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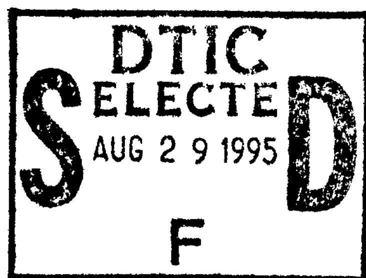

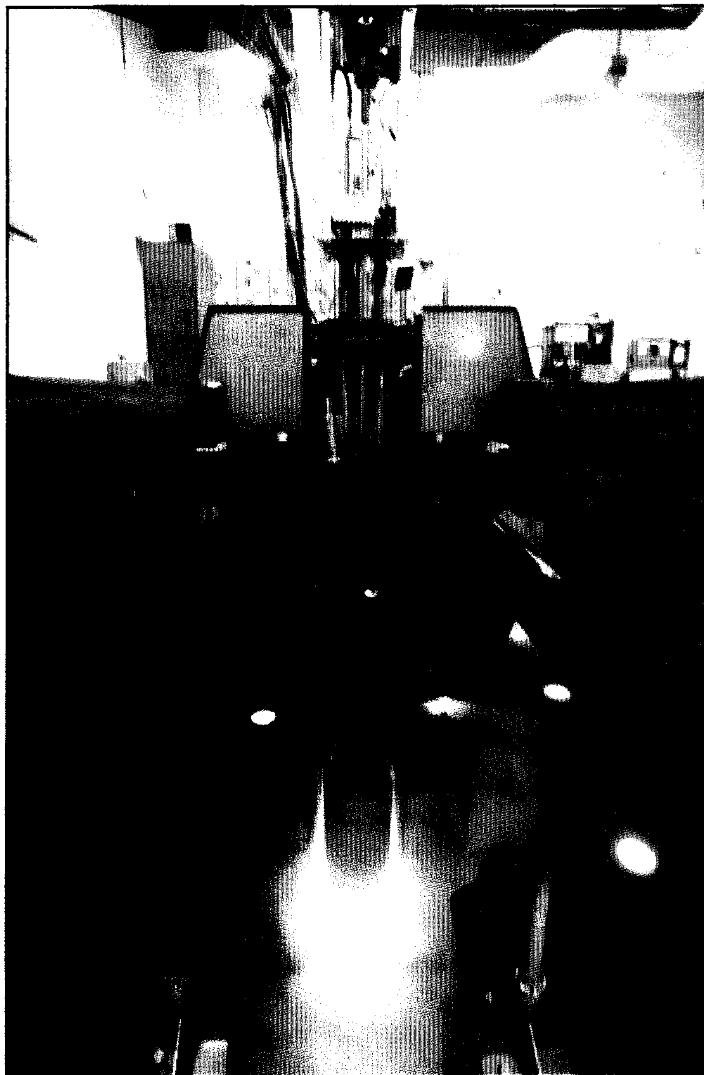

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CONTENTS

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SR95-11: Dubois A, Fiala N, Weichbrod RH, Ward GS, Nix M, Mehlman PT, Taub DM, Perez-Perez GI, Blaser MJ (1995) Seroepizootiology of *Helicobacter pylori* gastric infection in nonhuman primates housed in social environments.

SR95-12: Farese AM, Hunt P, Boone T, MacVittie TJ (1995) Recombinant human megakaryocyte growth and development factor stimulates thrombocytopoiesis in normal nonhuman primates.

SR95-13: Kandasamy SB, Thiagarajan AB, Harris AH (1995) Possible involvement of prostaglandins in increases in rat plasma adrenocorticotropic hormone and corticosterone levels induced by radiation and interleukin-1α alone or combined.

SR95-14: Limanni A, Baker WH, Chang CM, Seemann R, Williams DE, Patchen ML (1995) C-*kit* ligand gene expression in normal and sublethally irradiated mice.

SR95-15: Walden Jr TL, Farzaneh NK (1995) Radioprotection by 16,16 dimethyl prostaglandin E_2 is equally effective in male and female mice.

SR95-16: Winsauer PJ, Bixler MA, Mele PC (1995) Differential effects of ionizing radiation on the acquisition and performance of response sequences in rats.

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Seroepizootiology of *Helicobacter pylori* Gastric Infection in Nonhuman Primates Housed in Social Environments

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We determined the seroepizootiology of *Helicobacter pylori* infection in rhesus monkeys. Plasma was obtained from 196 animals (age range, 1 to 22 years) that were housed in social environments, either in indoor gang cages, in outdoor corrals, or in free-ranging forested conditions. Plasma immunoglobulin G levels were determined with a specific enzyme-linked immunosorbent assay, and the cutoff immunoglobulin G value for *H. pylori* seropositivity was determined from a study of 25 monkeys whose infection status was assessed by light microscopy and culture. One-year-old animals of both genders in all housing conditions had the lowest rate of positivity (60% in monkeys 1 year old versus 81% in monkeys 2 to 10 years old, $P = 0.026$). In addition, females tended to have higher rates of positivity than males. Seroconversion during a 1-year observation period occurred in 7 (28%) of 25 seronegative animals. Seroreversion occurred in 3 (4%) of the 78 positive animals; all 3 of these animals had received antimicrobial agents during the year. These observations demonstrate that the epizootiology of *H. pylori* infection in rhesus monkeys may serve as a model for human infection.

Helicobacter pylori is a spiral gram-negative bacterium that frequently infects the gastric mucosa of humans and causes chronic superficial gastritis (1). It now is clear that *H. pylori* infection is involved in the pathogenesis of peptic ulcer disease (22) as well as in adenocarcinoma of the distal stomach (17, 24, 26). The presence of *H. pylori* may be demonstrated by histology and culture of gastric mucosal biopsies or, noninvasively, by the urea breath test (29). In addition, determination of the presence of specific serum antibodies that consistently accompany the infection (2) has allowed the conduct of large epidemiologic studies (8, 28).

Ethical considerations make difficult the performance of studies attempting to clarify the pathophysiology of *H. pylori* infection in humans, and it is therefore important to develop and validate a relevant animal model. Although many animal species may be naturally or experimentally infected with various *Helicobacter* species that are similar to *H. pylori* isolated from patients (11, 13, 21), important phenotypic and genetic differences have been observed. In addition, the gastric pathology in these animal models is not identical to that present in humans.

Natural infection by *H. pylori*-like organisms has been reported to occur in several nonhuman primates (3–5, 10), and PCR amplification and partial 16S rRNA gene sequence analysis of spiral bacteria cultured from gastric biopsies of rhesus monkeys have indicated 98 to 100% homology with human strains of *H. pylori* (7, 15). Furthermore, this infection is persistent and is associated with acute as well as chronic gastritis and elevated specific immunoglobulin G (IgG) levels, all of which may be suppressed by therapy (9). Taken together, these data indicate that, as with humans, rhesus monkeys naturally have persistent *H. pylori* infection. Therefore, this animal appears to represent a good model to evaluate the role played by *H. pylori* in the production of gastritis and of the associated immune response. Furthermore, the model also permits the evaluation of novel therapies (9). However, the epizootiology of *H. pylori* in this species is at present unknown. The goal of our study, therefore, was to evaluate the seroepizootiology of *H. pylori* infection in rhesus monkeys of both genders across different age groups and different types of social housing conditions and habitat.

(This work was presented in part at the American Gastroenterological Association Digestive Diseases Week, Boston, Mass., May 1993 [9a].)

MATERIALS AND METHODS

Animals. Domestic, colony-reared, male and female rhesus monkeys (*Macaca mulatta*) were randomly selected among different groups as indicated below. They were grouped as yearlings (0.5 to 1.5 years), juveniles/subadults (2 to 6 years), prime adults (7 to 10 years), and older adults (11 to 22 years). All experiments were conducted according to the principles set forth in reference 23a.

Housing conditions. Animals were bred in three types of housing conditions. The first type of housing was gang cages (10 by 11 by 10 ft [ca. 3.0 by 3.4 by 3.0 m]) with sealed cement floors, which contained an average of one adult male and five adult females. The total gang cage population consisted of approximately 319 animals. The second housing type was corrals (60 by 100 ft [ca. 18 by 30 m]) with a grass and dirt substrate and a large arboreal jungle gym. The average group was composed of 3 adult males and 23 adult females. The total corral population consisted of approximately 355 animals. The third environment was a 475-acre South Carolina sea island in which approximately 4,200 animals were free ranging in forested conditions. There were 34 groups on the island, each composed on average of 51 adults (range, 14 to 325 animals; means, 6 males and 45 females per group).

Sampling. At the time of the physical examination given annually to the rhesus monkeys, a total of 202 animals of both genders and various ages in the three housing conditions described above were randomly selected (gang-caged social groups, 36 males and 37 females; corralled social group, 30 males and 34 females; free-ranging social group, 23 males and 43 females). Five milliliters of EDTA-treated blood was obtained from each animal, and the plasma was frozen at −70°C. One year later, plasma was again obtained from 106 (77%) of the 137

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TABLE 1. Seropositivity for *H. pylori* in the three housing conditions

Housing condition and age range (yr)	Females			Males			Males and females		
	Total no.	No. positive	% Positive	Total no.	No. positive	% Positive	Total no.	No. positive	% Positive
Gang caged									
≤1.5	2	2	100	4	1	25.0	6	3	50.0
2–6	17	12	70.6	14	13	92.9	31	25	80.6
7–10	6	5	83.3	2	2	100	8	7	87.5
11–22	12	10	83.3	16	11	68.8	28	21	75.0
All	37	29	78.4	36	27	75.0	73	56	76.7
Corralled									
≤1.5	6	4	66.7	6	2	33.3	12	6	50.0
2–6	10	9	90.0	11	7	63.6	21	16	76.2
7–10	5	5	100	4	3	75.0	9	8	88.9
11–22	13	11	84.6	9	6	66.7	22	17	77.3
All	34	29	85.3	30	18	60.0	64	47	73.4
Free ranging									
≤1.5	6	4	66.7	6	5	83.3	12	9	75.0
2–6	14	11	78.6	11	9	81.8	25	20	80.0
7–10	12	10	83.3	2	2	100	14	12	85.7
11–22	10	10	100	4	3	75.0	14	13	92.9
All	43	35	81.4	23	19	82.6	65	54	83.1

monkeys from the gang cages and the corrals sampled previously (53 monkeys each from gang cages and corrals).

Measurement of *H. pylori*-specific plasma IgG. Plasma IgG levels were determined blindly by using a previously described enzyme-linked immunosorbent assay (ELISA) (8, 28). The *H. pylori* antigen used in the ELISA was prepared from five *H. pylori* strains and represents a pool of sonicated whole cells composed of a mixture of protein and lipopolysaccharide antigens, as described previously (28). The sonicates from each strain were pooled and diluted in 0.05 M carbonate buffer (pH 9.6) to yield the optimal protein concentration of 10 μg/ml. A 0.1-ml aliquot of this solution was added to each well of a flat-bottom Immulon 2 plate (Dynatech Laboratories, Alexandria, Va.). The screening serum dilutions were 1:800, while peroxidase conjugates of goat anti-human IgG (Tago, Inc., Burlingame, Calif.) were diluted 1:2,000. Results were corrected for day-by-day variation of the ELISA and were expressed as ratios, i.e., the fraction of the mean plus three standard deviations of results for 40 healthy U.S. children. All assays were run in duplicate on two or more days (i.e., at least four times). Tests for possible cross-reactivity of *H. pylori* antibodies had been done by absorbing sera from *H. pylori*-infected persons that had high values in the IgG ELISA with cells of other enteropathogens (28). The assay was >95% sensitive and specific for human infection when an IgG ratio of >1.0 was considered indicative of the presence of anti-*H. pylori* antibodies. In monkeys, the ELISA was 83% specific and 69% sensitive with a ratio cutoff for positivity of 0.6 (9). In earlier studies, the correlation when anti-monkey and anti-human IgG conjugates were used was highly significant ($r = 0.80$; $P < 0.001$) (9).

Serological definition. Seropositivity and seronegativity were defined as ratios of ≥0.6 and <0.6, respectively, as previously described (9). Seroconversion was defined as a >100% increase in the IgG ratio to >0.6, and seroreversion was defined as a >50% decrease of IgG to a ratio of ≤0.6.

Statistical analysis. Results were expressed as means ± 1 standard error of the mean. Fisher's exact test and the Mantel-Haenszel corrected chi-square test were performed to test differences between groups.

RESULTS

Seroprevalence. The overall frequency of seropositivity increased with age, and there were no statistically significant differences between the three housing conditions (Table 1). Animals of both genders under 2 years old in all housing conditions tended to have the lowest rates of positivity (60% in monkeys under 2 years old versus 81% in monkeys 2 to 10 years old; $P = 0.026$). However, this difference was entirely due to the difference among the males (50 versus 82%; $P = 0.021$); the difference among the females (71 versus 81%) was not significant. Except for this difference, gender did not significantly affect the rate of *H. pylori* infection, although males of all age classes tended to have lower rates of positivity than females (73 versus 82%; not significant), with the difference being particularly clear in the younger (50 versus 71%; not significant) and in the older (69 versus 88.5%; not significant) age groups (Fig. 1). Finally, 11- to 22-year-old males had a significantly lower rate of infection than did 4- to 10-year-old males (64 versus 91%; $P = 0.01$).

Follow-up at 1 year. At the 1-year follow-up, seroconversion was observed in 7 of 24 previously seronegative animals; 7 of 12 of those monkeys that were ≤6 years old seroconverted, versus 0 of 12 of those that were >6 years old (Fig. 2; $P < 0.001$). The mean (± standard error of the mean) age of animals that seroconverted was 3.3 ± 0.8 years, versus 11.3 ± 1.5 years for those that did not seroconvert (Fig. 2; $P < 0.001$). Five of six seronegative males among corralled animals seroconverted, while two of five seronegative females among gang-caged monkeys seroconverted. The number of seronegative animals in each gender and type of housing was too small to allow evaluation of statistical differentiation of the rate of seroconversion among these subgroups. Of 78 animals that were seropositive

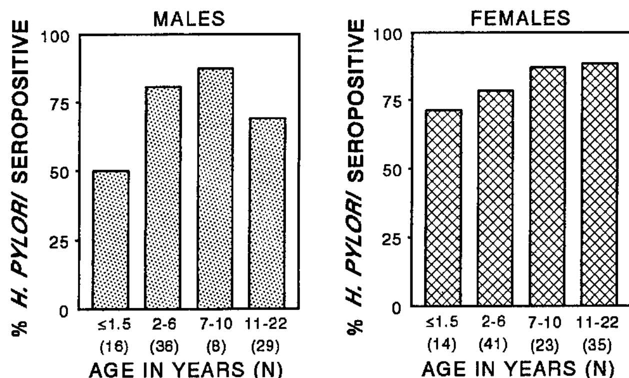

FIG. 1. Mean prevalence of seropositivity by age and gender among the 202 monkeys studied. The bars illustrate the percentage of *H. pylori*-positive animals in each age group in each gender. The numbers in parentheses represent the number of animals in each group.

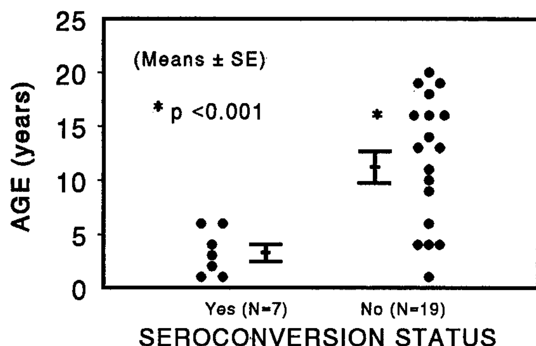

FIG. 2. Ages of previously seronegative monkeys that seroconverted (left) and did not seroconvert (right) at the 1-year follow-up. Each point illustrates the age of one animal, and the bar represents the mean age (± standard error of the mean) in each group. Two of the 26 seronegative animals were not tested at 1 year and do not appear on the figure.

at the first sampling, 3 (4%) had seroreverted at resampling 1 year later. Two were corralled females (ages 6 [odds ratio, 1.872 to 0.552] and 7 [odds ratio, 2.558 to 0.538] years), and one was a 20-year-old gang-caged male (odds ratio, 2.395 to 0.188). All three of these animals had received antimicrobial agents (erythromycin and/or Peptobismol) during the previous year, versus 27 of the other 78 persistently seropositive animals ($P = 0.047$).

DISCUSSION

The present studies demonstrate a high prevalence of seropositivity for *H. pylori* in the monkeys in each of the three housing conditions. This high seroprevalence is entirely consistent with our knowledge of the endemicity of *H. pylori* infection among humans in developing countries (23, 27). In addition, as often occurs in human populations in developing countries (23, 27), *H. pylori* infection was acquired before the age of 2 years by >50% of the rhesus monkeys housed in social environments. Although it was high from an early age, the seroprevalence of *H. pylori* in these animals further increased with age, as has been reported for humans (8, 14), and this effect may be due to a continuous risk of infection (32). However, there was little variation in the high (50 to 93%) positivity rate, similar to what is observed in developing countries in persons between the ages of 10 and 60 years. The lower rate of infection in older males may reflect a cohort effect or a decline in prevalence, possibly due to gastric atrophy (18). This latter possibility is supported by our recent longitudinal study of patients who developed severe atrophic gastritis with pernicious anemia and in whom serum antibody responses to *H. pylori* diminished and disappeared (4a).

Females tended to become infected at younger ages, and this could be related to their having more social interactions than males. In humans, no consistent difference between males and females has been reported (14, 23). *H. pylori* infection occurred as frequently in housing conditions in which there was little contact with humans (that is, in free-ranging animals) as in gang-caged and corralled monkeys, which were in closer contact with humans. Thus, as expected, the environment or the proximity to humans does not appear to play a substantial role in the epizootiology of the infection, and *H. pylori* is enzootic among the monkeys.

Only 4% of *H. pylori*-positive animals seroreverted during a 1-year period, and these three animals had received antimicrobial agents. This finding is consistent with our previous observation that, as it does in humans, *H. pylori* persists in naturally infected monkeys (9). In humans, the spontaneous rate at which infection is cleared in the absence of antibiotics also appeared to be <1%/year (6, 20, 25, 30).

In contrast, seroconversion occurred at a very high rate (28%) among seronegative animals. Furthermore, all of those monkeys that seroconverted were less than 6 years old, which means that the rate of seroconversion was >50% among the 1- to 6-year-old monkeys. This high rate is entirely consistent with the progressive age-related change in seropositivity until all the animals that are susceptible to the infection are infected, possibly by the age of 6 years under these conditions. Thus, the young monkeys were susceptible but had not yet been exposed, while the older monkeys no longer were susceptible as a consequence of *H. pylori*-related antral gastritis (9, 18). The small size of each subgroup of seronegative animals precluded analysis of the effect of gender or housing conditions on the rate of seroconversion. However, the observation that the seroprevalence remained higher among the older females suggests that this group may be continually more exposed to transmission of *H. pylori* or that atrophic changes progressed more slowly in females. Biopsies of older monkeys will be needed to address this question. Technical considerations prevented our sampling free-ranging animals at 1 year. However, the observation that the type of housing condition did not influence the seroprevalence suggests that similar conversion rates may be present in this latter group.

Like that in humans, the route of transmission of *H. pylori* infection in rhesus monkeys is unknown. Contamination of the water supply may play a role in certain developing countries (19), but this possibility appears to be excluded for our study because the water supply to the monkeys housed in gang cages and corrals was the chlorinated municipal water supply. Since the frequency of contacts with humans did not appear to substantially modify the seroprevalence of infection, it is likely that *H. pylori* is transmitted from monkey to monkey, possibly via the fecal-oral or the oral-oral route (12, 31). Consumption of feed contaminated by saliva (12) or feces (31) could be the vehicle of this transmission, as was recently demonstrated for vegetables (16).

In conclusion, our observations demonstrate that the epizootiology of *H. pylori* in rhesus monkeys is similar to the epidemiologic characteristics reported for human infection, especially in developing countries and in groups at low socioeconomic levels. Thus, this animal model may provide important and relevant information by allowing modifications of the environment that are not possible in studies involving humans. Correlation with gastric pathology should permit investigations and interventions under way that are relevant to both peptic ulcer disease and gastric cancer.

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RAPID COMMUNICATION

Recombinant Human Megakaryocyte Growth and Development Factor Stimulates Thrombocytopoiesis in Normal Nonhuman Primates

By Ann M. Farese, Pamela Hunt, Thomas Boone, and Thomas J. MacVittie

Megakaryocyte growth and development factor (MGDF) is a novel cytokine that binds to the c-*mpl* receptor and stimulates megakaryocyte development in vitro and in vivo. This report describes the ability of recombinant human (r-Hu) MGDF to affect megakaryocytopoiesis in normal nonhuman primates. r-HuMGDF was administered subcutaneously to normal, male rhesus monkeys once per day for 10 consecutive days at dosages of 2.5, 25, or 250 μg/kg of body weight. Bone marrow and peripheral blood were assayed for clonogenic activity and peripheral blood counts were monitored. Circulating platelet counts increased significantly ($P < .05$) for all doses within 6 days of r-HuMGDF administration and reached maximal levels between day 12 and day 14 postcytokine administration. The 2.5, 25.0, and 250.0 μg/kg/d doses elicited peak mean platelet counts that were 592%, 670%, and 449% of baseline, respectively. Bone marrow-derived clonogenic data showed significant increases in the concentration of megakaryocyte (MEG)-colony-forming unit (CFU) and granulocyte-erythroid-macrophage-megakaryocyte (GEMM)-CFU, whereas that of granulocyte-macrophage (GM)-CFU and burst-forming unit-erythroid (BFU-e) remained unchanged during the administration of r-HuMGDF. These data show that r-HuMGDF is a potent stimulator of thrombocytopoiesis in the normal nonhuman primate.

This is a US government work. There are no restrictions on its use.

MEGAKARYOCYTOPOIESIS is comprised of a series of events that involve both the proliferation and maturation of megakaryocyte progenitors toward production of mature platelets.[1-3] Several cytokines, interleukin-3 (IL-3), IL-6, IL-11, leukemia inhibitory factor (LIF), and granulocyte macrophage colony-stimulating factor (GM-CSF) have been shown to promote megakaryocytopoiesis at different stages of development and platelet production.[4-13] A novel protein, megakaryocyte growth and development factor (MGDF), also described as the c-Mpl ligand (Mpl-L, ML) and thrombopoietin (TPO), has been identified, cloned, and showed megakaryocytopoietic activity both in vitro and in vivo in rodent models.[14-18] This study is the first to describe the efficacy of recombinant human (r-Hu) MGDF on megakaryocytopoiesis and platelet production in a large animal model, the nonhuman primate.

MATERIALS AND METHODS

Animals. Domestic born male rhesus monkeys, *Macaca mulatta,* mean weight 3.8 ± 0.2 kg, were housed in individual stainless steel cages in conventional holding rooms at the Armed Forces Radiobiology Research Institute in an animal facility accredited by the American Association for Accreditation of Laboratory Animal Care. Monkeys were provided 10 air changes/h of 100% fresh air, conditioned to 72° ± 2°F with a relative humidity of 50% ± 20% and maintained on a 12-hour light/dark full spectrum light cycle, with no twilight. Monkeys were provided with commercial primate chow, supplemented with fresh fruit and tap water ad libitum. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals,* prepared by the Institute of Laboratory Animal Resources, National Research Council.

Study design. The animals were randomly assigned to one of four treatment groups. Cohorts of animals were administered *Escherichia coli*–derived r-HuMGDF (Amgen Corp, Thousand Oaks, CA) subcutaneously (sc), once per day (QD) for 10 consecutive days at dosages of 2.5 (n = 3), 25 (n = 3), or 250 (n = 2) μg/kg/d. Control animals (n = 3) received 25 μg/kg/d of human serum albumin (HSA [Plasbumin-5; Miles Inc, Cutter Biological]) sc, QD for 10 days. The level of endotoxin in r-HuMGDF was undetectable (<0.06 EU/mg: Limulus amebocyte lysate assay [Associates of Cape Cod, Inc, Woods Hole, MA]). HSA was not assayed for endotoxin content; however, pyrogenicity tests performed were negative (Miles Pharmaceutical Division, Biological Products, Elkhart, IN).

Hematologic evaluations. Peripheral blood (PB) was obtained from the saphenous vein to assay complete blood (Baker, System 9000; Serono-Baker, Allentown, PA) and differential counts (Wright-Giemsa Stain; Ames Automated Slide Stainer, Elkhart, IN). Approximately 1.5 mL of bone marrow (BM) was aspirated from the humerus and/or iliac crest of anesthetized (ketamine, 10 mg/kg intramuscular [im], 21-gauge needle in 0.3 mL vol) primates into heparinized syringes. Mononuclear cells (MNC) were separated using Histopaque (Sigma, St Louis, MO) and 5×10^4 cells/mL were assayed using MethoCult H4230 (Stem Cell Tech, Vancouver, BC, Canada). A combination of cytokines (G-CSF [5 ng/mL], MGDF [20 ng/mL] [Amgen], MGF [50 ng/mL] [Immunex R & D Corp, Seattle, WA], erythropoietin [2 U/mL] [Behringwerke, Marburg, Germany], IL-3 [20 ng/mL], IL-6 [40 ng/mL], GM-CSF [5 ng/mL], Sandoz Pharm, East Hanover, NJ]) and transferrin (Sigma) (10 μg/mL) were added to all 1-mL culture plates. Colony growth was assessed after a 10-day incubation at 37°C in a fully humidified; 5% CO_2 atmosphere. Results were expressed as number of colony-forming cells (CFC) per 10^5 MNC.

Statistical analysis. Statistical analysis of PB platelet counts were evaluated using a nonparametric *t*-test. Clonogenic data were analyzed using one-way Analysis of Variance (ANOVA) with a repeated measure on data collected over days. Further analysis followed when significance of dose and/or day effects were found. A log transform was used in analyzing granulocyte-macrophage (GM) and granulocyte-erythroid-macrophage-megakaryocyte (GEMM)-

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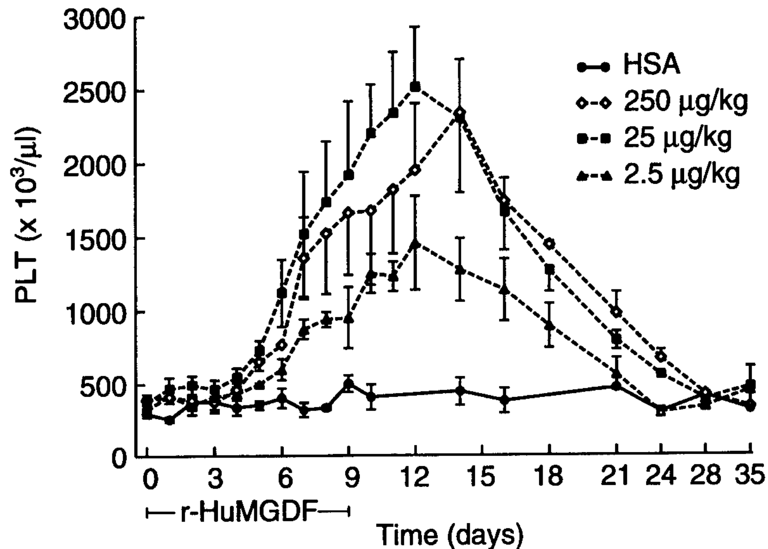

Fig 1. Effects of r-HuMGDF administration on PB platelet counts in normal primates. The platelet counts (PLT) observed in normal rhesus primates after r-HuMGDF or HSA administration that was administered sc, QD for 10 consecutive days at dosages of 2.5 (n = 3), 25 (n = 3), or 250 (n = 2) μg/kg/d. Control animals (n = 3) received 25 μg/kg/d of human serum albumin sc, QD for 10 days. Data represent mean ± SEM of the absolute platelet counts for the cytokine- or HSA-treated animals.

colony-forming unit (CFU) data to correct for variance differences. These tests were performed using the software package SYSTAT (SYSTAT, Inc, Evanston, IL).

RESULTS

Administration of r-HuMGDF for 10 days at 2.5, 25, or 250 μg/kg/d resulted in significant increases in circulating platelet counts in normal primates (Fig 1). At these doses a gradual, yet significant increase ($P < .05$) (range 770 to 1,121 $\times$ 10^3 μL) in platelet counts from baseline values (range 297 to 440 $\times$ $10^3/\mu$L) occurred during the first 6 days of r-HuMGDF administration and was followed by a rapid increase to mean maximum platelet counts between days 12 and 14 ($P < .001$ for all doses). The peak response in the mean platelet count (2,511 $\times$ $10^3/\mu$L) was elicited with the 25 μg/kg/d r-HuMGDF dose at day 12, 3 days after cessation of treatment (Fig 1). This response was significantly greater ($P = .05$) than that elicited by the 2.5 μg/kg dose but not significantly different ($P = 0.4$) than the maximum level attained with the 250 μg/kg dose. The respective maximum mean platelet counts for animals receiving 2.5 or 250 μg/kg/d r-HuMGDF were 1,458 $\times$ $10^3/\mu$L on day 12 and 2,337 $\times$ $10^3/\mu$L on day 14 (Fig 1). The mean platelet counts in all treatment groups returned to within baseline levels by day 28 of the experimental protocol. Neither the red nor white blood cell counts were significantly modulated by the administration of r-HuMGDF at these doses throughout the observation course (Fig 2, A and C). A mild yet statistically significant increase in the absolute neutrophil count, which occurred early in the protocol (day 1 to day 5), was only observed in the animals that received the 250 μg/kg/d dose of r-HuMGDF (Fig 2B). The absolute basophil and eosinophil counts remained within baseline values (0.05 $\times$ $10^3/\mu$L ± .02 and 0.29 $\times$ $10^3/\mu$L ± .05, respectively) for all three treatment groups throughout the experimental course (data not shown).

BM-derived megakaryocyte (MEG)-CFU, GM-CFU, burst-forming unit-erythroid (BFU-e), and GEMM-CFU were assayed from marrow aspirates obtained on days 3, 10, 16, and 28 of the experimental protocol. BM-derived MEG-CFU concentrations were significantly ($P \leq .05$) increased above time-matched HSA-treated control values on days 3, 10, and 16 by the administration of r-HuMGDF at all dosages (Fig 3A). MEG-CFU per 10^5 MNC increased as early as 3 days after initiation of r-HuMGDF administration with peak values observed at day 10, 1 day after cessation of treatment. The largest increase in the concentration of MEG-CFU (68.3 MEG-CFU/10^5 MNC) ($P = .000$) was observed in the group that received 25 μg/kg/d of r-HuMGDF relative to time-matched HSA-treated control values. It is of interest that the marrow-derived GEMM-CFU were also increased in concentration in a similar response pattern (Fig 3D). Neither the MEG-CFU nor the GEMM-CFU per 10^5 MNC derived from HSA-treated animals vary significantly with time over the experimental protocol ($P > .81$ and .619, respectively). The concentration of GM-CFU and BFU-e remained unchanged throughout r-HuMGDF administration regardless of dose (Fig 3, B and C). GM-CFU did not vary significantly ($P = .608$) from each other within each dose group nor were they significantly different ($P = .08$) from their time-matched HSA-treated controls. BFU-e concentrations were not significantly different ($P = .187$) from one another over time within each r-HuMGDF dose group. The BFU-e day 10 and day 16 values for the r-HuMGDF–treated animals were significantly less ($P < .034$ and .04, respectively) than their time-matched HSA-treated controls because of the increased concentration of BFU-e in the HSA-treated marrow.

PB-derived clonogenic concentration was assessed at days 7 and 49 postcytokine administration. Significant ($P \leq .05$) increases in MEG-CFU (1.9/10^5 MNC) but not GM-CFU were observed at day 7 in animals treated with either 2.5 or 25 μg/kg/d r-HuMGDF. The MEG-CFU and GM-CFU per 10^5 MNC observed in untreated animals (n = 8) were 0.4 and 3.2/10^5 MNC, respectively. MEG-CFU were not observed in any dosage group at day 49 (Fig 4).

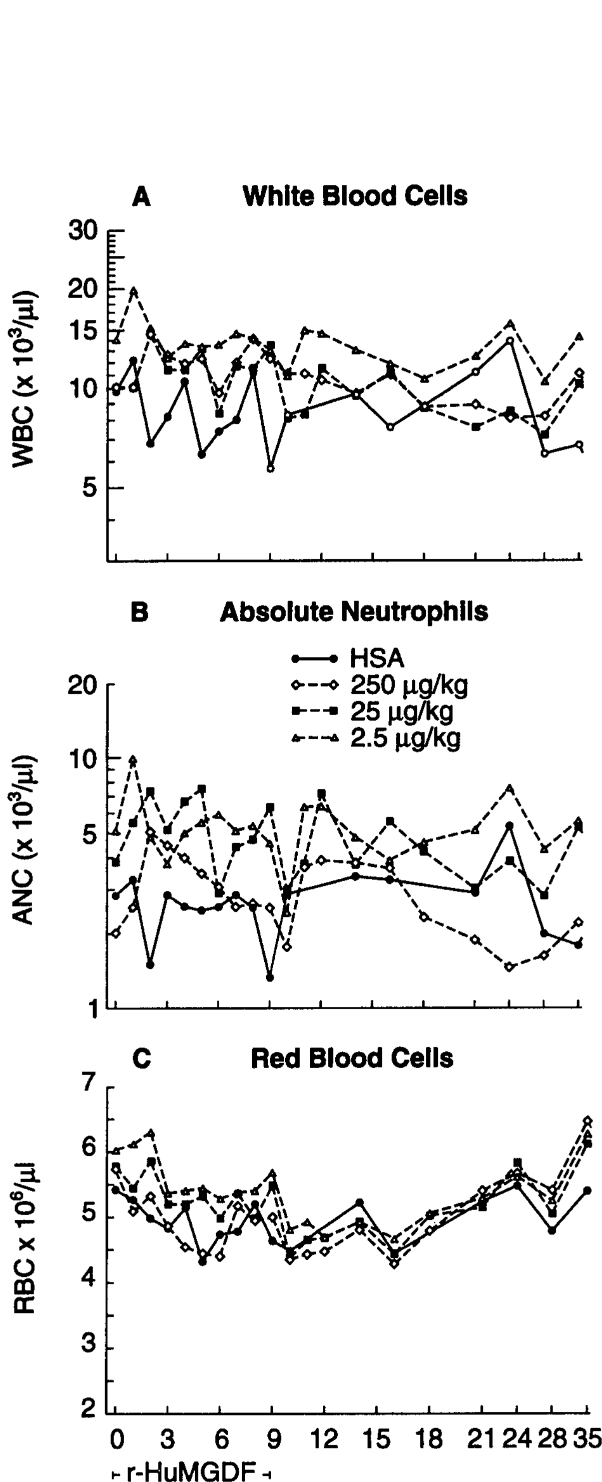

Fig 2. Effects of r-HuMGDF administration on PB counts in normal primates. (A) White blood cells (WBC), (B) absolute neutrophil count (ANC) and (C) red blood cells (RBC) observed in normal rhesus primates after r-HuMGDF or HSA administration which was administered sc, QD for 10 consecutive days at dosages of 2.5 (n = 3), 25 (n = 3), or 250 (n = 2) μg/kg/d. Control animals (n = 3) received 25 μg/kg/d of human serum albumin sc, QD for 10 days. Data represent mean RBC, WBC, ANC for the cytokine or control-treated animals.

DISCUSSION

The magnitude of the megakaryocytic/thrombopoietic response attained in the normal rhesus monkeys to r-HuMGDF is several-fold greater than the platelet increases observed in primates administered variable doses of IL-3, IL-6, IL-11, or LIF.[19-28] Platelet levels in response to r-HuMGDF were increased approximately 600% of baseline within several days following a 10-day protocol. A dose effect was noted between the 2.5 and 25 μg/kg/d dosages but the effect of the 250 μg/kg/d dose was not significantly different from 25 μg/kg/d dose. The optimum dose for increasing platelet production and stimulation of MEG-CFU may be at 25 μg/kg/d or greater but less than the 250 μg/kg/d. The largest dose of r-HuMGDF may provide ligand beyond the saturation binding point for available c-mpl on CFCs and megakaryocytes. The higher dose may also induce downmodulation of c-mpl in a negative feedback loop.

IL-3 administration to normal primates resulted in modest although significant twofold increases in circulating platelets that were somewhat delayed in occurrence until the second or third week after initiation of treatment.[19,21] In other reports IL-3 administration was not associated with any consistent change in platelet levels.[20] The increases in platelet levels were also associated with variable responses in the frequency of hematopoietic progenitor cells (HPC). Such variability in the platelet response from primate preclinical studies predicted similar responses in humans treated with IL-3.[29,30] Platelet responses were modest, delayed, and usually associated with increased cycling of marrow-derived HPC, such as GM-CFU, BFUe, and MEG-CFU. In contrast, platelet responses in primates administered IL-6, IL-11, and LIF have been consistent.[20,22-28] IL-6 and IL-11 initiated an early increase in platelet counts within the first week with peak responses, approximately twofold or greater, noted within 2 weeks of the treatment schedule.[20,22-27] Whereas, the onset of a dose-dependent increase in platelets after LIF administration occurred during the second week of a 14-day administration period with peak values occurring several days after cessation of treatment.[28]

The data presented herein suggests a stimulatory effect of r-HuMGDF at two levels, the MEG-CFU and the production of platelets from the megakaryocytes. Although it is presumed that MGDF and TPO are lineage-specific in action, and responsible for the megakaryocyte colony-stimulating activity and platelet production, Zeigler et al recently showed that c-mpl ligand acts directly on murine hematopoietic stem cell populations isolated from fetal liver and BM.[14-16,18,31,32] In addition, Methia et al[31] have shown that c-mpl is expressed in $CD34^+$ purified cells, megakaryocytes, and platelets. Furthermore, the addition of c-mpl antisense oligodeoxynucleotides inhibited megakaryocytic colony formation but not that from BFU-e or GM-CFU. Our results are consistent with this role of r-HuMGDF in the normal primate. Marrow-derived CFC assays performed from MGDF-treated primates showed stimulation of MEG-CFU and GEMM-CFU but not that of GM-CFU and BFU-e. Possibly the exogenous MGDF acted in concert with endogenous IL-3, c-*kit* ligand (KL), or

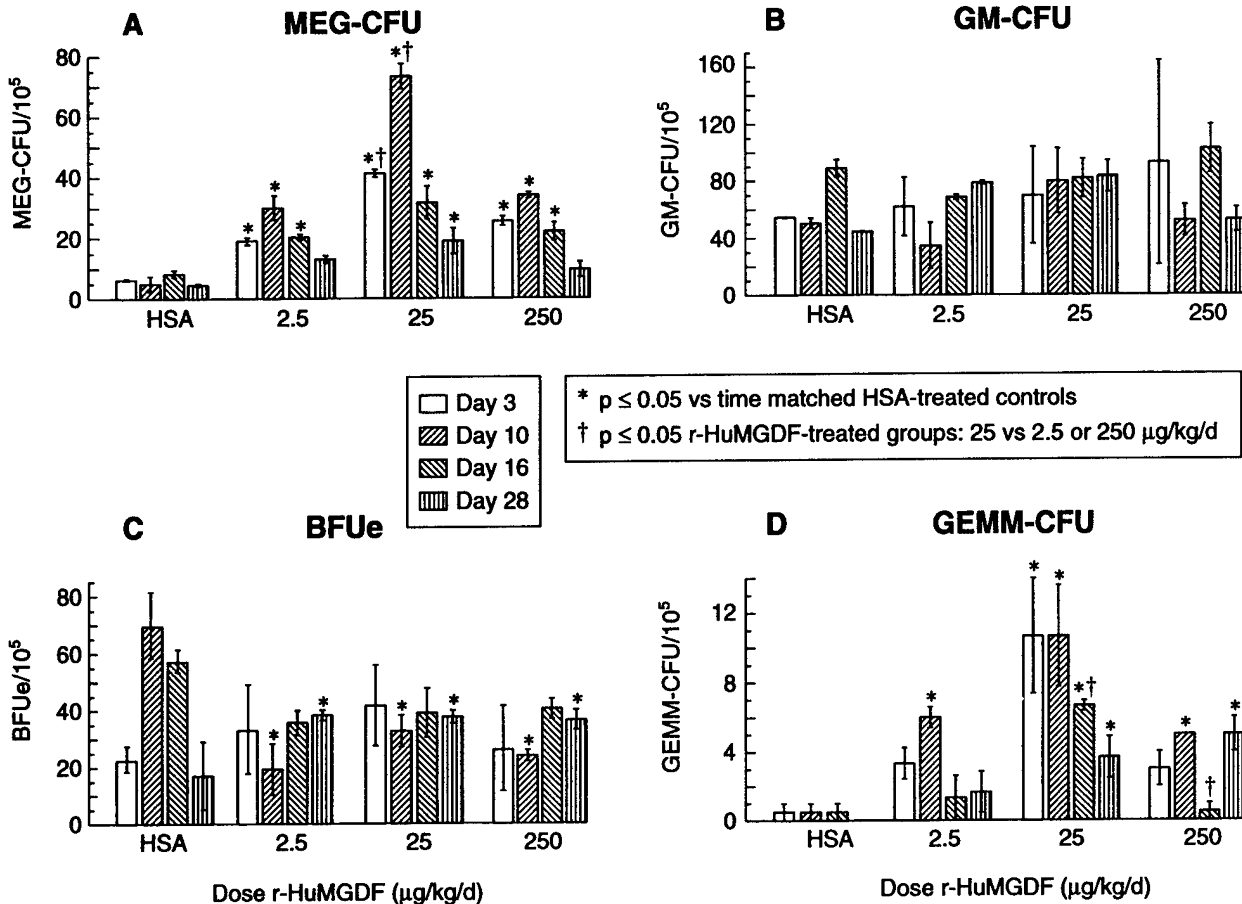

Fig 3. BM-derived concentration of MEG-CFU, GM-CFU, BFU-e, and GEMM-CFUs in normal rhesus primates. (A) MEG-CFU, (B) GM-CFU, (C) BFU-e, and (D) GEMM-CFU concentrations observed in normal rhesus primates on days 3, 10, 16, and 28 after r-HuMGDF or HSA administration as described in Materials and Methods. Clonogenic concentrations are reported as the mean ± SEM of the CFU per 10^5 MNC BM cells.

Flt-3/flk-2 ligand to increase the frequency of multipotent GEMM-CFU. It is not known whether MGDF can induce secretion of cytokines from megakaryocytes, but recently Jiang et al[33] and Wickenhauser et al[34] have shown megakaryocytes capable of spontaneous secretion of IL-3, GM-CSF, IL-6, and IL-1. These data suggest that autocrine and paracrine mechanisms are available to not only influence maturation of megakaryocytes but other more primitive CFC through direct or indirect action through the hematopoietic microenvironment.

Early preclinical and in vitro studies suggested that megakaryocyte and platelet production were regulated at several hierarchical cellular levels by a number of hematopoietic growth factors such as IL-1, IL-3, GM-CSF, KL, IL-6, IL-11, and LIF.[11-17,31,32,35,36] Preclinical and clinical protocols have been conducted based on such results. Combination protocols of IL-3 and GM-CSF, and IL-3 and IL-6 follow from the concept that IL-3 acted as a primer and stimulated proliferation of early MEG-CFU, which are then driven to the production and maturation of megakaryocytes and platelets by such factors as IL-6, IL-11, and LIF.[19-21,37-39] The present results are not disconcordant with the concept that a combination of growth factors can contribute to the regulation of the production of megakaryocytes and platelets. Additional factors such as IL-3, IL-6, IL-11, and LIF may play roles in stress-related production of platelets. In support of this concept, megakaryocytes have been shown to spontaneously secrete several cytokines known to affect multipotent CFCs as well as MEG-CFU and megakaryocytes. Additionally, stimulation of megakaryocytes with IL-3, IL-11, or IL-1 significantly increased the secretion of IL-3, IL-6, GM-CSF, and IL-1.[33,34] The recent example that c-*mpl*–deficient mice are not devoid of megakaryocytes and platelets is also consistent with the data that other growth factors are involved in regulating normal thrombocytopoiesis.[40]

The data presented herein support the recent in vitro and in vivo observations that suggest the single protein MGDF is a major physiological regulator of thrombocytopoiesis. This action may be mediated through stimulation of MEG-CFU to maturation and production of platelets.[14-18,40] The efficiency of this process as noted by the increased concentration of marrow-derived MEG-CFU in association with the approximate sixfold

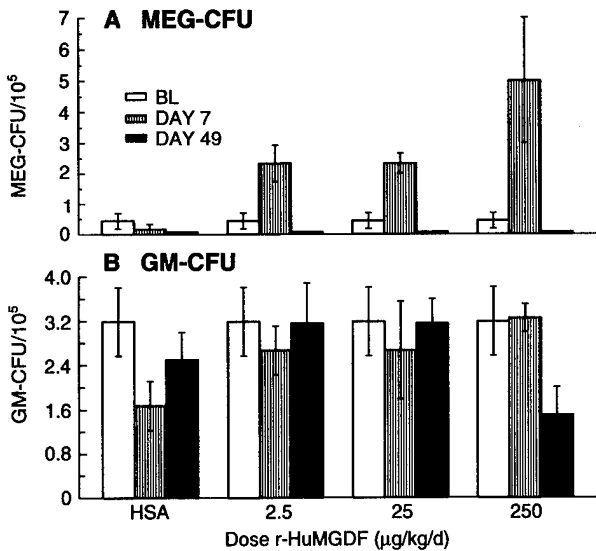

Fig 4. PB-derived concentration of MEG-CFU and GM-CFU in normal rhesus primates. (A) MEG-CFU and (B) GM-CFU concentrations observed in normal rhesus primates on days 7 and 49 after r-HuMGDF administration as described in Materials and Methods versus untreated animals (n = 8). Clonogenic concentrations are reported as the mean ± SEM of the CFU per 10^5 PB-derived MNC.

increase in platelets within 12 days of r-HuMGDF administration, as well as its lack of adverse clinical effects predict significant therapeutic efficacy in enhancing recovery of platelet production in the myelosuppressed host.

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Possible Involvement of Prostaglandins in Increases in Rat Plasma Adrenocorticotropic Hormone and Corticosterone Levels Induced by Radiation and Interleukin-1α Alone or Combined

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Exposing rats to 1–10 Gy of ionizing radiation increased plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) levels. In both irradiated and nonirradiated rats, recombinant human interleukin-1α (rhIL-1α; 1 hr before radiation/sham exposure) enhanced plasma ACTH and CORT levels. Indomethacin, a cyclooxygenase inhibitor, attenuated plasma ACTH and CORT levels induced by radiation. Indomethacin also attenuated ACTH and CORT levels induced by radiation and interleukin-1α alone or combined. These results suggest that prostaglandins are involved in the increase in plasma ACTH and CORT levels induced by radiation and rhIL-1α alone or combined. © 1995 Society of Toxicology.

Cytokines have been shown to play a key role in mediating communication between the endocrine and immune systems. In particular, the leukocyte-derived interleukin-1 (IL-1) polypeptide appears to be an important messenger between the immune system and the hypothalamic–pituitary–adrenal (HPA) axis (Bateman *et al.*, 1989; Tsagarakis *et al.*, 1989; Sapolsky *et al.*, 1987). Treatment with recombinant human interleukin-1α (rhIL-1α) before irradiation attenuates radiation-induced hyperthermia in rats by stimulating antioxidant enzymes (Kandasamy *et al.*, 1993). Adrenocorticotropic hormone (ACTH) and corticosterone (CORT) are important for thermoregulation because administration of these peptides lowers body temperature and inhibits both experimental and clinical fevers (Glyn and Lipton, 1981; Kandasamy and Williams, 1984). It has been reported that rhIL-1 increases plasma ACTH and CORT levels (Dinarello, 1988) but it is not known whether attenuation of radiation-induced hyperthermia by rhIL-1α involves increases in plasma ACTH and CORT levels.

The levels of ACTH, CORT, and catecholamines in plasma are increased in acute stress and in endocrine changes that accompany an immune response (Besedovsky *et al.*, 1983; Besedovsky and Sorkin, 1975; Blalock *et al.*, 1985). Radiation is a stressor, and little is known about the effect of ionizing radiation on stress hormone levels. Preliminary results demonstrate an increase in ACTH, CORT, and β-endorphin (BE) levels in plasma after radiation exposure (Kandasamy *et al.*, 1991).

The purpose of this study was to determine the effect of ionizing radiation, rhIL-1, and a combination of rhIL-1 and radiation on plasma ACTH and CORT levels, and to elucidate the mechanism involved in the increase in plasma ACTH and CORT levels by radiation, rhIL-1, and rhIL-1 and radiation combined.

MATERIALS AND METHODS

Drugs. rhIL-1α was a gift from Dr. P. Lomedico of Hoffman–LaRoche (Nutley, NJ) and was diluted to the desired concentration in pyrogen-free saline before injection. Indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in a mixture of 1% sodium hydroxide and sterile norpyrogenic saline.

Experimental animals. Male Sprague–Dawley rats (outbred) weighing 200–300 g (Crl:CD(SD)BRD; Charles River Breeding Laboratories, Kingston, NY) were quarantined on arrival and screened for evidence of disease by serology and histopathology. The rats were housed individually in polycarbonate Micro-Isolator cages (Lab Products, Maywood, NJ) on autoclaved hardwood contact bedding (Beta Chip, Northeastern Products Corp., Warrenburg, NY) and were provided commercial rodent chow (Wayne Rodent Blok, Continental Grain Co., Chicago, IL) and water *ad libitum*. Animal holding rooms were kept at 21 ± 1°C with 50 ± 10% relative humidity on a 12-hr light:dark cycle with no twilight.

Radiation exposure. Rats were placed in clear plastic well-ventilated containers for approximately 5 min before irradiation or sham exposure. The animals were exposed bilaterally to gamma rays using a ^{60}Co source at a rate of 10 Gy/min to a total dose of 1–10 Gy. Prior to irradiation, the radiation dose rate at the midline of an acrylic rat phantom was measured

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using a 0.05-cc tissue-equivalent chamber manufactured by Exradin, Inc. The dose rate at the same location with the phantom removed was measured using a 50-cc ionization chamber fabricated at AFRRI. The ratio of these two dose rates, the tissue–air ratio, was 0.93. All ionization chambers that were used have calibration factors traceable to the National Institute of Standards and Technology. Dosimetry measurements were performed following the AAPM Task Group 21 protocol for determining the absorbed dose from high-energy photon and electron beams (Task Group 21, 1983).

Levels of ACTH and CORT in plasma. Rats were handled twice a day for 7 days, with a mock decapitation procedure also included. After 7 days, groups of rats were pretreated ip with 3 μg/kg of rhIL-1α or saline 1 hr before radiation exposure [the dose of rhIL-1α was chosen from previous data (Kandasamy *et al.,* 1991) and preliminary experiments]. Rats were terminated by decapitation 1 hr after radiation/sham exposure, and trunk blood was collected into prechilled tubes containing EDTA. Plasma was obtained from blood by centrifugation and was stored frozen at −70°C for estimating the levels of hormones. Plasma samples were measured for ACTH and CORT by radioimmunoassay (RIA) using duplicates of samples as described previously (Thiagarajan *et al.,* 1989). Prior to determining ACTH level, plasma samples were extracted as reported previously (Dave *et al.,* 1985) with certain modifications. In short, disposable syringes were placed on C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA), activated by passing 50 ml of HPLC-grade methanol, and washed by passing 25 ml of triple-distilled water. Frozen plasma samples were thawed and rapidly aliquoted (0.25–2.5 ml plasma) into the syringes. The plasma samples were immediately pushed through the Sep-Paks at a flow rate of approximately 1.5 ml/min. Each plasma sample-loaded Sep-Pak was washed with 10 ml of triethylamine formic acid (TEAF) solution, which was prepared by adding 10.5 ml of reagent-grade formic acid to 1 liter of triple-distilled water, followed by adjusting the pH of this solution to 3.2 with triethylamine. The plasma sample-loaded TEAF-washed Sep-Paks were eluted with 6 ml of a 60% acetonitrile:40% TEAF solution, which was collected into polystyrene test tubes (17 × 100 mm). The eluate was vigorously mixed and aliquoted into two to four polystyrene test tubes (12 × 75 mm) and evaporated to dryness in a *vacuum concentrator* (Speed-Vac, Savant Instruments, Hicksville, NY). Dried aliquots were stored for subsequent RIA analysis. Peptide recoveries ranged from 88 to 92%, and results presented are not corrected for these minor recovery losses. After extraction, plasma levels of ACTH and CORT were quantified under assay conditions presented in an earlier publication (Dave *et al.,* 1985).

The antiserum used for the determination of ACTH was a gift from Charles Oliver, Marseilles, France, and was generated against ACTH (1–4) coupled to bovine serum albumin (BSA) via carbodiimide (Ustegi *et al.,* 1976). The ACTH antiserum was used at a final dilution of 1:125,000. At this dilution, the antiserum bound 35–40% of 6000–8000 cpm [^{125}I]ACTH (NEN, Boston, MA) added to each assay tube when no unlabeled peptide was present. Under optimal assay conditions, the binding characteristics of the ACTH antiserum were such that, at 4–6 pg/tube of unlabeled ACTH, a 20% displacement of maximally bound [^{125}I]ACTH was observed. The specific details of the ACTH assay procedure are as outlined previously (Dave *et al.,* 1985), with the exception that the diluent or assay buffer consisted of 0.02 M barbital buffer, pH 8.6, and contained 0.2% BSA and 0.2% mercaptoethanol. The percentage cross-reactivity of the ACTH antiserum with the various peptide fragments or analogs of ACTH was 100% with ACTH and ACTH (1–24) and <0.001% with α-melanocyte stimulating hormone (α-MSH), β-MSH, γ-3-MSH, BE, β-lipotropin, ACTH (17–39), and ACTH (1–10). [^{125}I]ACTH was obtained from Incstar (Stillwater, MN). Synthetic peptides used for standard curve preparation and recovery experiments were obtained either from Peninsula Labs (Belmont, CA) or Bachem (Torrance, CA). Plasma CORT concentrations were determined by RIA using an antiserum obtained from Hazelton Labs (Rockville, MD). [1,2,6,7-^{3}H(N)]CORT (NEN) and CORT (Sigma Chemicals) were used as labeled ligand and standard, respectively. In short, 10 μl of plasma was added to 1 ml of 95% ethanol, and each sample was vortexed and centrifuged at 6000*g* for 10 min. Aliquots of the supernatant fluid were dried down for subsequent analysis by RIA. The experiments for the levels of ACTH and CORT in plasma were repeated twice. The precision of each assay was similar, with an intraassay sample variation from 3 to 5% and an interassay sample variation from 6 to 8%.

Statistical analysis. Statistical analysis of data was performed using analysis of variance with a significance level of $p < 0.05$. Intergroup comparisons were performed using Tukey's test (18).

RESULTS

Exposure of rats to 1–10 Gy of radiation increased plasma ACTH and CORT levels (Figs. 1 and 2). In both irradiated and nonirradiated rats, 3 μg/kg of rhIL-1α (sp act 8.8×10^8 units/mg) administered ip 1 hr before irradiation/sham exposure enhanced plasma ACTH and CORT levels (Figs. 3 and 4). Administration of 1 and 2 mg/kg of indomethacin ip 1 hr before irradiation/sham exposure moderated radiation-induced plasma ACTH and CORT levels, rhIL-1α, and rhIL-1α plus radiation-induced ACTH and CORT levels (Figs. 3 and 4). Data for 2 mg/kg of indomethacin are not presented because there was no significant difference between the two doses. Administration of 0.5 mg/kg of indomethacin moderated radiation-induced plasma ACTH and CORT levels but the effects were not statistically significant. Administration of 0.5–2 mg/kg of indomethacin ip did not affect plasma ACTH and CORT levels in control rats.

DISCUSSION

Irradiation increases the levels of ACTH and CORT in plasma, and pretreatment with rhIL-1α enhances both their

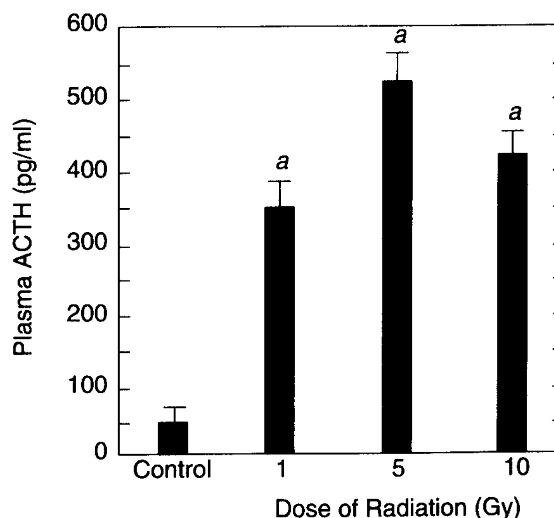

FIG. 1. ACTH levels in plasma following irradiation. Values are expressed as mean of levels from eight rats ± SEM. [a]Significantly different from control values: $p < 0.05$.

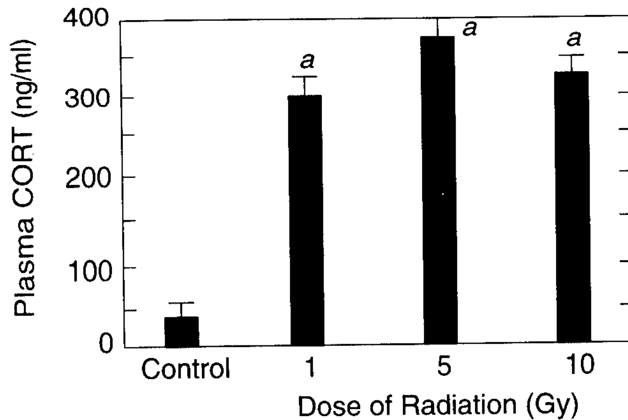

FIG. 2. CORT levels in plasma following irradiation. Values are expressed as mean of levels from eight rats ± SEM. [a]Significantly different from control values: $p < 0.05$.

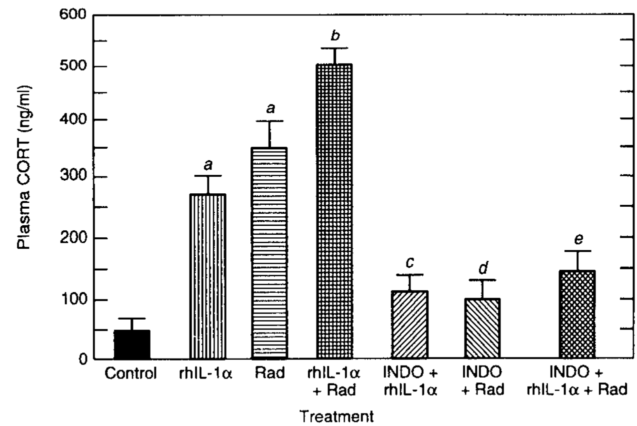

FIG. 4. Effect of 1 mg/kg indomethacin (INDO) on 3 μg/kg rhIL-1α-induced CORT levels in nonirradiated and irradiated (1 Gy) rats. Values are expressed as mean of levels for eight rats ± SEM. [a]Significantly different from control values: $p < 0.05$. [b]Significantly different from irradiated values: $p < 0.05$. [c]Significantly different from rhIL-1α-treated values: $p < 0.05$. [d]Significantly different from irradiated values: $p < 0.05$. [e]Significantly different from rhIL-1α-treated and irradiated values: $p < 0.05$.

irradiated and nonirradiated plasma levels. The mechanism by which radiation, rhIL-1α, or rhIL-1α and radiation combined produce neuroendocrine effects is not known. However, it has been suggested that the central nervous system and neuroendocrine effects of rhIL-1α are produced through stimulation of the corticotropin-releasing hormone (CRH) and prostaglandins (PGs) (Berkenbosch *et al.*, 1991; Tsagarakis *et al.*, 1989; Katsuura *et al.*, 1988; Navarra *et al.*, 1991). It has also been reported that rhIL-1α stimulates the secretion of ACTH from the corticotropic cells of the anterior pituitary gland either by stimulating the release of CRH from hypothalamic neuroendocrine cells, which in turn stimulates the anterior pituitary gland, or by acting directly on the normal pituitary cells. The subsequent role of ACTH is to stimulate secretion of CORT from

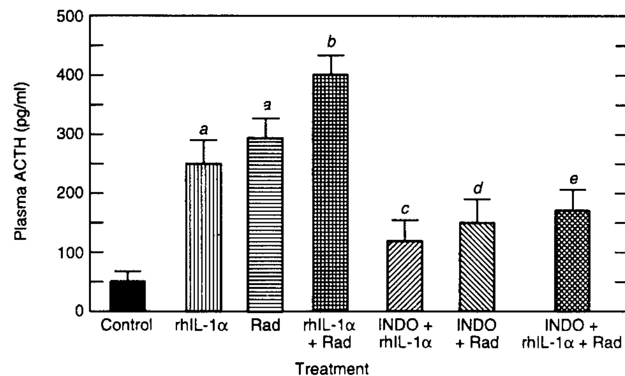

FIG. 3. Effect of 1 mg/kg indomethacin (INDO) on 3 μg/kg rhIL-1α-induced ACTH levels in nonirradiated and irradiated (1 Gy) rats. Values are expressed as mean of levels for eight rats ± SEM. [a]Significantly different from control values: $p < 0.05$. [b]Significantly different from irradiated values: $p < 0.05$. [c]Significantly different from rhIL-1α-treated values: $p < 0.05$. [d]Significantly different from irradiated values: $p < 0.05$. [e]Significantly different from rhIL-1α-treated and irradiated values: $p < 0.05$.

the cortex of the adrenal gland. There are reports suggesting that direct action of rhIL-1α on the pituitary gland is unlikely (Uehara *et al.*, 1987). In addition, direct stimulation of the adrenal cortex by rhIL-1β to increase the serum CORT levels in rats has been reported (Roh *et al.*, 1987).

It should be noted that systemic or central injections of rhIL-1 are less effective in nonhypothalamic sites, while low doses of rhIL-1 injected intraventricularly or intrahypothalamically released ACTH quite readily (Sapolsky *et al.*, 1987; Brekenbosch *et al.*, 1987). Although rhIL-1 receptors have been identified throughout the brain (Breder *et al.*, 1988; Farrar *et al.*, 1987; Katsuura *et al.*, 1988) as well as in a variety of immune cells (Scapigliati *et al.*, 1989), the ability of rhIL-1 to cross the blood–brain barrier is still being debated. It has been suggested that lymphokines may pass through areas where the blood–brain barrier is absent, such as the median eminence, subfornical organ, organum vasculosum of the laminae terminalis of the hypothalamus, and the area postrema at the base of the fourth ventricle (Pardridge, 1983).

RhIL-1 increases PG synthesis in the hypothalamus (Berheim *et al.*, 1980). PGs themselves stimulate the HPA axis (Bernardini *et al.*, 1990). PGs stimulate the HPA axis (Bernardini *et al.*, 1990) and are involved in the development of ACTH response induced by physical (Kluger *et al.*, 1987) and psychological (Morimoto *et al.*, 1991) stresses. Prevention of plasma ACTH and CORT release induced by radiation exposure, rhIL-1α-enhanced, or rhIL-1α and radiation-induced ACTH and CORT releases by indomethacin, a cyclooxygenase inhibitor, suggests that PGs are involved in the mechanism of the release of these hormones. It has been reported that pretreatment with indomethacin com-

pletely prevented ACTH response induced by either iv or icv injection of rhIL-1β, indicating that PGs are involved in the mechanism of ACTH release (Katsuura *et al.,* 1988; Morimoto *et al.,* 1989). In addition, it has been demonstrated that injection of PGs into the median eminence stimulates ACTH release, whereas indomethacin injected into the hypothalamus suppresses ACTH release (Hedge and Hanson, 1972; Thompson and Hedge, 1978). During stressful conditions, activation of the sympathetic nervous system stimulates the release of norepinephrine (NE), and the ACTH release induced by NE is mediated by PGs (Watanabe *et al.,* 1991). Although rhIL-1 acts through the PG system, there are reports indicating that rhIL-1 stimulates the HPA axis via the catecholaminergic pathway (Dunn, 1988; Weidenfeld *et al.,* 1989).

It has been reported that all psychological stress-induced responses are not necessarily inhibited by indomethacin. For example, indomethacin suppressed the increase in body temperature and plasma ACTH levels induced by psychological stress but had no effect on blood pressure and heart rate induced by this stress (Morimoto *et al.,* 1991). Likewise, indomethacin attenuated radiation-induced hyperthermia but not the decreased locomotor activity due to radiation (Ferguson *et al.,* 1990).

At present, the mechanisms involved in inhibiting ACTH and CORT release by indomethacin are not well understood. It should be noted, however, that inhibition of ACTH release by indomethacin may involve a mechanism other than PG synthesis. Using indomethacin, Rivier and Vale (1991) investigated the role of PGs in stimulating the effect of rhIL-1α on ACTH and CORT secretion in intact rats, adrenalectomized (ADX) rats without steroid replacement, and ADX rats treated with exogenous corticosterone or dexamethasone. Based on their experiments, they concluded that the ability of indomethacin to block rhIL-1α-induced ACTH secretion in intact rats appears to be mediated through corticosteroid feedback because rhIL-1α increases CORT levels. However, results obtained when a similar experiment was carried out in ADX/corticosteroid-treated rats suggest that the ability of rhIL-1α to activate ACTH secretion may be partially dependent on the release of PGs. In ADX rats with no corticoid therapy, indomethacin did not measureably alter rhIL-1α-induced ACTH release, suggesting that PGs were not involved in mediating the effects of rhIL-1α. In ADX and ADX/corticosteroid-treated rats, plasma CORT levels remained undetectable at all times.

Our experiments in normal rats demonstrated that PGs are involved in rhIL-1α-enhanced ACTH and CORT releases, which is in contrast to the findings of Rivier and Vale (1991). We have no explanation at this time for the discrepancy except to suggest that the dose of indomethacin used by Rivier and Vale (1991) in their experiments was 10 times the dose we used. Experiments are in progress to determine whether high doses of indomethacin moderate ACTH and CORT levels differently in rhIL-1α-enhanced, radiation-induced, or rhIL-1α- and radiation-induced plasma levels of rats.

It is now widely recognized that deficiency of one or more anterior pituitary hormones may follow therapeutic external irradiation, administered for a variety of conditions such as pituitary and other central nervous system tumors, when the hypothalamic–pituitary axis falls within the radiation therapy fields (Larkins and Martin, 1973; Perry-Keene *et al.,* 1976; Samaan *et al.,* 1982; Lam *et al.,* 1987). The investigation of combined exposures to radiation and chemical agents has been considered important with regard to the enhancement of radiation risk (Streffer and Mueller, 1984). Chemicals with a potential to provide increased radio response deserve particular attention if they are used frequently as therapeutic agents. RhIL-1 is a radioprotector and as mentioned earlier it increases plasma ACTH and CORT levels (Dinarello, 1988). Our results demonstrate that although exposure to ionizing radiation is toxic, the capacity of rhIL-1 to enhance ACTH and CORT levels in plasma is unaffected in irradiated rats. In addition, the increases in ACTH and CORT levels may explain the attenuation of radiation-induced hyperthermia by rhIL-1 because both ACTH and CORT are antipyretic.

Our results suggest that ionizing radiation and rhIL-1α treatment increase ACTH and CORT levels in plasma. PGs are involved in the increased release of ACTH and CORT induced by irradiation and treatment with rhIL-1α in irradiated and nonirradiated rats.

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c-*kit* Ligand Gene Expression in Normal and Sublethally Irradiated Mice

By A. Limanni, W.H. Baker, C.M. Chang, R. Seemann, D.E. Williams, and M.L. Patchen

The c-*kit* ligand (KL; Steel factor, mast cell growth factor, stem cell factor) is a hematopoietic factor that has been shown to act as a potent cofactor for hematopoietic growth and differentiation in vitro. The in vivo effects of KL, however, have been variable. To study the hematopoietic role of KL in vivo, we evaluated KL gene expression in both normal mice and mice recovering from myelosuppressive radiation exposure using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. In a single RNA sample, we found that the RT-PCR technique has high precision (coefficient of variation, 15.7%). Amplifications of serial 1:2 dilutions of template RNA precisely correlated with starting RNA concentrations at 20 cycles or at 25 cycles, depending on the level of expression. Amplification of individual normal bone marrow and spleen cell RNA showed basal expression in all normal bone marrows but irregular expression in normal spleens. On day 2 after a sublethal 7.75-Gy (0.4 Gy/min) ^{60}Co irradiation, splenic KL gene expression increased approximately 2.5-fold (*P* = .011), and bone marrow expression increased 15-fold (*P* = .004). During a 28-day postirradiation recovery period, KL expression increased in bone marrow on days 2 through 7. Splenic expression during the same period was more variable. In conclusion, the KL gene is invariably expressed in all murine bone marrows and variably expressed in normal murine spleens. Postirradiation, recovering bone marrow and spleen both express increased levels of KL mRNA at day 2 and continue to express increased levels for several days postexposure. These data support a role for KL in the endogenous recovery of hematopoiesis after hypoplastic injury.

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THE c-*kit* LIGAND (KL) is a cytokine known to be involved in both hematopoietic and nonhematopoietic development. This is evidenced by the fact that mice with viable mutations at the Steel genetic locus are sterile, have deficient melanin production, and exhibit macrocytic anemia as well as decreased megakaryocyte and granulocyte numbers. When KL is administered to Steel mice, partial reversal of the hematopoietic defects is observed. Multiple cell types have been shown to produce KL, including hematopoietic stromal cells, fibroblasts, hepatic parenchyma, and gonadal tissues.[1-6] The active factor exists in both membrane-bound and soluble forms, with the soluble form representing a cleavage product of a surface-bound protein.[5] KL binds the c-*kit* protooncogene product, the c-*kit* receptor, which among other cells is expressed on primitive hematopoietic progenitor cells.[7,8] Using cloned stromal cells from Steel mice that had been transformed with either membrane-bound or soluble KL, Toksoz et al[9] showed that stromal cells expressing membrane-bound KL were more capable of inducing erythroid burst-forming unit (BFU-E), granulocyte-macrophage colony-forming unit (CFU-GM), and mixed colony-forming unit (CFU-Mix) proliferation in liquid culture than stromal cells expressing only soluble KL.

Because of the apparent role of KL in hematopoietic regulation, KL represents a factor that might aid in recovery of hypoplastic bone marrow (BM) or support engraftment in BM transplantation. In vitro, KL alone has little hematopoietic effect but synergizes with multiple cytokines, including interleukin-1 (IL-1), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (Epo). Such effects have been shown with unfractionated as well as fractionated BM cells.[10-18]

When administered to normal primates, KL enhances hematopoiesis, as evidenced by increased BM CFU-GM and BFU-E and peripheral neutrophil, lymphocyte, and red blood cell counts.[19] Andrews et al[20] showed that KL administration in normal animals stimulated the circulation of cells capable of engrafting lethally irradiated baboons. Radiation studies suggest involvement of KL in the regulation of hematopoietic regeneration after BM injury. In studying the role of KL in the radioprotectant effect of IL-1, Neta et al[21] incidentally showed that an antibody to KL increased the radiation sensitivity of irradiated control mice, strongly suggesting that endogenous KL plays a role in recovery from radiation-induced hematopoietic hypoplasia. In a series of experiments using a murine radiation model, Patchen et al[22] have shown that recovery of splenic colony-forming units (CFU-S) and CFU-GM, as well as BM CFU-GM, can be accelerated by postirradiation KL administration. Furthermore, they have shown that transplantation of lethally irradiated mice with BM from recovering irradiated mice treated with KL increases survival compared with cells from recovering saline-treated controls.[22] The ability of KL to accelerate postirradiation hematopoietic recovery in canine radiation models has also been shown.[23,24]

Consequently, there is significant data to support the conclusion that KL is a major stromal factor responsible for growth and differentiation in normal hematopoiesis and in recovery from hypoplastic injury. However, as KL is a stromal factor, whether radiation or cytotoxic regimens may cause stromal injury and alter expression of this important factor is not known. In our study we used a mouse model of sublethal radiation-induced hypoplasia to evaluate the effects of radiation on KL ligand expression and the potential involvement of KL in the recovery of radiation-induced hematopoietic injury. This study shows that, after sublethal radiation injury, increases in KL gene transcripts can be detected in murine spleen and BM.

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MATERIALS AND METHODS

Experimental design. BM and splenic KL expression was first studied in normal animals to determine if KL expression was constitutive in either organ. After establishing the pattern of BM and splenic expression in normal animals, BM and splenic KL expression in mice 48 hours after sublethal 7.75-Gy irradiation was evaluated to determine the acute effects of radiation injury. Four to six sets of pooled samples at each time point were analyzed for KL expression. Each set represented RNA from three normal animals or seven irradiated animals (because of the differences in cellularity after irradiation). Later experiments were designed to look at the expression of KL during prolonged recovery from sublethal irradiation. In each of two experiments, BM or splenic RNA from three to seven animals on days 1, 2, 3, 4, 7, 10, 14, 17, 21, 24, and 28 after irradiation was pooled and analyzed.

Mice. $B_6D_2F_1$ female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for experimentation at 8 to 10 weeks of age (weighing approximately 20 g). Mice were maintained in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in Micro-Isolator cages on hardwood chip contact bedding and were provided commercial rodent chow and acidified (pH 2.5) water ad libitum. Animal rooms were maintained on a 12-hour light/dark cycle at 70° ± 2°F and 50% ± 10% relative humidity, with at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were tested for Pseudomonas infection and were quarantined until test results were obtained. Only healthy mice were used for experiments. All animal experiments were approved by the Institute's Animal Care and Use Committee. Research was conducted according to the principles of the Institute for Animal Resources, National Research Council.[25]

Irradiation. Mice were irradiated (whole-body) in the bilateral gamma field of the Armed Forces Radiobiology Research Institute (AFRRI) ^{60}Co facility. During irradiation, animals were confined within individual cubicles of a plexiglass restrainer to restrict movement. The midline tissue dose to animals was 7.75 Gy (delivered at a dose rate of 0.4 Gy/min). Before animal irradiations, dosimetry was performed in an acrylic mouse phantom with a 0.5-cc tissue equivalent ionization chamber (calibration traceable to the National Institute of Standards and Technology). The tissue-to-air ratio was 0.96, and the dose variation within the exposure field was less than 3%. Dosimetric measurements were made in accordance with the American Association of Physicists in Medicine (AAPM) protocol for the determination of absorbed dose from high-energy photon and electron beams.[26]

Cell suspensions. Femurs and tibia were obtained by cervical dislocation after mice had been killed. Cell suspensions for each assay represented pooled cells from either three normal animals or seven irradiated animals at each respective time point. Cells were flushed from bones with McCoy's 5A medium (Flow Labs, McLean, VA) containing 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT). Spleen cells were obtained by pressing spleens on a stainless steel mesh screen and washing with McCoy's 5A medium. The nucleated cells were counted with a Coulter counter (Coulter Corp, Luton, UK).

RNA preparation. Total cellular RNA was obtained from intact spleens and single-cell suspensions of BM, using the RNAzol (Tel-Test Inc, Friendswood, TX) modification of the Chomczynski method according to manufacturer's directions.[27] Each spleen was lysed in 2 mL RNAzol and homogenized with a tissue homogenizer. BM cells were lysed in 1 to 2 mL RNAzol. Lysates were extracted with chloroform, and the extract was centrifuged at 12,000*g* to collect the aqueous supernatant. Supernatants were precipitated with isopropanol on ice and centrifuged to pellet the RNA. The pellets were washed with 75% ethanol and recentrifuged. The ethanol was decanted, and pellets were air-dried. Pellets were resuspended in Tris-EDTA buffer (20 mmol/L Tris, pH 8.0; 1 mmol/L EDTA), and samples were quantified in a Beckman DU-65 ultraviolet spectrophotometer (Beckman Industries, Fullerton, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). At each time point, 0.5 μg of pooled splenic RNA or 0.1 μg of pooled BM RNA was reverse-transcribed to single-stranded cDNA in 50 mmol/L Tris-HCL (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 1 mmol deoxynucleoside triphosphate (dNTP) mix, and 50 U of Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) at 37°C for 1 hour. The reaction was stopped by heating to 90°C for 10 minutes, and the mixture cooled on ice for 10 minutes. PCR master mix was added to the final concentration of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 1 μmol/L 5′ primer, 1 μmol/L 3′ primer, and 1.25 U Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). Samples were amplified for 20 to 25 cycles with 1-minute denaturation at 94°C, annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes. A final extension at 72°C for 7 minutes was performed after the last cycle. In each set of reactions, RNA samples were subjected to PCR without undergoing the RT procedure to exclude genomic DNA contamination.

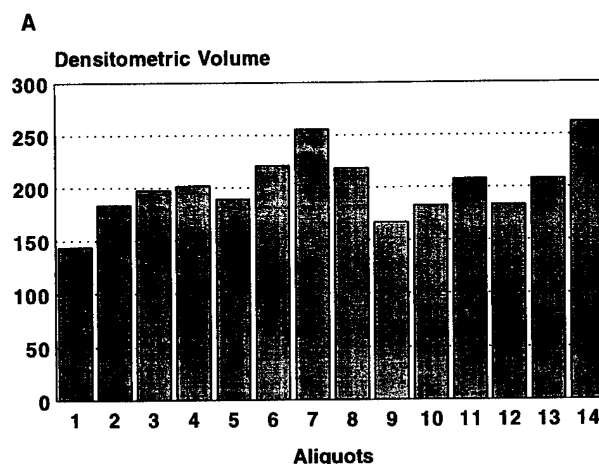
PCR primers and probes. PCR primers for murine KL and glyceraldehyde-phosphate dehydrogenase (GAPDH) were obtained commercially (Synthetic Genetics, San Diego, CA). The following were the sequences for each primer: KL sense, AAG-GAG-ATC-TGC-GGG-AAT-CCT-GTG-A; KL antisense, ACT-GCT-ACT-GCT-GTC-ATT-CCT-AAG-G; GAPDH sense, CCA-TGG-AGA-AGG-CTG-GGG; and GAPDH antisense, CAA-AGT-TGT-CAT-GGA-TGA-CC. The GAPDH probe was obtained from American Type Culture Collection (ATCC; Rockville, MD). The KL probe was a gift from the Immunex Corporation representing the *Sal* I fragment of murine mast cell growth factor (MGF) cloned into the *Sal* I site of pBluescript (Stratagene, La Jolla, CA).

Southern blots and hybridization. Each PCR product (10 μL) was electrophoresed in a 1× Tris-borate-EDTA agarose gel (89 mmol/L Tris (pH 8.0), 89 mmol/L boric acid, 2 mmol/L EDTA) and transferred onto Nytran membrane (Schleicher and Schuell, Keene, NH). Blots were prehybridized overnight at 42°C in 6× saline sodium citrate (1× SSC: 150 mmol/L sodium chloride; 15 mmol/L sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution: 0.2 mg/mL Ficoll, 0.2 mg/mL polyvinylpyrolidone, 0.2 mg/mL bovine serum albumin), 0.1 mg/mL sheared denatured salmon sperm DNA, 50% formamide, 1% sodium dodecyl sulfate (SDS), and 50 mmol/L Tris (pH 8.0). Blots were then hybridized overnight in the same solution after addition of nick-translated, radiolabeled plasmid probes to a final concentration of 1×10^6 to 2×10^6 cpm/mL of prehybridization fluid. Blots were then washed three times at 42°C in 2× SSC/0.5% SDS and finally washed at 62°C for 1 hour in 0.1× SSC/0.1% SDS. Excess fluid was removed, and the blots were exposed to Kodak XAR autoradiography film (Eastman Kodak, Rochester, NY).

Quantitation and analysis. Using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA), each sample's densitometric volume was measured. PCR product quantification was based on relative expression differences between irradiated and control samples. GAPDH was used to normalize for tube-to-tube variations in RNA loading due to pipette error or reaction efficiency. To normalize, the densitometric volume of each set of GAPDH RT-PCR reactions was averaged, and each specific GAPDH densitometric volume was expressed as a percent of the average to obtain a correction factor (CF_n). Each KL densitometric volume was then divided by the corresponding GAPDH correction factor to obtain a corrected KL densitometric volume (ie, $GAPDH_n/GAPDH_{average} = CF_n$; $KL_{corrected} = KL_n/CF_n$). All statistical comparisons were made using the Mann-Whitney U test.

RESULTS

Cellular response to irradiation. The cellular response to radiation was a predictable decrease in cellularity on the



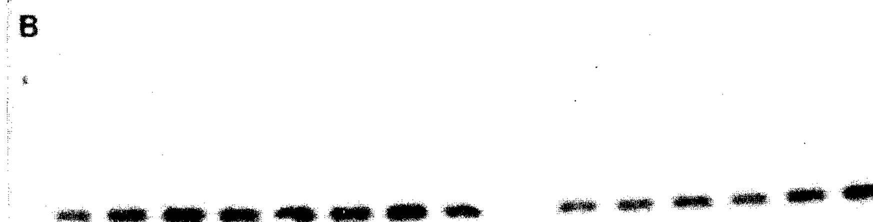


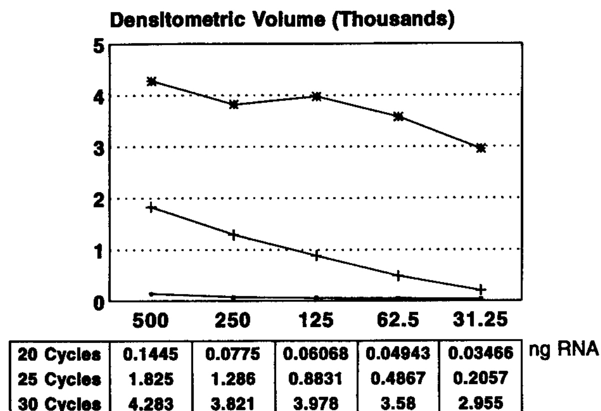
Fig 1. Precision of the RT-PCR assay in the measurement of c-*kit* ligand expression. (A) Data represent the simultaneous amplification of 14 equal aliquots of a single sample of splenic RNA (mean, 201.7; median, 199.85; SD, 30.55; coefficient of variation, 15.71%). (B) Photograph represents the corresponding autoradiograph. Exposure time was 4 hours.

second day postirradiation. In BM, the cellularity decreased from $9.93 \times 10^6 \pm 0.45 \times 10^6$ cells per bone in normals to $0.60 \times 10^6 \pm 0.03 \times 10^6$ cells by the second day after irradiation. Splenic cellularity decreased from $131.98 \times 10^6 \pm 11.98 \times 10^6$ cells per spleen in normals to $19.99 \times 10^6 \pm 0.38 \times 10^6$ cells on the second day postirradiation.

RNA yield postirradiation. The total RNA yield per animal also decreased after irradiation. The BM RNA yield per animal decreased from 11.48 ± 0.89 μg in normals to 2.90 ± 0.24 μg on the second day after irradiation. In whole spleens, the RNA yield decreased from 180.99 ± 16.96 μg to 59.95 ± 4.48 μg per spleen. However, the degree of decrease in BM RNA did not correlate with the decrease in BM cellularity, as the RNA yield per cell rose from 0.31 ± 0.22 $\mu g/10^6$ cells to 1.21 ± 0.06 $\mu g/10^6$ cells. Comparable data are not available in spleens, as the RNA yields were obtained from whole-spleen lysates and not from single-cell spleen suspensions.

Reaction efficiency. To evaluate the efficiency of the combined RT and PCRs, splenic RNA from a single subject was aliquoted into 14 separate reaction tubes, reverse-transcribed, and then amplified using substrate, buffer, and enzymes prepared as RT and PCR master mixes. The resulting blot is shown in Fig 1. Absolute densitometric volume averaged 201.7 ± 8.5. Although the absolute densitometric volume is a function of exposure time and film saturation kinetics, the sample showed remarkably little variation (coefficient of variation, 15.7%).

Quantitation efficiency. To assess the effects of amplification cycle number and exposure duration on quantitation efficiency, serial twofold dilutions of a single sample of splenic RNA were amplified simultaneously under identical conditions as described above at 20, 25, and 30 cycles. The resulting blots were first autoradiographed for 4 hours and then overnight to compare differences in observed results. The short-exposure (Fig 2) blot easily displays expression at 30 cycles but not in a serial twofold fashion, reflecting either saturation of the reaction or the film. While the 20-cycle samples were detected, the densitometer could not differentiate results at the lower dilutions in a twofold manner. However, the 25-cycle PCR products did show serial twofold differences at the lower concentrations of RNA but not at the higher concentrations. As shown in the accompanying data table to Fig 3A, the long-exposure blots show near-linear twofold decreases in densitometric volume at 20 cycles. Similar linear twofold decreasing results were observed at 25 cycles, but only at the lower concentrations. The results of 20- and 25-cycle long-exposure autoradiographs suggest that somewhat longer exposures can be used for the 20-cycle reactions without significant loss of quantification efficiency



	500	250	125	62.5	31.25	ng RNA
20 Cycles	0.1445	0.0775	0.06068	0.04943	0.03466	
25 Cycles	1.825	1.286	0.8831	0.4867	0.2057	
30 Cycles	4.283	3.821	3.978	3.58	2.955	

Fig 2. Densitometric volume of c-*kit* ligand PCR products in serial twofold dilutions of whole-spleen RNA after 20 (■), 25 (+), and 30 (*) cycles of amplification and an autoradiographic exposure duration of 4 hours. Table presents actual scanning data.

due to film saturation (Fig 3B). They also demonstrate that 20-cycle amplification would easily permit comparison between low-level normals and potentially stimulated gene expression as low as two to three times normal. At 30 cycles, saturation of the autoradiograph was exaggerated compared with both the short-exposure blot and the lower cycle amplifications at the same exposure length.

Normal KL expression. Bone marrow and splenic RNA from individual animals was amplified under identical conditions for each tissue to determine whether KL was constitutively expressed and if there were significant differences between individual animals. In BM, KL appeared to be regularly expressed at comparable levels in all animals (Fig 4A). However, in spleens the range of normal expression was quite broad, with levels in many individuals undetectable at amplification cycles and exposures designed to detect modest levels (Fig 4B).

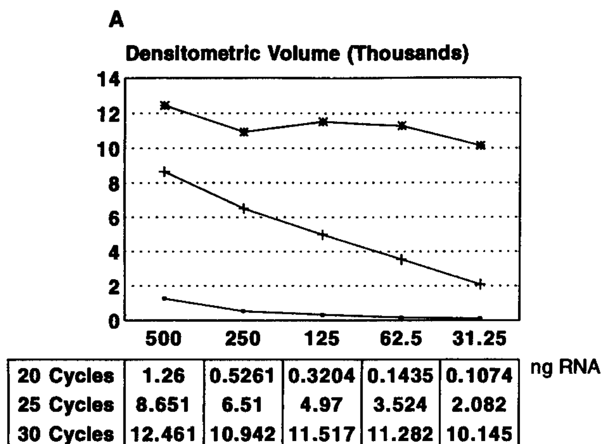
Expression on the second day postirradiation. To evaluate the acute effects of irradiation on KL expression, BM and splenic RNA from three sets of normal mice or mice 2 days after radiation exposure (each set of RNA was pooled from three to seven mice) was amplified in duplicate under identical conditions. An equal aliquot of RNA was separately subjected to RT-PCR for GAPDH as a control. Figure 5A and B shows that, after GAPDH correction, BM and splenic KL transcript levels in irradiated mice increased 15-fold and threefold, respectively. When evaluated without correcting for GAPDH expression, the difference in BM KL expression after 7.75-Gy irradiation increased 25-fold (Fig 6A), as BM GAPDH expression alone increased threefold in irradiated mice (Fig 6B). Splenic GAPDH expression did not change after irradiation (data not shown).

KL expression during prolonged recovery from sublethal irradiation. To evaluate the time course of KL expression during prolonged recovery from 7.75-Gy irradiation, RNA from three to seven animals was harvested at various times and subjected to RT-PCR. As shown in Fig 7A, BM transcript levels showed a uniphasic change in expression, with an increase on day 2 postirradiation and a return to normal on day 10. Splenic KL transcript levels increased on day 1 and returned to normal on day 4 postirradiation, only to increase again on day 10 and return to baseline on day 17 (Fig 7B).

DISCUSSION

This study shows that KL transcript levels increase in hematopoietic tissues immediately and for several days after sublethal radiation injury. This response to injury may be an integral part of the host response to hypoplastic injury and appears to partially explain previously reported data on the role of endogenous KL in radioprotection and the effects of exogenous KL in therapy for radiation injury.

A



	500	250	125	62.5	31.25	ng RNA
20 Cycles	1.26	0.5261	0.3204	0.1435	0.1074	
25 Cycles	8.651	6.51	4.97	3.524	2.082	
30 Cycles	12.461	10.942	11.517	11.282	10.145	

B

Fig 3. (A) Densitometric volume of c-*kit* ligand PCR products in serial twofold dilutions of whole-spleen RNA after 20 (■), 25 (+), and 30 (*) cycles of amplification and an autoradiographic exposure duration of 16 hours. Table presents actual scanning data. (B) Photograph represents actual autoradiograph.

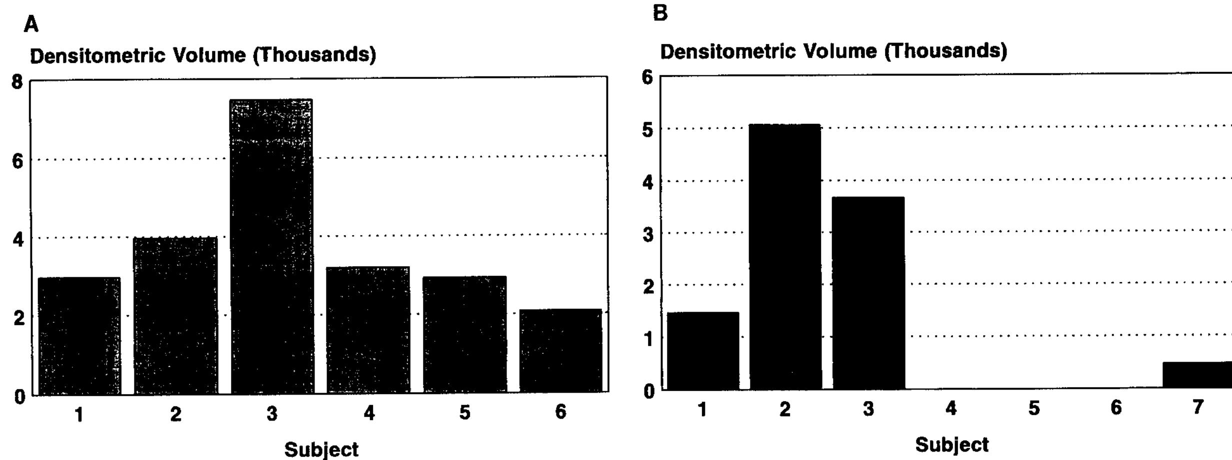


Fig 4. Expression of c-*kit* ligand in normal BM (A) and spleen (B). Each sample represents GAPDH-corrected relative expression found in individual subjects' BM and spleen after equal amounts of RNA were amplified in 20-cycle reactions under equal conditions. Exposure time: A, 2 days; B, 3 days.

As shown, KL transcript levels were uniformly present in normal BM but, although common, were not uniformly present in normal splenic tissue. Several possible explanations exist for this difference. Before RNA preparation, BM cells were extracted from femurs and tibias, and some activation of gene transcription could have occurred due to manipulation of the cells during the harvest process; in contrast, splenic RNA was prepared within seconds of spleen removal. However, if gene activation did occur it was not maximal as further dramatic increases in transcript levels were seen in irradiated mice. An alternate explanation is that basal hematopoiesis is the primary function of BM and is supported to some unknown extent by this basal level of the KL transcript. The variable KL expression of the normal spleen may represent its secondary role in basal hematopoiesis.

The postirradiation increases in BM and splenic KL transcript levels seen in this study may have numerous explanations. The existing data on the role of KL as a growth factor suggest that it is necessary for normal basal hematopoiesis on the basis of the interactions of KL with the c-*kit* product, as shown in the Steel and White mutant mouse model systems.[28] However, the in vitro data suggest that this role may be a necessary but insufficient one to drive maximal hematopoiesis. This appears to be borne out by in vivo data in murine hypoplasia models where exogenously administered KL produced, at best, modestly accelerated hematopoietic regeneration[22] compared with other growth factors evaluated in similar models.[29] If the transcript levels reported here are matched by protein expression, then these results may serve to explain some of the conflicts on the basis of the interaction between exogenous KL and the c-*kit* receptor in competition with endogenous KL produced after a hypoplastic injury. Recent data reported by Avraham et al[30] on the role of KL in the interaction between megakaryocytes and BM stroma, as well Caceres-Cortes and Hoang's[31] data on leukemic cell interaction, provide further evidence in support of this hypothesis. Neta et al's[21] data showing that anti-KL antibodies block the radioprotectant effect of IL-1 argue for an interme-

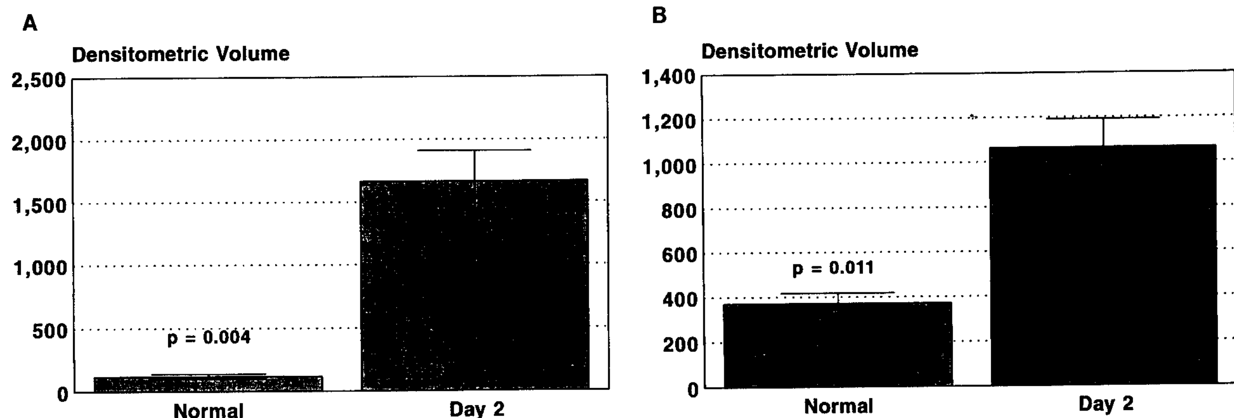


Fig 5. Expression of c-*kit* ligand in the BM (A) and spleen (B) of normal $B_6D_2F_1$ mice and of mice on day 2 after 7.75-Gy ^{60}Co irradiation. Data represent the mean ± SE of results from four to six BM and spleen samples and have been corrected for GAPDH expression. Exposure time: A, 24 hours; B, 16 hours.

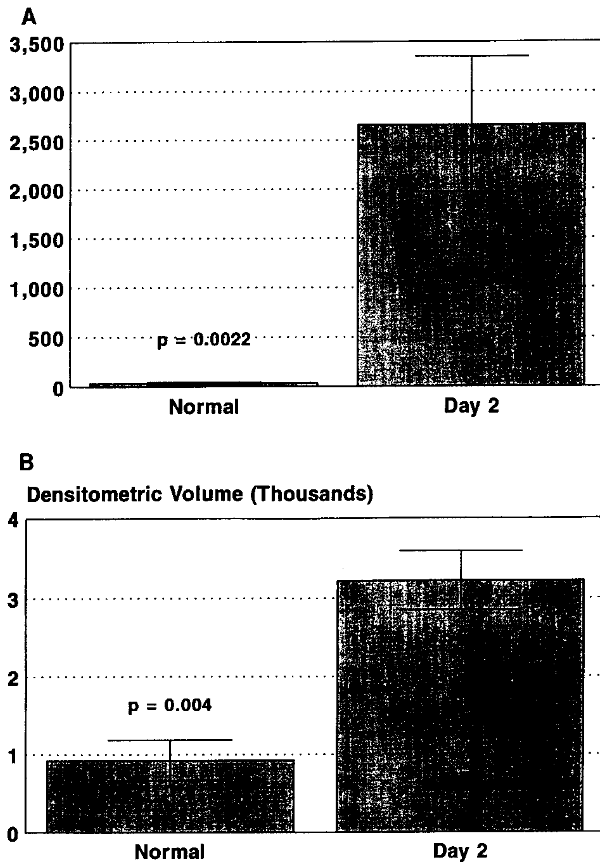


Fig 6. Expression of c-*kit* ligand (A) and GAPDH (B) in the BM of normal $B_6D_2F_1$ mice and of mice on day 2 after 7.75-Gy ^{60}Co irradiation. Data represent the mean ± SE of four to six marrow samples. Exposure time: A, 24 hours; B, overnight.

diary role of KL as an explanation for the radioprotectant effect of IL-1; however, an alternate explanation may be that the function of the radiation-induced expression of membrane-associated or -secreted KL was simply blocked by the anti-KL antibody. This is especially likely based on the increased radiation sensitivity observed in otherwise normal animals treated with the anti-KL antibody.[21] Furthermore, while early data suggested that basal endothelial production of KL was upregulated by IL-1, more recent data have shown that IL-1 has no direct effect on KL production by either cultured endothelium or stromal cells.[32,33] The overall apparent lack of specific effect on the BM CFU-S (but not CFU-GM) repopulation observed by Patchen et al[22] in irradiated animals treated with KL may be explained by exogenously administered KL in combination with increased endogenously produced KL saturating the c-*kit* receptor on primitive progenitor cells, thus preventing interaction of the progenitors with marrow stromal cells. Alternatively, the expression of KL and other pivotal factors or the expression of their receptors may be regulated by the pharmacologic effects of KL so that, in vivo, the dynamic interactions yield vastly different results than those noted in vitro.

Other than increased transcription or stabilization of transcripts, an alternative explanation for the increased KL gene expression observed in our study includes relative homogenization of the cell populations in the BM and the spleen postirradiation. As stromal cells are relatively resistant to the radiation doses used in this study, this is a reasonable explanation. More likely, however, is a combined explanation including some degree of cellular homogenization along with true increases in transcript levels. In similar radiation experiments studying BM and splenic transforming growth factor β (TGF-β) gene expression, another stromal cell product, transcript levels did not change.[34] The fact that no effect was seen on TGF-β suggests that the observed increases in KL expression cannot be explained simply on the basis of radiation-induced homogenization of radiation-resistant stromal and hematopoietic cell populations. In further support, Shirota and Tavassoli[35] have described putative radiation-induced histologic changes in BM microvasculature at doses as low as 1 Gy and more prominent changes at higher radiation doses well below those used in our study. As human umbilical vein endothelial cells have been shown to normally express KL, it is reasonable to hypothesize that a radioresistant stromal cell population would be directly or indirectly affected by radiation at these doses.[32]

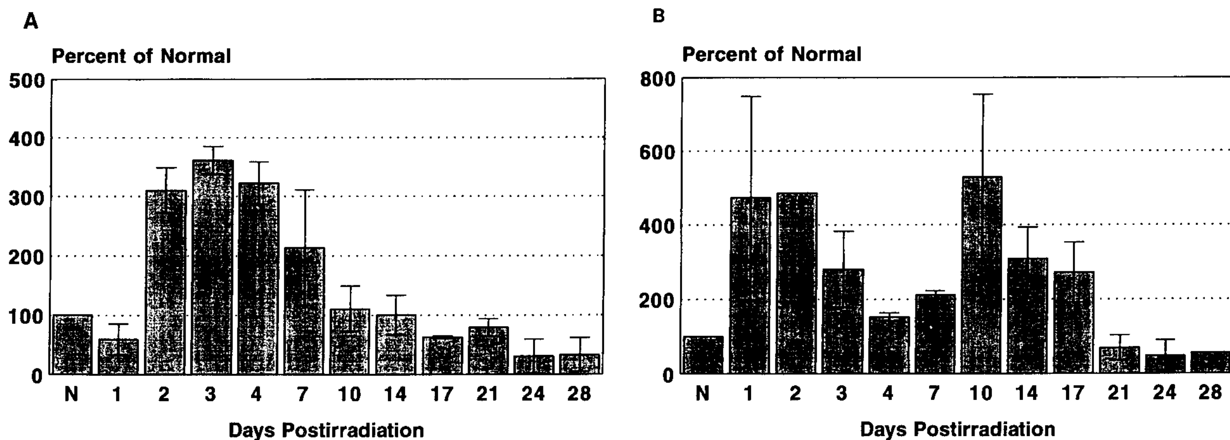


Fig 7. Changes in BM (A) and splenic (B) c-*kit* ligand expression in $B_6D_2F_1$ mice during a 28-day period after 7.75-Gy ^{60}Co irradiation. Data represent the mean ± SE of two separate experiments.

The use of the RT-PCR method to quantify levels of gene transcripts is a sensitive technique that must be carefully adapted to experimental conditions and the data requirements of the investigation. A number of methods to yield reliable results have aimed at controlling the supposed inherent variability of the RT-PCR process, including simultaneous amplifications of material, external standards for comparison, and various methods of internal standardization to control for variability. Additionally, the detection methods used to identify PCR products and the quantitation method to assign a value to the result must also be closely adapted to the data requirements to avoid saturation of the reaction, the film, or the scanning device. In this study, a semiquantitative method of transcript level comparison was obtained by simultaneous amplification of multiple sets of samples, each in duplicate. Attention was also directed to the length of exposure needed to achieve detection without causing film saturation to preserve the real differences between results. Consequently, the method chosen had a high degree of precision and was able to clearly differentiate relative differences in transcript levels.

In conclusion, this study shows that both BM and spleen express increased levels of KL transcripts after sublethal radiation injury and that the RT-PCR method, properly used, is a reliable method for semiquantitative analysis of changes in gene expression. The observed increases in KL expression may be an important response of hematopoietic stromal tissues in mediating hematopoietic recovery from hypoplastic injury.

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Radioprotection by 16,16 Dimethyl Prostaglandin E_2 is Equally Effective in Male and Female Mice

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Radioprotection/cytoprotecion/16,16 dimethyl prostaglandin E_2/sex factors/bone marrow aplasia

Pretreatment with 16,16 dimethyl prostaglandin E_2 ($DiPGE_2$) provides effective protection against radiation and chemical injury. Cytoprotection against chemical injury is known to be influenced by sex factors, and is more effective in females than males. Since prostaglandin metabolism and biological responses to prostaglandin may vary between sexes, studies were conducted to compare $DiPGE_2$-induced radioprotection in male and female mice.

Pretreatment with 400 μg $DiPGE_2$/kg body wt substantially enhanced 30-day survival in males and females. There was no significant difference in the $LD_{50/30}$ of male and female mice receiving vehicle alone prior to irradiation, 8.34 Gy versus 8.46 Gy, respectively. $DiPGE_2$ treatment increased the $LD_{50/30}$ in males to 12.1 Gy, providing a dose modification factor (DMF) of 1.45. Similar increases were observed in females, with a $LD_{50/30}$ of 11.6 and a DMF of 1.37. The reported difference in $DiPGE_2$-induced cytoprotection between males and females exposed to ethanol injury, and the lack of variation in the present radioprotection study suggests that separate mechanisms are involved in the two processes.

INTRODUCTION

Prostaglandins (PGs) mediate a diverse array of biological processes ranging from inflammation and cellular differentiation to shock. Yet, as a class, they contain some of the most potent radioprotective agents known[1-4]. They were initially examined as potential radioprotectants because of previous studies demonstrating that pretreatment with prostaglandins of the E series reduced injury of the gastric mucosa to chemical and physical insults[5,6]. This phenomena, as it relates to the gastric mucosa, has been termed "cytoprotection"[5]. Its mechanism of action is unknown.

Interestingly, the cytoprotective properties of prostaglandins are influenced by sex factors[6,7,8]. Two studies have shown that pretreatment of rats with $DiPGE_2$ significantly reduced the degree of gastric hemorrhage to a subsequent exposure of ethanol in females, compared to

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males[6,8]. In one of the studies, 59% less hemorrhages occurred in females. This has been related both to the 10-fold higher renal concentration of prostaglandin dehydrogenase in male rats[6], and to differences in the concentrations of alcohol dehydrogenase[8]. Renal PGDH concentration in rats has been shown to be influenced by estradiol concentration[9]. Differences between sexes have also been noted in prostaglandin synthesis. For example, the livers of female rats possess a sex specific cytochrome P-450 that produces a novel 17-hydroxylation of PGEs[10]. Variations in PG metabolism between sexes may be of clinical significance in areas other than reproduction, as illustrated by the fact that the incidence of gastric ulcers is higher in males than females[11]. In addition, indomethacin, a cyclooxygenase inhibitor, induces gastric tumors in female germ free rats, but not in males[7].

Beyond the descriptively limited bounds of cytoprotection which relegated that phenomena to the gastric mucosa; pretreatments with $DiPGE_2$ can induce protection from radiation injury in rats[12] and mice[2,3]. However, with the exception of two studies[12,13] the majority of the radioprotective work has been conducted on males. One study found that pretreatment with misoprostol, a PGE_1 analog, did not modify radiationinduced proctitis in female rats[12]. The other study showed a small radioprotective effect in female mice pretreated with $DiPGE_2$ prior to irradiation with fission neutrons[13]. Yet, no study has compared the radioprotective effects in both sexes. In light of the influence of sex differences on $DiPGE_2$-induced cytoprotection, a study was conducted to determine if sex factors were also important in $DiPGE_2$-induced radioprotection. As reported below, sex factors did not influence the radioprotective properties of $DiPGE_2$. This raises the possibility that the mechanism(s) for radioprotection differs from that of cytoprotection.

MATERIALS AND METHODS

Mice. CD21F1 male and female mice, 10 to 12 weeks old and weighing about 25 g, were obtained from Charles River Laboratories (Kingston, NY) and housed and cared for as previously described[2].

Prostaglandins. Prostaglandins were obtained from Biomol Research Laboratories (Philadelphia, PA). Mice received either 4, 40, 200, or 400 μg $DiPGE_2$/kg body wt in a volume of 100 μl by subcutaneous administration in the nape of the neck, as previously described[2]. Single treatments of $DiPGE_2$ were administered at 5, 10, 15, 30, 40, 60, 80, or 120 min prior to irradiation or at 15 min postirradiation. Control mice were administered an equal volume of saline 10 min prior to irradiation.

Irradiation. Groups of 10 mice each were placed in Plexiglas containers and irradiated in a bilateral cobalt-60 gamma field at a dose rate of 1.0 Gy/min as previously described[14]. Animals used in the radiation mortality curves received 7 to 15 Gy. The total numbers of animals irradiated per radiation dose and treatment ranged from 20 to 60 for males and 20 for females. In other radiation experiments, mice received 10 Gy. Survival was monitored on a daily basis, and the number of animals surviving 30 days postirradiation was recorded. The $LD_{50/30}$ and 95% confidence limits were determined from probit curve fitting of the 30-day mortality data were

fitted to probit curves[15]. At the conclusion of the experiments, surviving animals were asphyxiation in a charged CO_2 chamber. All experiments were repeated without attempt to standardize the female estrous cycles.

RESULTS

Optimum Time and Dosage. The optimal times and dosages for administration of DiPGE$_2$ to male mice have been previously determined[2]. That study showed that the optimal times for administration was 5 to 10 min prior to irradiation and that a "window" of protection existed

Table 1. DiPGE$_2$ Dose-Response on Radioprotection in Female Mice. Mice (n=20)[2] received saline or DiPGE$_2$ 10 min prior to 10 Gy γ-irradiation, as described in Methods

DiPGE$_2$ (μg/kg body wt)	30-Day mortality (%)
0	100
4	100
40	10
200	30
400	0

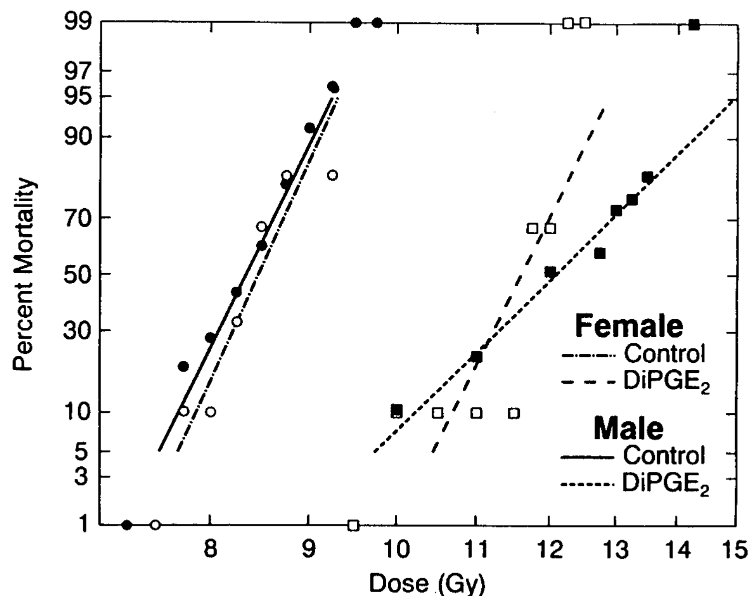

Fig. 1. Mortality curves for irradiated male and female mice. All mice with or without DiPGE$_2$ pretreatment received graded doses of bilateral ^{60}Co γ-irradiation at a dose rate of 1 Gy/min, as described in Methods and Materials. DiPGE$_2$-treated mice received 400 μg/kg body wt at 10 min prior to irradiation. The mortality at 30 days postirradiation was determined, and the data analyzed by probit analyses. The open symbols represent females and the closed symbols males.

which essentially decayed within 1 hr. In the present experiments, similar effects were observed for female mice in that the optimal time of administration was 10 min prior to irradiation and postirradiation treatments were not protective (data not shown). The degree of protection was dose dependent, as shown in Table 1. Doses as low as 40 μg $DiPGE_2$/kg body wt were radioprotective for female mice, yet were logistically limited in usefulness by the shorter "window" for protection. A dose of 400 μg/kg was selected for further comparison studies in order to minimize some of the behavioral side effects, observed in previous studies with male mice[1,2], while retaining a sufficient duration and degree of radioprotection. Diarrhea was a significant side effect in both males and females receiving $DiPGE_2$ with all doses used, including those as low as 4 μg/kg. Although decrements in locomotor behavior were not quantitated, male and female mice both exhibited detectable decreases in ambulation within min following $DiPGE_2$ administration. All mice recovered from the locomotor inactivity and diarrhea episodes, with no visually apparent deficits at 1 day postirradiation. No drug induced lethalities were observed in any of the male of female mice receiving $DiPGE_2$ treatments in this study.

Radiation Mortality Curve. Pretreatment with 400 μg $DiPGE_2$/kg was equally effective in enhancing the survival of male and female mice receiving otherwise lethal doses 10 Gy ^{60}Co γ-irradiation (Figure 1). The survival parameters of $DiPGE_2$-treated mice were determined by probit analyses (Figure 1) and summarized in Table 2. Untreated male mice had an $LD_{50/30}$ of 8.35 Gy compared to 8.46 Gy for the untreated female mice. Pretreatment with 400 μg $DiPGE_2$/kg provided a substantial degree of radioprotection, with a DMF of 1.45 for the male mice and 1.37 for the female mice. The 95% confidence intervals for these values are provided in Table 2. All $DiPGE_2$ treated males and females survived radiation treatments up to 9.5 Gy, an otherwise 100% lethal radiation dose in the absence of adequate $DiPGE_2$ pretreatment. The slopes of the probit lines for the male $DiPGE_2$-treated mice were significantly different from the control mice ($p=0.01$). The variations in the slopes of the probit analyses for the $DiPGE_2$-treated male and female mice (Figure 1) were not significantly different. The relative effectiveness of $DiPGE_2$ in female mice compared to male mice determined as the ratio of the DMFs was 1.04 [0.975, 1.222]. This difference was not significant.

Table 2. Probit Analysis of Lethality Data for $DiPGE_2$-Induced Radioprotection

Treatment	Sex	LD 50/30 Dose[a]	Dose Modification Factor[a]
Control	Male	8.35 [8.27, 8.43]	
	Female	8.46 [8.35, 8.58]	
$DiPGE_2$	Male	12.07 [11.79, 12.34]	1.446 [1.391, 1.503]
	Female	11.59 [10.80, 12.29]	1.370 [1.315, 1.427]

[a][95% Confidence Interval], $p<0.001$

DISCUSSION

The protective actions of eicosanoids on biological systems are arbitrarily divided into three

categories: cytoprotection, chemoprotection, and radioprotection. Prostaglandin-induced cytoprotection has been observed in diverse gastric injury processes including ethanol[1,5,8] acid, base, or thermal treatment[5]. They have been used clinically to protect pat ients from gastric injury associated with chronic non-steriodal antiinflammatory agent usage, and also appear to modify radiation-induced injury in patients with oral mucositis resulting from radiochemotherapy[16]. Since the mechanisms involved have not been clearly elucidated, overlap between categories may occur, particularly if similar mechanisms of injury are involved. In some instances, cytoprotection and chemoprotection are equivalent processes[5]. They jare both induced within similar time frames and require that prostaglandin treatment be initiated prior to injury.

There are however, distinct differences between cytoprotection and radioprotection. Contrary to observations on DiPGE$_2$-induced cytoprotection[6,8], DiPGE$_2$-induced radioprotective properties are not influenced by sex factors. Pretreatment with DiPGE$_2$ in the present study provides equal degrees of radioprotection from hemotopoietic death in male and female mice. In contrast, cytoprotection has been previously shown to yield a substantially higher degree of protection to females[6,8]. This difference may reflect different end-points for assessment, mechanisms of injury, or species differences. Cytoprotection and radioprotection also differ in the dosage of prostaglandin required to elicit an appropriate response. Cytoprotection can be elicited by lower doses of DiPGE$_2$, 5 μg/kg, compared to 40 to 400 μg/kg necessary for radioprotection. Since radioprotection requires larger doses of DiPGE$_2$ and since sex factors in the present study do not influence radioprotective properties, a more likely interpretation is that the two processes, cytoprotection and radioprotection occur by different mechanisms.

Misoprostol has been reported to protect mice from lethality induced by exposure to radiation or to doxorubicin[17]. The time courses for both types of protection are similar; however, the degree of chemoprotection observed following misoprostol pretreatment is greater than the radioprotective response[17]. The similarity between the time courses in that study suggested that a single mechanism may be responsible for chemoprotection and for radioprotection[17]. That conclusion, while at first glance opposite from the one derived in this paper, is not really at odds, when comparisons are made of the mechanisms of injury involved in the two types of treatments. Both doxorubicin and radiation treatments injure deoxyribonucleic acid, both are potent suppressors of myelopoiesis, and in the misoprostol study[17], a common end-point of hematopoietic death is being assessed. However, when comparing cytoprotection of gastric mucosa from ethanol injury with that of radioprotection to hematopoietic stem cells and animal survival, the end-point, though common in "protection", differs substantially. Thus prostaglandin-induced cytoprotection may differ from prostaglandin-induced radioprotection of hematopoietic stem cells, while permitting certain similarities between prostaglandin-induced radioprotection and chemoprotection. Cytoprotection may be a component of the radioprotective phenomena, particularly as it applies to radiation-induced gastrointestinal death. Radioprotection studies with prostaglandins and related compounds demonstrate that more than one protective mechanism is elicited[2,3,18], albeit, that one might be more important for overall protection than another. Studies of ^{3}H-misonidazole uptake into the bone marrow of DiPGE$_2$-treated mice indicate that tissue hypoxia is an important consequence of treatment[18], although other mechanisms must also play roles in radioprotection. It is possible that the role of hypoxia

in prostaglandin-induced radioprotection[18] might negate the influence of sex factor related modifications on other contributing radioprotective mechanisms. Given that prostaglandins protect both sexes equally, are more potent radioprotective agents than sulfhydryls such as WR-2721, are ubiquitous in mammals, and that their concentrations vary in response to the microenvironment, a better understanding of the mechanisms underlying their radioprotective properties would be beneficial.

In conclusion, cytoprotection, chemoprotection, and radioprotection are distinct processes, like large circles at the angles of a triangle, that may overlap depending on the type of injury and the end-point observed.

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Differential Effects of Ionizing Radiation on the Acquisition and Performance of Response Sequences in Rats[1]

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Abstract: PETER J. WINSAUER, MICHAEL A. BIXLER AND PAUL C. MELE. Differential Effects of Ionizing Radiation on the Acquisition and Performance of Response Sequences in Rats. *Neurotoxicology* **16**(2): 257-270, 1995. *To compare the effects of ionizing radiation on the acquisition and performance of response sequences, rats responding under a multiple schedule of food reinforcement were each administered 0.5-6 Gy of ^{60}Co gamma radiation. In one component of the multiple schedule, subjects acquired a different three-response sequence each session by responding sequentially on one of three response keys in the presence of three consecutively presented colors (repeated acquisition). In the other component, the three-response sequence was the same each session (performance). The response sequence in each component was maintained by food presentation under a second-order fixed-ratio (FR) 2 schedule. Errors in both components produced a 5-sec timeout but did not reset the sequence. In all subjects, 0.5-6 Gy of gamma radiation dose-dependently decreased response rates in both components for 1-5 days postexposure. These gamma-ray doses also produced dose-dependent increases in errors in both components, but only at doses that substantially decreased response rate. Unlike the effects on response rate in both components, which were comparable over the 5-day period after exposure, the effects on accuracy were generally different for the two components. More specifically, the largest increases in percent errors in the performance component occurred on day 2 postexposure, whereas the largest increases in percent errors in the acquisition component occurred on day 4 postexposure. Taken together, these results indicate that (1) acute sublethal doses of gamma radiation differentially affect the acquisition and performance of response sequences, (2) these doses of gamma radiation differentially affect the measures of rate and accuracy within each condition of behavior, and (3) using a sensitive baseline, which includes an accuracy measure, provides important information about the disruptive effects of radiation that could not be predicted from the effects on response rate alone.*
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Key Words: Repeated Acquisition, Multiple Schedule, Gamma Rays, Stimulus Control, Rats

INTRODUCTION

Exposure to ionizing radiation above background levels in humans has occurred through clinical treatment, industrial accidents (*e.g.*, Three Mile Island and Chernobyl), space travel and nuclear weapon detonations (King and Makale, 1991; Mele *et al.*, 1988; Mickley *et al.*, 1989). Not surprisingly, the behavioral and neurochemical data that have been collected after both accidental and experimental exposures in a large number of species have resulted in an understanding of the wide-ranging effects of radiation and variations in radiosensitivity across species. As evidenced by the distinct lack of data, however, it is clear that some of the most difficult effects to study have been the effects of radiation on learning or the acquisition of new behavior. Collecting data on these effects has been particularly difficult when the radiation exposures also

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[1]Early studies reported doses of ionizing radiation using the roentgen, which is a unit of exposure based on ionization measured free in air. Recent studies have used gray (Gy) or rad (1 Gy = 100 rad), which are units of absorbed dose (International Commission on Radiation Units and Measurements, 1980). For ease of comparison, tissue doses cited in this report are given in Gy. The roentgen-to-Gy conversion factor used was $9.66\ 10^{-3}$.

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produce sickness and affect the performance of the organism or the animal's ability to function. As one group of authors point out (Mickley *et al.*, 1989), human data on this subject is generally difficult to obtain and rarely unconstrained. Clinical irradiations, for example, tend to offer little data because precise measures are infrequently recorded, and patients are already behaviorally compromised by their illness or the therapy being used. Experimental exposures with animals, although conducted in a more controlled environment, have generally not controlled for distinctions between learning and performance or studied effects of acute sublethal exposures on learning in the 30 day LD_{50} range for humans (*i.e.*, 1.5-4.5 Gy (Anno *et al.*, 1989)).

Several studies examining the effects of ionizing radiation on learning in rodents have discussed the importance of the learning task itself when the intention is to show radiation-induced deficits (Burt and Ingersoll, 1965; Fields, 1957; Urmer and Brown, 1960; Winsauer and Mele, 1993). In fact, in the relatively few studies that have shown radiation-induced disruptions in learning after radiation exposure (cf. Mickley *et al.*, 1989), two critical factors have seemingly been the time of postexposure testing and the complexity of the task. For example, Fields (1957), who tested the effects of 0.97-5.8 Gy of x rays on rodents in an activity drum and in different maze patterns, reported that (1) no differences in the number of T-maze errors were observed until after the activity drum records ceased to differentiate the various radiation groups, (2) the most complex vertical-maze patterns produced the most significant error differences, and (3) the most difficult learning criterion (three consecutive errorless runs) revealed the greatest decrement. His observations about the delayed disruptions in learning and the critical nature of the methods used to measure these disruptions were verified in a later study by Urmer and Brown (1960), who tested the effects of gamma radiation on rats' ability to learn a reorganized maze. These investigators found that the effects of a 4-Gy dose of ^{60}Co gamma radiation delivered at a dose rate of 1 Gy/min were not evident until subjects were challenged or asked to "reorganize" a preirradiation response pattern into a new response pattern. Interestingly, they also found that subjects working on the training pattern or preirradiation maze pattern showed no effect at this same dose.

Despite the findings of Fields (1957) and those of Urmer and Brown (1960), relatively little has been done to characterize further the effects of radiation on learning in rodents. Moreover, the existing data on such effects are largely specific for only one type of experimental learning situation; that is, maze learning or spacial learning (see Arnold, 1952; Blair, 1958; Furchtgott, 1951). One exception was a learning study by Winsauer and Mele (1993), in which a repeated-acquisition procedure was used along with a single-subject design to demonstrate that graded sublethal doses of ^{60}Co gamma radiation (1-8 Gy) produced dose-dependent disruptive effects on both the rate and accuracy of responding. Similar to the study by Fields (1957), which used a maze, this investigation showed that ionizing radiation differentially affected the response rate and accuracy of a learning task over a 1- to 5-day postexposure period. More specifically, the effects of gamma radiation on accuracy (*i.e.*, percent errors) in each subject were generally greater at 72 hr postexposure than at 24 hr postexposure even though response rates were comparably decreased at these time points.

Although delayed disruptions in learning were reported in the studies by Winsauer and Mele (1993) and Fields (1957), the disruptive effects of gamma radiation on acquisition and performance have not been compared directly within a session or in the same subject. In other words, there is little, if any, within-subject experimental data that demonstrate the degree to which ionizing radiation might disrupt an individual performing distinctly different tasks (*i.e.*, tasks to be learned as well as tasks thoroughly rehearsed or overlearned). Therefore, the present research sought to extend the analysis of radiation effects on complex operant behavior by examining the effects of 0.5-6 Gy of gamma radiation, using a multiple-schedule baseline with both acquisition and performance components. This baseline also functions as a means of comparing the degree to which radiation can affect behaviors under differential stimulus control, a behavioral variable known to modulate the effects of a number of drugs (*e.g.*, Laties *et al.*, 1981; Thompson, 1975; Thompson and Moerschbaecher, 1979) and radiation (Mele and McDonough, 1990). Stimulus control generally refers to differential responding in the presence of different stimuli. In this case, the different stimuli associated with responses in the acquisition component were relatively weak because the response sequence was changed each session, whereas the different stimuli associated with responses in the performance component were relatively strong because the response sequence was the same across sessions.

MATERIALS AND METHODS

Animals

Six adult male Sprague-Dawley rats (RP-79, RP-80, RP-82, RP-83, RP-84, and RP-85) approximately 120 days old and maintained at 80% of their free-feeding weights (357 g, 368 g, 338 g, 416 g, 434 g, and 377 g, respectively) served as subjects. The 80% body weight for each subject was determined after a quarantine period of 10 days and another period of at least 20 days in which rodent chow and acidified water were provided ad libitum. After this time, food was earned during the experimental session and, if necessary, was provided in the home cage after the session in order to maintain subjects at that weight. Acidified water (pH 2.5-3) was routinely available in the home cage throughout the experiment to minimize the possibility of opportunistic bacterial infection (McPherson, 1963). In general, water acidified to a pH of 2.5 has been found to be a highly efficacious method for reducing specific types of potentially harmful bacteria while not affecting water intake or other growth factors even when it has been administered over an extended period of time (*e.g.*, Tober-Meyer *et al.*, 1981). All subjects were housed individually in plastic Micro-Isolator cages (Allentown Caging Equipment Co., Inc., Allentown, New Jersey) containing sterilized hardwood-chip bedding. The housing room was maintained at 21 ± 1°C with 50 ± 10% relative humidity on a 12-h light-dark cycle, which began at 0600 each day.

Apparatus

Four identical modular test chambers (Coulbourn Instruments, Inc., model E10-10TC) configured specifically for rodents were used. The front wall of each chamber contained a houselight, speaker, auditory feedback relay, pellet trough (10 cm above the floor and centered), and three response keys aligned horizontally (8 cm apart, center to center, and 4.5 cm above the floor). Each response key could be transilluminated by three Sylvannia 28ESB indicator lamps, one with a red plastic cap, one with a green, and one with a yellow. Response keys required a minimum force of 0.15 N for activation and each correct response produced an audible click of the feedback relay. Each chamber was enclosed within a sound-attenuating cubicle equipped with a fan for ventilation. White noise was continuously present in each chamber to mask extraneous sounds. The chambers were connected to a PDP11/73 computer (Digital Equipment Corp., Bedford, Massachusetts), programmed with SKED-11 software (State Systems, Inc., Kalamazoo, Michigan), and to cumulative recorders (Gerbrands Corp., Arlington, Massachusetts) located in a nearby room.

Behavioral Procedure

Preliminary training of the rats was similar to that described for pigeons by Thompson (1973). Briefly, this included magazine training, shaping of the nose poke, and reinforcing responses on each of the three keys, which were transilluminated with yellow light. When the subjects reliably responded in this single response sequence, another response (associated with a different colored stimulus) was added until each subject was able to respond in a three response sequence. The final baseline was a multiple schedule with acquisition and performance components. During the acquisition component, all three response keys were illuminated simultaneously with one of three colors: green, red, or yellow. The subject's task was to respond (nose push) on the correct key in the presence of each sequentially illuminated set of colors (*e.g.*, keys green - center correct, keys red - left correct, keys yellow - right correct). Completion of the sequence turned off the keylights, produced a 0.4-sec presentation of the pellet trough light, and reset the sequence. The same sequence (in this case, center-left-right or CLR) was repeated throughout a given session and maintained by food presentation under an FR-2 schedule; *i.e.*, every second completion of the sequence resulted in the delivery of a 45-mg food pellet. When the subject pressed an incorrect key (in the example, the left or right key when the green keylights were presented), the error was followed by a 5-sec timeout. During the timeout, the keylights were turned off and responses had no programmed consequence. An error did not reset the sequence, such that the stimuli were the same before and after the timeout.

To establish a steady state of repeated acquisition, the three-response sequence was changed from session to session. An example of a typical set of five sequences was CLR, RCL, LRC, CRL, and RLC, with the order of the color presentations always green, red, and yellow. The sequences were carefully selected to be equivalent in several ways and there were restrictions on their ordering across sessions. More specifically, each

sequence was scheduled with equal frequency and adjacent positions within a sequence for a given session were different. Occasionally, a correct sequence position for a given color was the same two sessions in a row.

During the performance component of the multiple schedule, the houselight above the keys was turned on. In this component, the three-response sequence remained the same (RCL) from session to session. In all other aspects (FR-2 schedule of food presentation, 5 sec timeout duration, etc.), the performance component was identical to the acquisition component.

Each session began in the acquisition component, which then alternated with the performance component after 40 reinforcers or 20 min, whichever occurred first. Each session was terminated after 200 reinforcers or 90 min, whichever occurred first.

Radiation Procedure. The baseline was considered stable when the total errors and overall response rates for both components no longer showed systematic change from session to session (approximately 42 sessions after the introduction of the multiple schedule). After the baseline had stabilized, dose-effect data for gamma-ray doses were obtained. Subjects received bilateral whole-body midline tissue doses of 0.5, 1, 3, 4.5, and 6 Gy administered at a fixed rate of 2.5 Gy/min from the Armed Forces Radiobiology Research Institute (AFRRI) ^{60}Co source (Carter and Verrelli, 1973). These doses were selected from a range of doses previously found to affect repeated-acquisition behavior (Winsauer and Mele, 1993). As shown in Table 1, all subjects received the same dose on a given day and these doses were given in a mixed order except for the 6-Gy dose, which was specifically administered last to each subject. The minimum time between exposures was 4 weeks. This interval was selected to allow for (1) complete baseline recovery (cf. Mele *et al.*, 1988) and (2) the collection of sufficient control data prior to the next exposure.

TABLE 1. Gamma Radiation Exposure History for Each of the Six Rats.

Exposure	Dose (Gy)	
	Acute	Cumulative
1	3	-
2	1	4
3	4.5	8.5
4	1	9.5
5	4.5	14
6	0.5	14.5
7	0.5	15
8	6	21

For irradiation, the subjects were placed in well ventilated clear plastic restraining tubes. A clear plastic stand, which held the tubes in a stacked position, allowed a total of four subjects to be exposed at one time. Dosimetry was completed prior to the irradiations. American Association of Physicists in Medicine (AAPM, 1983) protocol for the determination of absorbed dose from high-energy photon and electron beams was used. A 50-cm^3 ionization chamber was used to obtain the free-in-air (FIA) tissue dose rate at the appropriate exposure position. A 0.5 cm^3 tissue-equivalent ion chamber was also used to obtain the tissue dose rate in the same position in a tissue-equivalent rat phantom. The tissue-air ratio (TAR) was calculated by dividing the nominal 0.5-cm^3 chamber reading by the 50-cm^3 chamber reading. The administered dose (midline tissue at the abdomen) to each animal was determined by using the TAR value, the FIA dose-rate value, duration of the irradiation, and other correction factors based on temperature and pressure. Each irradiation was conducted on a Monday and required approximately 20 min. This included the exposure time and the time necessary for transporting the rats to and from the exposure area. Sham irradiations, which also included transport, consisted of the subjects' being placed in the restraining tubes for a comparable amount of time. Throughout the experiment, the day on which irradiation occurred and the four subsequent days were designated postexposure day 1 and postexposure days 2-5, respectively.

Data Analysis

The data from both components of the multiple schedule were analyzed for each session in terms of (1) the overall response rate (total responses/min, excluding timeouts) and (2) the overall accuracy, expressed as percent errors [(errors/total responses) x100]. In addition to these measures based upon session totals, within-session changes in responding were monitored by cumulative recorders and the computer. For example, acquisition of a response sequence was indicated by within-session error reduction; that is, a decrease in the number of errors between food presentations as the session progressed. The data for each subject were analyzed by comparing a given postexposure session with the control (sham irradiated) range of variability for that subject. A dose of radiation was considered to have an effect when the dose data fell outside the control range.

RESULTS

For all six subjects, stable responding occurred in each component of the multiple schedule during baseline sessions and control sessions when subjects were sham irradiated. Overall response rates and percent errors were consistent from session to session in both components, and acquisition of the response sequences was characterized by a steady state in terms of within-session error reduction. During the control sessions for all subjects, the mean number of errors in acquisition was 127 and all the data points fell within the range of 48-329 errors. In addition, acquisition was always indicated for each subject by the pattern of responding that occurred at the beginning of the initial acquisition component when, after a short period of time (5-10 min), the number of errors sharply decreased and correct (errorless) completions of the response sequence increased. After acquisition of the response sequence, this relatively errorless pattern of responding was present in the acquisition components for the remainder of the session. Moreover, under baseline and control conditions throughout the experiment, an essentially similar pattern of acquisition was obtained for each subject with each new sequence acquired. This pattern of responding in the acquisition component also accounted for the fact that the average percent errors in acquisition (15%) were typically larger than the average percent errors in performance (6.6%), even though overall response rates in both components were comparable.

For the five-day postexposure period, the effects of 0.5-6 Gy of gamma radiation were both time dependent and dose dependent for all subjects. These general effects are represented in Fig. 1 by the daily session data for RP-80. As shown, no effects on either overall response rate or percent errors were obtained with any dose tested on day 1. However, large dose-dependent decreases in overall response rate occurred on days 2-4 with responding in both components, at almost all doses except the high dose, returning to control levels on day 5. The effects on percent errors across this five-day period were more complex in that, compared to control levels of responding, the effects on percent errors in the acquisition and performance components had different time courses. On day 2, for example, the increase in percent errors tended to be larger in the performance component than in the acquisition component. On days 3 and 4, however, this was not true. In fact, at almost all doses for RP-80, percent errors in performance after day 2 approximated those for control sessions. The day-2 effects on percent errors were particularly evident at the three higher doses where increases in percent errors were evident in performance, but little or no increases were evident in acquisition despite the fact that overall response rates were comparably decreased under both schedule conditions

Figs. 2 and 3 show the radiation-induced disruptions of response rate and percent errors obtained in all subjects on days 2 and 4, respectively. On postexposure day 2 (Fig. 2), comparable dose-dependent decreases in overall rates of responding were evident in both components. Unlike the effect on response rate, the effects on percent errors in all subjects were variable both within and across the two components. That is, in two subjects (RP-80 and RP-81) percent errors tended to be larger in the performance component than in the acquisition component if one considers the fact that in these subjects percent errors in performance show a larger absolute increase from control than errors in acquisition. At the 4.5-Gy dose, for example, both determinations produced decreases in response rates in both components, only a selective increase in percent errors in performance, and no increase in percent errors in acquisition. In two other subjects (RP-83 and RP-84), a larger effect on performance errors can only be seen at the 6-Gy dose, and in the final two subjects, little or no effect on percent errors occurred in either schedule component.

Effects on accuracy on postexposure day 4 (Fig. 3) were different from those obtained on day 2, even though overall response rates were similarly decreased across components. At the 6-Gy dose in all subjects except RP-81, little or no effect was obtained on percent errors in the performance component, but a substantial increase in percent errors was obtained in the acquisition component. Again, these differential effects on accuracy across days 2-4 (see Figs. 2 and 3) occurred while response rates in both components were comparably affected.

Fig. 4 illustrates the within-session effects of a 4.5-Gy dose of gamma radiation in subject RP-80 on postirradiation days 1-5. Each cumulative record shows the first four components of the session for that day. As shown in the early portion of the control record (top row), errors in acquisition decreased in frequency as the session progressed, which indicated that the subject acquired the correct sequence of responses. Errors in performance, on the other hand, were fewer and occurred at about the same rate across each of the performance components. This difference in error patterns for the acquisition and performance components is clearly

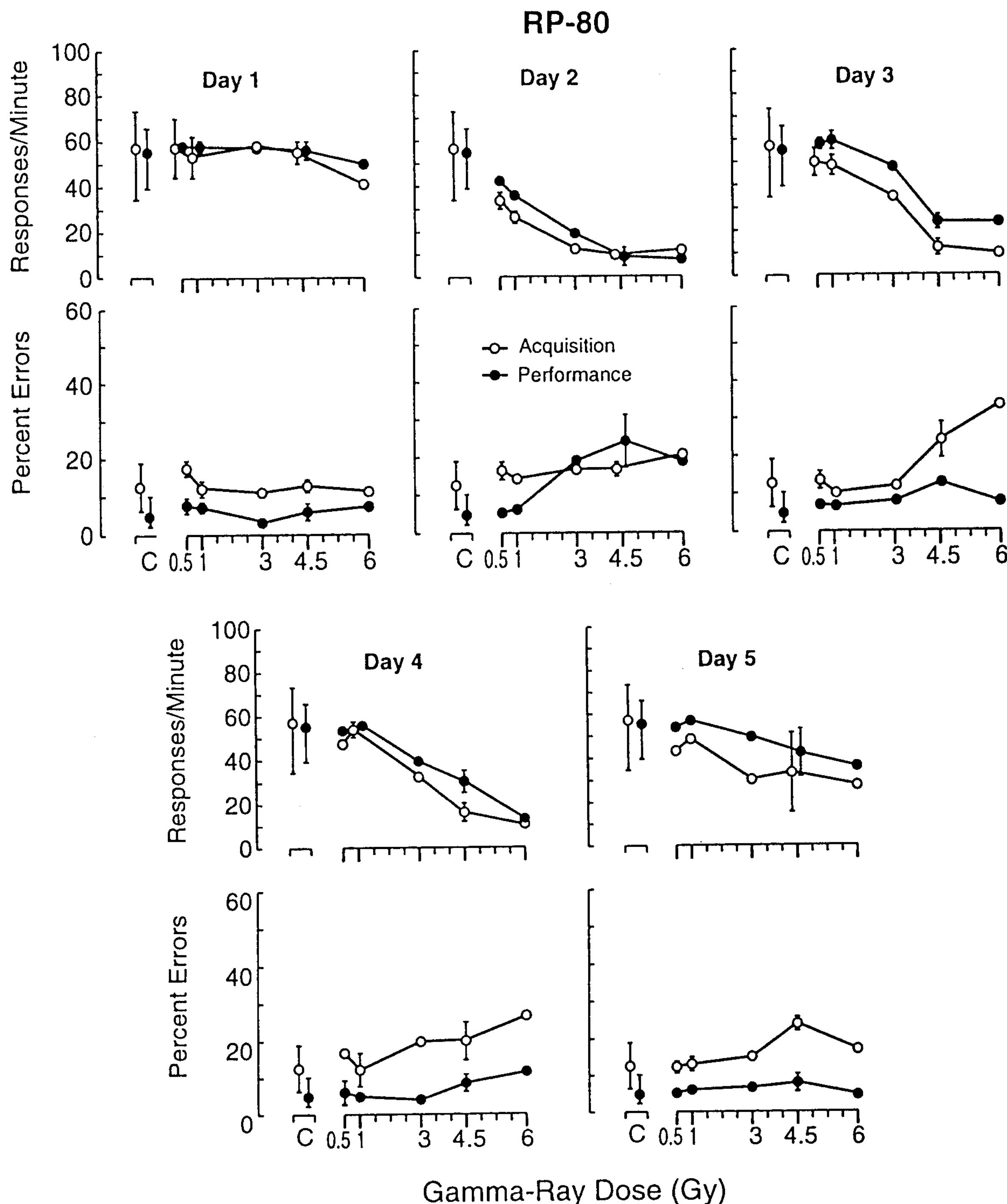

FIG. 1. Effects of different doses of ^{60}Co gamma rays on overall response rate and percent errors in the acquisition and performance components of the multiple schedule for rat RP-80. Each of the five panels represents dose-effect data obtained on each of the five days following exposure, which occurred 4 hr before the experimental session on day 1. The unconnected points with vertical lines at C indicate the mean and range for both components of the multiple schedule during 11 (RP-83, RP-84, and RP-85) or 19 (RP-79, RP-80, and RP-81) control sessions. The points with vertical lines in the dose-effect curves indicate the mean and range for two determinations; points without vertical lines indicate either a single determination (at 3 and 6 Gy) or an instance in which the range is encompassed by the point.

represented in the record for day 1 (second row) and by the two boxed insets (upper right-hand portion of the figure). The two insets reflect responding during the initial few minutes of the first occurrence of each

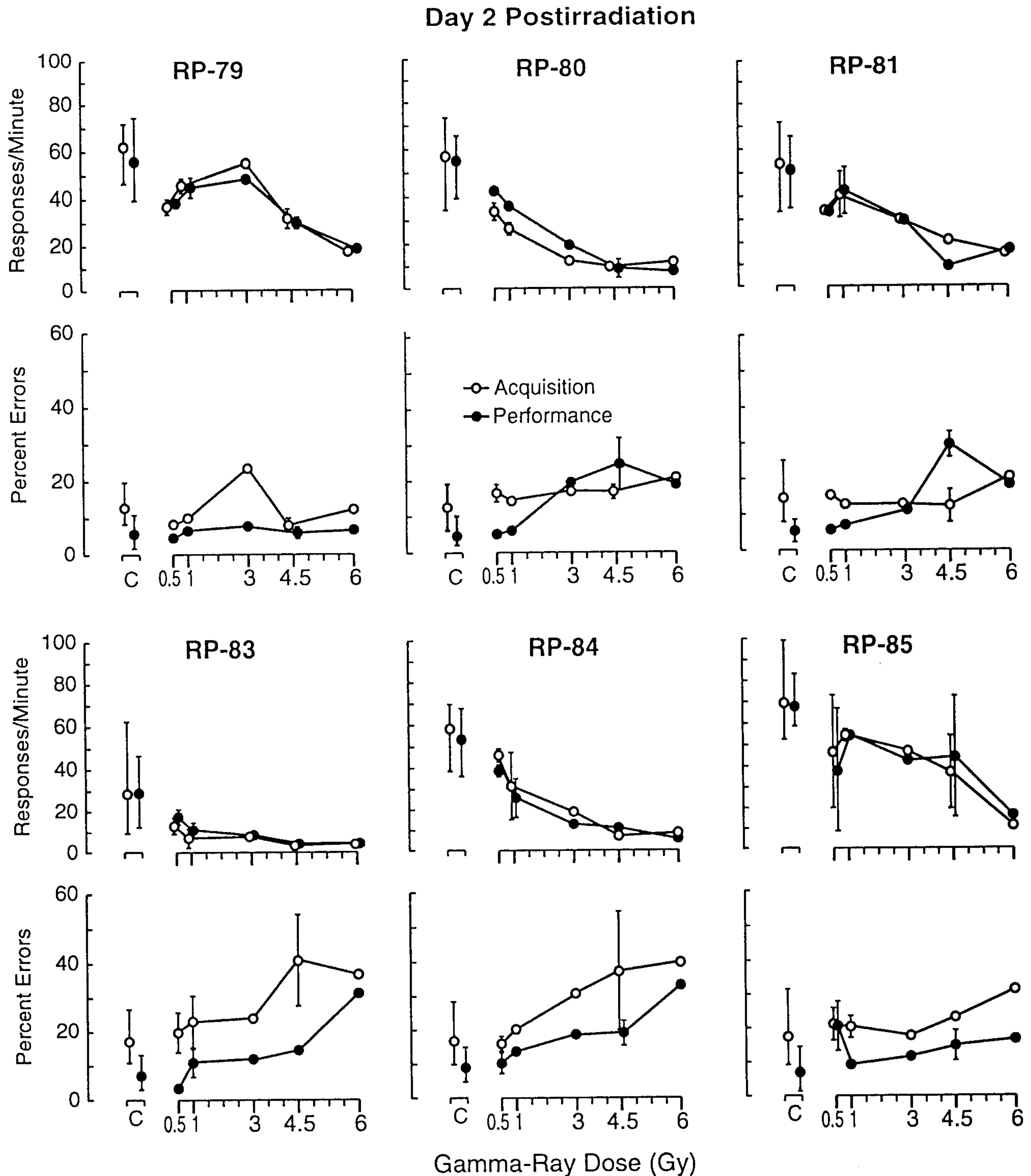

FIG. 2. Effects of different doses of ^{60}Co gamma rays on overall response rate and percent errors in the acquisition and performance components of the multiple schedule for all subjects on day 2 postexposure. For details, see Fig. 1 legend.

component during the day-1 session. At the beginning of acquisition, the error rate was very high and consecutive errorless completions of the response sequence were infrequent. At the onset of performance conditions, errors occurred at a lower rate and consecutive errorless completions of the response

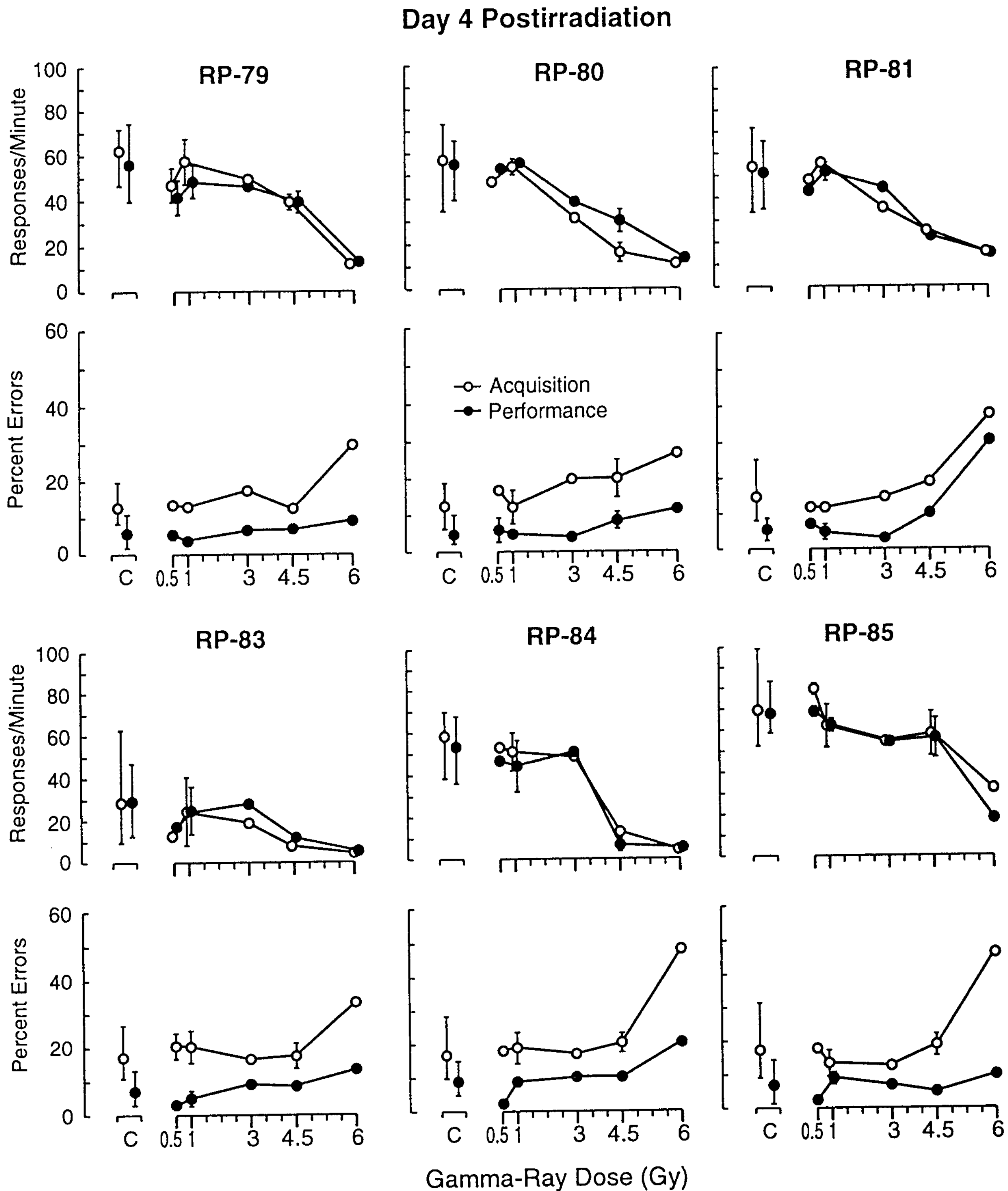

FIG. 3. Effects of different doses of ^{60}Co gamma rays on overall response rate and percent errors in the acquisition and performance components of the multiple schedule for all subjects on day 4 postexposure. For details, see Fig. 1 legend.

sequence were frequent. On day 2, however, the within-session pattern of responding reflected slowed responding in both schedule components along with substantial pauses during the initial performance component and the second acquisition component. Note, in contrast to an increase in performance errors,

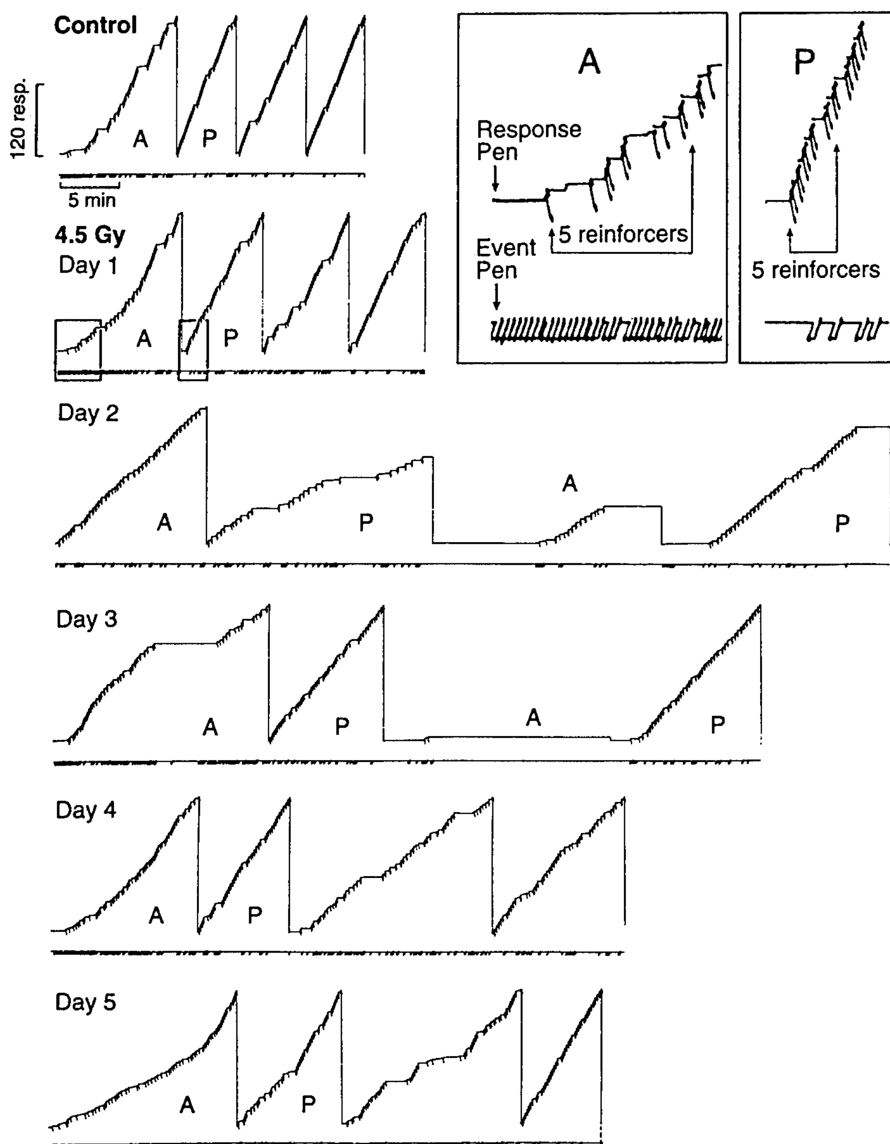

FIG. 4. Within-session effects of ^{60}Co gamma rays during a control session (top record) and five consecutive sessions conducted after a single 4.5-Gy dose on day 1 in subject RP-80. Each cumulative record shows the initial four components of that day's session and the pattern of responding that occurred in both acquisition (A) and performance (P) components of the multiple schedule. In each record during both components, as exemplified by the inset in the upper righthand corner of the figure, the response pen stepped upward with each correct response and was deflected downward each time the three-response sequence was completed. Errors in both components are indicated by the event pen (below each record), which was held down for 5 sec during each timeout. A change in components of the multiple schedule (after 40 reinforcements or 20 min) reset the stepping pen.

acquisition errors were not increased above control levels on this day. As indicated by the records for days 3 and 4, this error pattern was reversed even though response rates were still decreased in both components. In the records for these days, the increase in acquisition errors was particularly apparent during the first component on day 3 and the second acquisition component on day 4. The day-5 record shows that rates of responding in this subject were returning to control levels but that a slower rate of acquisition and an increase in errors still occurred.

As shown in Table 1, all six subjects received a cumulative gamma-ray dose of 21 Gy. Although this dose of radiation was lethal for four of six subjects (*i.e.*, RP-79, RP-80, RP-81, and RP-85), the time courses for the lethal effect in these subjects were markedly different. Specifically, subjects RP-79 and RP-81 died 8 and 6 days after the last exposure, respectively, whereas subjects

RP-80 and RP-85 died 29 and 27 days after the last exposure, respectively. Of note was the fact that both RP-80 and RP-85 died long after completely recovering control levels of responding for both response rate and accuracy in both components of the multiple schedule.

DISCUSSION

As in other repeated-acquisition studies involving rats (Cohn *et al.*, 1993) and other species (*e.g.*, pigeons (Moerschbaecher *et al.*, 1979), monkeys (Moerschbaecher *et al.*, 1983; Thompson and Moerschbaecher, 1979) and humans (Bickel *et al.*, 1990; Desjardins *et al.*, 1982), the multiple-schedule baseline of repeated acquisition and performance produced stable rates of responding in both components and produced error levels that were, as expected, higher in acquisition than performance. The long-term stability of this operant baseline also provided the necessary means for repeatedly assessing the behavioral effects of varying acute doses of whole-body gamma radiation, which often last 1-5 days at the doses tested (Jarrard, 1963; Mele *et al.*, 1988).

Administration of 0.5-6 Gy of gamma radiation produced comparable dose-dependent and time-dependent decreases in response rate in both components of the multiple schedule. This was clearly indicated, in the dose-effect data for days 2 and 4 postexposure, by the considerable overlap in the curves for response rate in both components (see Figs. 2 and 3, upper panels). These rate-decreasing effects were similar to effects found in other studies involving either x rays or gamma rays and schedule-controlled operant behavior (Brown, 1966; Brown *et al.*, 1960; Jarrard, 1963; Mele *et al.*, 1988; Wicker and Brown, 1965). In particular, the effects on response rate were similar to those effects found at comparable gamma-ray doses in an earlier repeated-acquisition study by Winsauer and Mele (1993). In that study, which involved only an acquisition task, whole-body gamma-ray doses of 1-8 Gy produced similar decreases in response rate across an equivalent postirradiation period (*i.e.*, 1-5 days). Such decreases are unique in that few studies examining complex behavior in rodents have obtained disruptions in rate after these relatively low gamma-ray doses. In the present study, a 1-Gy dose affected the rate of responding in either the acquisition or the performance component in more than half the subjects on day 2 postexposure.

Disruptions in the accuracy of responding in both components occurred only at doses that substantially decreased overall response rate. As in the previous repeated-acquisition study by Winsauer and Mele (1993), the effects on accuracy in the present study were different from those in many earlier radiation studies involving a learning task (for review, see Mickley *et al.*, 1989). Surprisingly, only a few of the earlier studies (Fields, 1957; Urmer and Brown, 1960) reported error-increasing effects at the doses and postirradiation times tested in the repeated-acquisition studies. However, the accuracy data in this study confirm our previous findings of disruptions in learning after exposure to whole-body sublethal doses of gamma radiation. Additionally, the present study produced new information about the differential sensitivity of acquisition errors and performance errors to acute gamma-ray exposure. On day 2 postexposure, for example, accuracy was disrupted to different extents both within and across components. In contrast, on day 4, accuracy in the acquisition component was more disrupted than that in the performance component. In fact, on day 4, percent errors in the performance component were generally at or near control levels for all subjects.

An important advantage gained by using this baseline involves the capability to compare the effects of radiation on behaviors under weak and strong stimulus control (*i.e.*, acquisition and performance, respectively). The degree to which effects of gamma radiation on complex behavior can vary, as a function of a behavioral variable such as stimulus control, is relatively unknown. Moreover, little research has compared (in the same subject) the effects of radiation exposure on behaviors under different conditions of stimulus control.

In one of the few early studies to compare the effects of radiation in groups of rats working on different tasks, Urmer and Brown (1960) found that the effects of 4 Gy of gamma radiation were most notable when subjects were challenged or required to "reorganize" a preirradiation response pattern into a new pattern. These investigators also reported that a group of rodents working on a maze pattern learned before irradiation were not affected by this dose. If learning the reorganized maze is considered a new task under weak stimulus control and if performance in the preirradiation or training maze is considered an established task under relatively strong stimulus control, then the results of Urmer and Brown compare favorably with the present results. However, an important difference exists between these two studies, in that the present study also found disruptions in the performance

component where behavior was under strong stimulus control. Whether or not this difference was a function of such experimental variables as task (elevated T-maze versus operant schedule), experimental design (groups versus single subject), deprivation level (23 hr deprived versus 80% free-feeding bodyweight), or even dose rate (1 Gy/min versus 2.5 Gy/min) remains to be determined.

That the behavior in the performance component in the present study was under relatively strong stimulus control was indicated by the fact that the baseline error levels were generally lower in performance than in acquisition. That behavior in both acquisition and performance components was differentially sensitive to gamma radiation was clearly indicated by the different time courses for radiation-induced effects on accuracy in each component. In one recent radiation study involving groups of rodents and similar conditions of deprivation and exposure, Mele and McDonough (1990) reported that single whole-body gamma-ray doses of 6 or 7.5 Gy produced decreases in both cued and noncued components of a multiple fixed-consecutive-number (FCN) schedule of milk reinforcement. As in the present study involving a multiple schedule, response rates and accuracies were reduced more under conditions of weak stimulus control (*i.e.*, the noncued condition by virtue of the absence of external stimuli) than of strong stimulus control (the cued condition). Certainly, these data agree with data from a variety of experimental situations involving many types of drug treatments in which behavior under strong stimulus control was more resistant to disruption than behavior under weak stimulus control (*e.g.*, Laties *et al.*, 1981; Thompson, 1975; Thompson and Moerschbaecher, 1979).

Differential effects on errors in the acquisition and performance components of a multiple schedule over days have been reported by Thompson (1974) in a study involving the chronic administration of five drugs. In this study, learning errors showed several patterns (*e.g.*, incremental trend, little or no change, tolerance) during the chronic administration of specific doses of each drug. The pattern following administration of methylphenidate and d-amphetamine, for example, was similar to the one in the present study; that is, after very few sessions, error-increasing effects in the performance component diminished whereas error-increasing effects in the acquisition component remained high. Although errors in acquisition actually increased over days in the present study, it is not unreasonable to suggest that behavioral variables such as stimulus control or reinforcement density, which selectively affect measures of rate and accuracy during chronic drug studies, selectively affect these same measures following radiation exposure. Unfortunately, any interpretation of the present data involving reinforcement density must be indirect because the conditions of stimulus control in part determined the differences in reinforcement frequency (cf. Moerschbaecher *et al.*, 1979). Only additional research, which specifically controls for these behavioral variables, can determine the extent of the function of stimulus control or reinforcement density in modulating radiation-induced effects on complex operant behaviors.

Just as it is difficult to attribute disruptions in complex operant behavior to changes in motivation resulting from radiation sickness (cf. Winsauer and Mele, 1993), it is difficult to attribute such disruptions to the radiation effects that produce lethality. Clearly, neither the lethal effect nor the time course for lethality were consistent for the six subjects tested, even though the radiation-induced behavioral disruptions were consistently dose dependent and time dependent in all subjects. This dissociation of radiation-induced behavioral disruptions and lethality is both important and problematic, since neither is predictive of, or necessarily coincident with, the other. Nevertheless, as was the case for other radiation studies involving groups of rats (Mele *et al.*, 1990; Paterson *et al.*, 1952), dose fractionation with an interirradiation interval of three weeks or greater, increased the total number of doses that could be tested as well as the total cumulative dose that could be administered before producing lethality. In fact, decreasing the highest dose tested from 8 Gy (Winsauer and Mele, 1993) to 6 Gy (present study) did not lessen the behavioral effects obtained, but did increase the cumulative dose from 16.5 Gy to 21 Gy, respectively. Thus, changing the highest dose tested by 2 Gy allowed for additional fractionated doses totaling 4.5 Gy to be tested without any additional loss of subjects.

In summary, the present research extends the analysis of radiation effects on learning by showing that behaviors in the same subject under different degrees of stimulus control are differentially sensitive to acute sublethal doses of gamma radiation. Another important finding is that this differential sensitivity is evident only in the accuracy data generated by the two components of the multiple schedule, whereas effects on response rate are comparable across components. Finally, the present study demonstrates

that the delayed effects of sublethal doses of gamma radiation on learning, reported in an earlier repeated-acquisition study (Winsauer and Mele, 1993), occur in the context of multiple behaviors.

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