its penetrability or quality is described in terms of its HVL. The material of choice for measuring beam quality of this irradiator is Al. Because of the space limitation inside the irradiator chamber, ideal conditions for measuring HVL’s as described by Trout (8) could not be attained. To approximate the ideal conditions, the distance between the source and the parallel-plate chamber was 36.8 cm with the filter placed halfway between. A collimator arrangement (figure 6) restricted the beam to the chamber’s window. Table 2 shows beam quality data for several thicknesses of added Al at 50 kVp and no added Al at 20 kVp. The approximate equivalent photon energy for each beam’s HVL was referenced from Johns and Cunningham (7).
Figure 6. Collimator arrangement used for half-value layer measurements.

Table 2. Parameters of Beam Quality

<table>
<thead>
<tr>
<th>kVp</th>
<th>Added filter (mm Al)</th>
<th>HVL (mm Al)</th>
<th>Homogeneity coefficient</th>
<th>Approximate equivalent energy (keV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>none</td>
<td>0.058</td>
<td>0.80</td>
<td>8</td>
</tr>
<tr>
<td>50</td>
<td>.012</td>
<td>.077</td>
<td>.70</td>
<td>9</td>
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<td>50</td>
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<tr>
<td>50</td>
<td>.633</td>
<td>.53</td>
<td>.63</td>
<td>19</td>
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</tbody>
</table>
The measured HVL values for the 20 kVp and 50 kVp/0.633 mm Al measurements are significantly lower than the respective NIST L20 and L50 HVL values shown in table 1. The differences, which indicate the AFRRI x-ray unit has a softer energy spectra, are likely due to the various ways the tube potential is supplied. The NIST power supply is constant potential whereas the laboratory x-ray irradiator potential is half-wave rectified single phase.

Parallel-plate Window Transmission

The ability of the parallel-plate chamber to transmit low-energy photons through the aluminized mylar window was tested by measuring x-ray transmission through mylar. The HVL of mylar for two different x-ray beams was measured with the collimator system. The two beams were 50 kVp with 0.633 mm of added Al and 20 kVp with no added filtration. The measured HVL's were 876 and 94.4 mg/cm² for the 50 kVp and 20 kVp beams, respectively. The calculated attenuation due to the chamber window is 0.04% and 0.30% for each of the respective beams. This method provided an approximation because the mylar attenuators were not aluminized.

Intercomparison

Intercomparison between the parallel-plate ionization chamber and the extrapolation chamber was made with AFRRI's laboratory x-ray irradiator to verify the parallel-plate chamber’s ability to measure low-energy exposures. The parameters measured were (a) exposure rate, (b) falloff with increasing vertical (Z axis) distance, (c) falloff in field size (X-Y axes), and (d) output charges with current and voltage. The amount of added filtration used was 0.633 mm of Al. Unless altered for experimental purposes, the tube voltage, current, and source-to-detector distance (SDD) were set at 50 kVp, 20 mA, and 19.2 cm, respectively.

Comparison of Exposure Rates

Ionization current measurements were made while changing the distance separating window and collecting plate of the extrapolation chamber. These measurements were repeated for both of the available plate collecting diameters on Elkind's extrapolation chamber. The results of these measurements are presented in figure 7. The slope of the fitted line for each set of measurements, when applied to equation 1 of appendix D, resulted in the exposure rate at the midpoint of the chamber. The intercepts of the extrapolated lines on the abscissa fell at about 0.09 cm.
Table 3 shows the values used to evaluate the exposure rate. The average exposure rate of the four extrapolation measurements was 10.5 R/sec, with a standard deviation representing 1.6% of the mean. The exposure rate as measured by the parallel-plate chamber was 10.6 R/sec. The method used to calculate the exposure rate to the parallel-plate chamber is equation 1 in appendix B. The excellent agreement between these two methods establishes a link between the landmark radiobiology experiments of Elkind and colleagues at the Argonne National Laboratory and those performed with the new unit at AFRI.

Table 3. Exposure Rate Evaluation of Extrapolation Chamber and Parallel-Plate Chamber

<table>
<thead>
<tr>
<th>Chamber*</th>
<th>Day</th>
<th>Diameter (cm)</th>
<th>Temperature (°C)</th>
<th>P (mm Hg)</th>
<th>Slope (ΔA/Δh)</th>
<th>X intercept (cm)</th>
<th>X (R sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>1</td>
<td>4</td>
<td>22.0</td>
<td>753.8</td>
<td>43.8</td>
<td>0.107</td>
<td>10.6</td>
</tr>
<tr>
<td>EC</td>
<td>1</td>
<td>1</td>
<td>22.0</td>
<td>753.8</td>
<td>2.78</td>
<td>0.033</td>
<td>10.7</td>
</tr>
<tr>
<td>EC</td>
<td>2</td>
<td>4</td>
<td>23.1</td>
<td>757.0</td>
<td>42.7</td>
<td>0.084</td>
<td>10.3</td>
</tr>
<tr>
<td>EC</td>
<td>2</td>
<td>1</td>
<td>23.1</td>
<td>757.0</td>
<td>2.74</td>
<td>0.033</td>
<td>10.5</td>
</tr>
<tr>
<td>PPC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10.6</td>
</tr>
</tbody>
</table>

*EC = extrapolation chamber; PPC = parallel-plate chamber.
Inverse Square Measurements

The measurement of beam falloff with increasing distance from the source was made with the parallel-plate and extrapolation (4-cm diameter only) chambers. Figure 8 shows the ratio of the observed to expected values at successive distances from the source. The point of normalization was 19.2 cm from the source. After 16 cm, the falloff of both chambers yielded an inverse square relationship to within 1%. Additional measurements were made by the parallel-plate chamber along the same axis with added filters of 0.180 mm and 0.012 mm Al. Figure 8 compares these data and shows that filtration with thinner filters caused greater deviation from the inverse square law. This was the expected result because air acted as an effective attenuator for the low-energy x rays transmitted through the thin filters. The slopes of the two lines were approximately 0.006 cm\(^{-1}\), which was consistent with the linear attenuation coefficient for 10 keV x rays in air [7].

![Figure 8. Radiation output measurements normalized to expected values. Expected values were calculated using the inverse square law referenced to the output at 19.2 cm distance.](image-url)
Beam Falloff in the X-Y Plane

Beam intensity measurements in the horizontal plane at 19.2 cm were made with the parallel-plate and extrapolation (4-cm diameter only) chambers. The measurements were made diverging from the geometrical center toward the front, back, left, and right. The values were normalized to the geometrical center. Figure 9 shows field distribution was in excellent agreement between the parallel-plate chamber and 4-cm-diameter extrapolation chamber. The center of the field was at the geometrical center of the beam for both chambers. The diameters of the fields for 95\(^\circ\), 92.5\(^\circ\), and 90\(^\circ\) uniformity are 4.7 cm, 6.3 cm, and 7.3 cm, respectively.

![Figure 9. Intercomparison of field uniformity measurements.](image)

Tube Current and Voltage

The linearity of the x-ray output with tube current over a range of 8 mA to 25 mA was evaluated by the parallel-plate chamber and both collection plate diameters of the extrapolation chamber. Figure 10 shows the tube current dependence of each ionization chamber's response as normalized to its sensitive volume. The results show excellent agreement and linearity over the entire range of x-ray tube current.
Figure 10. Linearity of x-ray output with tube current.

Figure 11 shows the effect of varying tube voltage on each chamber's volume-normalized response. Agreement between the two anode diameters of the extrapolation chamber was quite good. Agreement of the parallel-plate chamber was also good at lower voltages, but shows an overresponse at higher voltages, which peak at 5% at 50 kVp. The data in figure 11 were fitted with both linear and power function curves, as shown. Both the linear and power function correlation coefficients were greater than 0.995.

Figure 11. X-ray output as a function of tube voltage.
Dosimetry Measurements

The cellular dose was calculated from parallel-plate measurements taken along the Z axis for three beam parameters. The beam parameters were 50 kVp with added filtration of 0.633 mm, 0.180 mm, and 0.012 mm Al. Equation 2 of appendix B shows the method of dose calculation. Tables 4-6 show the dose rate at incremental distances from the source at the geometrical center for the single-plate arrays. The hole number is the position where the plate was placed to attain the respective source-to-cell distance. One shelf height was for the room temperature setup and the other was for the ice water setup. The uniformity measurements at each distance were derived from falloff measurements in the X-Y plane at several Z-axis positions for each beam. From those measurements, cones of uniformity were constructed to ensure 90%, 92.5%, and 95% uniformity across the plate. Thus, an investigator can use the dose rate and uniformity that best suits the experimental needs.

While taking beam profile measurements with the thinner filters, we observed that the point of maximum beam intensity was offset from the vertical axis towards the back of the cabinet (-Y direction). This effect was enhanced at greater source-to-detector distance (SDD). The displacement for the 0.180-mm Al beam was 0.3 cm at 15 cm SDD and increased to 1 cm at 40 cm SDD. The displacement of the 0.012-mm Al beam was 0.5 cm at 15 cm SDD and increased to about 2 cm at 40 cm SDD. While this effect was not fully characterized, it probably resulted from a variation in the x-ray energy spectrum across the x-ray beam in the direction of the anode-cathode axis. Because the offset varied with both the SDD and added filtration, the irradiation arrays were left in place at the X-Y plane origin. As a result, the cone of uniformity was slightly narrower than if it had been centered at the point of peak intensity. In the worst case, the deviation of the maximal intensity from the intensity at the geometric axis was 0.5%.

Incomplete aspiration of culture medium from the petri dishes before irradiation can markedly influence the uniformity of cellular x-ray dosage. This was demonstrated by the results of a pilot experiment on x-ray dose response and cell survival that used the 0.012 mm Al filter. An anomalous cluster of cell colonies around the periphery of the culture plate was observed in the high radiation dose samples. Investigation into this abnormality suggested that this effect was produced by the shielding of x rays by a meniscus of excess culture fluid that was created by not aspirating all of the excess cell culture medium from the petri dish. This was confirmed by repeating the experiment with complete medium aspiration (that is, all of the excess medium was removed), which resulted in the elimination of the ring of colonies. Upon the addition of incremental amounts of medium, the medium-induced shielding around the outer edge reappeared. Figure 12 shows cell survival populations after staining when the culture medium had been aspirated completely, followed by the addition of...
Table 4. Dose Rate Chart of 0.633-mm Al Beam

<table>
<thead>
<tr>
<th>Hole #</th>
<th>Hole #</th>
<th>SDD (cm)</th>
<th>Dose rate (cGy/s)</th>
<th>Uniformity of cell irradiation (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>60-mm dish</td>
<td>100-mm dish</td>
</tr>
<tr>
<td>Room temp</td>
<td>Ice water</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>9</td>
<td>10</td>
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Table 5. Dose Rate Chart of 0.180-mm Al Beam

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<th>Uniformity of cell irradiation (percentage)</th>
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Table 6. Dose Rate Chart of 0.012-mm Al Beam

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up to 0.5 ml of medium before irradiation. Notice that a shielding effect was present with as little as 0.1 ml of medium added to the dish. A similar effect was present for the triple-dish array.

![Figure 12. Meniscus effect for essentially unfiltered (0.012 mm Al) beam. The cell cultures were aspirated to completeness with increasing amounts of culture fluid added: (a) 0 ml, (b) 0.1 ml, (c) 0.3 ml, and (d) 0.5 ml.]

SUMMARY

Dosimetry measurements have been performed on the AFRRI laboratory x-ray irradiator for three different cell culture dish arrays positioned at a range of distances from the x-ray tube. The device used for these measurements was a parallel-plate ionization chamber with a thin aluminized-mylar window. Intercomparison with an extrapolation chamber showed excellent agreement between these independent measurement methods. Results are presented for radiation dose rate, uniformity, and beam quality for operation of the tungsten target, Be window x-ray tube at 50 kVp, 20 mA, and with filters either 0.012 mm, 0.180 mm, or 0.633 mm thick. The data in this report establish a dosimetric link between the cellular radiobiology research to be performed at AFRRI with the laboratory x-ray irradiator and the landmark work performed by Elkind and Sutton (1) with a similar device.
REFERENCES


ACKNOWLEDGMENTS

The authors would like to thank Dr. Colin K. Hill for his helpful discussions and advice in planning this project. We would like to especially thank Mr. Franklin M. Sharpnack for the construction of the petri dish holders and acrylic positioning devices for the x-ray irradiator. We would also like to thank Mr. Scott Hawkins for the copious dosimetry measurements he made, which are included in this report. Finally, we acknowledge and thank Dr. Michael P. Hagan for his invaluable assistance and guidance in numerous aspects of this project.
APPENDIX A. X-RAY ENERGY SPECTRA

Figure A-1. X-ray energy spectrum for 50 kVp and 0.65 mm Al filtration. The solid line shows the spectral distribution of x-ray energy, and the dash line shows that of x-ray fluence. Redrawn from reference (5).

Figure A-2. X-ray energy spectrum for 20 kVp and no added filtration. The solid line shows the spectral distribution of x-ray energy, and the dash line shows that of x-ray fluence. Redrawn from reference (5).
APPENDIX B. EXPOSURE AND DOSE CALCULATIONS FOR THE PARALLEL-PLATE IONIZATION CHAMBER

\[ \dot{X} = \left( \frac{\Delta Q}{\Delta t} \right) N_x k_{tp} \]  \hspace{1cm} (B-1)

where
- \( \dot{X} \) = exposure rate in R/s
- \( \Delta Q \) = charge collected over time interval \( \Delta t \) (nC/s)
- \( N_x \) = chamber calibration factor (see table 1). The calibration factor whose HVL and homogeneity factor most closely resembles the values measured for each field was chosen for that field. The uncertainty in applying this method is approximately 1%.
  - \( = 6.502 \text{ R/nC for 0.633 mm added Al beam} \)
  - \( = 6.349 \text{ R/nC for 0.180 mm added Al beam} \)
  - \( = 6.348 \text{ R/nC for 0.012 mm added Al beam} \)
- \( k_{tp} \) = correction to standard room temperature and pressure

\[ \bar{D} = \dot{X} f_{\text{water}} k_{bs} k_{sw} \]  \hspace{1cm} (B-2)

where
- \( \bar{D} \) = absorbed dose rate to cell medium (cGy/s)
- \( f_{\text{water}} \) = factor that converts exposure in air to dose in water, which approximates the cell media. This is an energy dependent value, although it varies less than 2.5% over the equivalent energy ranges involved (7).
  - \( = 0.886 \text{ for 0.633 mm added Al beam} \)
  - \( = 0.902 \text{ for 0.180 mm added Al beam} \)
  - \( = 0.905 \text{ for 0.012 mm added Al beam} \)
- \( k_{bs} \) = correction for backscatter. This correction was assumed to be 1.00 because the amount of plastic behind the ionization chamber was approximately the same as behind the cells.
- \( k_{sw} \) = correction for attenuation due to plastic wrap. This correction was only applied to the 0.633 mm Al beam. Plastic wrap was included in the measurements of the other fields.
  - \( = 1.02 \text{ for 0.633 mm added Al beam} \)
APPENDIX C. DERIVATION OF EXPOSURE RATE CALCULATION FOR EXTRAPOLATION CHAMBER (3)

a. Definition of Exposure:

1 R in dry air for x or γ rays = 1 esu charge / cm^3 at STP
∴ 1 R = 3.336 x 10^{-10} C/cm^3

b. Conversion of C/cm^3 to amp-s/cm^3 at STP

Given: 1 amp = 1 C/s
1 C = 1 amp-s
∴ 1 R = 3.336 x 10^{-10} amp-s/cm^3
and 1 R/s = 3.336 x 10^{-10} amp/cm^3

c. Define:

\[ K = \frac{1 \text{ R/s}}{3.336 \times 10^{-10}} = 2.998 \times 10^9 \left[ \frac{(\text{R/s})}{(\text{amp/cm}^3)} \right] \]

d. \[ \dot{\phi} = K \frac{dQ/dt}{V_{\text{STP}}} = K \left( \frac{\text{R/s}}{\text{amp/cm}^3} \right) \frac{I \ (\text{amp})}{V_{\text{STP}} \ (\text{cm}^3)} \]

= 2.998 x 10^9 (I/V_{\text{STP}})

where I = current of charge generated in air volume V at STP conditions
e. Volume of a right circular cylinder \( V = \pi r^2 h \)

where \( r \) is the radius and \( h \) is the height

Correct the air volume to room temperature and standard pressure.

\[
V_{\text{STP}} = \left( \frac{295.15}{T + 273.15} \right) \left( \frac{P}{760} \right) (\pi r^2 h)
\]

where \( T \) is the air temperature in °C and \( P \) is the atmospheric pressure in mm of mercury.

f. \[
\dot{X} = 2.457 \times 10^9 \left( \frac{T + 273.15}{P} \right) \left( \frac{I_c}{r^2 h} \right)
\]

where \( r \) = the extrapolation effective radius (cm)

\( I_c/h \) = slope of line from chamber response (amp) vs. separation of window-anode (cm)

\( h \) = separation distance of the extrapolation chamber window from the anode