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RADIATION RESEARCH 130, 61–64 (1992)



# Short and Long Courses of Ofloxacin Therapy of *Klebsiella pneumoniae* Sepsis following Irradiation

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BROOK, I., AND LEDNEY, G. D. Short and Long Courses of Ofloxacin Therapy of *Klebsiella pneumoniae* Sepsis following Irradiation. *Radiat. Res.* 130, 61–64 (1992).

Exposure to whole-body irradiation is associated with fatal gram-negative sepsis. The optimal length of therapy of such infection is not established. The effect of short and long courses of oral therapy with the quinolone ofloxacin for orally acquired *Klebsiella pneumoniae* infection was tested in B6D2F$_1$ mice exposed to 8.0 Gy of bilateral radiation from $^{60}$Co. A dose of $10^8$ organisms was given orally 4 days after irradiation, and therapy was started 1 day later. Cultures of the ileum 7 days after irradiation showed the recovery of *K. pneumoniae* in 7 of 10 untreated mice and in 3 of 20 treated with ofloxacin. However, 14 days after irradiation *K. pneumoniae* was isolated in 5 of 6 untreated mice, in 7 of 9 that received the short course of therapy, and in one of those that received the long course of therapy ($P < 0.05$). At Day 7, *K. pneumoniae* was isolated from the livers of 6 of 10 untreated mice, and from none of those receiving ofloxacin ($P < 0.05$). At 14 days, *K. pneumoniae* was isolated in 4 of 6 untreated animals, in 4 of 9 that received the short course of therapy, and in none of the mice that received the long course of therapy ($P < 0.05$). Only 3 of 20 (15%) untreated mice survived for 30 days as compared to 11 of 20 (55%) mice treated for 7 days with ofloxacin and 18 of 20 (90%) mice treated for 21 days with ofloxacin ($P < 0.05$). These survival data illustrate the efficacy of a 21-day course over a 7-day course of ofloxacin therapy for orally acquired *K. pneumoniae* infection in irradiated hosts. © 1992 Academic Press, Inc.

## INTRODUCTION

Exposure to ionizing radiation enhances the host's susceptibility to systemic infection due to endogenous and exogenous organisms (*1*, *2*). A possible source of endogenous infection in irradiated hosts is the gastrointestinal tract (*2*), which is colonized by aerobic and anaerobic organisms. Following irradiation, members of that flora may translocate to the liver and spleen, and thereafter can be associated with fatal septicemia (2, 3). The most important bacterial species isolated from septic animals are gram-negative enteric bacteria (*2*, *3*). *Klebsiella pneumoniae* is frequently linked to death from sepsis (*4*, *5*) and is especially prevalent in immunocompromised patients (*6*). In a preliminary report it was shown experimentally that the prevention of translocation of these organisms and the control of sepsis can reduce mortality.[1]

We found the quinolone antibiotics to be efficacious in reducing systemic infection due to *K. pneumoniae* following irradiation and oral feeding with *K. pneumoniae* (*7*, *8*). The efficacy of these antibiotics may be due to their selective ability to eradicate *Enterobacteriaceae* while preserving the anaerobic gut flora (*8*). However, animal mortality was not completely prevented and the duration of quinolone therapy necessary to eliminate the bacteria was not determined in these studies.

This study was designed to evaluate the optimal length of therapy required to treat irradiated mice that develop septicemia due to orally ingested *K. pneumoniae*.

## MATERIALS AND METHODS

### *Animals*

Female B6D2F1 mice approximately 12 weeks old were obtained from the Jackson Laboratory (Bar Harbor, ME). All animals were kept in quarantine for about 2 weeks before being transferred to a room with a 12-h (6 AM to 6 PM) light–dark cycle. Representative samples were examined to ensure the absence of specific bacteria and common murine diseases. Animals were maintained in a facility accredited by the American Association for Accreditation of Laboratory Animal Care, in Micro-Isolator cages on hardwood chip bedding and were provided commercial rodent chow and acidified water (pH 2.2) that was changed to tap water 48 h before irradiation. All experimental procedures were done in compliance with both the National Institute of Health and the Armed Forces Radiobiology Research Institute (AFRRI) guidelines regarding animal use and care.

### *$^{60}$Co Irradiation*

Mice were placed in Plexiglas restrainers and given a whole-body dose of 8.0 Gy from a $^{60}$Co source at 0.4 Gy/min. The dose is an $LD_{60/30}$ for B6D2F$_1$ female mice. Before the experiment, the dose rate at the midline of an acrylic mouse phantom was measured using a 0.5-cc tissue-equivalent ion-

[1] Brook and T. B. Elliott, "Pefloxacin Therapy in the Prevention of Mortality after Irradiation." Presented at the 16th International Congress of Chemotherapy, Jerusalem, Israel, 1989.



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ization chamber manufactured by Exradin, Inc. (Chicago, IL). The dose rate at the same location with the phantom removed was then measured using a 50-cc ionization chamber manufactured at AFRRI. The ratio of these two dose rates, the tissue-air ratio, was then used to determine the animal doses for routine experimental procedures. In this experiment the tissue-air ratio was 0.98.

All ionization chambers used have calibration factors traceable to the National Institute of Standards and Technology. The dosimetry measurements were performed following the AAPM Task Group 21 Protocol for the Determination of the Absorbed Dose from High-Energy Photon and Electron Beams (*9*).

### *Bacteria*

The strain used in this study was a human clinical isolate of *K. pneumoniae* with a type 5 capsule (AFRRI No. 7). The organisms were harvested in the logarithmic phase of growth in Bacto Brain Heart Infusion Agar (Difco, Detroit, MI). A concentration of $10^9$ organisms/1 ml saline was prepared, and a volume of 0.1 ml was fed to each animal by using a 20-gauge animal feeding tube fitted to a 1.0-ml syringe. We used this number of organisms because ingestion of fewer bacteria did not produce mortality in the animals.

### *Antimicrobials*

Ofloxacin was obtained from Ortho Pharmaceutical Corp. (Raritan, NJ) and was given once every 24 h in a dose of 40 mg/kg. Standard powder formulations with known potencies were used for *in vitro* and *in vivo* studies. Ofloxacin was given by gavage in a volume of 0.1 ml sterile saline. All control animals received 0.1 ml sterile saline by gavage.

### *Antimicrobial Serum Concentration*

Serum concentrations of the antimicrobials were determined in six irradiated mice each 1 and 23.5 h after the administration of the antimicrobials on the fifth day of therapy. *Bacillus subtilis* ATCC 6633 was used as a test organism, and Mueller–Hinton agar (pH 7.4) was used as a test agar. This method could not detect serum concentrations below 0.2 μg/ml.

### *Microbiological Methods*

Mice were observed for mortality and symptoms of disease for 30 days. Ten animals were selected at random from each group on Days 7 and 14 following irradiation. When fewer than 10 animals survived in a group, all were studied at that day. Animals were killed by cervical dislocation. Specimens of liver and ileum were processed for the presence of bacteria. No other organs were processed and no blood samples were obtained, because previous studies showed that liver cultures correlated best with sepsis (*2*). The livers were removed aseptically and homogenized immediately. The ileum was opened, and ileal content samples were obtained using a swab. The liver and stool specimens were swabbed onto blood and MacConkey agars, and the organisms were identified using conventional methods (*10*).

### *Experimental Design*

Each mouse was fed $10^8$ *K. pneumoniae* organisms 4 days after irradiation. Antimicrobial therapy was initiated 5 days after irradiation, and was administered orally for either 7 or 21 days. Survival experiments were performed three times with 60 mice, 20 mice for each group in each experiment. Microbial analysis of the liver and the ileal contents for bacteria colonization was done only twice with 75 mice, 25 mice for each group in each experiment. Each survival and microbial analysis experiment consisted of two antibiotic therapy groups and the untreated control group. The first group of antibiotic-treated mice received ofloxacin for 7 days (short course), the second group was given ofloxacin for 21 days (long course) and the third group was given sterile saline for 21 days.

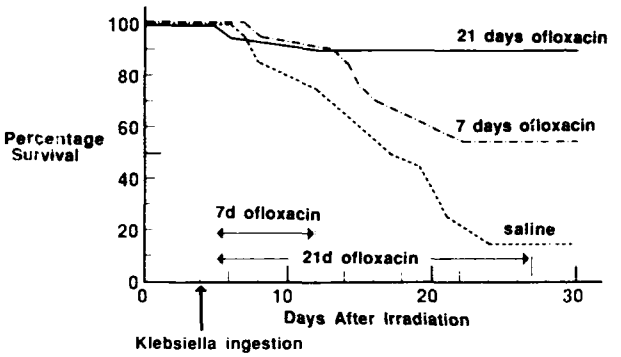


FIG. 1. Survival of 60 B6D2F1 female mice irradiated with 8.0 Gy of $^{60}$Co γ rays, fed with $10^8$ *K. pneumoniae*, and treated with ofloxacin. Twenty mice were included in each group. The data represent one experiment. A total of three experiments showed similar results; see text.

Samples of liver and ileum were obtained 7 and 14 days after irradiation. These sampling times correlated with mice receiving either two oral ofloxacin doses from the short course group or nine oral ofloxacin doses from the long course group. By 14 days after irradiation, mice on the short treatment schedule had not received ofloxacin for 2 days.

### *Statistical Methods*

Statistical analyses were done using the Cox–Mantel test (*11*).

## RESULTS

### *Mortality*

Mortality in the groups that received ofloxacin was significantly less in all three experiments ($P < 0.05$) than in the water-treated control groups. A further significant increase in survival was noted in all three experiments in the animals treated for 21 days compared to 7 days. The survival after 30 days in the first experiment (Fig. 1) was 3 of 20 (15%) of the water-treated control mice, 11 of 20 (55%) of the animals treated with ofloxacin for 7 days, and 18 of 20 (90%) of the mice treated for 21 days. In the second experiment, 4 of 20 (20%) water-treated control mice 10 of the 20 (50%) mice treated for 7 days with ofloxacin, and 17 of 20 (85%) mice treated for 21 days with ofloxacin survived. In the third experiment, 5 of 20 (25%) water-treated control mice, 12 of 20 (60%) mice treated for 7 days with ofloxacin, and 18 of 20 (90%) mice treated for 21 days with ofloxacin survived.

### *Isolation of Organisms in Liver*

In the first experiment, at Day 7 after irradiation, *K. pneumoniae* was isolated from the livers of 6 of 10 (60%) randomly selected control animals, and in none of those receiving ofloxacin ($P < 0.05$) (Table I). At day 14, *K. pneumoniae* was recovered in 4 of 6 (67%) water-treated animals, in 4 of 9 (45%) of those that received the short course

**TABLE I**
**Recovery of *K. pneumoniae* from Liver and Ileum of B6D2F$_1$ Mice Irradiated with 8.0 Gy $^{60}$Co $\gamma$ Rays**

| | Liver | | Ileum | |
|---|---|---|---|---|
| | Days after irradiation | | Days after irradiation | |
| Therapy group | Day 7[a] | Day 14[b] | Day 7[a] | Day 14[b] |
| First experiment | | | | |
| Control (water) | 6/10 | 4/6 | 7/10 | 5/6 |
| Short course | 0/10 | 4/9 | 2/10 | 7/9 |
| Long course | 0/10 | 0/10 | 1/10 | 1/10 |
| Second experiment | | | | |
| Control (water) | 5/10 | 4/7 | 5/10 | 4/8 |
| Short course | 0/10 | 3/8 | 0/10 | 3/5 |
| Long course | 0/10 | 0/10 | 0/10 | 0/10 |

[a] By Day 7, animals in both the short- and long-course therapy groups had received 2 days of treatment with ofloxacin.

[b] By Day 14, animals in the short-course group had been treated for 7 days; animals in the long-course group had been treated for 9 days.

of therapy, and in none of those that received the long course ($P < 0.05$).

In the second experiment, *K. pneumoniae* was recovered from the livers of 5 of 10 (50%) control animals, and in none of those receiving ofloxacin ($P < 0.05$). At Day 14, *K. pneumoniae* was isolated in 4 of 7 (57%) water-treated animals, in 3 of 8 (37%) of those that received the short course of therapy, and in none of those receiving the long course.

*Isolation of Organisms in the Ileum*

In the first experiment, at Day 7 after irradiation, *K. pneumoniae* was recovered in 7 of 10 (70%) water-treated mice and in 3 of 20 (15%) mice treated with ofloxacin ($P < 0.05$) (Table I). At 14 days after irradiation, *K. pneumoniae* was isolated in 5 of 6 (83%) water-treated mice, in 7 of 9 (78%) that received the short course of therapy, and in one of those that received the long course ($P < 0.05$).

In the second experiment, at Day 7, *K. pneumoniae* was isolated in 5 of 10 (50%) water-treated mice and in none of those treated with ofloxacin ($P < 0.05$). At Day 14, the organism was recovered in 4 of 8 (50%) water-treated mice, in 3 of 5 (60%) mice that received the short course of therapy, and in none of those that received the long course.

*Antibiotic Serum Concentration*

The mean serum concentrations of ofloxacin were 2.4 ± 0.3 μg/ml at 1 h and 0.4 ± 0.2 μg/ml at 23.5 h.

## DISCUSSION

The results demonstrate that a quinolone such as ofloxacin can reduce the colonization of the ileum and the development of subsequent septicemia with *K. pneumoniae* in $\gamma$-photon-irradiated mice. The results support the findings of Trautmann *et al.* (*12*), who observed the efficacy of ciprofloxacin in the management of systemic *K. pneumoniae* infection in neutropenic mice.

We have developed a model of acquired *K. pneumoniae* infection in irradiated mice that may represent the mode of invasion of external pathogens into an irradiated host. We showed previously that irradiated mice develop fatal septicemia due to orally administered *P. aeruginosa* (*13*). We also observed that the number of endogenous gastrointestinal tract aerobic and anaerobic bacteria declined 24 h following irradiation and that the decline was maximal at 7 days after irradiation (*14*). The decrease in the number of endogenous bacteria in the gut may make the host more susceptible to acquisition of external pathogens such as *K. pneumoniae*.

The ability of *K. pneumoniae* to cause systemic infection in irradiated mice may be due to the following factors: (1) the bacterial void created in the gut following the decrease in the number of endogenous organisms, (2) the increased permeability of the mucosal cells damaged by irradiation, and (3) the diminution of the local and systemic immunity.

The effectiveness of quinolones in the therapy of *K. pneumoniae* infection may be attributed to local inhibition of the organism's growth within the gut lumen, while preserving the anaerobic gut flora (*15*), and to their systemic antibacterial activity to prevent the infection from spreading to other sites within the body. We found that the optimum duration of quinolone therapy is a prolonged one that would provide adequate coverage against the offending organism for at least 21 days. The superior efficacy of a 21-day course of therapy over a shorter course may be due to the need to provide adequate therapy until the immune system recovers, and granulocytes are present in the circulation (*16*). Although a complete recovery of the granulocytes requires up to 6 weeks at this dose of radiation, a sufficient number of them may be present after 3 weeks to eradicate the *K. pneumoniae* infection completely (*16*). Immunomodulators such as synthetic trehalose dicorynomycolate (*16*), glucan (*17*), and colony-stimulating factor (*18*) may facilitate this process.

Selective decontamination of the gut with orally administered quinolones is used to prevent sepsis in immunocompromised hosts (*8, 15*). These agents were also found to be effective in the management of septic episodes in neutropenic patients (*19*). The availability of an oral route of administration, the advantage of achieving selective inhibition of potential pathogens in the gut, and the ability to treat systemic infection make the quinolones promising agents for oral therapy of orally acquired *K. pneumoniae* infection in irradiated hosts. Although the exact length of therapy with quinolones is yet to be determined, and may be shorter than 21 days, therapy with quinolones should be

idministered for a sufficient time, which will provide extended coverage until recovery of the immune system occurs.

## ACKNOWLEDGMENTS

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RADIATION RESEARCH 130, 88-93 (1992)



# Possible "Accelerated Striatal Aging" Induced by $^{56}$Fe Heavy-Particle Irradiation: Implications for Manned Space Flights

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JOSEPH, J. A., HUNT, W. A., RABIN, B. M., AND DALTON, T. K. Possible "Accelerated Striatal Aging" Induced by $^{56}$Fe Heavy-Particle Irradiation: Implications for Manned Space Flights. *Radiat. Res.* 130, 88-93 (1992).

The present experiments were carried out to determine the effects of energy deposition from energetic iron ($^{56}$Fe particles, an important component of cosmic rays) on motor behavioral performance and to determine if the observed deficits were caused by alterations in the neostriatum (an important motor control area). Neostriatal function was assessed with two correlated parameters, i.e., motor behavioral performance (wire suspension task), and oxotremorine-enhanced $K^+$-evoked release of dopamine from perifused striatal slices. Rats were exposed to one of several doses of $^{56}$Fe-particle irradiation (0.10–1.0 Gy) and tested on a wire suspension task at 3–180 days postirradiation. Results indicated that profound decrements occurred in both of these indices. The effects on $K^+$-evoked release of dopamine were evident for as long as 180 days after irradiation, and a subsequent experiment indicated that these effects appeared as early as 12 h postirradiation. Since similar findings have been observed in aged rats, the results are discussed in terms of these particles producing a possible accelerated striatal aging effect. 

## INTRODUCTION

It is well known that future astronauts on long-term space flights will be exposed to various types of radiation. Although the effects of factors such as weightlessness have been addressed extensively during previous orbital missions, the potential health hazards of long-term exposure to radiation in space have not been examined sufficiently with respect to brain and behavior (*1*). For example, the putative effects of radiation on behavioral performance have not been addressed. Exposure to particles of high charge and energy (HZE) could occur during major solar events in which the flux of heavy particles can increase rapidly by three or four orders of magnitude above the galactic cosmic-ray background (*2*). Also, heavy charged particles, with energies to 10 GeV/nucleon, constitute about 1% of galactic cosmic radiation. It has proven to be difficult to shield against these heavy charged particles. Curtis and Wilkinson (*3*) calculated that as much as 10 g/cm$^2$ of aluminum reduces the absorbed dose of heavy particles by only 20–40%. While a great deal of information has been generated about the mechanisms of the damage caused by HZE particles and its repair (*4*), few experiments have examined the early and late effects of these particles on motor behavior. The potential for HZE particles to alter central neuronal functioning and behavioral performance becomes increasingly important when one considers that space travelers, especially those performing tasks outside the shelter of a space vehicle, may be exposed to HZE particles that can create microscopic lesions in virtually all organs of the body [see (*5*) for review]. Moreover, additional studies showed that (a) mice given brief exposure to low doses of $^{40}$Ar particles showed time-dependent reductions in performance of a wire suspension task (*6*), and (b) rats acquired a conditioned taste aversion (a general measure of behavioral toxicity) after doses as low as 0.1–0.2 Gy of $^{56}$Fe particles (*7*). If one can extrapolate from these studies as well as those in which organisms were exposed to other types of radiation and examined for changes in behavioral performance, it could be postulated that decrements in indices such as motor behavior are a distinct possibility. Several studies indicate that motor performance of irradiated animals declines when they perform tasks requiring physical strength, endurance, and coordination (*8–11*). These studies generally used a variety of radiation sources, ranging from mixed neutrons/$\gamma$ rays (*11*) to high-energy electrons (*10*). The precise central locus of these deficits is still unknown. However, studies of nonirradiated organisms suggest that the nigrostriatal system may be important in mediating motor behavior.

The telencephalic end terminus of this system, i.e., the striatum, is one of the basic central processing areas involved in mediating motor behavior. This structure appears to control a variety of motor responses, ranging from the simple, balance and coordination (*12–14*), to the complex,

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e ordering and sequencing of intricate behavioral patterns rected by exteroceptive stimuli (*15, 16*). The neurotransitter dopamine (DA) appears to be very important in the gulation of these behaviors. The disruption of the striatal A system using pharmacological blockade (*17*) or lesions 8, *19*) results in a variety of motor impairments (e.g., loss motor coordination). Additionally, aged organisms, nich show profound reductions in striatal DA functiong, are also similarly impaired [see (*14*) for review].

At least one parameter of DA functioning has been own in previous experiments to be compromised followg irradiation, since exposure to 5.0 Gy of $^{56}$Fe particles ppears to reduce concentrations of striatal 3-methoxytyraine, an indicator of dopamine activity (*20*). A second imrtant indicator of striatal DA integrity can be assessed by amining DA release. Release of DA under the control of a oup of inhibitory muscarinic cholinergic heteroreceptors also important for regulating DA release (*21–23*). Muscanic agonists, such as oxotremorine, can activate the hetoreceptors, which potentiate the $K^+$-evoked DA release om perifused striatal slices (*21, 22, 24*). The present studs were conducted to determine if we could observe an ZE-particle-radiation-induced reduction in sensitivity of uscarinic cholinergic heteroreceptors to an applied muscanic agonist that would result in reduced striatal DA release id deficits in motor performance. These determinations ere made by irradiating rats with $^{56}$Fe particles and relatg the degree of change in motor behavioral performance any deficits in muscarinic enhancement of the $K^+$oked DA release from perifused striatal slices.

## MATERIALS AND METHODS

### imals

Male Sprague–Dawley Crl:CD(SD)BR rats (Charles River Laboratories, ngston, NY) weighing 200–300 g were used in these experiments. The s were housed at the vivarium at the Lawrence Berkeley Laboratory BL), Berkeley, California. The rats were maintained in polycarbonate ges that contained autoclaved hardwood contact bedding (Beta Chip rtheastern Products Corp., Warrensburg, NY). They were given food d water *ad libitum*. The animal holding rooms were kept at 21 ± 1°C th 50 ± 10% humidity.

### ysimetry

The rats were exposed to whole-body irradiation with high-energy $^{56}$Fe rticles (600 MeV/amu) in the BEVALAC at the LBL. In each experint rats were irradiated one at a time. This energy provided a Bragg curve th the plateau region extending 8 cm in water. Since the diameter of the (including the plastic restrainer) was 7 cm the animals were within this teau region of the curve. Entrance dose measurements were made by staff of the BEVALAC facility using parallel plate ionization chambers th Mylar windows and $N_2$ gas flow positioned in the beam line (*25, 26*). e rats were irradiated in well-ventilated Plexiglas holders. In experiment rats were given one of four doses (0.10, 0.25, 0.50, or 1.0 Gy) at a dose e that averaged 1 Gy/min. In experiment B, rats were given one dose 0 Gy). The specified doses were received by all organs. Control rats were en sham exposure, placed in the plastic restrainer for the same amount time as irradiated rats.

### *Procedure, Experiment A*

All behavioral and biochemical (see below) assessments were carried out at 3, 8, or 14 days for groups either irradiated with 0.10, 0.25, 0.50, or 1.0 Gy $^{56}$Fe particles or sham-irradiated. Numbers of rats assessed at each day were as follows: for sham-irradiated rats, 12 rats at 3 days, 10 rats at 8 days, and 10 rats at 14 days; for rats irradiated with 0.10, 0.25, or 1.0 Gy, 10 rats each day; for rats irradiated with 0.50 Gy, 18 rats each day. Also, 10 rats exposed to 0.50 Gy, 10 rats exposed to 1.00 Gy, and 18 sham-irradiated control rats were assessed 180 days postirradiation. Half of each group was tested in the morning, and the other half was tested in the afternoon.

*Behavior.* Previous research has suggested that one of the most sensitive motor behavioral tests for assessing decrements in striatal DA function is wire suspension (*27*). This test measures muscle strength by examining an animal's ability to grasp a horizontal wire with its forepaws and remain suspended. In this experiment each rat was raised to an elevated hanging wire (58 cm above the table top) and given one trial, with total hanging time (in s) recorded.

*Muscarinic control of dopamine release.* Following the behavioral tests, the animals were killed by decapitation; their brains were quickly removed, and the striata were dissected on ice.

1. Cross-cut striatal slices (300 μm) were prepared using a McIlwain tissue chopper, and the slices from each animal were placed into a small glass vial which contained a modified Krebs–Ringer basal release medium containing 21 m*M* $NaHCO_3$, 3.4 m*M* glucose, 1.3 m*M* $NaH_2PO_4$, 1 m*M* EGTA, 0.93 m*M* $MgCl_2$, 127 m*M* NaCl, and 2.5 m*M* KCl (pH 7.4). The medium had been bubbled for 30 min with 95% $O_2$/5% $CO_2$.
2. Slices from each vial were washed twice in this medium and aliquots were placed into two chambers of a perifusion apparatus. Thus the cross-cut slices from each animal went into a "pair" of perifusion chambers.
3. After being placed in the chambers, the tissue was allowed to equilibrate for 30 min while being continuously perifused with basal release medium at the rate of 124 μl/min. Gillson peristaltic pumps controlled the flow rate of the medium.
4. After the equilibration period, a 5-min baseline fraction was collected on ice.
5. The tissue was then exposed to a "HiKCl" (release) medium which contained 30 m*M* KCl, 1.26 m*M* $CaCl_2$, and 57 m*M* NaCl, as well as the other components described above (pH 7.4). One chamber of the pair for each animal received 500 μ*M* oxotremorine in this release medium, and the other chamber received only the release medium. Five-minute fractions continued to be collected on ice for 30 min. The fractions were collected into tubes containing 0.3 ml of cold 0.4 *N* perchloric acid, 0.05% sodium metabisulfite, and 0.10% EDTA.
6. These samples were then stored at −80°C for later DA analysis using high-performance liquid chromatography (HPLC) coupled to electrochemical detection.

The HPLC system consisted of a Varian Model 5000 ternary chromatograph, a Varian 401 data system, a Varian Model 8055 autosampler, and a Valco air-actuated injector with a 50 μl loop. The effluent was monitored with a bioanalytical systems LC-4B amperometric detector using a glassy carbon electrode. The detector potential was set at 0.72 V as a $Ag/AgCl_2$ reference electrode with a sensitivity of 10 Na/V. The mobile phase consisted of a filtered, degassed 100 m*M* $KH_2PO_4$ buffer containing 3 m*M* 1-heptanesulfonic acid, 100 μ*M* EDTA, and 8% (v/v) acetonitrile (pH 3.6). The components were eluted off a Waters 10-μm particle, μBondapak C18 reverse-phase column (30 cm × 0.39 cm; flow rate = 1 ml/min) maintained at 30°C. Results were calculated relative to known previous standards that were analyzed by HPLC under similar conditions. Data were expressed as pmol/mg protein as analyzed by the Lowry procedure (*28*).

*Data analysis.* Data from the behavioral experiments were analyzed by using five-dose (0, 0.10, 0.25, 0.50, or 1.0 Gy) by day (3, 8, or 14 days) analyses of variance. Data from the perifusion experiments were analyzed by first computing peak difference scores by subtracting the picomoles of DA released to 0 μ*M* oxotremorine from that released to 500 μ*M* for each fraction and for each "striatal-channel" pair. These differences scores were

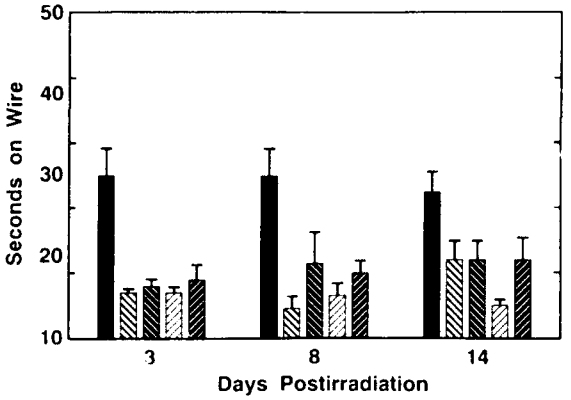


**FIG. 1.** The effects of exposure to graded doses of $^{56}$Fe-particle radiation on the wire suspension time of 200–300 g Sprague–Dawley rats are illustrated. Each rat was tested once, and sham-irradiated and irradiated groups were tested at 3, 8, and 14 days postirradiation. Each rat was placed on a wire suspended 54 cm above a table top, and the total time(s) that it remained suspended was recorded. All of the radiation groups significantly differed from the controls. Only the 0.25- and 0.50-Gy and the 0.50- and 1.0-Gy groups differed from each other (see Results). (■) Control, (▧) 0.10 Gy, (▨) 0.25 Gy, (▨) 0.50 Gy, (■) 1.0 Gy.

then analyzed by five dose (0, 0.10, 0.25, 0.50, or 1.0 Gy) by three-way analysis of variance and post hoc $t$ tests. Data from the 180-day animals were analyzed by a separate analysis of variance.

*Procedure, Experiment B*

This experiment examined the short-term effects of this exposure to $^{56}$Fe particles. One dose was used (1 Gy) and behavioral and biochemical procedures were carried out as described in experiment A. These parameters were assessed at 12 and 36 h after irradiation or sham irradiation. Times intermediate with those in experiment A were also used (60 and 84 h) to test the reliability of the results obtained in experiment A. Twelve irradiated rats and four sham-irradiated control rats were assessed at each of the four times (12, 36, 60, and 84 h). Data were analyzed as described in experiment A. Peak release differences between the oxotremorine-treated and untreated channels were computed for each rat and a one-way analysis of variance with post hoc $t$ tests was computed.

## RESULTS

### *Experiment A*

*Behavior.* The results of the wire suspension task are illustrated in Fig. 1, which shows that exposure to $^{56}$Fe particles induced deficits in performance. Irradiated rats remained suspended on the wire for a shorter time than sham-irradiated rats [$F(4, 159) = 39.46$, $P < 0.001$]. There were, however, no differences across days. The effects of $^{56}$Fe particles were the same at 3, 8, and 14 days postirradiation [days × radiation dose $F(8, 159) = 1.07$, $P > 0.05$]. Subsequent post hoc $t$ tests carried out for all days indicated that all of the irradiation groups differed significantly from the sham-irradiated groups [e.g., controls vs 0.10 Gy; $t(58) = 13.78$, $P < 0.001$]. Comparisons among the various irradiated groups indicated that the only overall differences were between the 0.25- and 0.50-Gy groups [$t(84) = 2.50$, $P < 0.01$] and the 0.50- and 1.00-Gy groups [$t(84) = 2.57$, $P < 0.01$].

*Release of DA.* Figure 2 shows that enhancement of the $K^+$-evoked release of DA paralleled the deficits observed in the wire suspension test. This enhancement was reduced in all the irradiated groups [$F(4, 161) = 12.50$, $P < 0.0001$], and there were no differences among days [$F(8, 161)$, $P < 1$]. The data from each group were pooled for all days and post hoc $t$ groups differed from all irradiated groups [e.g., sham vs 0.10 Gy, $t(60)$ 2.55, $P < 0.01$]. Additional tests indicated that the 0.10-Gy group differed from the 0.50-Gy group [$t(82) = 3.41$, $P < 0.01$] and the 1.00-Gy group [$t(58) = 3.11$, $P < 0.01$].

*Release of DA 180 days postirradiation.* The deficits in the oxotremorine enhancement of $K^+$-evoked release of DA were also seen 180 days after irradiation [$F(3, 44) = 19.23$, $P < 0.001$] (Fig. 2). Subsequent post hoc $t$ tests indicated that both irradiated groups had a lower enhanced release of DA than sham-irradiated groups [sham-irradiated vs 0.50 Gy: $t(26) = 3.34$, $P < 0.01$; sham-irradiated vs 1.00 Gy: $t(26) = 2.84$, $P < 0.01$], but did not differ from each other ($t < 1$). Large weight increases in all three groups precluded any behavioral tests on the wire suspension task in these animals.

### *Experiment B*

Because no differences were seen among days in experiment A, we surmised that the radiation effects occurred

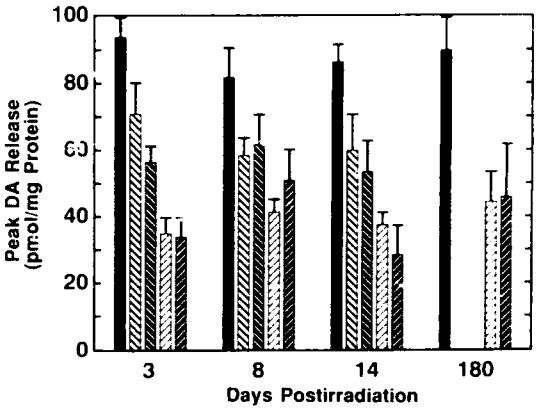


**FIG. 2.** The effects of exposure to graded doses of $^{56}$Fe-particle radiation on oxotremorine enhancement of $K^+$-evoked release of DA from perifused striatal tissue slices (see Materials and Methods). The figure illustrates the peak diffrence scores for each group. These scores were obtained by subtracting the DA release value (pmol/mg protein) under the 30 m*M* KCl conditions from the DA release value obtained under oxotremorine conditions for each rat and computing the means. The far right portion of the figure illustrates the long-term effects of exposure to 0.50 or 1.00 Gy of $^{56}$Fe-particle radiation on oxotremorine-enhanced striatal DA release. Enhancement of $K^+$-evoked DA release was significantly reduced in all the irradiated groups regardless of the number of days postirradiation (see Results). (■) Control, (▧) 0.10 Gy, (▨) 0.25 Gy, (▨) 0.50 Gy, (■) 1.0 Gy.

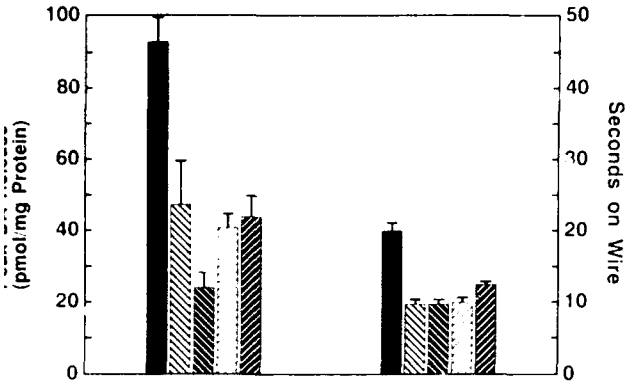


FIG. 3. The short-term effects of exposure to graded doses of $^{56}$Fe-particle radiation on striatal DA release (left) and wire suspension time (right). Data were collected and analyzed as described under Materials and Methods except that these parameters were assessed at (▨) 12, (▩) 24, (▨) 60, and (▩) 84 h after exposure and compared to data from sham-irradiated animals (■). As the figure shows, the radiation effects on both wire suspension and oxotremorine-enhanced DA release were observed as early as 12 h postirradiation, and the maximal effects on the latter parameter were seen at 36 h postirradiation. No differential effects of postirradiation time were observed on wire suspension performance.

fore the initial test at 3 days. Therefore, experiment B was carried out to determine possible radiation effects at earlier times.

*Behavior.* The behavioral analysis indicated that irradiation significantly decreased the time that the animals remained suspended on the wire [$F(4, 57) = 22.22$, $P < 0.001$] (Fig. 3, right). Subsequent post hoc $t$ tests indicated that the radiation effects began as early as 12 h postirradiation and that all the irradiated groups differed significantly from the sham-irradiated groups. No differences were seen among the various treated groups (e.g., 12 h vs 36 h, $t = 0.08$, $df = 24$, $P > 0.05$).

*Release of DA.* The results from the assessments of DA release paralleled behavioral determinations. Radiation significantly decreased $K^+$-evoked release of DA [$F(4, 57) = 5.58$, $P < 0.001$] (Fig. 3, left). As with the behavioral data, subsequent $t$ tests indicated that the effects of radiation on DA release were seen as early as 12 h postirradiation and that all irradiated groups differed significantly from the sham-irradiated group: (e.g., sham-irradiated vs 12 h = 3.16, $df = 26$, $P < 0.001$). In addition the 36-h irradiated group was significantly lower than all other irradiated groups (e.g., 12 h vs 36 h, $t = 4.34$, $df = 22$, $P < 0.05$).

## DISCUSSION

These experiments indicate that exposure to $^{56}$Fe-particle radiation effectively decreases the responsiveness of muscarinic cholinergic heteroreceptors to oxotremorine. Radiation doses ranging from 0.10 to 1.0 Gy were almost equally effective in reducing $K^+$-evoked release of DA to agonist stimulation. Moreover, these changes occurred in concert with decrements in motor performance, as assessed in the wire suspension test. There was not, however, a one-to-one relationship between these two parameters. It appeared that the effects on behavior were relatively independent of dose (at least within the range of doses employed here), while the effects of radiation on oxotremorine-enhanced $K^+$-evoked release of DA varied with the dose and postirradiation times. These findings suggest that there may be a threshold for muscarinic cholinergic heteroreceptor–DA responsiveness. Once this responsiveness falls below a certain level, behavior is disrupted.

Additional determinations indicated that lowered muscarinic cholinergic heteroreceptor responsiveness and motor behavioral decrements occurred as early as 12 h after irradiation. Once the decrease in muscarinic cholinergic heteroreceptor responsiveness occurred, it appeared to last as long as 180 days in the animals irradiated with 0.50 and 1.0 Gy.

When one considers that space travelers on long-term space flights may be exposed to $^{56}$Fe particles, these findings indicate that some motor impairment and decreased responsiveness of muscarinic cholinergic heteroreceptors may result from these exposures. One could speculate that, since the radiation was administered in very concentrated doses over a short period of time, the effects may be unrelated to the possible consequences that might occur from long-term exposure in space. However, there is an important consideration that should be mentioned in this regard. Although the mechanism of the $^{56}$Fe-particle-induced changes is presently being investigated, it could be hypothesized that these alterations are brought about by free-radical damage to the striatum. If this is the case, then there should be parallel observations in nonirradiated animals in which there is putative damage to the striatum from free radicals. One group of organisms in which damage from free radicals has taken its toll on the function of the central nervous system (CNS) includes those that are senescent. It is believed that life-span effects of free radicals produced during normal metabolism are responsible for the alterations in membrane structure and function seen in senescence (*29*). In the CNS, the differences in transbilayer fluidity induced by peroxidation may be intimately involved in factors such as loss of neurotransmitter receptor function (*30*). Striatal and motor behavioral deficits similar to those found in the present experiments have been reported to occur as a function of aging. These experiments have indicated that there are age-related deficits in balance, strength, and coordination as assessed with the wire suspension task and other motor behavioral tasks (*14, 31*). Moreover, these alterations occur in concert with specific striatal changes, such as decreases in tyrosine hydroxylase activity (*32*), striatal $D_2$ concentrations (*33–36*), and the concentration of muscarinic cholinergic heteroreceptors (*36, 37*). Additional experiments indicate that increases in $K^+$-evoked release of DA was reduced in response to the application of several differ-

ent muscarinic cholinergic heteroreceptor agonists, including oxotremorine, in the striatal slices obtained from senescent (24-month-old) animals. This reduction appears to be specific for muscarinic cholinergic heteroreceptors, since direct enhancement of $K^+$-evoked release of DA through inhibition of the dopamine $D^2$ receptor or stimulation of the nicotinic receptor reveals no age-related deficits (*38*). A finding of particular relevance in these experiments is that the loss in responsiveness of muscarinic cholinergic heteroreceptors to some agonists appeared in striatal tissue obtained from middle-aged rats [12 months old (*38*)]. In fact, changes in several important striatal parameters appear in 12-month-old animals [e.g., loss of DA receptors and decreased adenylate cyclase activity (*31*)] and in middle-aged humans [e.g., decreases in striatal tyrosine hydroxylase activity (*32*, *39*)].

If one postulates that at least some subset of those who are chosen for long-term space flights were middle-aged or approaching middle age, then these heavy particles could impinge upon areas of the brain, such as the striatum, that are already experiencing the effects of aging, and the effects of these two perturbations may be synergistic.

There is one other consideration that is important in this regard. If exposure to heavy particles reduces sensitivity of muscarinic cholinergic heteroreceptors, then deficits in cognitive performance could occur as well. The proper functioning of the muscarinic cholinergic systems, especially in brain areas such as the hippocampus, has long been recognized as being critical to cognitive performance (*40*). The hippocampus shows several age-related functional alterations [e.g., reduced sensitivity to basal and stimulated ACh release (*41–43*)] that accompany these cognitive deficits.

Similarly, Philpott and his associates (*7*) found that both the synaptic density and the synaptic spine length in the CA-1 area of the hippocampus were lower in mice irradiated with 0.005 or 0.5 Gy than in controls. These morphological assessments were made at 6 and 12 months after irradiation. The effects of $^{56}Fe$ on cognitive performance and hippocampal functioning in rodents are presently being examined. Although many experiments using different doses, times, species, radiation sources, and other factors must still be done, the extant findings suggest that under the right conditions long periods in space could accelerate the aging process and attendant motor and cognitive problems.

Finally, these experiments provide additional support for the "age–radiation parallel" hypothesis, which has been suggested for over 30 years. These studies suggest that radiation may have a life-shortening effect (*46*) and may change biochemical (*45*, *47*) and cellular (*46*) parameters. Indeed, one study (*48*) indicated that radiation exposure ($^{60}Co$ $\gamma$-ray source) enhanced the accumulation of lipofuscin (the age pigment) in the brain, heart, and intestine of mice. Increases in lipid peroxidation in the liver, and in the activity of acid phosphatases and cathepsin accompanied these alterations. All of these changes are associated with free-radical damage and are similar to those that occur during aging (*49*), and they further support the hypothesis that changes induced by aging and by radiation may share a common chemical/biochemical mechanism.

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Chapter 13

# Modulation of Mortality by Tissue Trauma and Sepsis in Mice after Radiation Injury

G. DAVID LEDNEY, THOMAS B. ELLIOTT, AND MARCUS M. MOORE

## ABSTRACT

The nuclear disasters at Hiroshima, Nagasaki, and Chernobyl underscore the need for useful animal models to (*a*) evaluate the combined effects of radiation and tissue trauma and (*b*) develop successful therapeutic modalities for sepsis in irradiated individuals inflicted with tissue trauma. In mice, mortality subsequent to radiation and tissue trauma depends on the (*a*) timing of the trauma relative to radiation, (*b*) dose and quality of radiation, (*c*) nature of the inflicted trauma (burn or wound), (*d*) genetic makeup of the host, and (*e*) microbiologic agents associated with the host. Therapies for sepsis after wound trauma were developed in gamma ray- and neutron-irradiated mice. Single-agent therapy for infections with antimicrobials or immunomodulators is not as useful as combined modality therapy with antimicrobials and immunomodulators. Topical treatment of the injury with antimicrobials in addition to systemic therapy with antimicrobials or immunomodulators is necessary to effect survival. Sepsis in mice subsequent to neutron irradiation and wound trauma was more difficult to treat than sepsis after gamma ray exposure and wounding. The increased biological effectiveness of neutrons compared to gamma rays for radiosensitive tissues makes therapy for sepsis less successful in neutron-irradiated hosts.

## INTRODUCTION

Approximately 100 years ago Roentgen discovered x-rays and Becquerel discovered radioactivity. Since that time man has attempted to harness the atom for civilian as well as military purposes. The promise of the expanded peaceful use of nuclear energies for diagnosis of disease, therapy for malignancies, and unlimited electrical power has been clouded by major accidents where radionuclides have been released into the environment from power reactors (Three Mile Island, United States, and Chernobyl, U.S.S.R.) and from abandoned medical radiation devices (Juarez, Mexico, and Goiania, Brazil). The increased risk to human health as well as the loss of life in Chernobyl and Goiania have had sobering influences upon the world.

Nearly 50 years ago nuclear weapons were developed in the United States and employed in Hiroshima and Nagasaki with devastating effects. Today many nations control nuclear weapons or reactors capable of producing weapon-grade material. In addition to these devices there are numerous medical centers and power plants using radiation for peaceful purposes. While these radiation resources are under apparent control, the possibility that accidents will occur or that terrorists will obtain and use these devices is of increasing concern.

The expanded probability of exposure to radiation environments requires the development of understanding of both the damage induced by radiation and the treatments available to counteract that damage. Further, the treatments provided must be tailored for the additional specific injuries expected or associated with radiation damage (Browne et al. 1990). For example, burn trauma in the individuals exposed to radiation at Chernobyl complicated therapeutic efforts (Baranov et al. 1989). Burns and mechanical traumas to the irradiated inhabitants of Hiroshima and Nagasaki contributed heavily to mortality (Fujita et al. 1990). In retrospective analyses of mortality after the Hiroshima event, burn and wound traumas in irradiated casualties may have resulted in underestimates of the $LD_{50}$ (Fujita et al. 1989).

Irradiated individuals with mechanical or burn injuries are termed "combined injured." Combined injury is defined here as the infliction of tissue damage on an individual when (*a*) one of the injuries is radiation and (*b*) the recovery and repair from it or the other injury has not taken place before the occurrence of the second. In this chapter, we summarize our findings on factors contributing to mortality after combined injury in mouse models. In addition, we show that survival is possible in lethal models of combined injury in mice if hematopoietic recovery is augmented and if overwhelming sepsis is controlled.

## MATERIALS AND METHODS

### Animals

B6CBF1/CUM female mice were obtained from Cumberland View Farms in Clinton, Tennessee. B6D2F1/J female mice were obtained from Jackson Laboratory in Bar Harbor, Maine. C3H/HeN female mice were obtained from the National Cancer Institute Animal Breeding Facility in Frederick, Maryland. The mice were maintained as previously described (Madonna et al. 1991). Research was conducted in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). All procedures involving animals were reviewed and approved by an institutional animal care and use committee.

### Radiation

Detonation of a nuclear device will result in the release of neutrons and gamma rays. The proportion of each of these received by an exposed individual will depend on such factors as type and yield of the weapon and distance from the epicenter. To simulate nuclear detonation environments, irradiations with mixed fission neutron and gamma photons were performed using the Armed Forces Radiobiology Research Institute (AFRRI) Training, Research, and Isotope, Gen-

eral Atomics (TRIGA) Mark-F research reactor. This reactor is a movable-core pool-type facility with maximum operational steady-state power of 1 MW. All reactor irradiations were performed at a total dose rate of 38 cGy/min by altering either experimental animal placement in the radiation field, reactor power, or shielding design. Desired neutron to gamma ratios were obtained by varying shielding configurations of water, lead, borated polyethylene, and paraffin at selected reactor powers. The total dose rate varied less than 2% over the entire radiation field. Mice were irradiated in aerated aluminum tubes that rotated at 1.5 rpm.

Gamma ray exposures such as that possible at a reactor site accident or in a fallout zone were simulated by irradiation with cobalt-60. B6CBF1 and B6D2F1 mice were irradiated bilaterally at 40 cGy/min in the AFRRI $^{60}$Co radiation facility. C3H/HeN mice were irradiated unilaterally at 40 cGy/min with a $^{60}$Co Theratron unit. All irradiations of mice were done in aerated Plexiglas restrainers. The tissue/air ratio was 0.988 for bilateral $^{60}$Co irradiation and 0.98 for unilateral $^{60}$Co irradiation. Dosimetric techniques for measuring reactor- and $^{60}$Co-produced radiations were described elsewhere (Zeman and Ferlic 1984).

## Skin Injuries

Mice were anesthetized by inhalation of methoxyflurane before injury. Full-thickness, nonlethal skin wounds of varying sizes, from 7% to 15% of the total body surface area (TBSA), were inflicted by removing a section of dorsal skin fold and underlying panniculus carnosus muscle with a steel punch. Details for inflicting skin wounds have been previously published (Madonna et al. 1991). Full-thickness burns were inflicted on the shaved dorsal surface area by a 12-second ignition of 95% ethanol (Steritz et al. 1982). The burn site varied in size from 7% to 15% of the TBSA.

## Bacteria

All endogenously acquired bacteria found in either normal, injured, irradiated, or combined-injured mice and isolated on phenylethanol agar or MacConkey's agar were identified by using combinations of Gram's stain, colony morphology, and specific biochemical tests (Lennette et al. 1985).

## Treatment Agents

Immunomodulators are substances that, when used, alter nonspecific or specific immune functions within the host. Synthetic trehalose dicorynomycolate (S-TDCM) was a gift of Ribi ImmunoChem Research, Inc., in Hamilton, Montana. S-TDCM activates nonspecific host resistance against induced bacterial infections in gamma ray- (Madonna et al. 1989) and neutron-irradiated mice (McChesney et al. 1990). Therefore, we examined its usefulness in endogenously acquired infections in combined injured mice. The method of S-TDCM preparation was previously described (Madonna et al. 1991). A dose of 200 $\mu$g S-TDCM was given intraperitoneally (ip) in 0.5 ml of 0.2% Tween-80 saline.

Oxacillin sodium, gentamicin sulfate, ofloxacin (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ), and ceftriaxone sodium (Hoffman-LaRoche,

Inc., Nutley, NJ) were used systemically. All these antimicrobials were prepared in pyrogen-free water, and 0.1 ml was injected subcutaneously (sc) above the right or left gluteus medius of each mouse daily for 10 consecutive days. The daily dose of oxacillin was 150 mg/kg; gentamicin, 7.5 mg/kg; ofloxacin, 40 mg/kg; and ceftriaxone, 75 mg/kg. Garamycin cream (0.1% gentamicin sulfate) was obtained from Schering Corp. in Kenilworth, New Jersey; a generic 0.1% gentamicin sulfate was obtained from Goldline Labs in Fort Lauderdale, Florida. The creams were applied once daily for 10 consecutive days in 0.5 to 0.7-g amounts sufficient to cover the wounded sites.

### Statistical Analyses

Survival data of mice in experimental groups were obtained for 30 days after irradiation. Comparisons were made by the generalized Savage (Mantel-Cox) procedure (Lee 1980). Probit analysis of numbers of mice surviving 30 days was made on log-transformed doses (Finney 1971, 1979).

## RESULTS

### Influence of Radiation Quality, Radiation Dose, and the Type of Skin Trauma on Survival of Combined-Injured Mice

In uncontrolled nuclear radiation environments, individuals may be exposed to either alpha, beta, gamma, or neutron particles. External contamination of the injury site with alpha and beta emitters does not normally constitute an immediate life-threatening situation for the individual. Internal and external contamination with radionuclides, while presenting a possibility of increased risk for life shortening and malignancy, does not usually preclude immediate lifesaving treatments for personnel with combined injury. Rather, burn or wound trauma in conjunction with exposure to neutron or gamma ray irradiation or combinations thereof pose the more serious life-threatening situation.

To simulate a variety of radiation environments, groups of B6D2F1 mice were irradiated with three different neutron/gamma dose ratios ($n/\gamma$) produced by the reactor. The $n/\gamma$'s employed were 0.33, 1, and 19. To simulate exposure in a high gamma ray fallout field, groups of mice were irradiated with "pure" gamma radiation either from a $^{60}Co$ source or the reactor. Nonlethal 2.5-cm by 3.8-cm (15% TBSA) burns or wounds were inflicted 1 to 2 hours after each exposure, and 30-day survival responses were compared to control mice that were irradiated but uninjured. Thus complete dose-response survival curves were obtained at each radiation quality with each type of injury. Radiation doses lethal to 50% of mice in 30 days ($LD_{50/30}$) are presented in Figure 13.1. In irradiated animals, the $LD_{50/30}$ is one endpoint used to compare relative sensitivity or resistance to radiations of different quality. In all groups of mice, i.e., control, irradiated, irradiated and burned, and irradiated and wounded, the $LD_{50/30}$ decreased as the proportion of neutrons in the total dose increased. At each $n/\gamma$, postirradiation burn trauma and wound trauma reduced the $LD_{50/30}$ from the comparable radiation control group about 10% and 20%, respectively.

Injury subsequent to radiation not only increases the mortality incidence (i.e.,

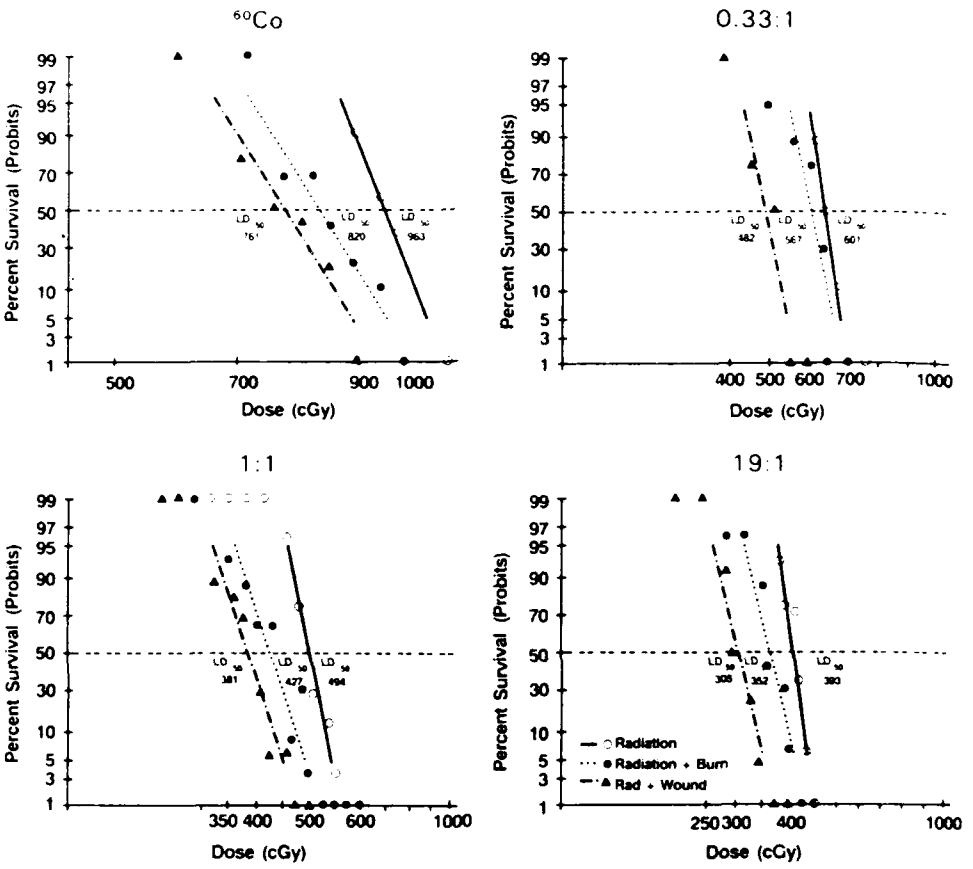


FIG. 13.1. Radiation doses required to produce 50% lethality in mice. All B6D2F1 mice were irradiated at 0.4 Gy/min, and $LD_{50/30}$ values were statistically determined from complete dose-response survival curves. The *open circles* indicate survival for irradiated mice. The *closed circles* indicate survival for mice given 15% TBSA burn trauma 1 to 2 hours after irradiation. The *triangles* indicate survival for mice given 15% TBSA wound trauma 1 to 2 hours after irradiation.

reduces the $LD_{50/30}$) but also reduces the survival time of mice that died. Plotted in Figure 13.2 are the mortality percentages of mice dying after irradiation with various proportions of neutron and gamma rays and or irradiated mice inflicted with burn or wound trauma. Except in isolated instances, the greatest mortality occurred during the 1st week after receiving a high proportion of neutrons or when wound trauma was inflicted after irradiation. When mice were irradiated with an $n/\tau = 1$ or less, or when burn trauma had been inflicted after irradiation, the majority of mortality occurred in the 2nd week postirradiation.

## Influence of Time of Injury Relative to Irradiation on Survival of Combined-Injured Mice

In a series of studies, three groups of B6CBF1 mice were irradiated with three (9, 10, or 11 Gy) lethal doses of $^{60}$Co, respectively. At eight time points before

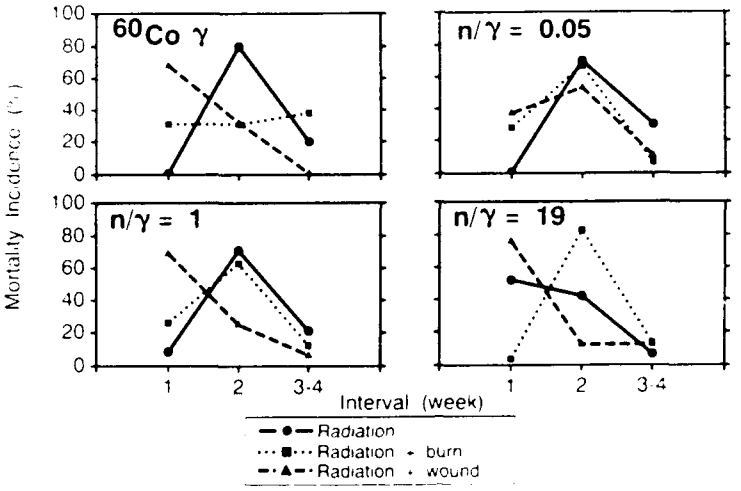


FIG. 13.2. Incidence of mortality of mice irradiated with $^{60}$Co-produced gamma rays or reactor-produced neutrons and gamma rays. The mortality percentages are based on a total of 30 to 60 mice irradiated, irradiated and burned, or irradiated and wounded at their respective $LD_{50,30}$'s at the indicated gamma ray or neutron to gamma ratios.

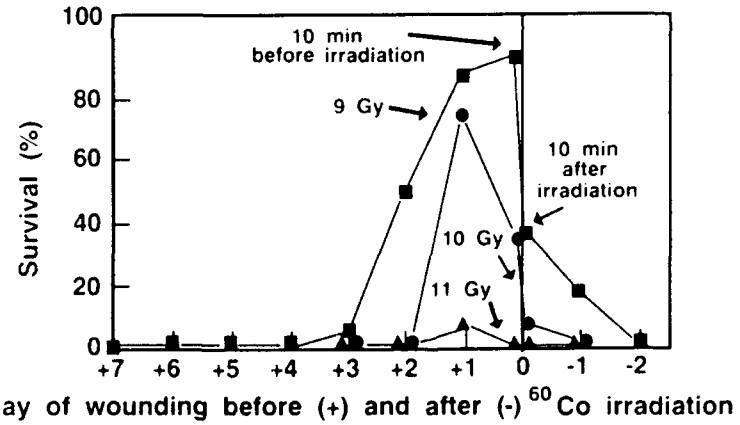


FIG. 13.3. Thirty-day survival of B6CBF1 female mice (n = 16/time point) after receiving 1.3-cm by 1.9-cm skin wounds and $^{60}$Co irradiation. All control irradiated mice died; all control wounded mice lived. (From Ledney, G. D., Exum E. D., Jackson, W. E., III. Wound-induced alterations in survival of $^{60}$Co irradiated mice: importance of wound timing. Experientia 1985;41:614–616.)

irradiation and three time points afterward, 1.3-cm by 1.9-cm skin wounds were inflicted. Survival data are shown in Figure 13.3. The 30-day survival of animals wounded before irradiation increased as the time interval between injury and irradiation shortened. Further, the number of 30-day survivors increased when mice were wounded within 10 minutes after a nominally lethal radiation dose (9 Gy). Survival times of all control-irradiated mice that died within the 30-day period were 11 to 14 days. However, survival was extended to 17 to 20 days for mice irradiated with nominally lethal doses (9 Gy) when wounds were inflicted

from 2 days before to 10 minutes after exposure. When wounds were inflicted 1 or 2 days after irradiation, the survival times for all groups of mice with combined injury were decreased to about 7 days.

In a second series of experiments, a comparative mortality study was done with B6D2F1 mice irradiated with sublethal doses of either $^{60}$Co (7 Gy) or with an $n/\tau = 19$ (3 Gy). Groups of mice were injured at each of three time points either before or after irradiation. Mortality data are shown in Figure 13.4. The incidence of death from combined injury was greater for animals irradiated with the high $n/\tau$ (19) than with $^{60}$Co. In all irradiated mice, wound injury resulted in more deaths than burn injury. Burn or wound injury after irradiation resulted in more deaths than injuries given before exposure. As seen in the first series of experiments, injuries occurring shortly before (10 minutes) sublethal irradiation resulted in fewer mortalities than injuries inflicted 1 or 2 days before radiation exposure. In the second group of studies, a relatively similar decrease in mortality incidence was noted for animals injured soon after (10 minutes) irradiation compared to injuries inflicted 1 or 2 days later.

In a third set of experiments, we determined if there was a positive correlation between wound-induced survival and the number of endogenous colony-forming units found on the spleen (E-CFU-S). The E-CFU-S assay is based on the survival and proliferation of hematopoietic cells forming discrete nodules on the surface of the spleen in an appropriately irradiated mouse. Approximately 8 to 14 days after irradiation the spleen is removed and histochemically stained and

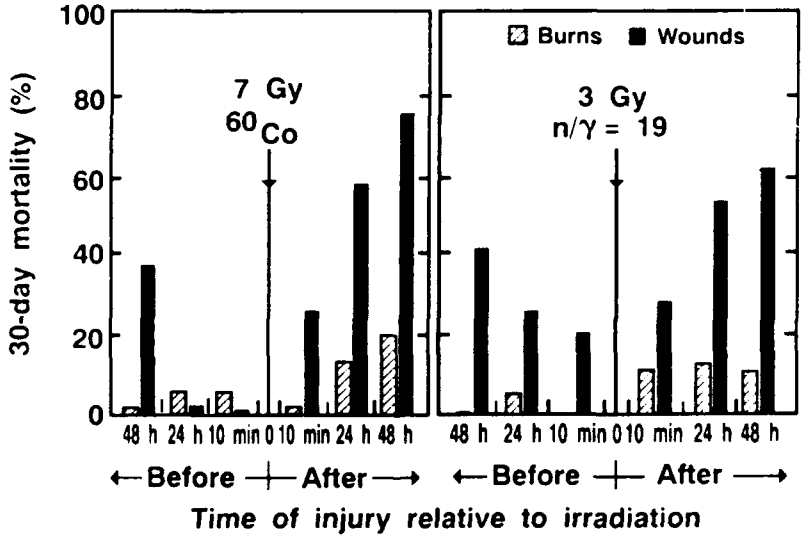


FIG. 13.4. Thirty-day mortality of B6D2F1 female mice after either 7 Gy $^{60}$Co (20 mice/time point) or 3 Gy mixed field (neutron/gamma ratio of 19; 24 mice/time point) irradiation and administration of 2.5-cm by 3.8-cm skin wounds or skin burns. In these experiments, less than 4% of mice died in burned, wounded, gamma-irradiated, or neutron-irradiated control groups. (From Ledney, G. D., Madonna, G. S., McChesney, D. G., Elliott, T. B., and Brook, I. Complications of combined injury. Radiation damage and skin wound trauma in mouse models. In: Treatment of radiation injuries. New York: Plenum Press, 1990)

preserved. The number of nodules are then counted. The E-CFU-S assay has been used as an indicator for chemical agents useful for protecting against radiation-induced hematopoietic failure in mice (Kinnamon et al. 1980). Along these lines S-TDCM increased E-CFU-S in both gamma ray and neutron-irradiated mice (Stewart et al. 1991). However, no quantitative relationship between colony number and survival is suggested. Thus to determine if survival from combined injury positively correlated with increases in E-CFU-S, groups of B6CBF1 mice were irradiated with either 9, 10, or 11 Gy. Skin wounds (1.3 cm by 1.9 cm) were inflicted either 2 days, 1 day, or 10 minutes before irradiation, or 10 minutes or 1 day after irradiation. The mean values of 10-day E-CFU-S for 12 to 16 mice per treatment group are reported in Figure 13.5. A positive correlation was noted between the survival from combined injury (Fig. 13.3) and the number of spleen colonies. Thus in combined-injury situations employing nominally lethal radiation doses (9 Gy), trauma increased the hematopoietic proliferative compartments of irradiated mice.

## Influence of Genetic Strain on Survival of Combined Injured Mice

The survival-mortality responses of mice to x-irradiation is controlled by their gene makeup (Kohn and Kallman 1956). We irradiated inbred C3H/HeN mice and hybrid B6D2F1 mice with mixed field ($n/\tau = 1$) or $^{60}Co$ gamma radiation to

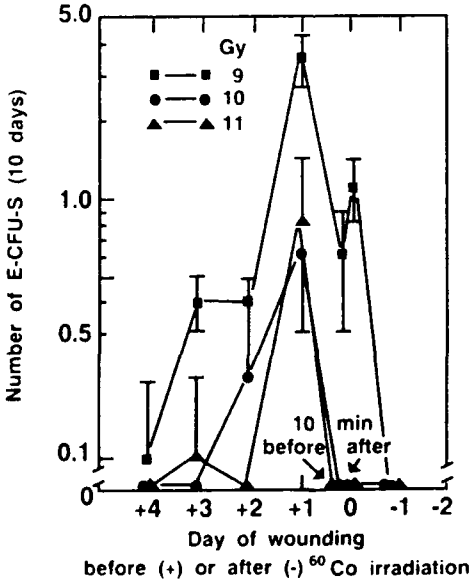


FIG. 13.5. Number of endogenous E-CFU-S in wounded irradiated mice. The time points tested were based on the survival studies in Figure 13.3. No E-CFU-S were found in control irradiated mice or in control wounded mice. Data presented in Figures 13.3 and 13.5 were obtained from all mice exposed to radiation at the same time. (From Ledney, G. D., Exum E. D., Jackson, W. E., III. Wound-induced alterations in survival of $^{60}Co$ irradiated mice: importance of wound timing. Experientia 1985;41:614–616.)

determine if survival responses to subsequent wound trauma were different in these two mouse strains.

Groups of B6D2F1 and C3H/HeN mice were given doses ranging from 250 cGy to 600 cGy ($n/\tau = 1$). A comparative study was done with these mouse strains given $^{60}$Co gamma photons in doses ranging from 550 cGy to 1100 cGy. In both sets of experiments, additional groups of mice received skin wounds 1 hour after irradiation. Figure 13.6 presents the $LD_{50/30}$ and relative biological effectiveness (RBE) for all of these experiments. $LD_{50/30}$ values for B6D2F1 mice were higher than for C3H/HeN mice, indicating resistance to the lethal effects of radiation or radiation wounding. Wound trauma significantly ($p<.009$) decreased slope values for a $n/\tau = 1$ and gamma-irradiated B6D2F1 mice and for gamma-irradiated C3H/HeN mice. The slope for $n/\tau = 1$ irradiated C3H/HeN mice (29.1) was not changed ($p<.22$) by inflicting wound trauma. Slope values for B6D2F1 and C3H/HeN mice were similar after gamma irradiation (35.8 and 37.6, respectively) and gamma irradiation with subsequent wound trauma (22.3 and 20.8, respectively). Slope values for these strains of mice were significantly different after $n/\tau = 1$ irradiation (40.3 versus 23.4, $p = .0002$). The $p$ value was 0.096 between the strains given $n/\tau = 1$ radiation and wound trauma.

## Sepsis in Irradiated Mice after Wound Trauma

Antimicrobial defenses are compromised by radiation, and death from sepsis (i.e., spread of bacteria or their products from a focus of infection) may occur if injury is severe. We have demonstrated that antimicrobials are useful in managing endogenously derived (Brook and Elliott 1991) or exogenously induced (Brook and Ledney 1990) sepsis in irradiated mice. In addition, we recently reported our findings on the use of the immunomodulator S-TDCM for managing sepsis in gamma-irradiated mice (Madonna et al. 1989) and neutron-irradiated mice (McChesney et al. 1990). Compared to animals irradiated only, little research

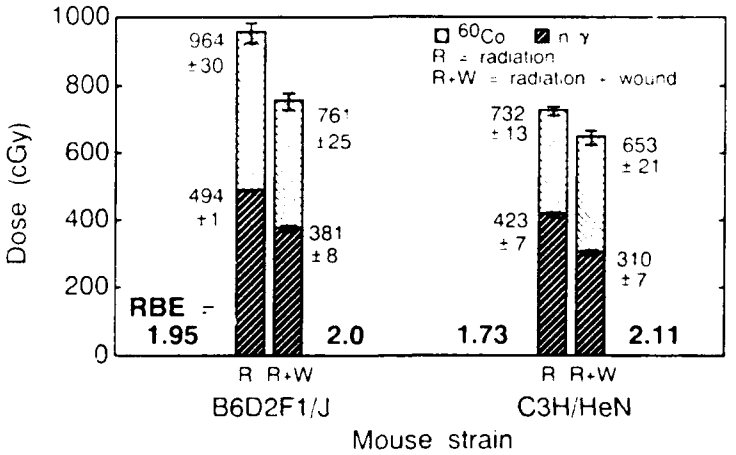


FIG. 13.6. $LD_{50/30}$'s and relative biological effectiveness (RBE) values of normal and skin-wounded mice after mixed-field ($n/\tau = 1$) or $^{60}$Co irradiation. The RBE values were calculated at $LD_{50/30}$.

has been done on the management of sepsis in combined-injured animal models (Elliott et al. 1990).

In the first series of studies, we determined the species and relative incidence of bacteria on the wound and in the liver of irradiated-wounded, wounded, and irradiated mice (Table 13.1). S-TDCM alone and combined with the antimicrobial ofloxacin, a new fluoroquinolone, were evaluated as therapies for infections. We previously demonstrated that ofloxacin (40 mg/kg/day per os (po) for 7 consecutive days starting 1 day after po challenge with *Klebsiella pneumoniae* was effective against exogenously induced infection in lethally irradiated (8.0 Gy) B6D2F1 female mice (Brook and Ledney 1991).

Gram-negative and gram-positive bacteria were found on the wound site of irradiated-wounded mice treated with S-TDCM or treated with saline and were also found on the wound site of irradiated mice, while only gram-positive bacteria were found on the wound site of nonirradiated animals. No gram-negative bacteria were found either on the wound site or translocated to the liver in mice treated with ofloxacin or ofloxacin and S-TDCM–early (day 4 to 5) mortality

TABLE 13.1. ISOLATION OF BACTERIA FROM WOUNDS AND LIVERS OF GAMMA-IRRADIATED-WOUNDED MICE AFTER S-TDCM AND OFLOXACIN THERAPY[a]

| Experimental Group | 4–5 Days of Culture | | 6–11 Days of Culture | |
|---|---|---|---|---|
| | Wound | Liver | Wound | Liver |
| Treatment | | | | |
| Saline or S-TDCM | *S. aureus*<br>*S. faecium*<br>*E. coli*<br>*P. mirabilis* | *P. mirabilis* | [b] | [b] |
| Ofloxacin | *S. aureus*<br>*S. faecium* | [c] | *S. aureus*<br>*S. xylosus* | *S. aureus*<br>*A. viridans*<br>*S. faecium*<br>*S. faecalis* |
| S-TDCM/ofloxacin | *S. aureus*<br>*S. faecium* | *S. faecium*<br>*S. aureus* | *S. aureus*<br>*S. xylosus*<br>*S. faecium* | *S. aureus*<br>*S. faecium*<br>*A. viridans*<br>*S. faecalis* |
| Control | | | | |
| Wounded | *S. aureus*<br>*S. xylosus*<br>*Streptococcus spp.*<br>*S. epidermidis* | [c]<br><br>*Streptococcus spp.* | *S. aureus*<br>*S. xylosus* | [c] |
| Irradiated | [d] | [c] | [d] | *S. aureus*<br>*E. coli*<br>*K. oxytoca* |

[a] C3H/HeN mice were wounded 1 h after 8.0 Gy gamma-irradiation, and antimicrobial therapy began 4 h later. S-TDCM (200 μg) was given i.p. 1 h after irradiation, immediately after wounding. Mice in each group were euthanized either on day 4, 5, 6, 8, or 11 after irradiation and injury, and the wound site and liver were cultured to identify the bacteria. Bacteria are listed in order of frequency of isolation in each group/time. Control mice received 9.0 Gy irradiation.

[b] No mice available for testing because of mortality.

[c] No bacteria isolated.

[d] Mice not wounded in this group.

from gram-negative sepsis was prevented by ofloxacin. However, gram-positive bacterial species were found on the wound site and in the liver of all mice treated with ofloxacin. From these findings we concluded that irradiated-wounded mice were dying with gram-positive bacterial sepsis, and that the source of the infections was the wound, because the bacteria found in the liver were similar to those bacteria colonizing the wound site.

### Survival from Sepsis in Irradiated Mice Inflicted with Wound Trauma and Given Systemic Combined Modality Therapy with Antimicrobials, S-TDCM, and Topical Antimicrobials

Bacteria colonizing the wound site were systemically disseminated in irradiated mice, as noted in Table 13.1. Because topical and systemic antimicrobials applied together are more effective in treating wound infections (Stringel 1989), we evaluated several common topical antimicrobial preparations for their efficacy in treating wound infections in mice injured after irradiation. Common disinfectants were also evaluated. In normal, nonirradiated mice inflicted with wounds, treatment with several disinfectants did not alter bacterial colonization of the wound site. The agents tested were 10% povidone-iodine (Pharmadine ointment, Sherwood), 0.5% povidone-iodine (Operand aerosol, Redi-Products), and 0.25% sodium hypochlorite (diluted Dakin's solution, Clorox). Because wound colonization by bacteria was not altered by these disinfectants in normal mice, they were not evaluated in irradiated mice. The effective agents tested and the survival data obtained are presented in Figure 13.7. In this experimental series, as well as in other tests, gentamicin cream increased survival time most ($p<.05$) and hence was used in later experiments with systemic antimicrobials and S-TDCM alone or in combination.

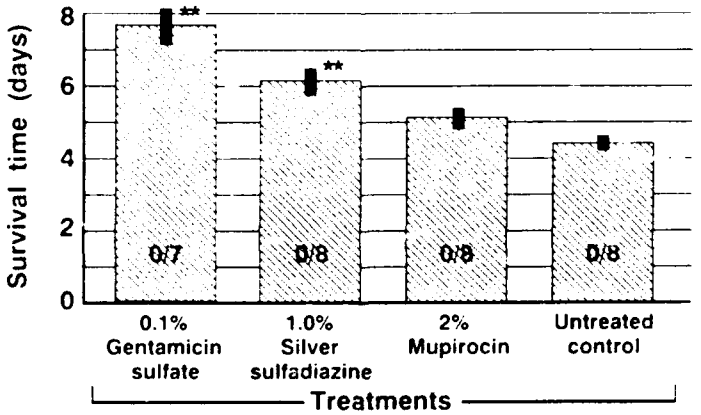


FIG. 13.7. Survival of irradiated wounded mice after topical application of antimicrobials. Mice were wounded 1 hour after 8 Gy gamma irradiation. Treatment with either gentamicin sulfate, silver sulfadiazine, or mupirocin commenced 4 hours after wounding and was applied for 5 consecutive days. Topical application of gentamicin sulfate and silver sulfadiazine significantly increased ($p<.05$) the survival of treated mice compared to untreated control mice. The mean survival time for gentamicin-treated mice differed significantly from silver sulfadiazine-treated mice ($p = .0213$).

Systemic antimicrobial therapy included sc treatments with oxacillin, ofloxacin, and gentamicin in both gamma-irradiated and $n/\tau = 1$ irradiated mice. Systemic ceftriaxone was evaluated only in $n/\tau = 1$ irradiated mice. The survival data for topical and systemic antimicrobial treatments are presented in Figure 13.8.

In mice wounded after 8.0 Gy gamma irradiation, topical gentamicin sulfate treatments with or without systemic gentamicin resulted in about 50% survival. When topical gentamicin was used with oxacillin, all mice survived. The increased survival was statistically significant at $p<.05$ when compared to the survival obtained for topical gentamicin treatments and $p<.01$ for all other comparisons. In mice wounded after 3.8 Gy $n/\tau = 1$ irradiation, topical gentamicin sulfate in combination with all the antimicrobials tested systemically increased survival significantly ($p<.01$). There were no significant differences in survival between the antimicrobial treatment groups ($p>.05$). The enhancement of survival with oxacillin and 0.1% gentamicin sulfate cream may be due to the synergistic action between the semisynthetic penicillin and the aminoglycoside gentamicin in *Staphylococcus aureus* infections (Rahal 1978). *S. aureus* was frequently found colonizing the wound and disseminating to the liver in irradiated-wounded mice (Table 13.1).

To demonstrate the efficacy of S-TDCM treatments with combination antimicrobial therapies (topical plus systemic), we used 0.1% topical gentamicin sulfate in combination with systemic gentamicin. In models of lethal $n/\tau = 1$ and gamma irradiations followed by skin wound trauma, topical gentamicin and S-TDCM therapy with or without systemic gentamicin treatment resulted in

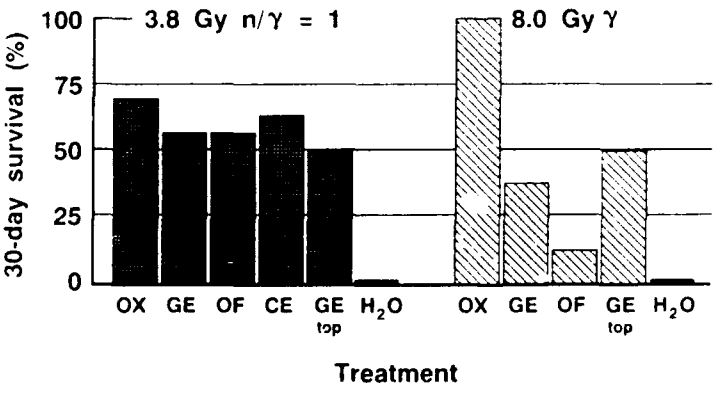


FIG. 13.8. Survival in irradiated mice inflicted with wound trauma after combined therapy with topical 0.1% gentamicin sulfate and systemic antimicrobials. C3H/HeN mice were wounded 1 hour after irradiation. Antimicrobial therapies commenced 4 hours after injury and were provided daily for 10 days. Groups of 20 mice ($n/\tau = 1$ irradiated) and groups of 16 mice (gamma irradiated) were treated topically with 0.1% gentamicin sulfate (GE, top) and systemically (sc) with either oxacillin (OX), GE, ofloxacin (OF), or ceftriaxone (CE). Control groups were treated with 0.1 ml sterile water plus gentamicin cream or were given no antimicrobial therapy. In $n/\tau = 1$ irradiated mice, all antimicrobial treatments were equally effective ($p>.05$) and increased survival ($p<.05$) over water-treated controls. In gamma-irradiated mice, topical gentamicin alone or with systemic oxacillin and gentamicin significantly increased survival ($p<.05$) compared to all other treatments.

approximately 60% survival (Fig. 13.9). S-TDCM effectively increased survival ($p<.05$) when combined with antimicrobial treatments, compared to antimicrobial treatments given without S-TDCM. Only S-TDCM combined with gentamicin produced 30-day survival approximating 50% in both $n/\tau = 1$ irradiated and gamma-irradiated mice. In this combined modality treatment series for sepsis in combined-injured mice, 0/20 survived after oxacillin treatment, 3/20 survived after ceftriaxone injection, and 1/20 survived the 30-day observation period after ofloxacin application. We did not test systemic antimicrobials without topical gentamicin sulfate in S-TDCM-treated, irradiated-wounded mice nor the combination gentamicin treatments in $n/\tau = 1$ irradiated-wounded mice.

## DISCUSSION

Our data confirm the complex issues involved in developing appropriate animal models for variables associated with combined injury. In radiation casualties where there are associated tissue injuries, the primary concern is medical stabilization of the patient followed by repair of life-threatening tissue injuries. Schemes for treating victims of radiation accidents have been published elsewhere (Browne et al. 1990). Emergency care to the irradiated-traumatized patient is of greater concern than decontamination of non-life-threatening internally or externally deposited radioactive isotopes or activation products (sodium-24 $T_{1/2}$ = 15 hours; phosphorus = 32 $T_{1/2}$ = 14.3 days). Eventually, decontamination must be done to reduce the body burden of contaminating or induced isotopes. The removal of radioactive material reduces the radiation dose absorbed by the individual.

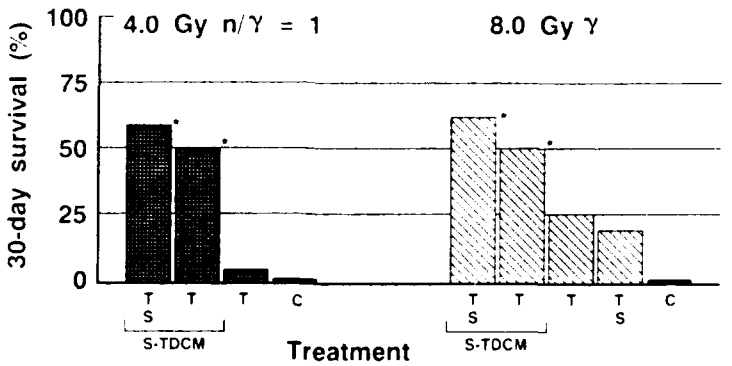


FIG. 13.9. Survival in irradiated mice inflicted with wound trauma and given combined therapy with topical 0.1% gentamicin sulfate and S-TDCM. C3H/HeN mice were wounded 1 hour after irradiation. S-TDCM (200 μg ip) was given immediately after wound trauma. Gentamicin therapy was applied topically and/or given sc 4 hours after injury and daily thereafter for 9 days. Groups of 20 mice ($n/\tau = 1$ irradiated) and groups of 16 mice (gamma irradiated) were treated as indicated. T = topical 0.1% gentamicin sulfate; S = systemic 7.5 mg/kg gentamicin sulfate; C = control. Topical gentamicin with systemic gentamicin was not tested in $n/\tau = 1$ irradiated mice. Systemic gentamicin sulfate with S-TDCM but without topical gentamicin was not evaluated in gamma-irradiated or $n/\tau = 1$ irradiated mice. In both $n/\tau = 1$ irradiated and gamma-irradiated mice, gentamicin therapy with S-TDCM and with or without systemic gentamicin therapy significantly increased (* = $p<.05$) survival, compared to all other treatment groups.

Once a radiation accident victim is stabilized, the second major concern is treatment for infection. Infection is a leading cause of death in otherwise survivable trauma incidents or radiation exposures. Severe immunological suppression induced by the combination of radiation and tissue injury makes the treatment for infection more difficult, as is noted in the present work in mice and in man (Baranov et al. 1989, Champlin 1990). Successful therapy for infections with topical antimicrobials, as reported here, suggests that bacterial colonization of the wound site must be controlled if further advances in therapy for combined injury are to be made (Kaplan 1985). Effective antibacterial therapies will allow sufficient time for induced hematopoietic proliferation or endogenous hematopoiesis to restore the aplastic condition induced by radiation. Possible increases in hematopoiesis by S-TDCM (Stewart et al. 1991) in consort with topical gentamicin sulfate increased survival from combined injury. The advantage of using specific cell growth factors, i.e., G-CSF and GM-CSF for treating sepsis and hematopoietic aplasia in combined-injured hosts remains to be determined. GM-CSF was used in several patients exposed to radiation in the accident at Goiania (Brandao-Mello et al. 1991, Butturini et al. 1988). Several patients responded to GM-CSF with increased peripheral granulocyte numbers; survival may have been increased by this cell growth factor. The animal models of combined injury discussed in this chapter could be used to evaluate the efficacy of cell growth factors prior to their use in radiation accidents.

In previous work, we observed that wound closure takes 1 to 2 weeks in unirradiated mice. Sublethal doses of 7.0 Gy $^{60}$Co gamma rays further delayed wound closure by 1 week, while 2.5 Gy of reactor-produced neutrons ($n/\tau = 19$) further delayed wound closure by 2 weeks. Thus not only does the delay in wound closure promote continued contact with bacteria but also the unhealed site may come under continued exposure to nuclear fallout.

In radiation accidents, significant time and energy may be expended in determining the absorbed dose. The immediate concern is proper triage; a later concern is effective long-term therapy. Biological dosimeters, as well as physical dosimeters, have been used to reconstruct absorbed doses. Biological dosimeters, as adjuncts to measuring radiation doses by other means, may be more difficult in the combined-injured patient than in the individual receiving radiation only. Trauma is well known to change general cell populations (white blood cells) as well as specific cell populations (macrophages and suppressor T lymphocytes). Trauma in irradiated mice significantly altered the white blood cell and platelet patterns from that observed in irradiated animals (Madonna et al. 1991). Likewise, trauma in irradiated mice reduced the possibility of using diamine oxidase (DAO) as an indicator of radiation damage (DeBell et al. 1987). DAO is an enzyme found in high concentrations in intestinal epithelial cells, a target nearly as radiosensitive as bone marrow cells. Perturbations induced by trauma in other cell systems used as biological dosimeters is a possibility and should be examined in combined injury models. In managing dose estimations of irradiated patients, staff charged with reviewing biological and physical dosimetry may need to take these concerns into account.

In conclusion, the combined-injured individual represents a greater challenge

to medical staff than the irradiated individual not compromised by additional tissue trauma. The issues of constructing radiation dose, decontamination of internal and external radioactive isotopes, activation products, and providing emergency and definitive care will all be made more difficult by the complex interactions of tissue trauma, radiation injury, and bacterial infection.

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## Cytokine Therapy in Canine and Primate Models of Radiation-Induced Marrow Aplasia

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### Introduction

Lethality consequent to radiation exposure throughout the hemopoietic syndrome dose range results from the combination of inopportune hemorrhage and overwhelming sepsis. We hypothesized that the radiation-induced pathogenesis is dependent upon the lack of cytokine-mediated self renewal of respective surviving stem cells coupled with their inability to generate functional end cells, e.g., neutrophils and platelets in a timely manner. The production of functional cells must occur within a critical, clinically manageable period of time, limited by the ability to support the lethally irradiated host with antibiotics, platelets and whole blood transfusions, and fluid administration[19]. Until recently, we could not, with the exception of bone marrow transplantation, therapeutically manipulate hemopoietic reconstitution.

The hemopoietic system has been responsive to a number of cytokines or growth factors. The cytokines most studied, both *in vitro* and *in vivo*, have been those acting more distal to the hemopoietic stem cell. These are the lineage-specific granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF, GM-CSF)[1,6,9,12,16-18,22-24,32,35,36], Interleukin 3 (IL-3), while receiving less attention appears to act on target cells more proximal to stem cells and provides stimulation of multilineage progenitor cells[7,11,13,14,16]. Data in normal animals and *in vitro* results suggest that this factor would be more effective acting in combined cytokine protocols[7,16,27,28,33]. *Donahue* et al.[7], and *Krumwieh* et al.[16], demonstrated synergy in the production of neutrophils using a sequential protocol of IL-3 followed by GM-CSF in normal primates. Whereas, *Monroy* et al.[25], demonstrated an additive effect in platelet production following a sequential protocol of Interleukin 1 (IL-1) and IL-3, again in normal primates. Even though IL-1, and IL-6 are not growth factors for hemopoietic cells, they are examples of regulatory cytokines that may act indirectly to release other regulatory cytokines and/or act as co-mitogens to stimulate proliferation of specific cells when in the presence of other cytokines[5,25]. Two recently identified cytokines, IL-11[4,26] and the ligand for the c-kit locus (KL, MGF, SCF)[12,37-39] also appear to be more efficient when used in combination with a second or even third cytokine[3,15,29]. Information available from *in vitro* and preclinical data from normal and hemopoietically compromised animals suggests that the most effective therapeutic protocols would be a combination of cytokines proven to act on

target cells more proximal to the stem cell and thus synergize with those lineage-specific cytokines that would drive expansion and differentiation of the committed progenitors toward increasing levels of mature cells.

Five cytokines, G-CSF, GM-CSF, IL-3, IL-1 and IL-6 have been shown to be therapeutically effective in enhancing recovery of sublethally irradiated or drug-treated mice[10,14,21,25,30,31,35], canines[20,32] or nonhuman primates[18,24,36]. They have effectively increased production of progenitor cells (CFU-s, GM-CFC). However, only the lineage specific cytokine G-CSF and GM-CSF have been effective in increasing survival of otherwise lethally irradiated and clinically supported canines[20,32] and primates[24]. Platelet production was unaffected and the animals remained at risk for spontaneous hemorrhage. These successes were dependent upon both the ability of the cytokine protocol to significantly decrease the duration of neutropenia and the clinical efficacy of platelet transfusions in preventing hemorrhage.

These results highlight the ability of single agent protocols of lineage specific factors to rescue lethally irradiated animals. The kinetics also suggest that early stem cells and progenitor cells are not responsive to these cytokines and that the animals remain at risk for infection and hemorrhage for extended periods of time. Further progress in decreasing the duration of neutropenia and thrombocytopenia while increasing survival of lethally irradiated animals will require combinations of existing cytokines and the testing of new candidate mediators. It appears within reason that radiation damage across the hemopoietic syndrome will be manageable based on the continued progress in cytokine development and knowledge of the regulatory mechanisms surrounding the stem cell and its progeny.

The ability to evaluate the kinetics associated with production of neutrophils, platelets, and lymphocytes in otherwise lethally irradiated preclinical models will provide valuable information on regulation of stem cell renewal and differentiation vis-a-vis specific cytokine action within the network supporting hemopoietic reconstitution.

## Material and Methods

### *Animals*

Domestic born male rhesus monkeys, Macaca mulatta, mean weight 2.9 ± 0.3 kg, were used in these studies. They were housed in individual stainless steel cages in conventional holding rooms of an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited animal facility. Monkeys were provided 10 air changes/hr of 100% fresh air, conditioned to 21 °C ± 2 °C with a relative humidity of 50% ± 10% 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*.

Purpose bred beagles, mean weight of 10.0 kg ± 0.8 kg were housed in individual stainless steel cages in conventional holding rooms of the AAALAC-accredited animal facility at the Armed Forces Radiobiology Research Institute. Canines were provided 10 air changes of 100% fresh air, conditioned to 21 °C ± 2 °C, with a relative humidity of 50% ± 10%. They were maintained on a 12 h light/dark full spectrum lighting cycle with no twilight, and provided with tap water *ad libitum* and commercial canine chow.

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

### *Irradiation and clinical support*

Monkeys, placed in an aluminium restraining chair, following a prehabituation period were total body irradiated (TBI) in a posterior-an-

terior direction using the AFRRI TRIGA reactor. They were exposed to a pulse (< 50 msec) of mixed (1:1, free in-air) fission neutron and gamma radiation to a total free-in-air-surface dose of 450 cGy. All exposures were monitored using ionization chambers, sulfur activation foils and radioluminescent glass and silicon diodes.

Dogs were bilaterally exposed to uniform, homogeneous, whole-body $^{60}$Co radiation from the AFRRI opposing $^{60}$Co sources, at a dose rate of 40 cGy/min; total doses, measured at midline tissue depth, were 350 cGy to 450 cGy. Radiation exposure took place in well ventilated Plexiglas restraint boxes (after prior acclimatization).

An antibiotic regimen was initiated prophylactically when the white blood cell count (WBC) dropped below 1000/μl and continued daily until the WBC rose above that value for 3 consecutive days. Gentamicin (1.5 mg/kg q12) and rocephin (100 mg/kg/day) were both given intramuscularly.

Fresh, irradiated (1500 cGy Co-60) platelets, from a random donor pool, of canines and nonhuman primates (> 10 kg bw) were administered when the platelet count dropped below 30,000/μl and continued every day until platelet levels rose above that value. The volume and concentration of platelets infused depended on the yield from donor primates and canines.

*Recombinant cytokine administration protocol*

Cytokines utilized in these studies were: recombinant human (rh) and canine (rc) G-CSF, rh GM-CSF and rh IL-3. All cytokines had no measurable endotoxin on the limulus amebocyte assay. The rh IL-3 and rh GM-CSF supplied by Immunex Corp. were yeast derived. Cytokines were supplied by Behringwerke AG, Marburg Germany (rh GM-CSF), Immunex Corp. (rh GM-CSF, rh IL-3), Genetics Institute, Cambridge, MA (rh GM-CSF), and AMGen Corp. Thousand Oaks, CA (rh G-CSF, rc G-CSF).

Normal canines: Canines were injected with rh GM-CSF subcutaneously (sq) in two equally divided doses for total dose of 100, 20, and 2 μg/kg/day. The rh G-CSF was administered in one dose sq at 10, 2, and 1 μg/kg/day. The rc G-CSF was injected in one dose sq at 5, 1, and 0.1 μg/kg/day.

The protocols involved administration of each agent alone and in combination. The combination protocols consisted of a.) simultaneous administration of rh GM-CSF and rh G-CSF or rc G-CSF, b.) sequential administration of rh GM-CSF followed by rh G-CSF.

Irradiated canine: The cytokines, rh-G-CSF, (10 μg/kg) and rh GM- CSF (100 μg/kg) were administered daily beginning on d1 through 21 or 24, s.c., in single or twice divided doses respectively.

Normal nonhuman primates: Primates were injected with rh GM-CSF (50, 25, 2 μg/kg/day), rh IL-3 (25 μg/kg/day), and the simultaneous injection of rh GM-CSF plus rh IL-3 (25 μg/kg/day, each).

Irradiated nonhuman primates: Beginning on d1 after irradiation monkeys were administered twice daily subcutaneous injections of either rh GM-CSF or rh IL-3 (25 μg/kg/day), rh GM-CSF + rh IL-3 (25 μg/kg/day each) or human serum albumin (HSA) (25 μg/kg/day) as a control for 21 days. In studies of sequential growth factor administration, IL-3 was given for 7 days (beginning on day 1) at a dose of 25 μg/kg/day, followed by 14 days of GM-CSF (25 μg/kg/day). Another cohort of animals received HSA for days 1–7, followed by 14 days of GM-CSF at 25 μg/kg/day.

*Peripheral blood analysis*

Peripheral blood was sampled at various times to assay complete blood counts (Model S Plus II, Coulter Electronics, Hialeah, FL) and differential counts (Wright-Giemsa Stain, Ames Automated Slide Stainer, Elkhart, IN).

*Neutrophil activation studies*

Peripheral blood was drawn by venipuncture into syringes containing perservative free heparin (10 U/ml). Contaminating red blood cells (RBCs) were lysed with 0.83% $NH_4Cl$ (10 minutes, 21 °C). Leukocytes were pelleted (400 xg, 10 min. 4 °C) and the lysate was decanted. The leucocytes were resuspended and washed in Hank's Balanced Salt Solution (HBSS) without $Ca^{++}$ and $Mg^{++}$ (GIBCO, Grand Island, NY) and re-pelleted (400 xg, 10 min. 4 °C).

The cell pellet was resuspended in Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO, Grand Island, NY) ($5-10 \times 10^6$) and maintained at 4 °C. Cell viability was determined to be $\geq$ 95% by trypan blue exclusion. Wright-Giemsa stained blood smears were prepared for differential and morphological examination.

$H_2O_2$ production was measured as described by *Bass* et al[2]. 2',7', dichlorofluorescin-diacetate (DCFH-DA) (Kodak, Rocchester, NY), a nonpolar, nonfluorescent compound diffused through cell membranes, was hydrolysed by cellular esterases to nonfluorescent, intracellularly trapped 2',7'-dichlorofluorescin (DCFH). When activated, the PMNs produced $H_2O_2$ which oxidizes DCFH to the fluorescent analogue 2',7'-dichlorofluorescein (DCF). Flow cytometric determination of $H_2O_2$ production of PMNs was determined by incubating the leukocytes ($10^6$ cells/ml) with 5 μM DCFH-DA in PBS for 10 minutes at 37 °C to allow for dye equilibrium.

Cells were then placed on ice to inhibit spontaneous $H_2O_2$ production and assayed immediately. Cells were stimulated with phorbol-12-myristate, 13-acetate (PMA) (Sigma, St. Louis, MO) (100 ng/ml) for 15 minutes and the fluorescence was measured flow cytometrically on a FACS analyzer interfaced to a Consort 30 computer system (Becton Dickinson, San Jose, CA). Green fluorescence was monitored between 515 and 545 nm after excitation by a mercury arc lamp with a 485/22 nm excitation filter. PMNs were differentiated by Coulter volume(s) and right angle light scatter properties. The percent change in $H_2O_2$ production was calculated as follows:

$$\frac{\text{Mean Fluorescence (FL) Intensity Experimental} - \text{Mean FL Intensity Control}}{\text{Mean FL Intensity Control}}$$

## Results

*Canine: Radiobiology and cytokine therapy*

The lethality dose response curve for canines exposed to Co-60 radiation without and with clinical support resulted in LD50/60 values of 260 cGy and 335 cGy respectively (Fig. 1). The use of therapeutic protocols for rh G-CSF or rh GM-CSF resulted in a shift of the LD50/60 to 450 cGy. Either cytokine administered from day 1 to 21 consecutively, reduced an LD100/60 (400 cGy) with clinical support only, to an LD0/60 (Fig. 1).

The lethality dose response curve indicated that therapy with rh G-CSF or rh GM-CSF would not be able to effect an increase in survival if the canine had received 600 cGy. Clinical support would not maintain the animals long enough for regeneration of appropriate target cells. The ability to titrate the radiation dose and reduction in numbers of surviving stem cells with the kinetics associated with specific cytokine therapy will provide valuable insights into regulation of the long term reconstitutional stem cell.

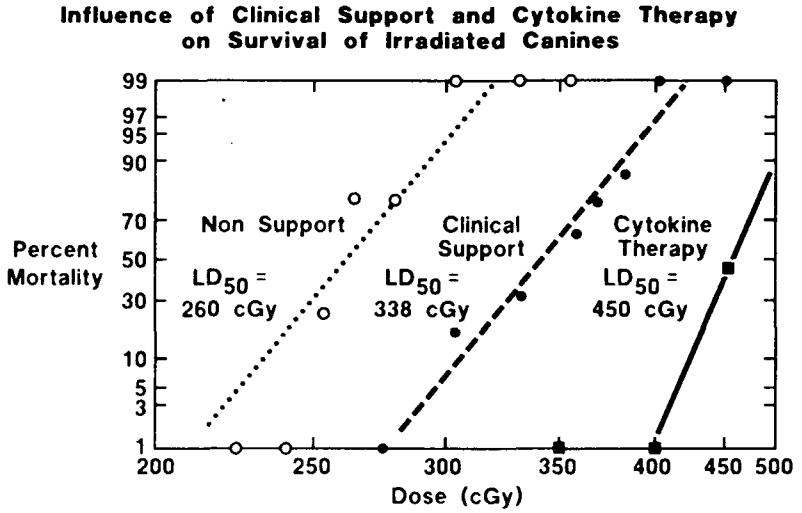



Fig. 1: Increase in survival (LD50/60 values) for Co-60 irradiated canines with addition of clinical support (fluids, antibiotics, platelet transfusions) (···●···) and clinical support plus cytokine therapy (-■-) over control, non supported animals (——●——).

The neutrophil and platelet recovery kinetics (Fig. 2) suggested that delayed administration of either cytokine would be as effective as the protocol initiated at day 1 post exposure. We tested this hypothesis by delaying administration of rh G-CSF until day 9, 12, or 15 after exposure at the LD100/60 of 400 cGy. The rh G-CSF begun at d9 was as effective as d1 initiation, with no lethality observed, whereas the d12 and d15 protocols resulted in 60%, and 50% survival, respectively. The delayed protocols were associated with longer durations of neutropenia and thrombocytopenia.

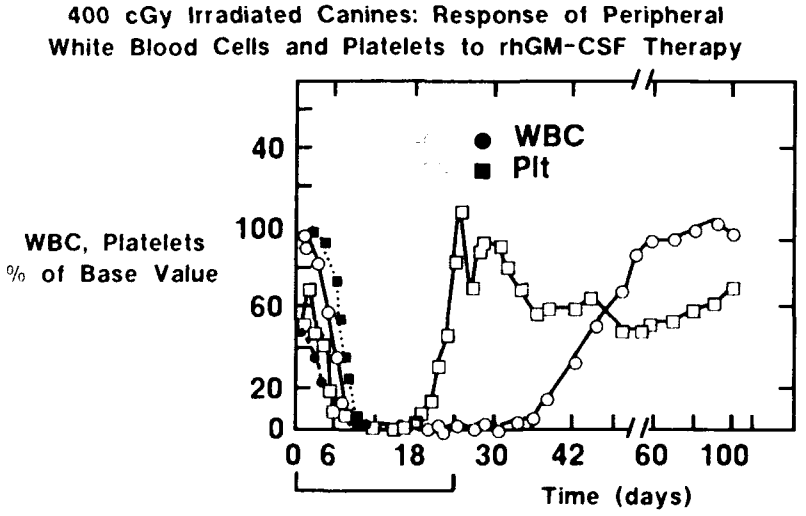



Fig. 2: Recovery kinetics of white blood cells (WBC) (——●——) and platelets (Plt) (——■——) in 400 cGy Co-60 irradiated canines treated with rh GM-CSF from d1-21 post exposure. WBC (●) and platelets (■) in control treated canines.

Neutrophil produced in cytokine-treated canines were primed for an enhanced respiratory burst to PMA. This increased ability to produce $H_2O_2$ may correlate with increased microbridal activity.

*Conclusion*

Therapy with single cytokine protocols using G-CSF or GM-CSF can increase survival in lethally irradiated animals. The calculated DRF value for the lethality dose response was 1.73. Lineage-specific cytokines do not act on stem cells. There is no apparent risk of stem cell exhaustion.

*Canine: Cytokine protocols in normal animals*

The rescue of otherwise lethally irradiated canines or primates with single agent recombinant therapy is still associated with a significant period of neutropenia. It requires at least 16–18 days after exposure to generate enough neutrophils to prevent sepsis-associated lethality (Fig. 2).

These data indicate that it will necessitate either a combination protocol approach or the use of factors not yet available for testing in large animal.

The recent availablity of recombinant *canine* G-CSF has made it practical to test this factor with recombinant *human* GM-CSF and compare it to the response generated with recombinant *human* G-CSF.

Single agent administration of rh GM-CSF, rh G-CSF or rc G-CSF is effective in producing a significant increase in peripheral neutrophils (Fig. 3a, b, c). We evaluated the efficacy of combined administration of rh GM- and G-CSF, and rc G-CSF in simultaneous and sequential protocols for stimulation of production and function of systemic neutrophils.

The most effective combination for production of neutrophils required simultaneous administration of GM and G-CSF; less effective were sequential injections with GM-CSF followed by G-CSF. G-CSF followed by GM-CSF was the least effective. A synergistic increase in peak response was observed with the simultaneous protocols (Fig. 4). The peak responses were at least 1.5-fold the additive responses. Neutrophil function was enhanced in a manner equivalent to that observed for the *in vivo* effect of G-CSF alone.

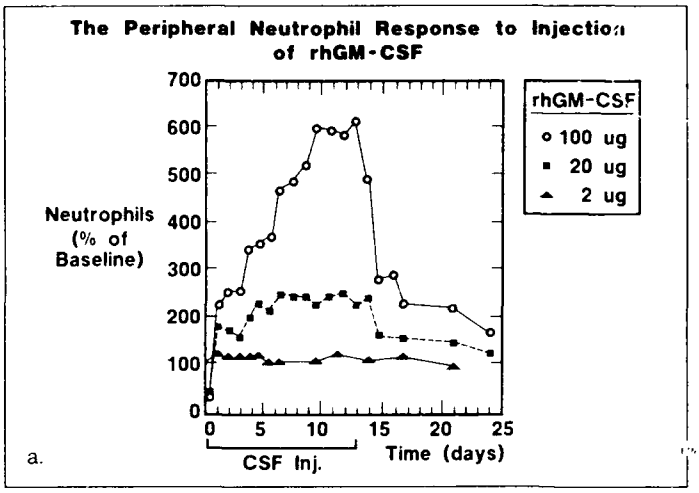
*Conclusion*

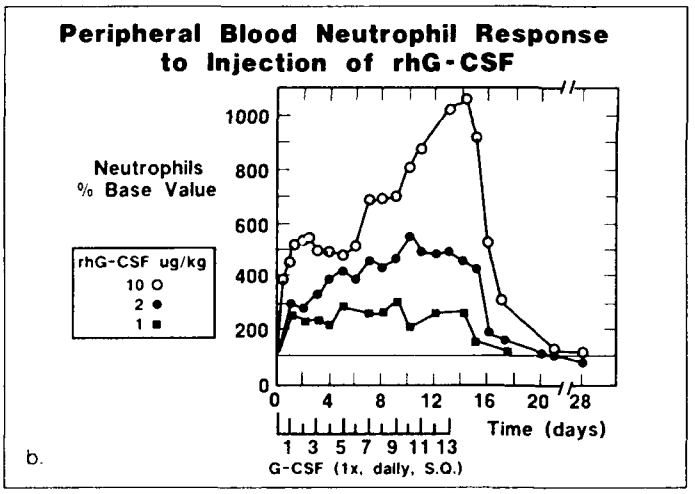
Simultaneous administration of recombinant cytokines, GM-CSF and G-CSF in normal canines was more efficient in producing a synergistic increase in neutrophils than a sequential protocol. This combined protocol may potentiate the therapeutic efficacy of these factors in reducing the critical duration of neutropenia following lethal irradiation or chemotherapy.

*Primate: Radiobiology and cytokine therapy*

The 450 cGy dose was associated with periods of neutropenia and thrombocytopenia through 16–18 days post exposure (Fig. 5a, b). The cytokines rh GM-CSF and rh IL-3 were evaluated for their ability to elicit neutrophil (PMN) and platelet production respectively. The rh GM-CSF accelerated PMN recovery (d15 versus d20) but not platelet recovery, whereas IL-3 significantly shortened the duration of thrombocytopenia (d14 versus d18) with a modest 2 day decrease in the neutropenia duration. The rh GM-CSF and rh IL-3, respective neutrophil and platelet responses were chosen as our standards against which to evaluate other cytokines or combination protocols.

We have recently completed evaluation of the fusion protein of IL-3 and GM-CSF called PIXY321 (Immunex Corp.), the combination protocol of IL-3 plus GM-CSF given simultaneously from d1 to d21, the sequential protocol of IL-3 and GM-CSF where IL-3 was administered from d1 to d7 and GM-CSF from d7 to d21, in addition to the delayed administration of GM-CSF from d7 to d21[37].



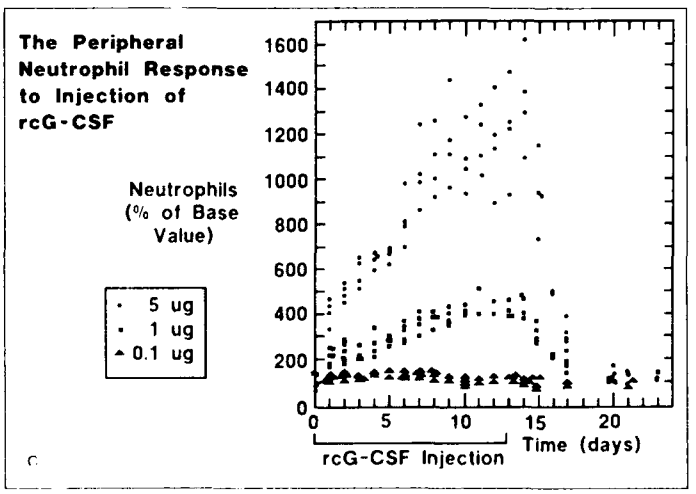




Fig. 3a, b, c: Changes in peripheral neutrophil (% of base value) in normal canines following subcutaneous administration of cytokines for 14 consecutive days; a) rh GM-CSF injected at doses of 100, 20, and 2 ug/kg/d, b) rh G-CSF injected at doses of 10, 2, and 1 ug/kg/d, c) rc G-CSF injected at doses of 5, 1, and 0.1 ug/kg/d.

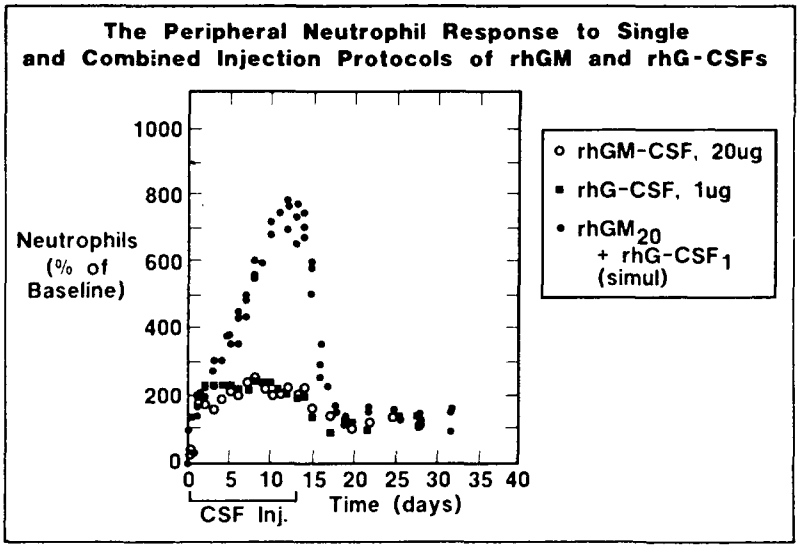



Fig. 4: Changes in systemic neutrophils (% of base value) in normal canines following subcutaneous administration of rh GM-CSF (20 ug/kg/d) and rh G-CSF (1 ug/kg/d) for 14 consecutive days.

In short, the best protocols were the use of the PIXY321 fusion protein and the simultaneous administration of IL-3 and GM-CSF. Each protocol resulted in a further decrease of the periods of neutropenia and thrombocytopenia (Fig. 6a, b). These results translated into fewer platelet transfusions and less days on antibiotics than any of the other protocols. It was of interest that the sequential protocol was no more effective than GM-CSF alone in the production of neutrophils. The data from the use of this protocol in normal primates[5] would have predicted a significant beneficial effect over the GM-CSF alone or the simultaneous protocol.

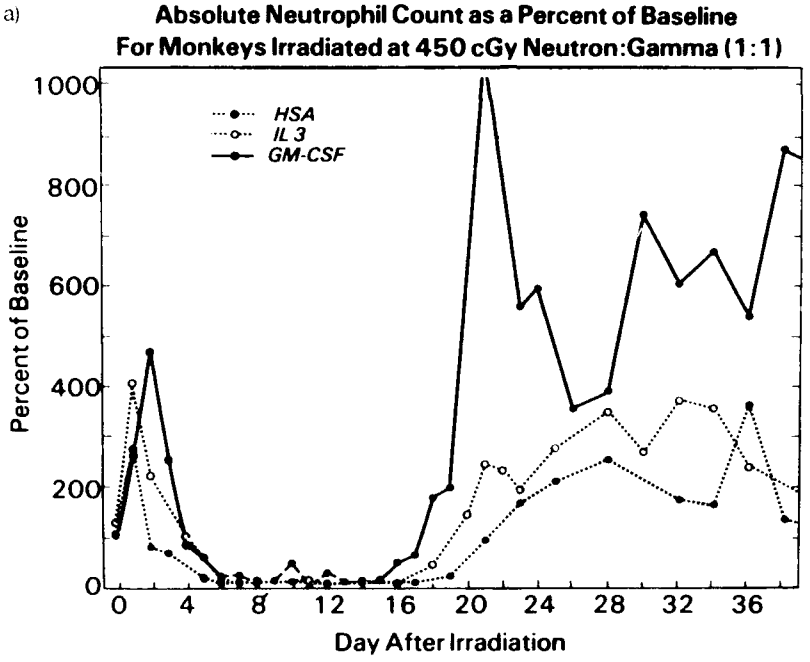
This underscores a cautionary note applied to the interpretation of cytokine effects in the normal animal with all of its regulatory controls (positive and negative) intact. Our recent data evaluating the fusion protein PIXY321 and the simultaneous IL-3 plus GM-CSF protocol in normal primates would have predicted less than optimal responses in the compromised animal[8]. PIXY321 and the simultaneous protocol (IL-3 plus GM) were less effective than GM-CSF alone in producing neutrophils.

Analysis of neutrophil function in the normal primates showed that all cytokines or cytokine combinations with the exception of IL-3 primed systemic neutrophils to undergo an increased respiratory burst. This held true for the irradiated and treated primates to include those neutrophils elicited by treatment with IL-3. This is probably the consequence of endogenous GM-CSF production in the irradiated and recovering animals.

*Conclusion*

The PIXY321 protein and the simultaneous administration of rh IL-3 and rh GM-CSF were the most effective protocols for decreasing both the periods of neutropenia and thrombocytopenia in high dose, sublethally irradiated nonhuman primates. A cautionary note must be extended to extrapolation of results in normal animals to proposed action in compromised models.

a)




b)

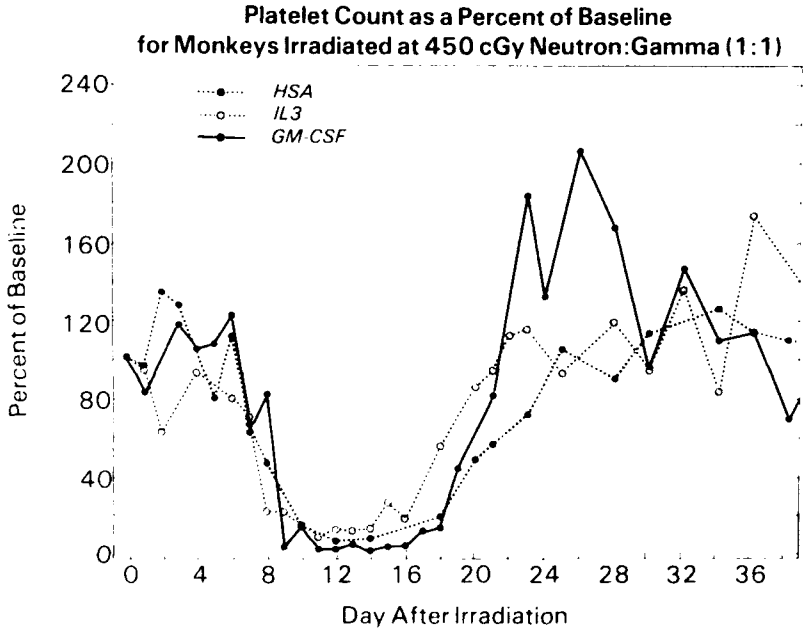



Fig. 5a, b: Recovery kinetics of (a) neutrophils (PMN) and (b) platelets as percent of baseline values in nonhuman primates with respective administration of GM-CSF and IL-3 from d1–21 consecutively after 450 cGy radiation exposure.

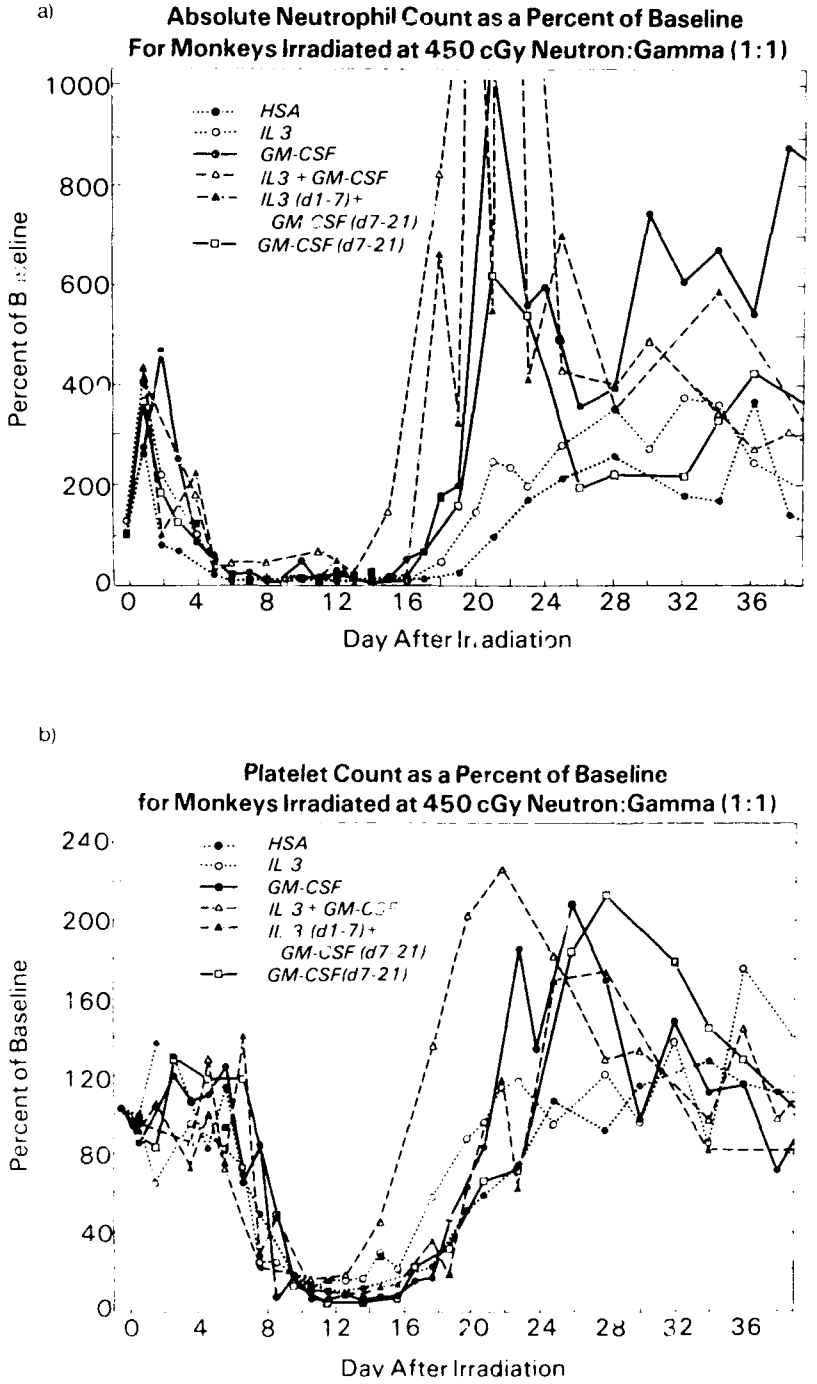



Fig. 6 (a, b). Recovery of (a) neutrophils and (b) platelets as percent of baseline in 450 cGy irradiated rhesus primates with single cytokine protocols for IL-3 and GM-CSF, and the combination protocols, IL-3 plus GM-CSF and IL-3 followed by GM-CSF as indicated in the legends.

## Discussion

Recovery from the lethal effects of irradiation to the hemopoietic system requires at least three key events. The first is the self-renewal of several populations of pluripotential and multipotential stem cells that will eventually lead to both short term recovery from immediate radiation effects and the long term reconstitution of the hemopoietic system. The second is the generation of functional end cells, i.e. the neutrophil and platelet, that will prevent the morbidity and mortality associated with the consequent hemorrhage and sepsis. The third is that the production of these functional cells must occur within a critical clinically manageable period of time defined by the capacity to support the irradiated host with antibiotics and platelet transfusions.

A number of cytokines have been shown effective as single agents, in reducing the duration of neutropenia in sublethally irradiated or drug-treated mice[12,14,21,25,32,34,35], canines[22,32], or nonhuman primates[15,24,36]. Enhancing the production of platelets in compromised animals has been more difficult. The effectiveness of the lineage specific cytokines rh G-CSF and rh GM-CSF has been underscored by their ability to increase survival in otherwise lethally irradiated and clinically supported canines[2,3,5] and primates[24]. We have extended these observations and utilized both rh G-CSF and rh GM-CSF in canines that have received supralethal doses of radiation. Data presented here illustrated the therapeutic potential of the lineage specific cytokines in enhancing survival. The dose reduction factor was increased from 1.3 with clinical support to 1.7 with support plus cytokine therapy. Current results in our laboratory (data not presented) indicate that the DRF can be extended to 2.0. It appears that the upper limit of whole body, uniform radiation exposure from which spontaneous regeneration of marrow stem cells cannot be induced with current cytokine protocols is 6.0 cGy. Neutrophils cannot be produced within that critical time frame (18–22 days post exposure) to prevent sepsis from opportunistic antibiotic resistent pathogens. Nor can the platelets be produced early enough to prevent spontaneous hemorrhage.

New protocols and/or cytokines will be required to induce expansion of the depleted progenitor pool with subsequent differentiation into neutrophils and platelets within the critical time frame defined by our ability to clinically support the animals. We are currently investigating the combined protocols of rh GM-CSF and rh G-CSF in the lethally irradiated canine model. Preliminary results suggest that combination protocols of these cytokines, although capable of inducing a synergistic response in normal canines, cannot produce neutrophils earlier than the single cytokine protocols shown here. The target cell must be available for the cytokine to act upon. Apparently the combination of the lineage specific cytokines cannot affect the production of target cells that would allow for an earlier production of neutrophils.

The high dose, sublethally irradiated nonhuman primate model allows us to evaluate the efficacy of therapeutic protocols for reducing durations of neutropenia and thrombocytopenia in a model requiring clinical support to achieve 100% survival. Data shown here and in publication[3,37] show the increased efficacy of the PIXY321 molecule and the simultaneous administration of IL-3 and GM-CSF in production of both neutrophils and platelets in the irradiated primates when compared to respective single factor protocols of GM-CSF or IL-3 alone. It was also demonstrated that the simultaneous protocol was better than the sequential protocol of IL-3 followed by GM-CSF.

This review of recent data from our laboratory can serve to illustrate several points: the radiation models allow us to titrate radiation dose with bioeffect (survival and duration of cytopenia) and thus analyze stem and progenitor cell recovery kinetics in response to cytokine therapy; recovery after otherwise lethal radia

tion exposure requires cytokine therapy; the use of lineage specific cytokines G-CSF and GM-CSF do not "exhaust" low numbers of surviving stem or progenitor cells after otherwise lethal radiation exposure; recovery from otherwise lethal radiation exposure requires at least 18–20 days to generate neutrophils using G-CSF or GM-CSF; cytokine therapy can be delayed at least 9–12 days and still produce neutrophils within the 18–20 day time frame indicating that the early time frame post irradiation is required for the generation of cytokine-specific target cells; combination protocols using IL-3 and GM-CSF or the PIXY321 molecule are the most effective to-date in reducing both the neutropenia and thrombocytopenia following high dose sublethal radiation exposure; the response to cytokine in normal animals cannot be directly extrapolated to their effect in radiation compromised animals.

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# Comparison of Behavioral and Radioprotective Effects of WR-2721 and WR-3689

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McDONOUGH, J. H., P. C. MELE AND C. G. FRANZ. *Comparison of behavioral and radioprotective effects of WR-2721 and WR-3689.* PHARMACOL BIOCHEM BEHAV **42**(2) 233-243, 1992.—The behavioral effects of the radioprotectant agents ethiofos, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) and *S*-2-(3-methylaminopropyl)aminoethylphosphorothioic acid (WR-3689) were evaluated in rats trained to respond under a multiple fixed-interval 120-s, fixed-ratio 50-response (mult FI FR) schedule of milk reinforcement. Each compound produced dose-dependent reductions in responding under both schedules over the same dose range (100-180 mg/kg, IP); $ED_{50}$s indicated that WR-3689 was slightly more potent than WR-2721. On several performance measures, WR-3689 produced greater decrements during a second dose-effect determination, whereas WR-2721 had more pronounced effects during the initial one. In a second series of studies, low (56 mg/kg) and high (180 mg/kg) doses of both drugs were tested for radioprotective effects in rats responding under an FR-50 schedule of milk reinforcement and exposed to a nonlethal (5 gray, Gy) or lethal (10 Gy) dose of ionizing radiation ($^{60}$Co gamma rays). Neither dose of radiation altered FR response rates on the day of exposure (day 1). Five Gy of gamma radiation produced a 25-40% reduction in response rates on days 2-5 (24-72 h) after exposure. Neither dose of WR-2721 or WR-3689 provided significant protection against these performance decrements. All groups exposed to 10 Gy experienced a progressive decline in FR responding on days 2-5 after exposure. Performance of groups that received pretreatment with the 180-mg/kg dose of either drug or the 56-mg/kg dose of WR-3689 was maintained at significantly higher levels than saline-treated controls on days 4-5 after exposure to 10 Gy; however, even at these higher levels of performance response rates remained below 50% of preirradiation control levels. Subsequently, 56 and 180 mg/kg WR-3689 and 180 mg/kg WR-2721 were found to provide protection against the lethal consequences of the 10-Gy exposure. Thus, neither WR-2721 nor WR-3689 afforded any significant short-term protection against radiation-induced performance decrements when these drugs were administered at either behaviorally ineffective or behaviorally disruptive doses. Rather, the beneficial effects of these drugs paralleled their ability to antagonize radiation-induced lethality.

Ionizing radiation  Radioprotection  WR-2721  WR-3689  Operant behavior
Performance decrement  Rats

THE radioprotective compound ethiofos [*S*-2-(3-aminopropylamino)ethylphosphorothioic acid] (WR-2721) is generally considered the most efficacious drug for protection against the lethal effects of ionizing radiation (6,9,10,26). Under optimal conditions, animals treated with WR-2721 survive exposure to almost twice the lethal dose of radiation (3,6,26), and WR-2721 is currently used clinically as an adjunct to both radiotherapy and chemotherapy treatment for cancer (10,25). However, WR-2721 has several drawbacks for use in other than clinical settings. First, WR-2721 is ineffective when administered orally. Second, it produces significant behavioral side effects at doses that provide maximal protection against radiation-induced lethality (1,2,13,15,16). For example, in animal studies, doses from 200-400 mg/kg WR-2721 provide progressively greater levels of radioprotection, but these doses also produce progressively greater and prolonged reductions in trained or spontaneous motor activity (2,13,16). In humans, hypotension, gastrointestinal disturbances, and hypocalcemia are the most serious and most frequently reported side effects (9).

*S*-2-(3-Methylaminopropyl) aminoethylphosphorothioic acid (WR-3689) is another radioprotectant drug that is a close structural analog of WR-2721. Although the radioprotectant capabilities of WR-3689 are reported to be less than those achieved with WR-2721, WR-3689 is less toxic and retains its radioprotective effect when administered orally (3,7,9,10). For these reasons, WR-3689 may have use in a wider variety of situations than WR-2721. The behavioral side effects of WR-3689, however, have not been systematically evaluated. Therefore, the first goal of this investigation was to evaluate the behaviorally disrupting effects of these two radioprotectant drugs on a multiple fixed-interval 120-s, fixed-ratio 50-response (mult FI FR) schedule of reinforcement. This operant conditioning procedure has been used routinely to char-

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acterize the behavioral effects of a wide variety of compounds (14).

Exposure to ionizing radiation produces disruptions of schedule-controlled behavior that are both dose and time dependent (4,5,11,18-20). The ability of radioprotectant agents to moderate these radiation-induced performance decrements has not been extensively investigated; only four studies have been reported that evaluated the ability of radioprotectants such as WR-2721 to counteract the effects of radiation exposure on performance (1,2,13,21). Sharp et al. (21) reported that pretreatment of rhesus monkeys with *n*-decylaminoethanethio-sulfuric acid (WR-1607) prevented the immediate but temporary degradation in performance that occurs following exposure to rapidly delivered, supralethal doses of radiation (10-40 gray, Gy). (The Gy is a unit of absorbed dose of ionizing radiation.) However, WR-1607 provided no protection against the lethal effects of these high levels of exposure. Bogo et al. (1,2) studied the ability of WR-2721 to protect rodents and nonhuman primates from the early performance decrements produced by exposure to high, supralethal doses of radiation. Unlike WR-1607, which protected against such decrements, the combined effects of WR-2721 and radiation exposure produced more severe performance decrements than either treatment alone. In these three studies, the radiation challenge doses that were used were well in excess of levels against which lethality protection can be provided with these compounds. In contrast, Landauer et al. (13) reported that mice protected with WR-2721 against the lethal effects of lower doses of radiation displayed suppressed levels of spontaneous activity for up to 6 months after exposure. Thus, a second goal of the present work was to determine whether either WR-2721 or WR-3689 could provide protection against the behavioral as well as lethal effects of radiation exposure. The approach taken, however, differed from that used in previous studies. First, both low as well as high doses of the radioprotectants were used to determine whether drug doses that have minimal behavioral effects may afford any protection. Second, two radiation doses were used: a low (5 Gy) dose that produces moderate performance decrements but is not lethal, and a high (10 Gy) dose that produces pronounced performance decrements and is ultimately lethal but is still within the protective range of these drugs.

## EXPERIMENT 1

### METHOD

#### *Animals*

Seven adult, male Sprague-Dawley rats, weighing 275-350 g at the start of the study, were used. They were quarantined on arrival and screened for evidence of disease. They were housed individually in plastic Micro-isolator cages containing sterilized woodchip bedding. Acidified water (pH = 2.5-3.0), commonly used to reduce the possibility of infection in irradiated organisms, was provided ad lib. Access to food was restricted to maintain animals at 80% of their free-feeding body weights established prior to behavioral testing. Animal holding rooms were maintained at 21 ± 1°C with 50 ± 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. A 12-h lighting cycle was in effect with full-spectrum lights on from 0600-1800.

#### *Apparatus*

Identical operant conditioning chambers (Coulbourn Instruments, Inc., Lehigh Valley, PA) were used. The front wall of each chamber contained a response lever, three cue lights mounted above the lever, a house light, a Sonalert speaker, and an opening that allowed access to a dipper that presented 0.06 ml sweetened condensed milk (a 1:1, v:v, mixture of Bordens Eagle Brand and tapwater). Each chamber was enclosed in a sound-attenuating cubicle that was equipped with an exhaust fan for frequent air exchange. The testing room containing the cubicles had masking noise present continuously. Control of experimental stations and recording of data were accomplished with a PDP 11/73 computer using SKED-11 software (State Systems, Inc., Kalamazoo, MI) and cumulative recorders (Gerbrands Corp., Arlingtion, MA) located in a separate room.

#### *Behavioral Procedure*

Rats were trained to lever press using an automated procedure consisting of two schedules of milk delivery that were in effect simultaneously. When the house light was illuminated, a variable-time (VT) schedule presented the dipper automatically on the average of once every 60 s, while a fixed-ratio 1 (FR 1) schedule presented the dipper after each lever press. The dipper was presented for 4 s and was signalled by illuminating a light over the dipper; the house light was extinguished during dipper presentation. The VT schedule was discontinued after 10 lever-press responses had been made in a single daily session. Sessions lasted 60 min or until 100 responses had been made, whichever occurred first. Rats that did not acquire the lever-press response after five to seven training sessions were shaped by the method of successive approximations.

After an additional one or two sessions under FR 1, rats were exposed to a series of increasing fixed-interval (FI) schedules over the next 10-15 training sessions until an FI 120-s schedule was in effect. Training on the FI 120-s schedule was continued for another 10-15 sessions, and then the FR component of the multiple schedule was introduced. The FR value was gradually raised over 10-15 training sessions until the terminal FR 50 schedule was reached. Only the house light was illuminated during the FI component. During the FR component, the three cue lights were illuminated and a 60-dB, 2.8-KHz tone was sounded. Each component schedule was presented three times during a daily session; the FI schedule component was always presented first, and the two schedules alternated throughout the session. Components ended with the first reinforcer delivered after 10 min or automatically if a reinforcer was not obtained after 12.5 min (2.5 min limited hold on component duration). There was a 10-s time out (TO) between components when all environmental cues were extinguished. Total session time was approximately 1 h. Rats were trained for approximately three months on the final schedule to establish stable baselines before drug testing was begun.

#### *Drugs*

WR-2721 (lot BL20103) and WR-3689 (lot BL08385) were obtained from the Department of Experimental Therapeutics, Walter Reed Army Institute of Research. The drugs were dissolved in saline immediately before injection. Injection volume was 1 ml/kg. Drugs were injected IP 15 min before behavioral testing. All rats were tested at each drug dose and each dose was tested twice. Doses were given in an ascending and then a descending order. Drugs were usually administered on Tuesdays and Fridays. Dose-effect curves were determined first for WR-2721 and then for WR-3689.

*Data Analysis*

Overall response rate, postreinforcement pause duration, and running response rate were calculated for both FI and FR responding for each session. Overall response rate was calculated by dividing the total number of responses in the three FI or FR components by the total duration of the respective components (excluding the time the dipper was raised). Postreinforcement pause duration was defined as the time from the end of a dipper presentation until the first response of the next ratio or interval. Running response rate was the response rate calculated with the postreinforcement pause time omitted. Index of curvature was also calculated for the FI data (8) to provide a measure of the temporal pattern of responding, often positively accelerated, that typically occurs under this schedule of reinforcement. For each behavioral measure, the data were analyzed using a within-group design with both drug dose and replication considered repeated-measures factors. The criterion level for significance was set at $p < 0.05$. In addition, dose estimations for drug effects on performance were determined using the following procedure. For each rat, the FI and FR response rate data from each baseline session prior to each drug session were used to calculate 95% confidence limits for nondrug performance. Then, for each drug session the performance of each rat was dichotomously categorized as "not decremented" (within the 95% confidence limits) or "decremented" (below the lower 95% confidence limit); performance after drug administration never exceeded the upper 95% confidence limit. These data were then used to determine $ED_{50}$ using standard probit analysis procedures.

## RESULTS

WR-2721 decreased FI response rates in a dose-dependent manner (Fig. 1, top); significant decreases occurred at the 133- and 180-mg/kg doses, $F(6, 36) = 6.20$, $p < 0.01$. There was a significant dose × replication interaction on FI responding, $F(6, 36) = 3.23$, $p < 0.05$. This was due to a greater decrease in FI response rates the second time rats received 56 mg/kg and less of a decrease in FI rates the second time they received 180 mg/kg.

There was also a significant dose × replication interaction for the effect of WR-2721 on the number of FI reinforcers earned, $F(6, 36) = 4.03$, $p < 0.01$. On the ascending series, there was a reduction in the number of FI reinforcers earned after the 133- or 180-mg/kg doses, while almost all possible reinforcers were earned during the descending drug series (Fig. 1, middle). Analysis of the index of curvature data revealed that there were no dose-related changes in this measure of FI performance (not shown).

The 133- and 180-mg/kg doses of WR-2721 also produced significant decreases in FR response rates [$F(6, 36) = 7.46$, $p < 0.01$; Fig. 1, bottom]; the replication and dose × replication factors were not significant. The decrease in FR responding produced by WR-2721 was due to a reduction in FR running response rate, $F(6, 36) = 7.15$, $p < 0.01$, and a concurrent increase in postreinforcement pause time [$F(6, 36) = 4.59$, $p < 0.01$; not shown]. In addition, there was a significant dose × replication effect for FR running response rates $F(6, 36) = 2.64$, $p < 0.05$; these rates were decreased less the second time rats received the 180-mg/kg WR-2721 dose.

Like WR-2721, WR-3689 significantly decreased FI response rates, $F(5, 30) = 4.09$, $p < 0.05$, at doses of 133 and 180 mg/kg (Fig. 1, top). However, unlike WR-2721, WR-3689 did not reduce response rates in a manner that varied significantly across replications of the dose-effect function, even though response rates tended to be decreased to a greater degree during the second (descending) than the first (ascending) determination.

WR-3689 also decreased the number of FI reinforcers earned [$F(5, 30) = 10.57$, $p < 0.01$; Fig. 1, middle]. This effect was significantly greater on the descending-dose series than on the ascending series, $F(1, 6) = 6.45$, $p < 0.05$, which was the reverse of effects observed with WR-2721. On the FI index of curvature measure, WR-3689 produced dose-dependent decreases that paralleled the effect on overall FI responding [$F(5, 30) = 7.45$, $p < 0.01$; not shown]. However, because reductions in the index of curvature occurred only when response rates were severely suppressed this effect cannot be considered particularly meaningful.

WR-3689, at 133 and 180 mg/kg, produced significant reductions in overall responding in the FR component [$F(5, 30) = 9.42$, $p < 0.01$; Fig. 1, bottom]; this effect did not vary significantly between the ascending- and descending-dose series. The reduction in overall FR responding was due to a reduction in running response rate, $F(5, 30) = 11.67$, $p < 0.01$, and an increase in postreinforcement pause time [$F(5, 30) = 7.65$, $p < 0.01$; not shown]. Moreover, there was a significantly greater ($p < 0.05$) increase in total FR postreinforcement pause time on the descending-dose series (mean = 221.2 s) compared to the ascending series (mean = 74.4 s).

A comparison of the effects of each drug on rates of responding expressed as a percentage of baseline control rates is presented in Fig. 2. Analysis of these data showed that WR-2721 produced equivalent rate-decreasing effects under both the FI and FR schedules, whereas WR-3689 produced significantly greater decreases in overall FR than FI response rates ($p < 0.01$). Probit analysis estimates of the median effective doses (and the lower and upper confidence limits) for producing rate-decreasing effects on each schedule for each drug were: FI: WR-2721 $ED_{50}$ = 99.5 mg/kg (74.3–144.2 mg/kg), WR-3689 $ED_{50}$ = 80.6 mg/kg (59.0–105.9 mg/kg); FR: WR-2721 $ED_{50}$ = 78.2 mg/kg (57.3–108.9 mg/kg), WR-3689 $ED_{50}$ = 55.7 mg/kg (31.3–76.7 mg/kg). Probits indicate that WR-3689 was slightly more potent than WR-2721 in reducing response rates.

## EXPERIMENT 2

The first experiment demonstrated that both WR-2721 and WR-3689 reduced schedule-controlled performance at doses (133–180 mg/kg) lower than those reported to provide maximal protection against radiation-induced lethality [200–400 mg/kg; (3,6,22,24)]. Although high doses of these drugs appear to provide maximal protection, lower drug doses that produce minimal behavioral effects may also provide some protection against radiation-induced lethality. This may be especially relevant because dose-effect curves for radiation-induced lethality are typically very steep, and even small shifts in these curves may result in protection of significant numbers of animals. In addition, radiation exposure produces decrements in the performance of trained behaviors. At sub- and near-lethal exposure levels, these performance decrements become most evident in the days immediately following exposure (11,18–20). Most previous behavioral studies of radioprotectants in conjunction with radiation exposure have dealt primarily with the ability of the drug to antagonize performance

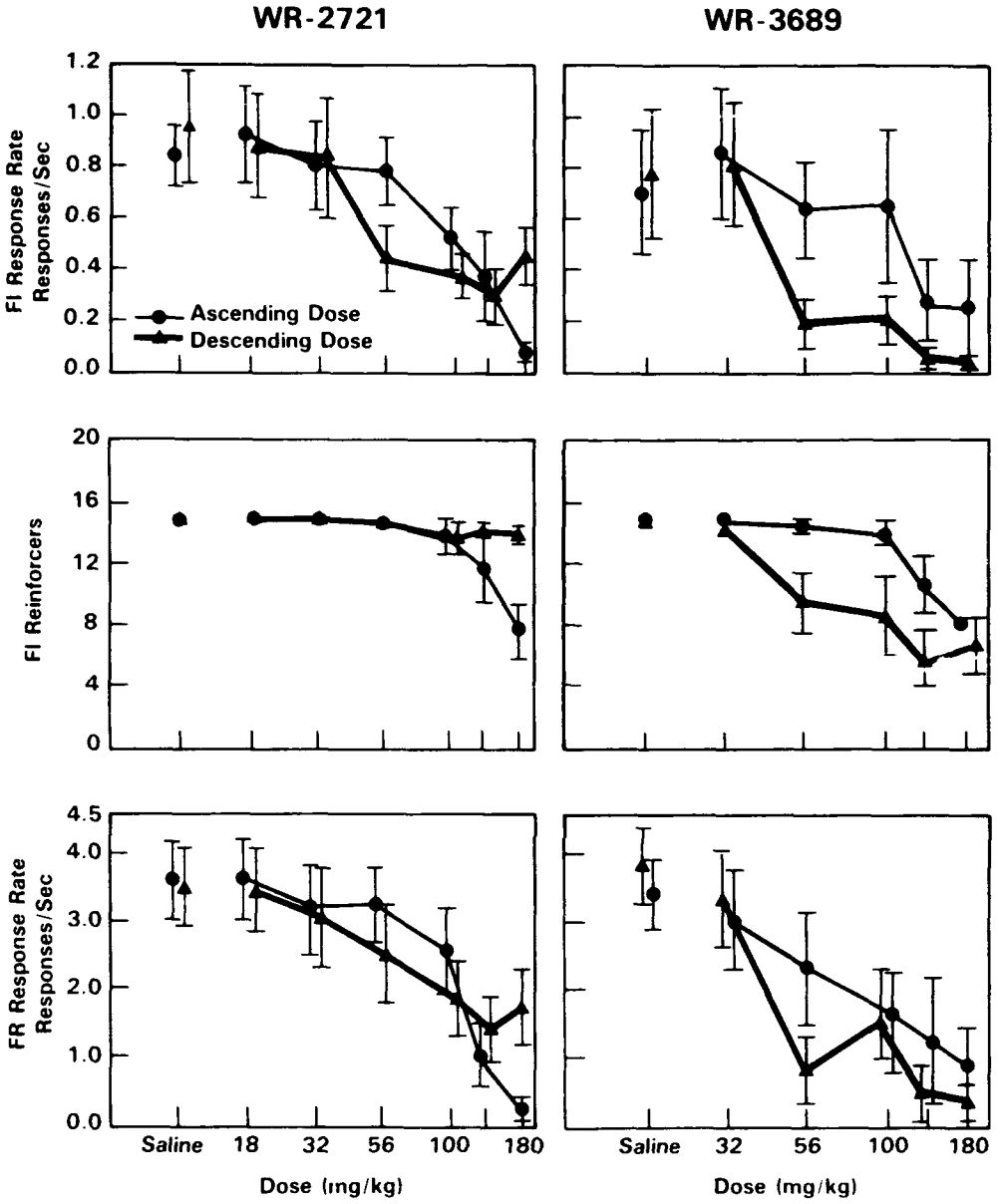


FIG. 1. Effects of WR-2721 (left) and WR-3689 (right) on selected measures of performance under a multiple FI 120-s, FR 50-response schedule of milk reinforcement. Top: FI response rate; center: FI reinforcers; bottom: FR response rate. (●), ascending dose-effect determination; (▲), descending dose-effect determination. Each point represents the mean ± SEM of seven rats.

decrements that occur within the first hour following rapidly delivered, supralethal levels of exposure (1,2,21). These high levels of exposure are well in excess of the protective capabilities of these drugs against radiation-induced lethality, and the performance decrements they produce are distinctly different from the types of behavioral decrements that occur following sub- or near-lethal levels of exposure. Only one study has investigated the ability of a radioprotectant to moderate radiation-induced behavioral decrements following lethal (yet survivable when given the radioprotectant) exposure. Landauer et al. (13) reported that mice protected with WR-2721 from the lethal effects of a 14-Gy exposure displayed significantly reduced levels of spontaneous locomotor activity for almost 6 months following exposure.

Experiment 2 followed a similar approach and was designed to extend these findings by directly comparing WR-2721 and WR-3689 for radioprotective efficacy against radiation-induced performance decrements and lethality. In this experiment, rats responded under an FR 50 schedule of milk reinforcement, and both high and low doses of the two radio-

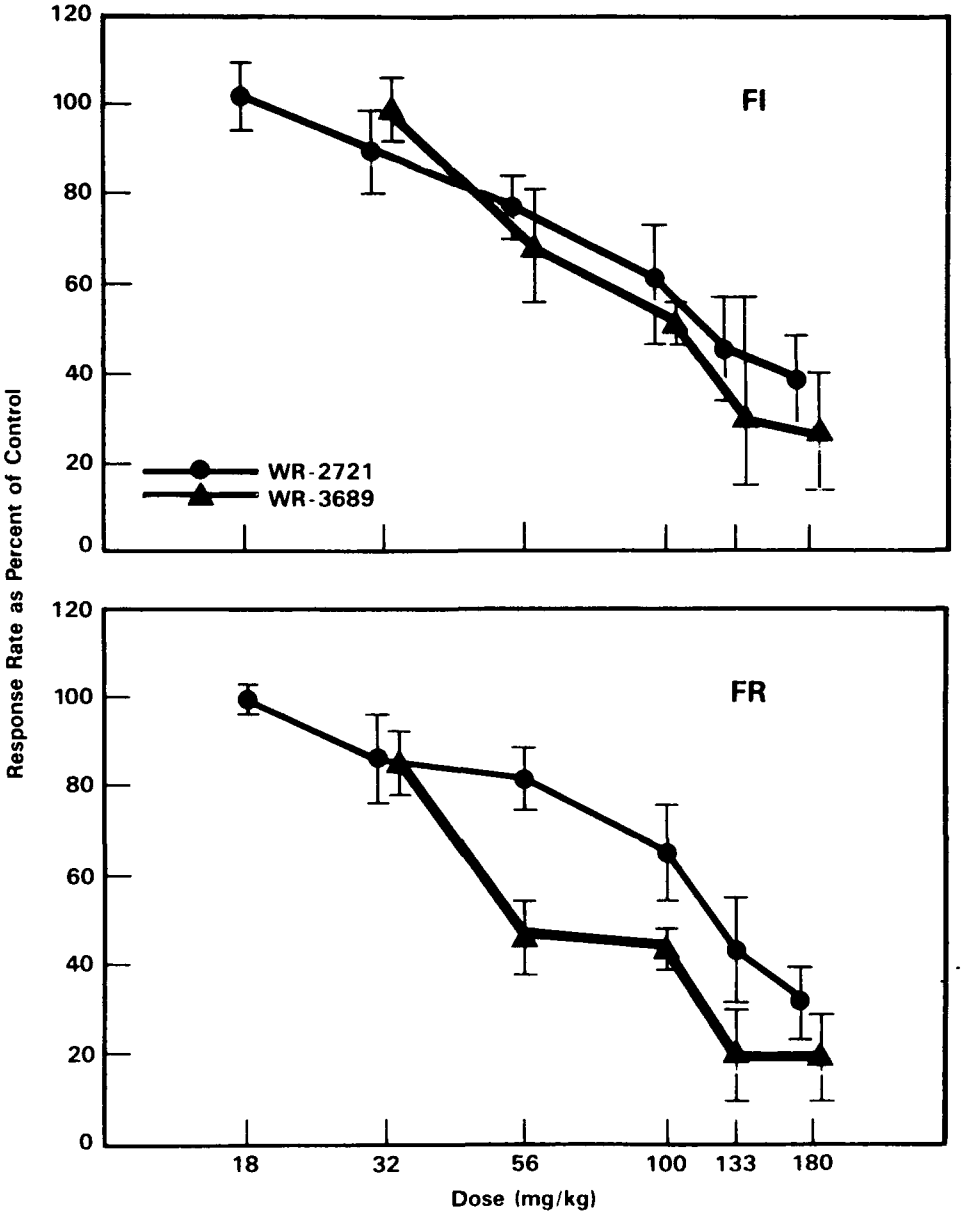


FIG. 2. Comparison of the rate-decreasing effects of (●) WR-2721 and (▲) WR-3689 under the multiple FI 120-s (top), FR 50-response (bottom) schedule of milk reinforcement. Response rates are expressed as a percentage of baseline control rates. Each point represents the mean ± SEM of seven rats.

protectant drugs were tested for their ability to moderate performance decrements that occur following either a nonlethal or lethal exposure to ionizing radiation.

## METHOD

### *Animals*

Adult, male Sprague-Dawley rats were used. Animals were maintained under identical conditions as described for the first experiment.

### *Behavioral Procedures*

Rats were trained to lever press as described above. After one or two sessions under FR 1, the FR value was gradually increased over 15–20 sessions until an FR 50 schedule was in effect. Sessions ended with the first milk delivery after 30 min or automatically if a reinforcer was not obtained after 30.5 min (0.5 min limited hold on session duration). Only the house light was illuminated during the session. Training on the FR 50 schedule continued for 30–40 sessions to

stabilize responding before experimental treatments were begun.

*Radiation Exposure*

Rats were placed in well-ventilated, clear-plastic, restraining tubes for irradiation. Rats were habituated to the tube-restraint procedure over several weeks. They were restrained and transported to the exposure room on at least five occasions before either the sham test or the actual exposure day. Approximately 1 week before radiation exposure, rats were assigned to test groups ($n$ = 6-7/group) and a sham-exposure test was conducted the next day. For the sham-exposure test, each rat was injected with its assigned drug dose, immediately placed in a restraining tube, and then transported to the exposure facility where, 15 min after injection, a sham exposure took place (rats were placed in the exposure room but were not irradiated). Rats were then returned immediately to the behavioral laboratory for testing. This series of manipulations was performed to determine the combined effects of drug injection, restraint, and transportation on performance of the FR 50 task. The procedure on the day of irradiation was identical to that just described with the exception that 15 min after drug injection rats were given a bilateral, whole-body exposure to gamma rays from a $^{60}$Co source at a rate of 2.5 Gy/min to a total dose of either 5 or 10 Gy. Prior to irradiation, the dose rate at the midline of an acrylic rat phantom was measured using a 0.5-cc tissue-equivalent ionization chamber (Exradin, Inc., Lisle, IL). The dose rate at the same location with the phantom removed was measured using a 50-cc ionization chamber fabricated in-house. The ratio of these two dose rates, the tissue-air ratio (TAR), was used to determine the doses for irradiated rats. In these experiments, the TAR was 0.93. All ionization chambers have calibration factors traceable to the National Institute of Standards and Technology. Dosimetry measurements were performed following the AAPM Task Group 21 *Protocol for the Determination of the Absorbed Dose from High-Energy Photon and Electron Beams* (22). In each experiment, three doses of each radioprotectant drug (saline control, 56 and 180 mg/kg) were tested against one of two radiation challenge doses (nonlethal dose = 5 Gy; lethal dose = 10 Gy). Therefore, in each study there were six experimental groups. The first FR test session began approximately 5 min after exposure ceased, and testing continued 5 days per week for 30 days, the standard duration used to assess rodent survival following radiation exposure.

*Data Analysis*

Overall FR response rate was considered the best indicator of performance and was the only measure analyzed in detail. The last 10 days of preirradiation baseline performance (exclusive of the days rats were placed in restraining tubes and the sham-exposure day) were averaged for each rat: these individual means were used to calculate baseline performance for each group and determine change from baseline data. For the 5-Gy exposures, separate analyses were performed for the first 5 days after exposure and for the entire 30-day period after exposure. Because of deaths within the various 10-Gy treatment groups, formal statistical analysis was only performed on the performance data from the days when within-group $n$s were constant. Data were analyzed using two-way repeated measures analyses of variance (ANOVAs), with drug dose being the between-groups factor and days the repeated measure. Significant effects were further evaluated using Dunnett's multiple comparison test or $t$-tests with Bonferroni corrections. The criterion for significance was set at $p < 0.05$.

## RESULTS

*WR-2721*

There were no between-group differences in response rates on baseline or "tube-restraint" days before exposure. Response rates on tube-restraint days did not differ significantly from baseline control days for individual groups. Baseline control response rates (mean ± SEM in responses per second) for the groups exposed to 5 Gy gamma radiation were 2.13 ± 0.52 (saline), 1.80 ± 0.26 (56 mg/kg), and 1.89 ± 0.27 (180 mg/kg). Baseline rates for the groups exposed to 10 Gy were 1.64 ± 0.31 (saline), 2.08 ± 0.30 (56 mg/kg), and 1.57 ± 0.32 (180 mg/kg). On the sham-exposure day, only the performance of the group given 180 mg/kg WR-2721 was significantly affected; responding was reduced to less than 50% of the preirradiation baseline rate (Fig. 3). Saline and 56 mg/kg WR-2721-treated groups performed at levels that were not significantly different from their baseline response rates.

*WR-2721 + 5-Gy Exposure*

Drug dose did not differentially affect performance, nor were there any significant dose × day interactions after the 5-Gy exposure. However,there were significant within-group changes in response rates. The performance of the saline-treated group was normal on the day of exposure, but was reduced significantly relative to baseline on days 2-5 following exposure. Performance of the saline-treated group had recovered by day 8 and did not vary significantly from baseline for the remainder of the study. Performance of the 56-mg/kg WR-2721 group was reduced significantly relative to its baseline on days 1-3 following exposure, and then recovered to baseline levels for the remainder of the study. The group treated with 180 mg/kg WR-2721 showed only a slightly different pattern of results: a nonsignificant reduction in responding on the day of exposure, significant reductions in performance on days 2-5 following exposure relative to baseline, and then recovery to preirradiation baseline performance levels for the remainder of the study.

*WR-2721 + 10-Gy Exposure*

The pattern of results was markedly different in groups that received the 10-Gy radiation exposure. On the day of the 10-Gy radiation exposure, there were no statistically significant decrements in performance from baseline within each drug treatment group, nor were there any significant differences between groups. On days 2-5 postexposure, the performance of all treatment groups deteriorated progressively from their preirradiation baselines, reaching a nadir on day 5. There were no significant differences between groups in the magnitude of the performance decrement or the rate of decline over these days.

Throughout the week after the 10-Gy exposure, all rats maintained their body weights within ± 15 g of target weight. Two rats in the 180-mg/kg WR-2721 ± 10-Gy dose group left a small amount (mean = 4.8 g) of food over the first 24 h (days 1-2) after exposure. In all groups, entire food rations were consumed on days 2-3 and only small amounts (mean = 3.1 g) were left on days 3-4; in contrast, performance had declined 65-72% from baseline over this time. On days 4-5,

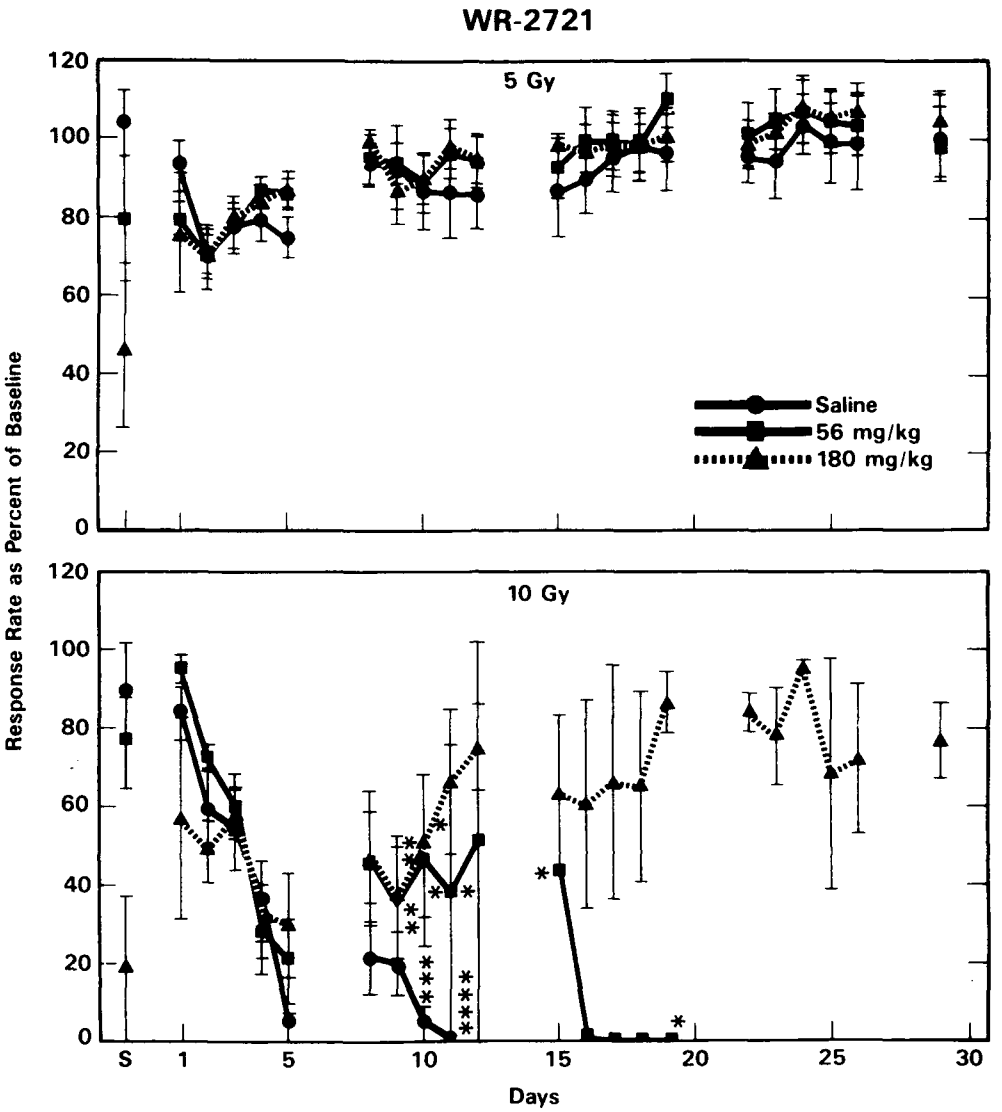


FIG. 3. Effects of (●) saline, (■) 56 mg/kg WR-2721, or (▲) or 180 mg/kg WR-2721 on FR 50 response rates and survival following exposure to 5 Gy (top) or 10 Gy (bottom) of [$^{60}$Co] gamma radiation. Response rates are expressed as a percentage of baseline control rates. Each point represents the group mean ± 1 SEM. Points at S represent the performance of the respective treatment groups on the day of sham exposure ($n$ = 6-7 per group). Radiation exposure occurred on day 1 for all groups 15 min prior to the behavioral testing. Asterisks that accompany curves on the 10-Gy exposure condition indicate deaths in these groups.

the time of maximal performance decrement in the first week, substantial amounts of food were left by all three 10-Gy treatment groups (saline: mean = 9.9 g; 56 mg/kg: mean = 12.8 g; 180 mg/kg: mean = 9.5 g).

On test sessions 8 and 9, performance of all treatment groups recovered somewhat relative to their performance on day 5. However, analysis of recovery was confounded after this point by varying mortality rates across groups. Saline-treated rats began dying between days 10-11, and all rats in this group died by day 12. Rats that received 56 mg/kg WR-2721 began dying after day 9; five of the six rats died between days 9 and 14, and the last rat died on day 23. In contrast, only three of the six rats that received 180 mg/kg WR-2721 died, and these deaths occurred between days 10-11. As might be expected, the performance of all three treatment groups became quite variable during the time that rats were showing signs of radiation toxicity. Three rats that received 180 mg/kg WR-2721 survived; their performance recovered to 80-90% of preirradiation baseline levels by the end of the third week after exposure. Subsequently, daily response rates of one rat were quite variable, while response rates of the other two rats generally remained near preirradiation baseline levels for the remainder of the study.

*WR-3689*

There were no significant between-group differences in response rates on baseline or tube-restraint days before expo-

sure. Baseline control response rates (mean ± 1 SEM in responses per second) for the groups exposed to 5 Gy gamma radiation were 2.15 ± 0.25 (saline), 2.22 ± 0.52 (56 mg/kg), and 2.07 ± 0.39 (180 mg/kg). Baseline rates for the groups exposed to 10 Gy were 2.32 ± 0.39 (saline), 1.97 ± 0.23 (56 mg/kg), and 1.69 ± 0.16 (180 mg/kg). On the sham-exposure test, the 180 mg/kg dose of WR-3689 decreased FR responding significantly relative to this groups's own baseline performance, the performance of the saline-treated group, and the performance of the group given 56 mg/kg WR-3689 (Fig. 4). The 56-mg/kg dose of WR-3689 produced small but nonsignificant reductions in responding.

*WR-3689 + 5-Gy Exposure*

The only significant between-groups effect occurred on the day of exposure when the 180-mg/kg WR-3689 treatment group responded less than either the saline or 56-mg/kg WR-3689 treatment groups (Fig. 4, top). Moreover, the saline, $F(5, 75) = 4.69$, $p < 0.01$, and 180-mg/kg WR-3689, $F(5, 75) = 9.25$, $p < 0.01$, treatment groups showed significant changes in response rates over the 5 days following the 5-Gy exposure. Relative to their baseline levels of performance, saline-treated rats showed significant decreases in FR performance on days 2, 3, and 5 postexposure, while the group treated with 180 mg/kg WR-3689 displayed significant decreases in performance on postexposure days 1, 2, and 3. In contrast, response rates were reduced to a small but nonsignificant degree (the largest reduction occurred on day 2) from baseline control values in the group given 56 mg/kg WR-3689 prior to irradiation. By the beginning of the second week after exposure, the performance of each group had recovered to within its preexposure baseline range, where it remained for the duration of the study.

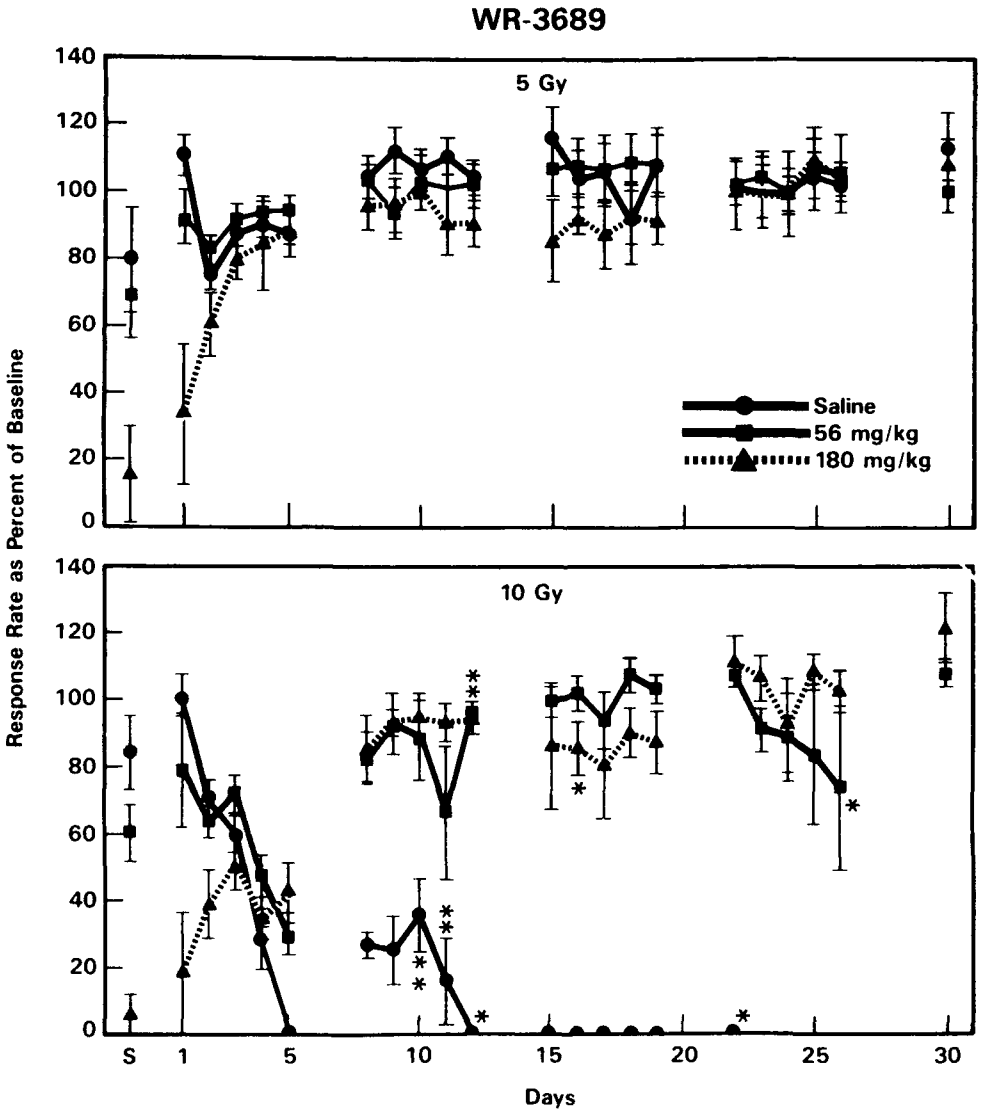


FIG. 4. Effects of (●) saline, (■) 56 mg/kg WR-3689, or (▲) 180 mg/kg WR-3689 on FR 50 response rates and survival following exposure to 5 Gy (top) or 10 Gy (bottom) [$^{60}$Co] gamma radiation. Presentation format is identical to that of Fig. 3. Asterisks that accompany curves on the 10-Gy exposure condition indicate deaths in these groups.

*WR-3689 + 10-Gy Exposure*

Following the 10-Gy exposure, each treatment group showed a distinct pattern of performance changes (Fig. 4, bottom). Saline-treated rats performed normally on the day of irradiation; performance then declined progressively to virtually negligible levels on day 5. On days 8 and 9 postirradiation, the performance of this group showed a modest recovery. However, performance rapidly declined again over the next two sessions as rats in this group began to die. Five of the rats in this group died between days 10 and 12 after exposure and one rat died on day 23 (this rat never performed more than 1-3 ratios per session after day 11).

Rats receiving the 56-mg/kg WR-3689 treatment also performed within their baseline range on the day of exposure to 10 Gy. Their performance progressively declined over days 2-5 to a level that was 30% of their preexposure baseline. Their performance recovered to levels not significantly different from their preexposure baseline on day 8. The "recovered" performance of this treatment group was maintained throughout the remainder of the study, except during the sessions that immediately preceded death for two rats (days 12 and 27).

For rats that received the 180-mg/kg WR-3689 treatment, performance during the test session immediately following the 10-Gy exposure was suppressed to the same extent that had occurred on the sham-exposure test. Performance improved somewhat but remained significantly below baseline over days 2-5. On day 5, the performance of this treatment group was 34% of baseline, a level not significantly different from the 56-mg/kg WR-3689 treatment group but significantly greater than the saline-treated group. On day 8, the performance of the 180-mg/kg WR-3689 group recovered to levels not significantly different from their preirradiation baseline values; performance was maintained at this level throughout the remainder of the study. Only one rat in this group died; this death occurred on day 16.

Rats in various treatment groups left different amounts of their daily food ration over the first 5 days after irradiation with 10 Gy. Five rats in the 180-mg/kg WR-3689 group left substantial amounts of chow (9-12 g) on the day after exposure and small amounts of food (<3-4 g) on each of the following 3 days. Similarly small amounts of food were left by three rats in the 56-mg/kg WR-3689 treatment group and by one saline-treated rat during the first week (days 4-5) after exposure. These results are the inverse of the performance data: Saline-treated subjects left the least food yet experienced the most pronounced performance decrement over this period, while the group that received 180 mg/kg WR-3689 left substantial amounts of food but continued to perform (especially on day 5).

One unexpected finding of these studies was the failure of 56 mg/kg WR-2721 to provide any protection against the lethal effects of 10 Gy radiation, whereas the same dose of WR-3689 protected 50% of 10 Gy-exposed rats. It has been reported that WR-2721 provides a greater shift to the right in the radiation-induced lethality function than WR-3689 (3). Thus, the failure of 56 mg/kg WR-2721 to provide any protection against this level of radiation challenge, while WR-3689 did, was not anticipated and raised the question whether some procedural variable may have contributed to this finding. To address this possibility, a replication of the WR-2721 + 10 Gy study was performed ($n$ = 6/group: saline, 56 and 180 mg/kg WR-2721). Operant training, drug dosing, and radiation exposures were conducted in an identical fashion to that already described. The results, both the survival data and the operant-performance data, were virtually identical to those of the initial WR-2721 experiment.

Saline-pretreated animals performed normally on the day of exposure, then showed a progressive decline in responding over the next several test sessions to near-zero performance on day 5 postexposure. This was followed by minimal improvement in performance on days 8-9 before a decline associated with mortality occurred; all saline-pretreated subjects died between days 8-11. Similarly, the group treated with 56 mg/kg WR-2721 performed at baseline levels on the test immediately after exposure, and their performance declined to near-zero levels on day 5 postexposure. The 56 mg/kg WR-2721-treated group did show a more pronounced recovery of performance than saline-treated rats over sessions 8-12, but performance then declined again and all rats died within the 30-day postexposure testing period. Rats in this group began dying on day 10, five of six of them had died by day 14, and the final rat died on day 24. The performance of the group treated with 180 mg/kg WR-2721 was significantly decremented on the day of exposure, primarily because of the high dose of the drug. Their performance improved toward baseline on day 2 to a level not different from the other two treatment groups. Over days 3-5 their performance declined, but on day 5 their performance began to recover and was significantly greater than either of the other two treatment groups. By day 8, the performance of the 180-mg/kg WR-2721 pretreatment group had recovered to preirradiation baseline levels, and despite one death (day 10) it remained in a range not significantly different from baseline for the remainder of the 30-day testing period. This mortality rate (1/6) was not substantially different from the 3/6 deaths experienced by the comparable treatment group in the initial study. Thus, in terms of protection against radiation-induced lethality and both the magnitude and duration of the postexposure performance decrements the results of this second experiment with WR-2721 replicated the findings of the initial study.

## GENERAL DISCUSSION

The major finding of the present study was that neither WR-2721 nor WR-3689, at either a low or high dose, produced a substantial blockade of the decrements in FR performance that occurred following radiation exposure, regardless of whether the radiation exposure was lethal or not. However, several interesting differences between WR-2721 and WR-3689 were revealed. First, although high doses of both compounds were equally efficacious in protecting against the lethal effects of radiation exposure, only WR-3689 offered any protection at low drug doses. Second, the calculated $ED_{50}$s indicated that WR-3689 was somewhat more potent than WR-2721 in producing behavioral decrements as indexed by disruption of mult FI FR performance.

The results of the mult FI FR experiment clearly indicated that both WR-2721 and WR-3689 produced dose-dependent decreases in response rates during each of the component schedules. The rate-decreasing effects of WR-2721 were similar for both component schedules, while for WR-3689 the FR dose-effect curve was shifted to the left of that for FI when rates were expressed as percentage of control. Unlike the recent report by Liu et al. (15), the effects of neither radioprotectant appeared to be characterized by an "all or none" suppression of responding. In that study, WR-2721 was tested in rats performing under an FR 20 schedule of water reinforce-

ment; doses of 75 or 100 mg/kg WR-2721 produced almost total cessation of responding ($ED_{50}$ = 58.5 mg/kg). In the present study, in contrast, animals continued to perform under both component schedules at doses up to 180 mg/kg of either radioprotectant. Although total suppression of performance did occur in selected cases, this was not a consistent finding. More typically, animals continued to perform throughout the session, albeit at very low response rates. This is most readily demonstrated by the continued acquisition of reinforcers under the FI component of the schedule at the high doses of both drugs. This difference between these results and those of Liu et al. (15) could be due to one or more procedural differences such as the type of reinforcer used to maintain behavior, the use of a multiple vs. a simple schedule of reinforcement, or the FR value.

Although there were no major differences in the character of the performance decrements produced by each compound in the present study, there were several interesting differences between the two drugs in the pattern in which effects were manifested on the dose-effect replications. WR-2721 produced significantly less of an effect on several indices of FI performance and on FR running response rate on the descending-dose series relative to the ascending-dose series. In contrast, WR-3689 produced significantly greater effects on the number of FI reinforcers acquired and on FR postreinforcement pause duration on the descending-dose series. There is no evidence to indicate an enhanced metabolism of WR-2721 with repeated dosing at the intervals and dose ranges used in this study (17). Moreover, based on physiochemical properties, WR-3689 is very similar to WR-2721 and should be metabolized in the same fashion (7). Thus, it is unlikely that these differential behavioral effects on the ascending and descending dose-effect determinations were due to pharmacokinetic differences between the two drugs. In general, though, pronounced decreases in performance occurred with either compound at doses $\geq$ 100 mg/kg. Most studies of the radioprotective efficacy of these compounds have focused on drug doses substantially higher than those used here (e.g., $\geq$ 200 mg/kg), and several authors have ascribed at least part of the radioprotective effects of these drugs to the general behavioral depression that is produced (12,13,16,23).

The second series of experiments addressed the question of whether relatively low doses of these drugs provide any protection against the lethal and behavioral effects of exposure to gamma radiation. The results show that neither compound, at either the low or the high dose, was effective in totally protecting animals against the decreases in FR performance that occur in the initial days following either nonlethal or lethal radiation exposure. At the lower radiation dose (5 Gy), the magnitude and time course of the depression in performance was essentially equivalent for all treatment groups. One notable finding was that the performance of the 56-mg/kg WR-3689 group never dropped significantly below its preirradiation baseline in the week following exposure. At the lethal radiation dose, the radioprotective effects of each drug were clearly evident, yet again there was no clear drug-induced antagonism of the pronounced behavioral decrement. At best, treatment with a radioprotectant attenuated only the relative degree of the performance decrement. This pattern of performance change over days after the lethal 10-Gy exposure (i.e., a progressive decline in responding to near-zero levels on day 5, followed by a modest recovery on days 8–10) mirrors the performance changes that were reported after exposure to a high but generally nonlethal dose (i.e., a gamma-ray dose of 9 Gy produced 14% lethality over 30 days) (20). The most striking difference between the two patterns is that nearly total recovery of performance occurs in the second week of testing following exposure to the 9-Gy dose, while after 10 Gy performance remains severely disrupted. Behavioral recovery during the second week after exposure was observed in the present study in rats that survived the 10-Gy exposure when pretreated with either WR-2721 or WR-3689.

Both WR-2721 and WR-3689 were equally efficacious at high doses in protecting rats against the lethal effects of radiation exposure. All rats that received saline as a pretreatment died, and all but one of the deaths occurred on days 10–12 postexposure. This is consistent with the time frame associated with radiation-induced hematopoietic failure (9,25). The 180-mg/kg dose of either drug protected 50% (WR-2721, replication 1) to 86% (WR-3689; WR-2721, replication 2) of the rats, but only WR-3689 showed any protective effects (50%) at the lower 56-mg/kg dose. Rats that had received a radioprotectant and died did so primarily over the same time frame as unprotected animals (70% died on days 10–12), although there were four animals that survived 16, 22, 24, and 27 days, respectively. Thus, drug treatment did not substantially alter survival time in these animals. Perhaps the most interesting finding of the study was that a low dose (56 mg/kg) of WR-3689 provided significant protection against the lethal 10-Gy radiation challenge, whereas an equivalent dose of WR-2721 was ineffective. This result was not anticipated, but the replication of the WR-2721 study confirmed that 56 mg/kg of this drug fails to provide protection under these conditions.

In summary, the two radioprotectant drugs WR-2721 and WR-3689 produced highly similar, dose-dependent behavioral decrements. Neither drug was capable of effectively antagonizing behavioral decrements that occurred following radiation exposure, which indicates that the mechanisms responsible for the behavioral decrements may be independent from those responsible for radiation-induced lethality. WR-3689 was capable of providing significant radioprotective effects at a low dose that produced no behavioral disruption by itself; WR-2721 did not provide protection at the same dose. This finding, in conjunction with its reported oral effectiveness, suggests that WR-3689 is a compound that possesses a number of desirable features as a radioprotectant that WR-2721 does not.

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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
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# ENERGY TRANSFER MECHANISMS IN DNA: RELATIONSHIP TO ENERGY DEPOSITION IN SUB-MICROSCOPIC VOLUMES

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Energy transfer on the nanometer scale plays an important role in the calculation and interpretation of energy deposition in sub-microscopic volumes. Transport of energy by secondary electrons is a well-known example of this phenomenon. Intramolecular energy and charge transfer after collisional excitation and ionization of molecules have received considerably less attention in the radiological physics community. Nevertheless, recent findings at several laboratories (1-3) have raised questions about the common assumption that radiation-induced DNA damage remains localized on the nanometer scale during the formation of biologically significant lesions.

We have investigated (4-6) mechanisms for the observation (1) that DNA base radicals induced by neutrons are dependent on the orientation of DNA fibers relative to the neutron flux. Monte Carlo codes developed by Wilson and Paretzke (7) and scoring algorithms contributed by Charlton (8) were used to model energy deposition in oriented DNA under direct proton-beam irradiation. Table I summarizes our results for a 1 MeV proton flux incident on an oriented DNA sample either parallel or perpendicular to the fiber direction. Although the average amount of energy deposited in the

Table I
Energy Deposition in Oriented DNA by 1 MeV Protons

| Orientation | Energy Deposited (eV) | Event Separation (nm) |
|---|---|---|
| 0° | 293 | 224 |
| 0° ± 10° | 150 | 47 |
| 90° | 62 | 2 |
| 90° ± 10° | 62 | 2 |

parallel case is five times greater than in the perpendicular case, the average separation between the excitations or ionizations in the same DNA chain is about 100 times greater in the parallel case. The pattern of deposition in the parallel case is also more sensitive to uncertainty in the fiber orientation relative to the proton beam.

Only long-range modes of intramolecular energy or charge transfer could couple the diffuse pattern of energy deposition events along a DNA chain in the parallel case in ways that would make the yield of free radicals orientation dependent. Singlet and triplet excitons that migrate only a few nm

in DNA of heterogeneous base composition should have equivalent effects in both irradiation geometries. However, mechanisms of energy and charge transfer have been proposed for DNA that have ranges comparable to our estimates of the separation between energy deposition events on a DNA chain that is oriented parallel to the proton flux. Data obtained by van Lith et al. (2) showed that excess electrons in frozen DNA solutions have mobility similar to that in pure ice and longer lifetimes. They reported evidence for electron migration over distances of the order of 100 nm under conditions where lifetimes were limited by homogeneous recombination. Yomosa's model (9) for a soliton involving the disruption of hydrogen bonds between base pairs predicts that a few tenths of an eV of vibrational energy can be transported along DNA at a rate of about 100 nm/ns.

Figure 1 illustrates a mechanism by which solitons might influence free-radical yields when DNA at 77°K is exposed to a proton beam parallel to the molecular orientation. The interaction of DNA with protons and secondary

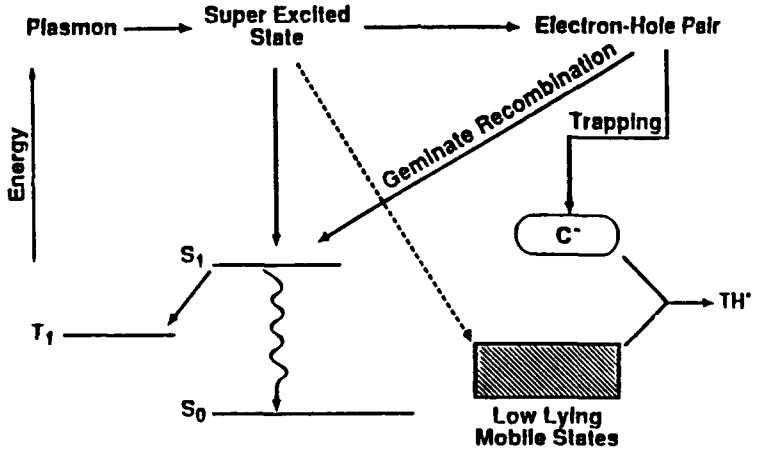


Figure 1. Decay modes of energy absorbed in solid DNA samples

electrons produces highly excited electronic states that decay primarily through formation of electron-hole pairs. Usually this decay is accompanied by some conversion of electronic to vibrational energy. Sufficiently large vibrational excitations in DNA can be self cohering (10). From recent work by Bernhard (11), the electron is expected to be trapped on cytosine by reversible proton transfer from guanine. If many super-excited states are produced in the same DNA chain by a proton flux that is parallel to the molecular orientation, then the probability of interaction between trapped electrons and solitons increases. This interaction may induce electron transfer to thymine where TH· is formed by irreversible protonation at C6.

The model illustrated in Figure 1 neglects the mobility of electrons ejected in the decay of super-excited states by ion-pair formation. The high mobility and long lifetime of excess electrons in the hydration layers of DNA (2) could also contribute to orientation effects in DNA damage by proton irradiation. This mechanism is illustrated in Figure 2, where the dashed lines

represent trajectories of ejected electrons that move primarily in hydration layers around DNA but occasionally are scattered between DNA chains. The squares represent fiber defects that mainly determine the mean free path of excess electrons when the sample is exposed to low-LET radiation or protons perpendicular to the fiber orientation. However, when proton tracks are

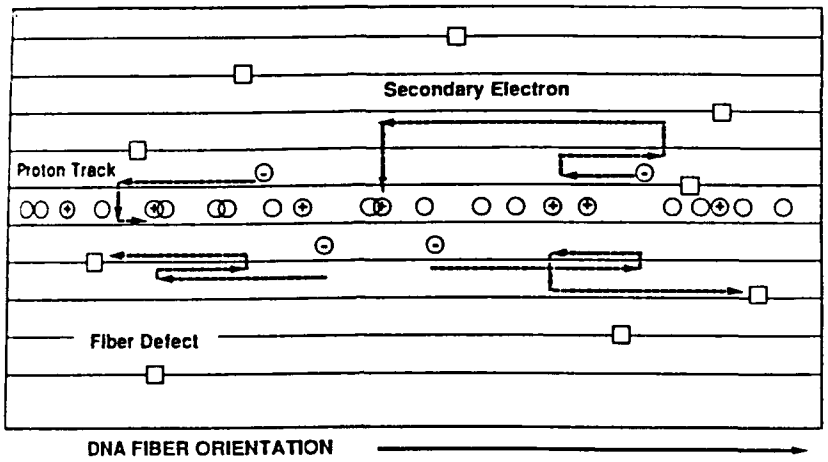


Figure 2. Schematic of electron-hole recombination in oriented DNA exposed to protons parallel to the fiber direction.

parallel to the DNA fibers, many positive ions lie in the high-mobility path of excess electrons. This increases the probability of electron-hole recombination and could reduce the yield of primary radical anions and cation in the parallel case.

Preparation of oriented DNA samples (12) to look for effects like those illustrated in Figures 1 and 2 involves wet spinning of high-molecular- weight DNA into fibers that are wound on a spool to form a thin sheet of oriented DNA. Samples for perpendicular irradiation were made by pressing together a sufficient number of sheets to give a thickness greater than the range of a 4 MeV proton, which is about 0.5 mm. Samples for parallel irradiation were sliced from a block of oriented DNA that had a thickness slightly less than the 3-mm inside diameter of quartz tubes used to transfer irradiated samples to the electron paramagnetic resonance (EPR) spectrometer. All samples were approximately 1 cm long and weighed about 15 mg.

For irradiation, the samples were placed on a copper block in contact with a reservoir of liquid nitrogen and held in place by a thin polyester film. After cooling to 77°K, the sample was placed in a vacuum chamber attached to the beam line of the accelerator. Samples were exposed to graded doses of 4 MeV protons in the range of 20 to 60 kGy at a dose rate of about 2.5 kGy/min. After irradiation, the samples were removed from the vacuum chamber and transferred to a precooled EPR tube. During this transfer, the sample lost contact with liquid-nitrogen-cooled surfaces for less than one

second as it fell through a funnel into the EPR tube. Several samples were exposed to γ rays for comparison with published data (13,14) and the results of proton irradiation. In this case the sample could be sealed into an EPR tube before irradiation due to the penetrating power of the radiation.

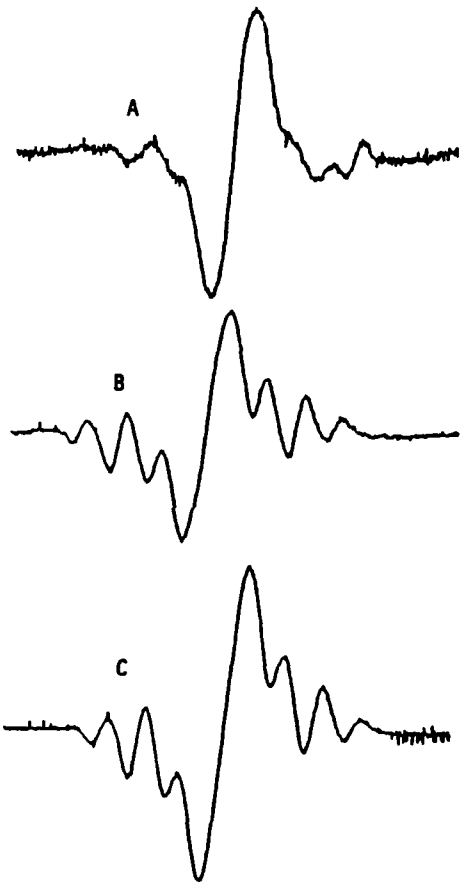


Figure 3. EPR spectra of oriented DNA exposed to γ rays (A) and protons perpendicular (B) or parallel (C) to the DNA fibers.

The EPR spectra shown in Figures 3A-3C were obtained on a VARIAN E112 EPR spectrometer with the sample held at 77°K in a quartz dewar. The doses were 4, 56, and 48 kGy, respectively, and the dose rate was less than the 10 kGy/min recommended by Henriksen and Snipes (15) to avoid heating of the sample.

All three spectra are composed of a central line that we associate with primary radical anion and cation species with varying amounts of modulation in the wings resulting from TH· production. The greater amount of TH observed with proton irradiation is not likely to be due to sample warming during the transfer from the proton beam line to the EPR spectrometer because

the irradiated samples were kept in contact with liquid-nitrogen-cooled surfaces, and the magnitude of the central line relative to the structure in the wings did not change as a function of proton dose. The greater proportion of TH· with proton irradiation may be due to the higher dose required to detect radicals since the total radical yield per unit of dose was more than an order of magnitude lower for protons than for γ rays; however, the shape of the EPR spectrum did not change significantly with proton dose in the range investigated. The recommendation of Henriksen and Snipes (15) regarding the dose rate was based on their experience with 6.5 MeV electrons which may not apply to proton irradiation; hence, the higher yield of TH· that we observed with protons may have resulted from sample heating due to a dose rate that was too large.

Unlike the results reported for neutrons (1), EPR spectra of radicals produced by direct proton irradiation of oriented DNA in parallel and perpendicular geometries were not significantly different. Figure 4 shows that, within experimental error, total radical yields were also independent

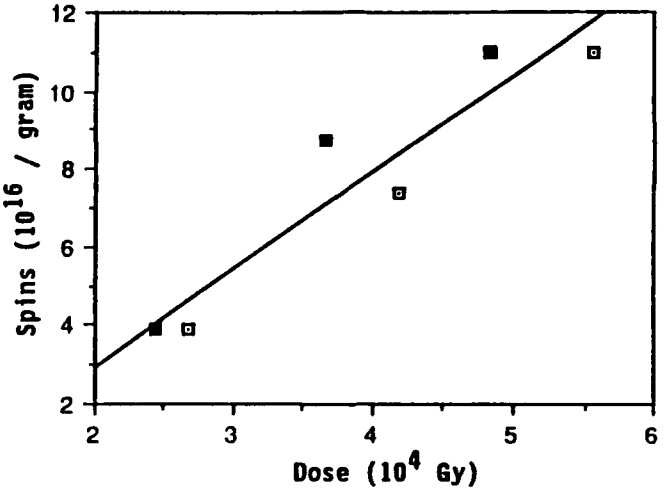


Figure 4. Total radical yields in parallel (■) and perpendicular (❑) proton irradiation of oriented DNA at 77°K.

of the orientation of DNA fibers relative to the proton flux. To obtain these results, differential EPR spectra were recorded digitally and double integrated to give the area under the absorption lines. This area was converted to number of spins by comparison with a standard sample of 2,2 diphenyl-1-picrylhydrazyl (DPPH) dissolved in paraffin.

In conclusion, we did not find any evidence for long-range energy or charge transfer in DNA from experiments in which oriented DNA was exposed to direct proton-beam irradiation. This may be due to the high doses and dose rates used in our experiments. The dose and dose rate could be reduced by redesigning the sample holder and transfer system to avoid the

limitations on sample size imposed by the present system. Experiments with larger samples, higher proton energies for greater penetration, and improved EPR detection sensitivity might reveal orientation effects that are not present in our data due to sample heating or other processes that destroy free radicals at high exposure levels (16).

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# Role of Interleukin 6 (IL-6) in Protection from Lethal Irradiation and in Endocrine Responses to IL-1 and Tumor Necrosis Factor

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## Summary

Primary responsibility for the induction of various acute phase reactions has been ascribed to interleukin 1 (IL-1), tumor necrosis factor (TNF), or IL-6, suggesting that these cytokines may have many overlapping activities. Thus, it is difficult to identify the cytokine primarily responsible for a particular biologic effect, since IL-1 and TNF stimulate one another, and both IL-1 and TNF stimulate IL-6. In this work, the contribution of IL-6 in radioprotection, induction of adrenocorticotropic hormone (ACTH), and induction of hypoglycemia was assessed by blocking IL-6 activity. Administration of anti-IL-6 antibody to otherwise untreated mice greatly enhanced the incidence of radiation-induced mortality, indicating that like IL-1 and TNF, IL-6 also contributes to innate resistance to radiation. Anti-IL-6 antibody given to IL-1-treated or TNF-treated mice reduced survival from lethal irradiation, demonstrating that IL-6 is also an important mediator of both IL-1- and TNF-induced hemopoietic recovery. A similar IL-1/IL-6 interaction was observed in the case of ACTH induction. Anti-IL-6 antibody blocked the IL-1-induced increase in plasma ACTH, whereas recombinant IL-6 by itself did not induce such an increase. Anti-IL-6 antibody also mitigated TNF-induced hypoglycemia, but did not reverse IL-1-induced hypoglycemia. It is, therefore, likely that TNF and IL-1 differ in their mode of induction of hypoglycemia. Our results suggest that an interaction of IL-6 with IL-1 and TNF is a prerequisite for protection from radiation lethality, and its interaction with IL-1 for induction of ACTH.

The ability to utilize cytokines clinically relies on identifying the specific pathophysiologic functions of individual cytokines in the host's defenses. With the availability of pharmacological quantities of recombinant cytokines, sufficient to be evaluated in animal models, it was originally anticipated that individual cytokines could be ascribed specific biologic activities. Indeed, initial studies documented that many inflammatory processes could be reproduced by systemic administration of rIL-1 or of rTNF-$\alpha$, establishing these two cytokines as major inflammatory mediators (1, 2). Subsequent studies, however, suggested that many of the actions of IL-1 or TNF, including stimulation of production of acute phase proteins by hepatocytes (3, 4), stimulation of the hypothalamic-pituitary-adrenal axis (5–7), or hematopoietin 1 effects (8, 9), can be mimicked by IL-6 (10–14).

Because IL-1 and TNF induce IL-6 (15, 16), the possibility that these relatively toxic cytokines can be replaced by the much less toxic IL-6 presents an obvious clinical advantage. To examine this possibility, we evaluated the contribution of IL-6 in several IL-1- and TNF-mediated activities, by testing the effect of anti-IL-6 antibody (20F3) in vivo. Our results show that IL-6 is an essential contributor to natural resistance to lethal irradiation, to IL-1- and TNF-induced recovery from lethal irradiation, to induction of ACTH[1] by IL-1, and to TNF-induced hypoglycemia. However, IL-6 did not induce any of the aforementioned effects when administered by itself to mice (15, 17, and R. Neta, unpublished results). Therefore, we propose that obligatory interaction of IL-1 or TNF with IL-6 may be a prerequisite for some of the biological effects of these inflammatory cytokines.

## Materials and Methods

*Mice.* $CD2F_1$ male and C3H/HeN female mice were purchased from the Animal Genetics and Production Branch, National Cancer Institute, National Institutes of Health (Frederick, MD). Mice were handled as previously described (15).

[1] *Abbreviation used in this paper:* ACTH, adrenocorticotropic hormone.

*Cytokines.* Recombinant human IL-1 (rHuIL-1α; 117–271 Ro 24-5008; lot IL-1 2/88; sp act 3 × $10^8$ U/mg) was kindly provided by Dr. Peter Lomedico, Hoffmann-La Roche, Inc. (Nutley, NJ). Recombinant murine TNF (rmTNF-α; lot 4296-17; sp act 2 × $10^8$ U/mg, as assayed on L929 cells in our laboratory) was kindly provided by Dr. Grace Wong, Genentech Inc. (So. San Francisco, CA).

*Antibodies.* Rat mAb to mouse rIL-6 (MP5 20F3) was prepared using semi-purified Cos-7 mouse IL-6 as an immunogen, as previously described (18). The isotype control, rat mAb to β-galactosidase (GL113) was used. A rat monoclonal IgG1, anti-IL-1 receptor antibody (35F5) was previously described (19). Chromatographically purified rat IgG (Sigma Chemical Co., St. Louis, MO) was used as an additional control. The antibodies and recombinant cytokines were diluted in pyrogen-free saline on the day of injection. Antibodies were given intraperitoneally 6–20 h before intraperitoneal injection of the cytokines.

*Irradiation.* Mice were randomized, placed in plexiglass containers, and were given whole-body irradiation at 40 cGy/min midline tissue dose, by bilaterally positioned $^{60}$Co elements. The radiation field was uniform within ±2%. The number of surviving mice was recorded daily for 30 d.

*Immunoenzymetric Assay for IL-6 Determinations in the Serum.* Serum samples obtained at 2–4 h after cytokine injections were assayed for IL-6 using a two-site sandwich immunoassay as described (18). The threshold sensitivity of this assay was 50 pg/ml.

*Measurements of ACTH in Plasma* ACTH was assayed in plasma from decapitated mice using an $^{125}$I RIA kit (INCSTAR Corp., Stillwater, MN) as previously described (17). The ACTH antibody used in this assay is derived from rabbits immunized against human ACTH1-24, a region identical in human and murine ACTH. The threshold sensitivity of this assay was 8 pg/ml.

*Glucose Determination.* Levels of glucose in serum samples were measured using glucose oxidase reagent kit (KODAK Ektachem Clinical Chemistry Slide) as described elsewhere (20). In this assay, hydrogen peroxide is detected based on its oxidant activity in a peroxidase-catalyzed oxidative-coupling reaction in the presence of chromogen to form a dye. The intensity of the dye is measured at 540 nm. The Kodak Ektachem-700 Analyzer was calibrated before each assay.

*Statistical Analysis.* Statistical evaluation of the results was carried out using $\chi^2$ analysis and analysis of variance followed by Fisher protected least significant difference test.

## Results

*The Role of IL-6 in Innate Radioresistance.* Previous work has indicated that administration of anti-IL-1R or anti-TNF antibody results in increased mortality of irradiated mice (21). To examine whether endogenously produced IL-6 contributes to innate radioresistance, anti-IL-6 antibody was given to normal, untreated mice before irradiation. Such treatment greatly enhanced the incidence of mortality, from 35 to 80% (Fig. 1). Thus, endogenously produced IL-6 clearly contributes to the survival of mice recovering from radiation injury.

*Comparison of IL-1 and TNF for Induction of IL-6.* We have previously shown that pretreatment with IL-1 or TNF results in enhanced survival of lethally irradiated mice. Since IL-1 and TNF are known to induce IL-6, we next sought to determine the relative contribution of IL-6 in TNF- and in IL-1-treated mice. We first determined the levels of IL-6 in circulation of IL-1- or TNF-treated mice (Table 1). Consistent with previous findings (15), detectable serum IL-6 levels appeared even after doses as low as 10 ng of IL-1, but microgram doses of TNF were required to induce measurable circulating IL-6. Consequently, a dose of 600 μg/mouse of anti-IL-6 antibody neutralized serum IL-6 in mice given 5 μg/mouse of TNF, but this dose was insufficient to completely block serum IL-6 in mice given 300 ng of IL-1 (Table 1). Therefore, in subsequent studies of the contribution of

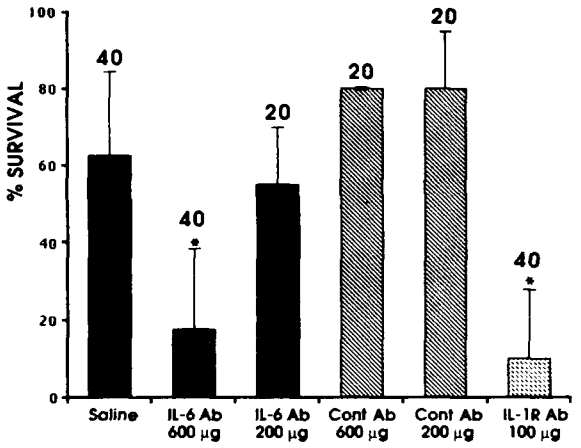


**Figure 1.** CD2F$_1$ mice received 0.5 ml/mouse i.p. of saline, anti-IL-6 Ab, control GL113 Ab, or anti-IL-1R Ab 16 h before exposure to 825 cGy of radiation. The number at the top of the bars indicates the total number of animals in four independent experiments. *Significantly different ($p < 0.001$) from saline control.

**Table 1.** *Effect of Anti-IL-6 Antibody on Serum Titers of IL-1- or TNF-induced IL 6*

| Cytokine treatment | Saline | Anti-IL-6 antibody | Control antibody |
|---|---|---|---|
| | | *pg/ml* | |
| Saline | <50 | <50 | <50 |
| IL-1 (20 ng) | 745 | - | 532 |
| IL-1 (50 ng) | 1,198 | <50 | - |
| IL-1 (100 ng) | 1,383 | <50 | - |
| IL-1 (300 ng) | 3,623 | 82 | - |
| TNF (5 μg) | 121 | <50 | 209 |

CD2F$_1$ mice were given saline injections, anti-IL-6 Ab (600 μg/mouse), or control Ab (GL113, 600 μg/mouse), followed by cytokine treatment 16 h later, and were bled at 2 and 4 h after cytokine administration. Sera from pools of three mice/group were assessed for IL-6. The levels of IL-6 are shown for 2 h after IL-1 injections and 4 h after TNF injections (since these were the optimal levels, with the exception, however, for anti-IL-6 and 300 ng of IL-1 where the results of two experiments of 4 h of bleeding and one experiment of 2 h of bleeding were combined). The results are means of two to four experiments (individuals values varied within ± 20%).

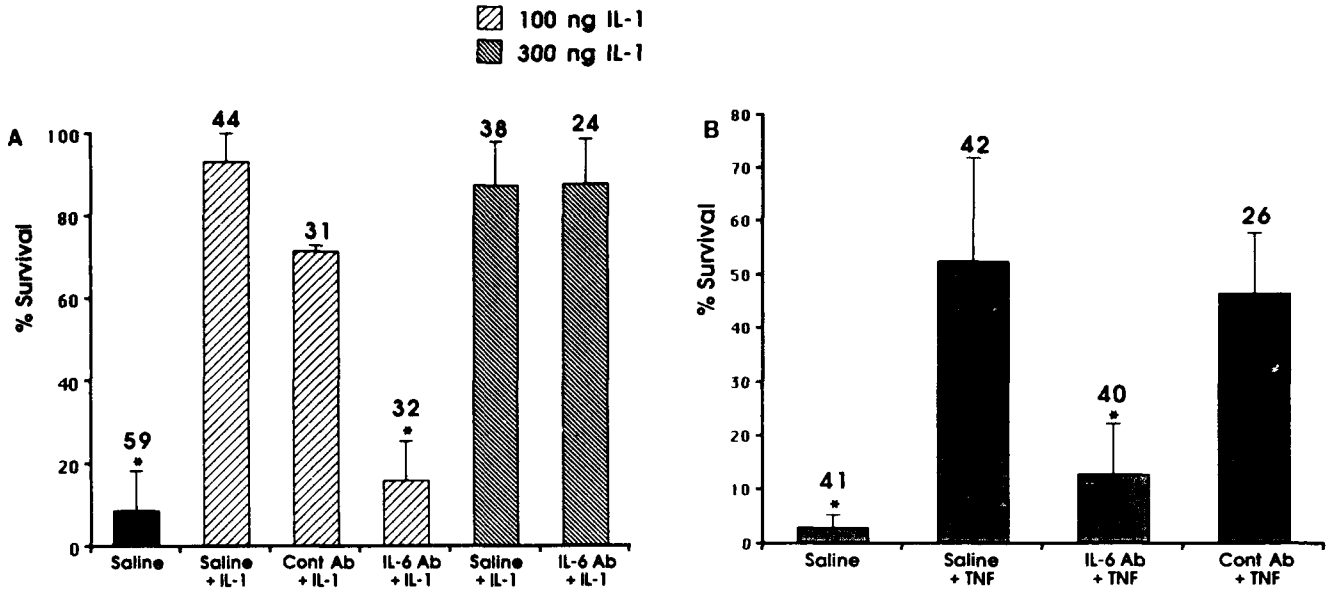


**Figure 2.** $CD2F_1$ mice received 600 $\mu$g anti-IL-6 or control Ab (GL113 or rat Ig) injections 16 h before treatment with IL-1 (*A*) or 5 $\mu$g TNF (*B*), and 20 h later were exposed to 950 cGy of radiation. *Significantly different ($p < 0.01$) from saline + IL-1 group, and saline + TNF group, respectively.

IL-6 in induction of protection from radiation lethality, hypoglycemia, and ACTH release, doses of IL-1 and TNF were used that could be neutralized by the anti-IL-6 antibody.

*The Role of IL-6 in IL-1- and TNF-enhanced Survival from Lethal Irradiation.* Both IL-1 and TNF promote survival from radiation lethality (22, 23). To assess the role of IL-6 in mediating the recuperative effects of these two cytokines, mice were given anti-IL-6 antibody 6–20 h before administration of IL-1 (100 ng) or TNF (5 $\mu$g). The results (Fig. 2, *A* and *B*) indicate that anti-IL-6 antibody can completely block both IL-1- and TNF-enhanced survival from lethal irradiation.

*The Role of IL-6 in IL-1- and TNF-induced Hypoglycemia.* As previously demonstrated, both IL-1 and TNF induce, within 2–4 h, hypoglycemia in mice (24). IL-1, however, is much more potent than TNF in inducing hypoglycemia, since as little as 20 ng of IL-1 induced hypoglycemia, whereas >5 $\mu$g of TNF was required to induce a similar decrease in blood glucose levels. Anti-IL-6 antibody did not change the magnitude of IL-1-induced hypoglycemia (Fig. 3 *A*). In contrast, the same dose of anti-IL-6 resulted in a significant reversal of TNF-induced hypoglycemia (Fig. 3 *B*).

*IL-1/IL-6 Interaction in ACTH Induction.* Our previous work

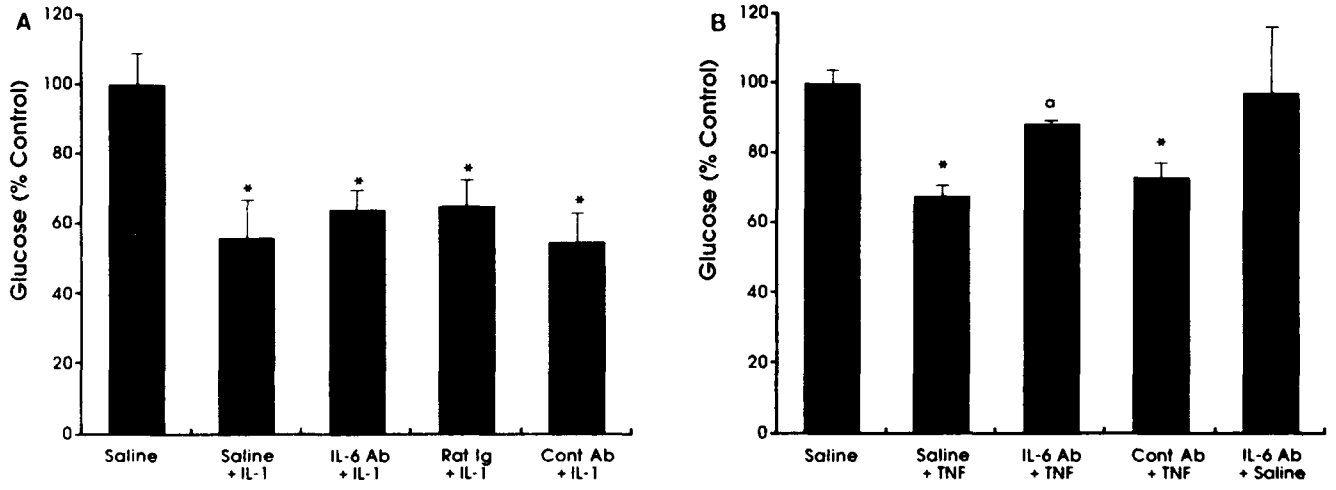


**Figure 3.** $CD2F_1$ mice received Ab (600 $\mu$g/mouse) 16 h before 20 ng IL-1 (*A*) or 5 $\mu$g TNF injection (*B*). The results are means ± SEM of three independent experiments, each one with a pool of three to five mice, for TNF and individual bleedings of seven mice from two independent experiments for IL-1. *Significantly different ($p < 0.05$) from saline control group. °Significantly different ($p < 0.05$) from saline + TNF.

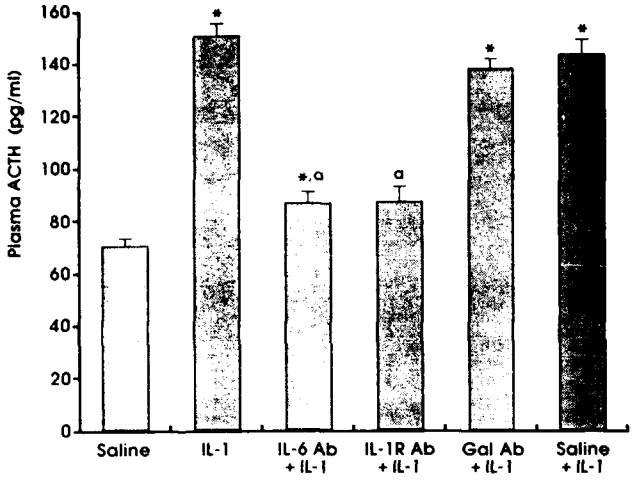


**Figure 4.** C3H/HeN mice were given Ab (600 $\mu$g/mouse) 16 h before 100 ng IL-1 injection. The results are means ± SEM of three independent experiments, each with five mice/group assayed individually. *Significantly different ($p < 0.01$) from saline control. [a]Significantly different ($p < 0.01$) from IL-1 treatment.

determined that IL-1 and IL-6 interact synergistically to induce circulating ACTH (17). To assess the contribution of IL-6 in IL-1-induced ACTH release, mice were given anti-IL-6 antibody before receiving IL-1. As shown in Fig. 4, anti-IL-6 antibody blocked the ACTH response to IL-1. Since no consistent elevation of plasma ACTH was detected in TNF-treated mice, similar experiments using anti-IL-6 antibody were not conducted in TNF-treated mice.

## Discussion

The availability of neutralizing antibody to cytokines allows the assessment of the contribution of individual cytokines and their interaction in host defenses in vivo. For example, our previous studies demonstrated that administration of anti-IL-1R and anti-TNF antibody to normal mice caused greater mortality upon exposure of these mice to lethal radiation (21). Moreover, anti-IL-1R antibody not only blocked IL-1-induced, but also TNF-induced protection from lethal irradiation. Conversely, anti-TNF antibody also reduced IL-1-induced protection from radiation death, suggesting that these two cytokines induce one another in vitro and in vivo (25–28), and that their interaction is necessary for enhanced survival from lethal irradiation.

IL-6 not only fails to induce IL-1 or TNF, but actually has been reported to suppress their production (29, 30). Thus, as we have previously observed, administration of IL-6 alone before irradiation would not be expected to lead to enhanced survival and would actually reduce survival from lethal irradiation (15). Despite this, injection of anti-IL-6 antibody in this study results in increased mortality of irradiated, normal mice as well as irradiated TNF- or IL-1-treated mice. These observations suggest that IL-6 participates in both innate as well as IL-1- and TNF-induced resistance to radiation lethality. The failure of IL-6 by itself to improve radiation survival indicates that the activity of IL-6 only becomes manifest in the course of interaction with other cytokines. This hypothesis is supported by our earlier report that IL-6 given together with suboptimal doses of IL-1 resulted in synergistically enhanced survival from lethal irradiation (15). Since radiation itself induces the production of endogenous IL-1 (31–33) and TNF (34, 35), their presence would be expected to result in endogenous production of IL-6. Consequently, the interaction of all three cytokines, i.e., IL-1, TNF, and IL-6, would contribute to innate resistance of normal mice to radiation lethality.

When given individually, IL-1 and TNF induce significant protection from radiation-induced lethality and induce hypoglycemia. One possible explanation for the observed lower potency of TNF in both of these biologic responses may be related to its much lower capacity to induce IL-6 (Table 1). Even 20 ng dose of IL-1 induced higher levels of IL-6 than 5 $\mu$g of TNF. Our results indicate that 600 $\mu$g of anti-IL-6 antibody blocked radioprotection and ACTH induction with 100 ng IL-1, whereas the hypoglycemia induced with 20 ng IL-1 was not significantly affected by this antibody. In contrast, TNF-induced hypoglycemia can be reduced by anti-IL-6 antibody. This suggests that the mechanisms of induction of hypoglycemia by these two cytokines differ. Alternatively, induction of IL-6 or distribution of antibody may vary in different tissues. Our previous work, showing a synergistic interaction of IL-1 and TNF in induction of hypoglycemia (24), supports the first of these two hypotheses.

The ability to block IL-1-induced release of ACTH with anti-IL-6 antibody further extends our previous findings, which showed that IL-1 and IL-6 synergize to induce ACTH (17). In that study, doses of 10 $\mu$g of IL-6 induced only minimal changes in plasma ACTH, whereas IL-1 alone induced ACTH within 2 h (a time interval also necessary for IL-1 induction of IL-6) (15). The combination of IL-1 and IL-6 induced optimal levels of ACTH within 30 min. Thus, together with our present results, these observations strongly support the hypothesis that the simultaneous presence of both IL-1 and IL-6 is necessary for release of ACTH in vivo. Our attempts to observe similar elevation of plasma ACTH after administration of 5 $\mu$g TNF to C3H/HeN mice were unsuccessful (Perlstein and Neta, unpublished results). Thus, TNF may not induce levels of IL-6 sufficient to induce ACTH. A previous report, which showed that rats given IL-6 alone have elevated plasma ACTH (13), may be explained either by a lower requirement for this interaction in rats or, alternatively, by the possible presence of sufficient endogenous levels of IL-1 in this species.

In conclusion, these results serve to reconcile the apparently conflicting observations by many laboratories concerning the relative importance of the contributions of IL-1, TNF, and IL-6 to a variety of pathophysiological reactions. The interdependence and synergistic interactions of these three cytokines are apparently mandatory for many of their biological effects.

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# SYNERGY BETWEEN G-CSF AND WR-2721: EFFECTS ON ENHANCING HEMOPOIETIC RECONSTITUTION AND INCREASING SURVIVAL FOLLOWING EXPOSURE TO IONIZING RADIATION

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## INTRODUCTION

WR-2721 is one of the most effective exogenous radioprotectants discovered (1, 2). Mechanisms through which WR-2721 protects cells from radiation-induced lethality include free radical scavenging, hydrogen atom donation, and induction of hypoxia. As a survival-enhancer in experimental animals, WR-2721 is extremely effective when administered ~30 min before radiation exposure. However, because the survival-enhancing effects of WR-2721 are dose dependent, WR-2721 doses that produce the greatest radioprotection are also the most toxic (3, 4). We previously demonstrated that an alternate approach to the use of high (potentially toxic) doses of single radioprotectants is the use of low doses of multiple agents that act by different survival-enhancing mechanisms (5, 6). For example, we demonstrated that the survival-enhancing effects of low-dose WR-2721 could be increased when glucan, an immunomodulator and hemopoietic stimulant, was administered following irradiation. The enhanced survival observed with WR-2721 + glucan was attributed to sequential effects of cell protection mediated by WR-2721 and increased hemopoietic proliferation mediated by glucan. Because glucan is a potent inducer of hemopoietic cytokines (7, 8), we hypothesized that hemopoietic cytokines may be used in place of glucan to stimulate hemopoiesis and to increase the survival-enhancing effects of WR-2721. In these particular studies, the ability of granulocyte colony-stimulating factor (G-CSF) to increase the survival enhancing effects of a low dose of WR-2721 are described.

## METHODS AND MATERIALS

### Mice

C3H/HeN female mice (~20 g) were purchased from Charles River Laboratories (Raleigh, NC). Mice were maintained at the Armed Forces Radiobiology Research Institute (AFRRI) in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Mice were housed 10 per cage in Micro-Isolator cages on hardwood-chip, contact bedding and were provided commercial rodent chow and acidified water (pH 2.5) ad libitum. Animal rooms were equipped with full-spectrum light from 6 a.m. to 6 p.m. and were maintained at 70°F +/- 2°F with 50% +/- 10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. Upon arrival, all mice were tested for Pseudomonas and quarantined until test results were obtained. Only healthy mice were released for experimentation. All animal experiments were approved by the Institute Animal Care and Use Committee prior to performance.

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## WR-2721 And G-CSF

Stock WR-2721 was obtained from Walter Reed Army Institute of Research (Washington, DC) and kept frozen at -20°C until use. Immediately before each use, WR-2721 was dissolved in sterile pyrogen-free saline Exposure of the material to light was minimized. Thirty minutes before irradiation, mice were injected intraperitoneally (i.p.) with 4 mg of WR-2721 in a 0.5-ml volume. WR-2721 control mice received 0.5 ml of saline. Recombinant human G-CSF was provided by AMGen (Thousand Oaks, CA). The G-CSF was E. coli-derived and had a specific activity of $10^8$ units/mg as assayed by a granulocyte-macrophage colony-forming unit assay using normal human bone marrow cells. Endotoxin contamination was less than 0.5 ng/mg protein as determined by the Limulus amebocyte lysate assay. G-CSF was administered subcutaneously (s.c.) once per day at a dose of 2.5 ug in a 0.1-ml volume on days 1-16 postirradiation. G-CSF controls were administered 0.1 ml of saline s.c.

## Irradiation

Mice were placed in ventilated Plexiglas containers and exposed to bilateral total-body gamma rays at a dose rate of 0.4 Gy/min. The AFRRI $^{60}Co$ source was used for irradiations. Dosimetry was determined by ionization chambers as previously described (9) with calibration factors traceable to the National Institute of Standards and Technology. Before initiating animal experiments, the dose rate at the midline of an acrylic mouse phantom was measured using a 0.5-$cm^3$ tissue equivalent ionization chamber manufactured by Exradin (Lisle, IL). Before each experimental irradiation, the dose rate at the same location with the phantom removed was measured using a 50-$cm^3$ ionization chamber fabricated at AFRRI. The ratio of these two dose rates, the tissue-air ratio (TAR), was then used to ensure delivery of the midline dose desired for each animal exposure. The TAR used in these experiments was 1.001.

## Survival Assays

Ten mice receiving each drug treatment were exposed to radiation doses ranging from 7 Gy to 14 Gy. Irradiated mice were returned to the animal facility and cared for routinely. Survival was monitored for 30 days; on day 31 surviving mice were euthanized by cervical dislocation. Experiments were repeated three times. The percentage of mice surviving each radiation dose at 30 days postexposure was used to construct a survival curve for each drug treatment. The radiation dose lethal for 50% of mice within 30 days postexposure (LD50/30) was extrapolated from each survival curve and used to calculate dose reduction factors (DRF's; LD50/30 of treated mice divided by LD50/30 of radiation control mice) for each drug treatment.

## Spleen Colony-Forming Unit Assays

Endogenous and exogenous spleen colony-forming units were used to evaluate regeneration of pluripotent hemopoietic progenitor cells. Endogenous spleen colony-forming units (E-CFU) were evaluated using the method of Till and McCulloch (10). Mice from various treatment groups were exposed to 10.75 Gy total-body irradiation. Twelve days after irradiation, mice were euthanized by cervical dislocation and their spleens were removed. Spleens were fixed in Bouin's solution, and the number of grossly visible spleen colonies was counted. Exogenous spleen colony-forming units (CFU-s) were also evaluated by a method of Till and McCulloch (11). Recipient mice were exposed to 9.25 Gy of total-body irradiation to eradicate most endogenous hemopoietic stem cells. Three to five hours later, 1-5 x $10^4$ bone marrow or 1-5 x $10^5$ spleen cells from normal or treated mice were intravenously injected into the lateral tail veins of irradiated recipient mice. Twelve days after transplantation, the recipients were euthanized by cervical dislocation, the spleens were removed, and spleen colonies were counted as described for the E-CFU assay. The cell suspensions used for each CFU-s assay represented tissues from three normal, irradiated, G-CSF-treated, WR-2721-treated, or combination-treated mice at each time point. Cells were flushed from femurs with 3 ml of McCoy's 5A medium (Flow Labs, McLean, VA) containing 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT). Spleens were pressed through stainless-steel mesh screen, and the cells were washed

from the screen with 6 ml medium. The total number of nucleated cells in the suspensions was determined by Coulter counter. Experiments were repeated three times.

Granulocyte-Macrophage Colony-Forming Cell Assay

Hemopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a previously described (5) agar granulocyte-macrophage colony-forming cell (GM-CFC) assay. Mouse endotoxin serum (5% v/v) was added to feeder layers as a source of colony-stimulating activity. Colonies (> 50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 5% $CO_2$. Triplicate plates were cultured for each cell suspension, and experiments were repeated three times. The cell suspensions used for each assay were prepared as described for the CFU-s assay.

Peripheral Blood Cell Counts

Blood was obtained from halothane-anesthetized mice by cardiac puncture using a heparinized syringe attached to a 20-gauge needle. White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts were performed using a Coulter counter.

Statistics

Results of replicate experiments were pooled and represent the mean +/- standard error of pooled data. Student's t-test was used to determine statistical differences in all but survival data. Survival data were analyzed using the method of Finney (12). Differences were considered significant when the p-value was less than 0.05.

RESULTS

Survival-Enhancing Effects of G-CSF, WR-2721, And WR-2721 + G-CSF

The ability of G-CSF, WR-2721, and WR-2721 + G-CSF to enhance survival in irradiated mice is illustrated in Figure 1. The LD50/30 for saline-treated mice was 7.85 Gy +/- 0.06 Gy. Preirradiation administration of 4 mg of WR-2721 significantly increased survival, resulting in an LD50/30 of 11.30 Gy +/- 0.15 Gy and a DRF of 1.44. Postirradiation treatment with G-CSF alone also slightly enhanced survival, yielding an LD50/30 of 8.30 Gy +/- 0.08 Gy and a DRF of 1.06. Administration of WR-2721 preirradiation and G-CSF postirradiation raised the LD50/30 to 12.85 Gy +/- 0.24 Gy, and resulted in a DRF of 1.64. This DRF was more than additive between the DRF's obtained with WR-2721 (1.44) and G-CSF (1.06) alone.

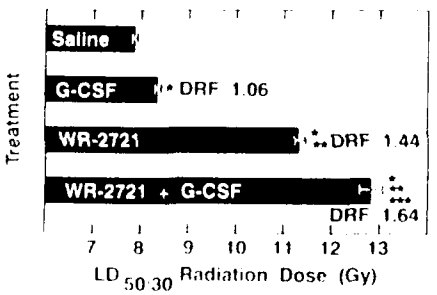


Figure 1. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on survival of irradiated mice. C3H/HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before $^{60}Co$ irradiation and G-CSF (2.5 ug/mouse/day, s.c.) on days 1-16 after irradiation. * $p < 0.05$, with respect to saline values; ** $p < 0.05$, with respect to G-CSF values; *** $p < 0.05$, with respect to WR-2721 values.

Hemopoietic Effects of G-CSF, WR-2721, And WR-2721 + G-CSF

The E-CFU assay was used in preliminary studies to identify hemopoietic differences among saline-, G-CSF-, WR-2721-, and combination-treated mice. In these studies a 10.75-Gy radiation dose was chosen because it was the highest radiation dose that would permit saline-treated mice to survive the 12 days postirradiation needed for performing the E-CFU assay. Figure 2 illustrates the E-CFU results obtained in mice exposed to 10.75 Gy. No E-CFU were detected in saline- or G-CSF-treated mice. WR-2721-treated mice exhibited only 1.8 +/- 0.1 E-CFU, while combination-treated mice exhibited 12.4 +/- 1.5 E-CFU. These data illustrate the ability of G-CSF to amplify the number of multipotent hemopoietic stem cells protected by preirradiation WR-2721 administration. The onset and duration of the hemopoietic responses induced by G-CSF, WR-2721, and WR-2721 +

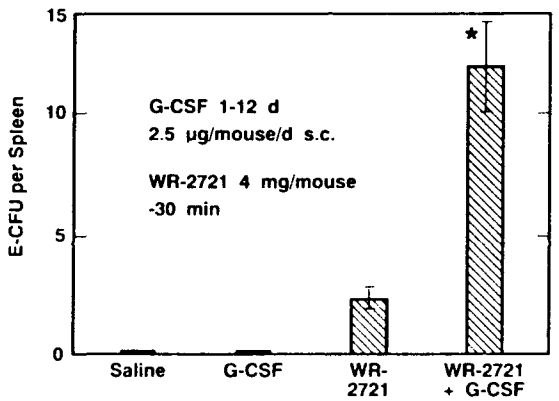


Figure 2. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on endogenous spleen colony formation (E-CFU) in mice exposed to 10.75 Gy. C3H HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before $^{60}Co$ irradiation and G-CSF (2.5 ug/mouse/day, s.c.) on days 1-12 (assay terminated on day 12) after irradiation. Data represented as the mean +/- standard error of values obtained from 15 mice in each treatment group. * $p < 0.05$, with respect to WR-2721 values.

G-CSF were further characterized by sequentially following the recovery of bone marrow (Table 1) and splenic (Table 2) cellularity, CFU-s, and GM-CFC on days 10, 17 and 24 after a 10.75-Gy radiation exposure. Although mice from all four treatment groups survived this radiation exposure to be evaluated at day 10, only WR-2721-treated and combination-treated mice survived to be evaluated on days 17 and 24 postexposure. Mice receiving WR-2721 + G-CSF consistently exhibited the most rapid and dramatic recoveries for all parameters evaluated. In addition to enhanced hemopoietic stem and progenitor cell recoveries, a more rapid recovery of mature peripheral blood WBC's, RBC's, and PLT's was also observed in mice receiving the combination WR-2721 + G-CSF treatment (Table 3).

Table 1

**Effect of G-CSF, WR-2721, and WR-2721 + G-CSF on Bone Marrow Cellularity, CFU-s, and GM-CFC Following a 10.75 Gy Radiation Exposure[1]**

| | Treatment | | | |
|---|---|---|---|---|
| | Saline | G-CSF | WR-2721 | WR-2721 + G-CSF |
| **Cells per Femur (x $10^6$)** | | | | |
| Day 10 | 0.37 ± 0.02 | 0.52 ± 0.09 | 1.46 ± 0.15 | 2.32 ± 0.25[3] |
| 17 | –[2] | –[2] | 2.14 ± 0.38 | 2.91 ± 0.16 |
| 24 | –[2] | –[2] | 2.23 ± 0.27 | 5.23 ± 0.25[3] |
| **CFU-s per Femur** | | | | |
| Day 10 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| 17 | –[2] | –[2] | 18.00 ± 4.00 | 84.00 ± 12.00[3] |
| 24 | –[2] | –[2] | 53.00 ± 11.00 | 381.00 ± 68.00[3] |
| **GM-CFC per Femur** | | | | |
| Day 10 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 16.00 ± 5.00[3] |
| 17 | –[2] | –[2] | 38.00 ± 11.00 | 353.00 ± 46.00[3] |
| 24 | –[2] | –[2] | 234.00 ± 53.00 | 510.00 ± 57.00[3] |

[1]Mean ± standard error of values obtained for three experiments.

[2]No mice survived to be assayed at this time point.

[3]$p < 0.05$, with respect to WR 2721 values.

(In nonirradiated $C_3H$/HeN mice, Cellularity = 6.59 ± 0.32, CFU-s = 2787 ± 423, GM-CFC = 3366 ± 263).

Table 2

**Effect of G-CSF, WR-2721, and WR-2721 + G-CSF on Splenic Cellularity, CFU-s, and GM-CFC Following a 10.75 Gy Radiation Exposure[1]**

| | Treatment | | | |
|---|---|---|---|---|
| | Saline | G-CSF | WR-2721 | WR-2721 + G-CSF |
| **Cells per Spleen (x $10^6$)** | | | | |
| Day 10 | 120 ± 02 | 130 ± 05 | 141 ± 06 | 152 ± 08 |
| 17 | –[2] | –[2] | 699 ± 19 | 223.3 ± 23.2[3] |
| 24 | –[2] | –[2] | 1946 ± 163 | 1534 ± 261 |
| **CFU-s per Spleen** | | | | |
| Day 10 | 08 ± 04 | 13 ± 02 | 47 ± 12 | 548 ± 68[3] |
| 17 | –[2] | –[2] | 4880 ± 256 | 1382 0 ± 341[3] |
| 24 | ?[2] | –[2] | 24730 ± 4900 | 41930 ± 5267[3] |
| **GM-CFC per Spleen** | | | | |
| Day 10 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| 17 | ?[2] | –[2] | 2490 ± 355 | 13044 0 ± 1040 0[3] |
| 24 | ?[2] | –[2] | 131320 ± 10150 | 17776 0 ± 1208 0[3] |

[1]Mean ± standard error of values obtained from three experiments.

[2]No mice survived to be assayed at this time point.

[3]$p < 0.05$, with respect to WR 2721 values.

(In nonirradiated $C_3H$ HeH mice, Cellularity = 147 ± 10, CFU-s = 6907 ± 779, GM-CFC = 1650 ± 118).

Table 3

**Effect of WR-2721 and WR-2721 + G-CSF on Peripheral Blood Cell Counts Following a 10.75 Gy Radiation Exposure[1]**

| | Treatment | |
|---|---|---|
| | WR-2721 | WR-2721 + G-CSF |
| **WBC per $mm^3$ (x $10^3$)** | | |
| Day 10 | 1.4 ± 0.3 | 1.4 ± 0.2 |
| 17 | 2.3 ± 0.1 | 18.3 ± 3.1[2] |
| 24 | 5.5 ± 0.3 | 28.4 ± 3.5[2] |
| **RBC per $mm^3$ (x $10^6$)** | | |
| Day 10 | 5.0 ± 0.5 | 4.3 ± 0.5 |
| 17 | 3.4 ± 0.3 | 4.0 ± 0.4 |
| 24 | 2.3 ± 0.6 | 6.0 ± 0.2[2] |
| **PLT per $mm^3$ (x $10^3$)** | | |
| Day 10 | 45.0 ± 9.0 | 170.0 ± 22.0[2] |
| 17 | 100.0 ± 18.0 | 177.0 ± 13.0[2] |
| 24 | 151.0 ± 15.0 | 435.0 ± 98.0[2] |

[1] Mean ± standard error of values obtained from 3 experiments.

[2] $p < 0.05$, with respect to WR 2721 values.

(*In nonirradiated* $C_3H$/HeN *mice*, WBC = 6.2 ± 0.7, RBC = 6.3 ± 0.5 and PLT = 950 ± 38)

## DISCUSSION

Hemopoietic injury and associated risks of infection and hemorrhage are common problems associated with radiation and radiomemetic drug therapies used in the treatment of cancer. Recent studies have demonstrated that the aminothiol WR-2721 can be administered prior to irradiation or chemotherapy to selectively protect normal cells, especially hemopoietic cells, and as a result reduce radiation- or drug-induced hemopoietic injury (14, 15, 16, 17, 18, 19, 20). Administration of the hemopoietic growth factor granulocyte colony-stimulating factor (G-CSF) after radiation or radiomimetic drug therapies also has been demonstrated to reduce the side effects of hemopoietic injury by accelerating hemopoietic regeneration (21, 22, 23, 24, 25, 26, 27, 28). In the studies reported here, we evaluated the survival and hemopoietic effects of WR-2721 and G-CSF used in combination in a murine radiation model.

In addition to reconfirming the ability of both WR-2721 and G-CSF to individually enhance survival in irradiated mice (2, 5, 6, 24), our data clearly illustrate the benefit of using these two agents in combination. The enhanced survival observed in combination-treated mice (Figure 1) appeared to result from the ability of G-CSF to accelerate regeneration of mature hemopoietic elements (Table 3) by amplification of multipotent and committed progenitor cells protected by the WR-2721 treatment (Tables 2 and 3).

In conclusion, we have demonstrated that the use of WR-2721 + G-CSF in combination can synergize to further mitigate hemopoietic injury and lethality associated with radiation exposure. Postirradiation use of hemopoietic growth factors in combination with classical radioprotectants may prove to be a valuable adjunctive therapy in the treatment of cancer.

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# *N*-(2-MERCAPTOETHYL)-1,3-PROPANEDIAMINE (WR-1065) PROTECTS THYMOCYTES FROM PROGRAMED CELL DEATH[1]

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**Gamma-irradiation, glucocorticoid hormones, and calcium ionophores stimulate a suicide process in thymocytes, known as apoptosis or programed cell death, that involves internucleosomal DNA fragmentation by a $Ca^{2+}$- and $Mg^{2+}$-dependent nuclear endonuclease. In this study we report that *N*-(2-mercaptoethyl)-1,3-propanediamine (WR-1065) blocked DNA fragmentation and cell death in thymocytes exposed to γ-radiation, dexamethazone, or calcium ionophore A23187. WR-1065 protected the thymocytes from radiation-induced apoptosis when incubated with cells after irradiation but not before and/or during irradiation. WR-1065 inhibited $Ca^{2+}$-and $Mg^{2+}$-dependent DNA fragmentation in isolated thymocyte nuclei. Our results suggest that WR-1065 protects thymocytes from apoptosis by inhibiting $Ca^{2+}$- and $Mg^{2+}$-dependent nuclear endonuclease action.**

Thymic small lymphocytes, commonly known as thymocytes, undergo a suicide process known as apoptosis or programed cell death in response to several stimuli, including exposure to γ-radiation (1–3), glucocorticoid hormones (4–6), calcium ionophores (6), antibodies to the CD3-TCR complex (7), or the environmental contaminant 2,3,7,8-tetrachlorodibenzo-γ-dioxin (8). The apoptotic death of thymocytes, lymphocytes, and intestinal crypt cells after clinically relevant doses of irradiation (2–5 Gy[3]) distinguishes them from most other cells, which undergo reproductive death at these radiation doses (9–14). In reproductive death the cell functions until it attempts one or more cell divisions, after which it dies (15). In apoptosis, however, the damage manifests itself in the absence of mitosis. Apoptosis is characterized by several morphologic and biochemical changes, including plasma and nuclear membrane blebbing, impairment in membrane permeability, chromatin condensation, DNA fragmentation, and impairment of ATP synthesis (9). The most characteristic biochemical marker for apoptosis is nuclear DNA fragmentation into oligonucleosomal subunits that can be recognized from random cleavage observed in cells undergoing necrosis (1–6).

The radioprotectant drug WR-2721 is a well-known protective agent that selectively protects normal tissues against cytotoxicities of radiation and chemotherapeutic alkylating agents (16–21). WR-1065 the dephosphorylated form of WR-2721 and generally considered to be the active form of the drug, has been shown to protect the mammalian cells in vitro from radiation-induced reproductive death (22–25). In this report we describe the protective effects of WR-1065 on apoptosis in thymocytes induced by γ-irradiation, dexamethazone, and calcium ionophore A23187.

## MATERIALS AND METHODS

*TCM.* RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-ME, 100 U/ml pencillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% heat-inactivated FCS was used in all the studies.

*Cell isolation.* CD2F1 male mice, 6 to 7 wk old, were asphyxiated with $CO_2$, and their thymuses were removed and placed in TCM on ice. Single cell suspensions were prepared by pressing the organs through wire mesh screens followed by passage through a 25-gauge needle. The suspensions were washed once in TCM and resuspended in cold Tris-buffered isotonic ammonium chloride to lyse the red cells (26). The cells were washed once in TCM and resuspended in TCM. Viable cell numbers were determined by trypan blue dye exclusion method (27).

*γ-irradiation.* Thymocytes ($2 \times 10^6$/ml) were exposed to 1.5 to 6.0 Gy $^{60}Co$ γ radiation at a dose rate of 1 Gy/min.

*Incubation of thymocytes.* Immediately after irradiation, cells were centrifuged at $200 \times g$ for 10 min, resuspended in fresh medium at $2 \times 10^6$ cells/ml, and incubated with WR-1065 in TCM containing 100 U/ml catalase at 37°C in a humidified incubator under an atmosphere of 5% $CO_2$ in air.

Dexamethazone was dissolved in a minimum volume of ethanol and diluted to the desired concentration with TCM. Thymocytes were incubated with different concentrations of dexamethazone in TCM with or without WR-1065 as described above. A similar quantity of ethanol was added to controls. The studies were repeated with different concentrations of calcium ionophore A23187.

*DNA fragmentation assay.* At selected times cells were harvested by centrifugation at $200 \times g$ for 10 min. The cells were lysed with 0.5 ml ice-cold hypotonic lysing buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100) and centrifuged at $13,000 \times g$ for 20 min to separate intact from fragmented DNA. The pellet was then sonicated for 10 s in 0.5 ml lysis buffer. DNA in the pellet and supernatant fractions was determined by an automated fluorometric method using Hoechst 33258 fluorochrome (28, 29), modified for our studies. This DNA analysis is based on the ability of Hoechst 33258 to bind DNA quantitatively to form a fluorescent complex.

A stock solution of Hoechst 33258 (1 mg/ml) was prepared in distilled water. This solution is stable for 2 wk if kept at 4°C in the dark. A 1 μg/ml working solution was prepared daily by diluting the stock with running buffer (0.5 M phosphate buffer, pH 7.0, containing 0.05% Brij S-35). The working dye container was wrapped in aluminum foil to protect it from ambient light during the analysis.

Continuous flow analysis was performed with Technicon Autoanalyzer II components (Technicon Instruments Corp., Tarrytown, NY), including an autosampler fitted with a 40-place sample tray, a single

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[3] Abbreviations used in this paper: Gy, gray; TCM, tissue culture medium; WR-1065, *N*-(2-mercaptoethyl)-1,3-propanediamine; WR-2721, S-2-(3-aminopropylamino) ethylphosphorothioic acid.

speed proportioning pump, and a fluoronephelometer. The fluorescence signal was directed to a Hewlett-Packard 3390A integrator (Hewlett-Packard, Downer's Grove, IL), which automatically identified and quantitated sample peaks. All tubes were flow-rated Tygon tubing (Fisher Scientific, Pittsburg, PA). We used a sampler cam that allowed the analysis of 40 samples/h with a 1 min running buffer wash between 30-s sample draws. A minimum sample volume of 0.35 ml was required with the pump tube used (0.6 ml/min).

The concentration of DNA corresponding to the peak height value of each sample was calculated from a curve of calf thymus DNA standards, by using a computer software. We found that fluorometric autoanalysis of DNA is more sensitive and reproducible than diphenylamine method. Sample concentrations ranging from 1 to 20 μg/ml were easily analyzed with the system. The sensitivity can be increased by increasing the volume of the sample draw and adjusting the sensitivity of the fluoronephelometer and integrator. Measurements were unaffected by the presence of cell homogenates or reagents in the sample.

Percentage of DNA fragmentation refers to the ratio of DNA in the 13000 × *g* supernatant to the total DNA in the pellet and 13000 × *g* supernatant.

*DNA electrophoresis.* The pellets and the supernatants were incubated with RNase (50 μg/ml) for 1 h at 37°C. After this incubation, 50 μg/ml proteinase K were added and the incubation continued for an additional 1-h period. The DNA was sequentially extracted with equal volumes of phenol and chloroform:isoamylalcohol (24:1). The aqueous phase was precipitated with two volumes of ethanol at -20°C overnight. Pellets were air dried and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 7.8, and 1 mM EDTA). Horizontal electrophoresis of DNA was performed for 2.5 h at 100 V in 0.75% agarose gel with 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0, as running buffer. DNA was visualized after electrophoresis by ethidium bromide staining.

*Nuclei isolation and endogenous nuclease activity.* Nuclei were prepared from the thymocytes by the method of Cohen and Duke (5). The nuclei were suspended in Tris-buffered (10 mM, pH 7.5) isotonic sodium chloride and incubated at 37°C for 4 h in the presence of different cations. After incubation, the nuclei were sedimented at 200 × *g* for 10 min. The supernatant was discarded because it contained no DNA. The nuclear pellet was lysed with lysis buffer, and intact DNA and fragmented DNA were estimated as described for whole cells.

*Materials.* RPMI 1640 medium, 2-ME, and the antibiotic mixture were purchased from GIBCO, Grand Island, NY; FCS was obtained from HyClone Laboratories, Logan, UT; Hoechst 33258 fluorochrome was purchased from Calbiochem-Behring, La Jolla, CA; Dexamethazone and calcium ionophore A23187 were purchased from Sigma Chemical Co., St. Louis, MO; and WR-2721 and WR-1065 were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

## RESULTS

*WR-1065 inhibits radiation-induced DNA fragmentation and cell death in thymocytes.* Single cell suspensions were prepared from thymuses and exposed to different doses of γ-radiation. After irradiation the thymocytes were resuspended in fresh medium containing 10 mM WR-1065 and incubated at 37°C as described in *Materials and Methods*. The level of DNA fragmentation was determined at various times postirradiation. DNA fragmentation increased with radiation dose and with time postirradiation. (Fig. 1, *A-D*). It is interesting to note that DNA fragmentation was completely blocked in cells incubated with WR-1065 after different doses of γ-radiation (Fig. 1, *A-D*). In all experiments, the background DNA fragmentation in unirradiated thymocytes increased with time to a maximum level of 10 to 15% at 8 h. There was no background DNA fragmentation in unirradiated thymocytes after WR-1065 treatment (Fig. 1*A*).

Electrophoretic analysis of pellet and supernatant DNA isolated from 6.0 Gy-irradiated thymocytes showed typical "ladder" pattern, consisting of DNA fragments of a size, multiple of 200 bp unit (Fig 2, *lanes D* and *H*, respectively). This pattern of DNA fragmentation has already been shown after γ-irradiation (1, 2) and glucocorticoid treatment (4) of thymocytes. The pellet DNA

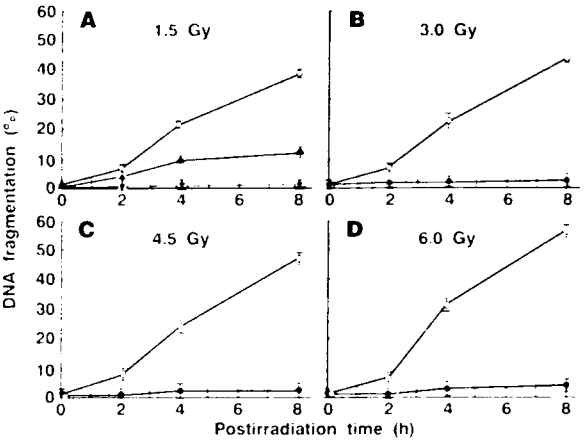


*Figure 1.* Effect of WR-1065 on DNA fragmentation in thymocytes exposed to increasing doses of γ-radiation. Thymocytes ($2 \times 10^6$) were irradiated in TCM at a dose rate of 1.0 Gy/min. The percentage of DNA fragmentation was measured after various times of incubation with or without 10 mM WR-1065, under the conditions mentioned in *Materials and Methods*. The results are mean ± SE from three experiments. ▲, unirradiated; △, unirradiated + WR-1065; ○, irradiated; ●, irradiated + WR-1065.

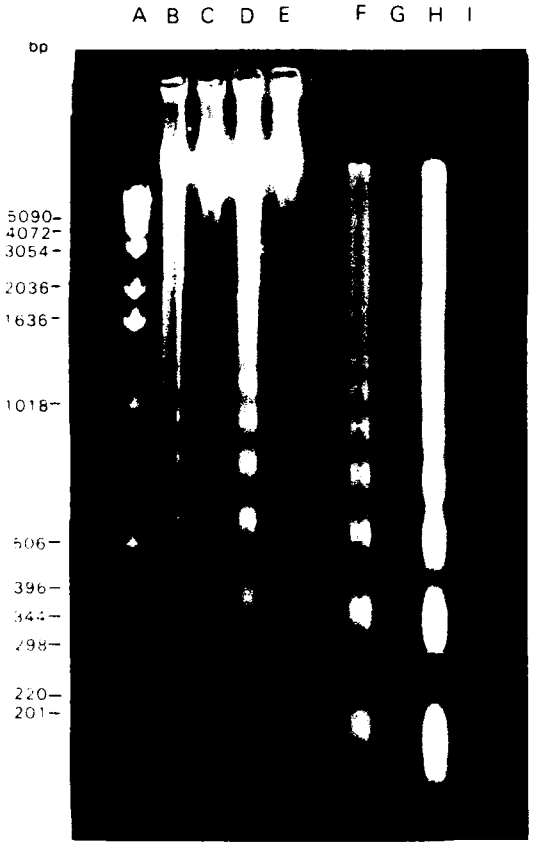


*Figure 2.* Agarose gel electrophoresis of pellet and supernatant DNA isolated from unirradiated or 6.0 Gy-irradiated thymocytes after 6 h incubation with or without 10 mM WR-1065. The molecular size standard DNA is a 1-kb DNA ladder purchased from GIBCO. *Lane A*, standard 1-kb DNA ladder; *lane B*, unirradiated-pellet; *lane C*, unirradiated + WR-1065-pellet; *lane D*, 6.0 Gy-pellet; *lane E*, 6.0 Gy + WR-1065-pellet; *lane F*, unirradiated-supernatant; *lane G*, unirradiated + WR-1065-supernatant; *lane H*, 6.0 Gy-supernatant; *lane I*, 6.0 Gy + WR-1065-supernatant.

isolated from irradiated thymocytes after WR-1065 treatment was of high m.w. and remained at the top of the gel, and there was no "ladder" pattern of DNA bands (*lane E*). The supernatant obtained from irradiated-thymocytes treated with WR-1065 had no DNA fragments (*lane I*). The pellet and supernatant DNA of unirradiated thymocytes contained a small amount of fragmented DNA

(*lane B* and *lane F*, respectively). The pellet DNA isolated from unirradiated cells treated with WR-1065 was of high m.w. (*lane C*). There were no DNA fragments in the supernatant isolated from unirradiated cells treated with WR-1065 (*lane G*). These results clearly indicate that WR-1065 protects the thymocytes from radiation-induced DNA fragmentation.

Figure 3 shows the effect of varying concentrations of WR-1065 on DNA fragmentation in thymocytes after different doses of $\gamma$-irradiation. The inhibition of radiation-induced DNA fragmentation depended on the concentration of WR-1065 during postirradiation incubation. At 2.5 mM WR-1065 there was 1 to 10% DNA fragmentation in thymocytes exposed to 1.5–6 Gy $\gamma$-radiation, and maximum inhibition of DNA fragmentation was obtained at 5 to 10 mM WR-1065. We used 10 mM WR-1065 in all our studies, and it was not toxic to the cells, as shown in Figure 4. Cell viability was assessed by trypan blue dye exclusion method. After irradiation the fraction of dead cells increased progressively with time. Addition of WR-1065 to unirradiated or irradiated thymocytes maintained their viability at 90 to 95% (Fig. 4). The results also indicate that DNA fragmentation in irradiated thymocytes precedes the loss of viability (Figs. 1 and 4), which is consistent with the results reported by others (1–6).

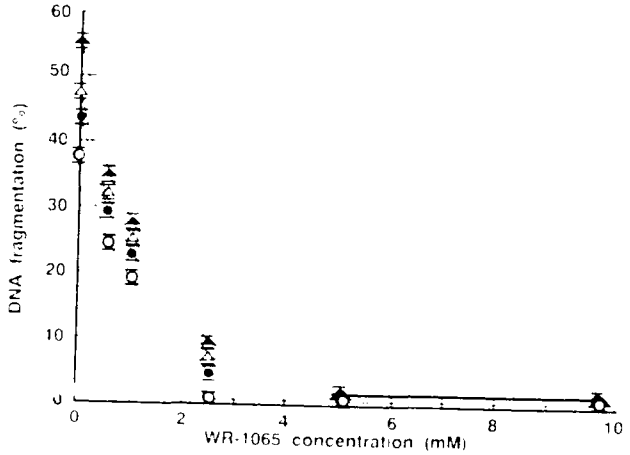


*Figure 3.* Effect of increasing concentrations of WR-1065 on radiation-induced DNA fragmentation in thymocytes. Thymocytes were exposed to different doses of $\gamma$-radiation, and percentage of DNA fragmentation was measured after 8 h incubation with increasing concentrations of WR-1065. The results are mean ± SE from three experiments. ○, 1.5 Gy; ●, 3.0 Gy; △, 4.5 Gy; ▲, 6.0 Gy.

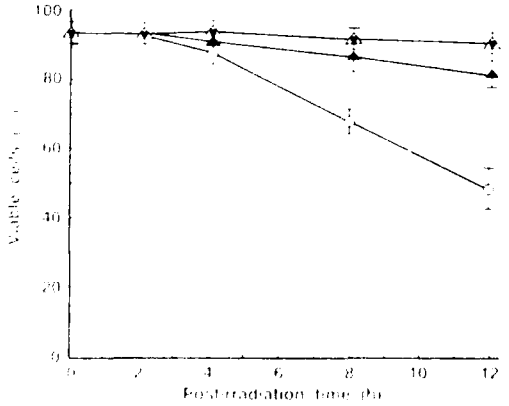


*Figure 4.* Effect of 10 mM WR-1065 on cell death at various times after irradiation at 6.0 Gy. Viability is expressed as the percentage of cells that excluded trypan blue. The results are mean ± SE from three experiments. ▲, unirradiated; △, unirradiated + WR-1065; ○, irradiated; ●, irradiated + WR-1065.

In the above-mentioned studies, thymocytes were incubated with WR-1065 after irradiation. Further studies were carried out to determine whether the addition of WR-1065 before irradiation protects the thymocytes from DNA fragmentation. Thymocytes were exposed to $\gamma$-radiation after 60 min incubation with 10 mM WR-1065. After irradiation, the cells were centrifuged, resuspended in fresh medium without WR-1065, and DNA fragmentation was measured after 8 h of postirradiation incubation. The results shown in Table I indicate that WR-1065 added to thymocytes before irradiation does not protect them from radiation-induced DNA fragmentation.

*WR-1065 blocks dexamethazone- and calcium ionophore A23187-induced DNA fragmentation in thymocytes.* Glucocorticoid hormones and calcium ionophores are known to stimulate apoptosis in thymocytes, which involves extensive DNA fragmentation by $Ca^{2+}$-and $Mg^{2+}$-dependent nuclear endonuclease (4–6). We studied the effect of WR-1065 on dexamethazone-induced and calcium ionophore A23187-stimulated DNA fragmentation in thymocytes. Thymocytes were incubated with increasing concentrations of either dexamethazone or calcium ionophore A23187 with and without WR-1065 in medium at 37°C for 8 h. Dexamethazone and calcium ionophore A23187 stimulated concentration-dependent DNA fragmentation and cell death (Table II) in thymocytes. WR-1065 blocked the dexamethazone-induced and calcium ionophore-stimulated DNA fragmentation and cell death (Table II) in thymocytes.

*WR-1065 inhibits $Ca^{2+}$- and $Mg^{2+}$-dependent DNA fragmentation in thymocyte nuclei.* Several studies indicate that DNA fragmentation observed during apoptosis

TABLE I

*Effect of preirradiation incubation of WR-1065 on DNA fragmentation in thymocytes*

| Treatment | DNA Fragmentation (%) | | |
|---|---|---|---|
| | 0 Gy | 3.0 Gy | 6.0 Gy |
| Control | 15.8 ± 0.5[a] | 43.7 ± 1.1 | 50.5 ± 0.8 |
| WR-1065[b] | 14.3 ± 1.5 | 40.5 ± 3.2 | 49.7 ± 1.7 |

[a] The results are mean ± SE from three experiments.

[b] Thymocytes were irradiated after 60 min incubation with 10 mM WR-1065 in TCM containing 100 U/ml catalase. After irradiation, cells were centrifuged, resuspended in fresh medium without WR-1065, and incubated for 8 h. Incubations and DNA analysis were carried out under the conditions mentioned in *Materials and Methods*.

TABLE II

*Effect of WR-1065 on cell viability and DNA fragmentation in thymocytes exposed to dexamethazone or calcium ionophore A23187*

| Treatments | Viability (%)[a] | | DNA Fragmentation (%)[b] | |
|---|---|---|---|---|
| | − WR-1065 | + WR-1065 | − WR-1065 | + WR-1065 |
| Dexamethazone | | | | |
| 0 nM | 95 ± 3 | 96 ± 2 | 12.5 ± 1.7 | 1.5 ± 0.6 |
| 100 nM | 78 ± 6 | 91 ± 3 | 40.0 ± 2.5 | 1.8 ± 1.2 |
| 200 nM | 69 ± 10 | 92 ± 5 | 47.2 ± 1.2 | 2.1 ± 1.3 |
| 1 μM | 40 ± 4 | 90 ± 4 | 53.1 ± 1.5 | 1.2 ± 0.9 |
| 10 μM | 25 ± 8 | 91 ± 6 | 57.5 ± 1.2 | 2.9 ± 1.2 |
| A23187 | | | | |
| 0 nM | 95 ± 3 | 96 ± 2 | 14.5 ± 1.6 | 1.0 ± 0.5 |
| 100 nM | 80 ± 7 | 91 ± 3 | 20.6 ± 1.8 | 1.0 ± 0.5 |
| 200 nM | 75 ± 5 | 89 ± 4 | 26.1 ± 0.9 | 1.7 ± 0.7 |
| 400 nM | 67 ± 6 | 90 ± 2 | 33.6 ± 1.8 | 1.9 ± 0.9 |
| 1 μM | 56 ± 7 | 87 ± 6 | 44.1 ± 0.7 | 1.9 ± 0.8 |

[a] Viability is expressed as the percentage of cells that excluded trypan blue after 8 h incubation with the indicated concentrations of either dexamethazone or calcium ionophore A23187 with or without WR-1065.

[b] DNA fragmentation was determined after 8 h incubation. The results are mean ± SE from three experiments.

of thymocytes is due to action of a $Ca^{2+}$- and $Mg^{2+}$-dependent nuclear endonuclease that cleaves host chromatin into oligonucleosome-length fragments (1–6). We tested the effect of WR-1065 on $Ca^{2+}$- and $Mg^{2+}$-dependent DNA fragmentation in thymocyte nuclei. Nuclei were isolated from thymocytes and incubated in Tris-buffered (10 mM, pH 7.5) isotonic sodium chloride with and without added $Ca^{2+}$ and $Mg^{2+}$. We found that most of the DNA remained intact in thymocyte nuclei incubated with $Mg^{2+}$ or $Ca^{2+}$ alone, but when both ions were present about 68% of DNA was fragmented (Table III). Interestingly, WR-1065 inhibited $Ca^{2+}$- and $Mg^{2+}$-dependent DNA fragmentation in thymocyte nuclei (Table III). The results of this study suggest that WR-1065 could be protecting the thymocytes from DNA fragmentation by inhibiting the action of $Ca^{2+}$- and $Mg^{2+}$-dependent nuclear endonuclease.

## DISCUSSION

The results of these studies clearly indicate that WR-1065 inhibits the internucleosomal DNA fragmentation and cell death in thymocytes exposed to γ-radiation, dexamethazone, and calcium ionophore A23187. WR-1065 is known to protect mammalian cells from radiation-induced reproductive death when incubated with the cells before and during irradiation; it does not inhibit reproductive death when added to cells after irradiation (22–25, 30–32). When mammalian cells are exposed to ionizing radiation, DNA damage occurs during irradiation due to direct interaction of free radicals with DNA and it can be measured immediately after irradiation (33). It is thought that the presence of WR-1065 in cells during irradiation prevents reproductive death by interferring with the interactions of radiation-induced free radicals with DNA (30–32).

The ability of WR-1065 to protect against free radical interaction with DNA is apparently not related to its ability to prevent apoptosis in irradiated cells. DNA fragmentation, a characteristic of apoptosis, is unaffected by incubating cells with WR-1065 before irradiation; fragmentation is at the same level in the irradiated cells pretreated with WR-1065 as it is in cells not pretreated with WR-1065 (Table I). In thymocytes there is no DNA fragmentation immediately after irradiation; fragmentation begins at 2 to 3 h postirradiation and increases with time (Fig. 1). In apoptosis, DNA fragmentation appears to be a distinctively postirradiation cellular process. Inasmuch as the presence of WR-1065 in the cell after irradiation completely blocks DNA fragmentation, it may be inhibiting a postirradiation cellular process responsible for DNA fragmentation. It has been reported that the magnitude of radioprotection against reproductive death depends on the intracellular concentration WR-1065 at the time of irradiation (32). The ability of WR-1065 to protect against DNA fragmentation during apoptosis depends on the concentration of WR-1065 during postirradiation incubation (Fig. 3).

TABLE III

*Effect of WR 1065 on activation of endogenous endonuclease in isolated thymocyte nuclei*[a]

| Treatments | $CaCl_2$, 5 mM | $MgCl_2$, 10 mM | DNA fragmentation (%) |
|---|---|---|---|
| Nuclei | – | – | 0.7 ± 0.4 |
| | – | + | 0.8 ± 0.1 |
| | + | – | 25.0 ± 1.6 |
| | + | + | 68.2 ± 1.3 |
| Nuclei + WR-1065[b] | – | – | 0.3 ± 0.3 |
| | – | + | 0.6 ± 0.1 |
| | + | – | 0.5 ± 0.1 |
| | + | + | 0.4 ± 0.1 |

[a] Thymocyte nuclei were incubated with different concentrations of cations at 37°C for 4 h and DNA was estimated as mentioned in *Materials and Methods*. –, without; +, with.

[b] 10 mM WR-1065. The results are mean ± SE from three experiments.

Dexamethazone and calcium ionophore A23187 induce a similar degree of DNA fragmentation in thymocytes as those observed after irradiation. WR-1065 inhibits DNA fragmentation in these cases also, suggesting a common mechanism of action. Our studies with thymocyte nuclei indicate that WR-1065 inhibits a $Ca^{2+}$- and $Mg^{2+}$-dependent process responsible for DNA degradation. In our studies, more than 50% of the DNA was fragmented in isolated nuclei incubated in the presence of $Ca^{2+}$- and $Mg^{2+}$ (Table III). DNA isolated from irradiated cells (Fig. 2, *lane D*) and cation-treated unirradiated nuclei (not shown) showed no difference in their electrophoretic patterns. The specific pattern of degradation of DNA into oligonucleosomal subunits suggests that an endonuclease may be involved in the process. It is possible that a $Ca^{2+}$- and $Mg^{2+}$-dependent nuclease may be constitutively present in an inactive form in thymocyte nuclei. When optimum concentrations of $Ca^{2+}$ and $Mg^{2+}$ are present, the enzyme may be activated to degrade DNA into oligonucleosomal subunits. A nuclease of similar specificity has been described in nuclei of thymocytes and other mammalian cells (34–37). Nuclei incubated with WR-1065 showed no cation-dependent DNA fragmentation (Table III), suggesting the inhibition of action of nuclease.

A variety of molecular and cellular mechanisms has been proposed to explain the ability of WR-1065 to protect mammalian cells from radiation-induced reproductive death (20, 38). The mechanism of protection offered by WR-1065 in our experiments is not clear. However, several possible mechanisms for the action of WR-1065 in thymocyte apoptosis may be suggested from the above results. First, WR-1065 may inhibit the DNA degradation by altering the structure of internucleosomal region in chromatin. WR-1065 binds to DNA and nuclear proteins in mammalian cells (39). This binding in thymocyte nuclei may alter the conformation of chromatin in such a way that internucleosomal region may not be available for degradation of chromatin into oligonucleosomal subunits. Second, WR-1065 may inactivate the enzyme responsible for DNA degradation. WR-1065 forms mixed disulfides with sulfhydryl groups in protein (20, 38). It is possible that WR-1065 may inactivate the nuclear endonuclease by forming mixed disulfides with sulfhydryl groups of the enzyme. Third, WR-1065 may regulate the cellular transport of cations necessary for DNA degradation. Studies indicate that WR-2721 and WR-1065 modulate calcium metabolism in chronic renal failure (40) and in hypercalcemia of malignancy (41). Recently, WR-1065 was shown to prevent calcium entry and cell death in U937 human premonocytic cell line exposed to hydrogen peroxide (42). The precise cellular mechanism by which WR-1065 regulates calcium transport is unknown. WR-1065 does not form chelation complexes with $Ca^{2+}$ and $Mg^{2+}$ (43). It may either act directly on calcium channels in the membrane or inhibit lipid peroxidation of membranes and prevent calcium entry. Lipid peroxidation alters membrane permeability and increases calcium influx, and it can be induced by several oxidants includ-

ing ionizing radiation (44–46). WR-1065 is known to inhibit the lipid peroxidation of membranes (47, 48). Studies are in progress to understand the precise cellular and molecular mechanism of action of WR-1065 in protecting thymocytes from apoptosis.

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RADIATION RESEARCH 130, 360–365 (1992)



# Radioprotection of Hematopoietic Tissues in Mice by Lipoic Acid

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RAMAKRISHNAN, N., WOLFE, W. W., AND CATRAVAS, G. N. Radioprotection of Hematopoietic Tissues in Mice by Lipoic Acid. *Radiat. Res.* 130, 360–365 (1992).

**Lipoic acid is a lipophilic antioxidant that participates in many enzymatic reactions and is used clinically to treat mushroom poisoning and metal toxicity. In this report the protective effect of lipoic acid (oxidized form) against radiation injury to hematopoietic tissues in mice was assessed by the endogenous and exogenous spleen colony assays and survival ($LD_{50/30}$) assay. Intraperitoneal administration of lipoic acid at a nonlethal concentration of 200 mg/kg body wt, 30 min before irradiation increased the $LD_{50/30}$ from 8.67 to 10.93 Gy in male $CD2F_1$ mice. Following a 9-Gy irradiation, the yield of endogenous spleen colony-forming units in mice treated with saline and lipoic acid was 0.75 ± 0.5 and 8.9 ± 1.6, respectively. Using the exogenous spleen colony assay, lipoic acid treatment increased the $D_0$ from 0.81 ± 0.01 to 1.09 ± 0.01 Gy, yielding a dose modification factor of 1.34 ± 0.01. Dihydrolipoic acid (reduced form) has no radioprotective effect in $CD2F_1$ mice.** © 1992 Academic Press, Inc.

## INTRODUCTION

Lipoic acid (6,8-thioctic acid) is a lipophilic, endogenous disulfide that can be reduced to dihydrolipoic acid with vicinyl thiol groups. The function of lipoic acid as a prosthetic group in the oxidative decarboxylation of the α-keto acids pyruvate and α-ketoglutarate in mitochondria is well known (*1–4*). Lipoic acid is used in the treatment of a wide variety of liver diseases (*5–7*) and dysfunctions (*8–12*) in which free radical-induced lipid peroxidation appears to be involved. Lipoic acid has been shown to provide protection against free radical-mediated injury both *in vivo* and *in vitro* (*13–17*). Recently, an interplay between lipoic acid and glutathione in the protection against microsomal lipid peroxidation has been demonstrated (*16*). The protective effects of lipoic acid against free radical-mediated injury interested us in examining whether lipoic or dihydrolipoic acid protects hematopoietic tissues in mice from free radical damage induced by ionizing radiation.

## MATERIALS AND METHODS

### *Animals*

The animals used were male $CD2F_1$ mice purchased from Charles River Laboratories (Kingston, NY). They were quarantined for 2 weeks and examined for pathological or serological indications of disease and *Pseudomonas* infection (representative sampling). The mice, weighing approximately 25 g, were housed eight to a plastic Microlsolator cage on hardwood chip, contact bedding in an AAALAC-accredited facility, and were provided commercial rodent chow and acidified water (pH 2.5) *ad libitum*. Animal rooms were maintained at 21 ± 1°C with 50 ± 10% relative humidity and 12 room changes of 100% conditioned fresh air per hour, with a 12-h light–dark cycle.

### *Lipoic Acid Treatment*

Lipoic and dihydrolipoic acids were obtained from Sigma Chemical Co. (St. Louis, MO). Immediately before use lipoic acid was dissolved in 1 *M* sodium bicarbonate and diluted to the desired concentration with sterile saline. The pH of the lipoic acid solution was 7.4. Lipoic acid was administered to mice intraperitoneally in a volume of 0.25 ml. Control mice received saline.

### *Survival Studies*

Mice were placed in ventilated Plexiglas containers and exposed unilaterally to $^{60}Co$ γ radiation at a dose rate of 0.2 Gy/min, using an Atomic Energy of Canada Limited Theratron-60 teletherapy unit. The total doses ranged from 6 to 12 Gy. The percentage of mice surviving each radiation dose 30 days following exposure was used to construct a probit plot survival curve for each treatment group. Probit analyses were done according to Finney (*18, 19*) to obtain $LD_{50/30}$ values. These values were used to determine dose reduction factors.

### *Endogenous Spleen Colony Assay*

The endogenous spleen colony assay was done according to the method of Till and McCulloch (*20*). Mice treated with lipoic acid and saline were exposed to total-body irradiation; 12 days following irradiation spleens were removed and fixed in Bouin's solution, and microscopically visible colonies were counted.

### *Exogenous Spleen Colony Assay*

The survival of hematopoietic stem cells following increasing doses of radiation was determined using the exogenous spleen colony assay (*21, 22*). Mice treated with lipoic acid and saline were exposed to $^{60}Co$ γ radiation (0–5 Gy), and 18 h later femoral bone marrow was removed and made into single cell suspensions in Hanks balanced salt solution containing 10% heat-inactivated fetal bovine serum. The cells were counted in a Coulter counter. Appropriate dilutions of cells were injected into the lateral tail veins of lethally irradiated recipient mice. The recipient mice received 9.4 Gy $^{60}Co$ γ radiation 2 h before the injection of test cells, and the number of colonies per spleen was determined 10 days later.

### *Measurement of Sulfhydryl Concentration*

Liver homogenate was prepared by homogenizing liver in phosphate-buffered saline to a final concentration of 1% (1 g liver/100 ml PBS). Dihydrolipoic acid was added to heparinized blood or liver homogenate to



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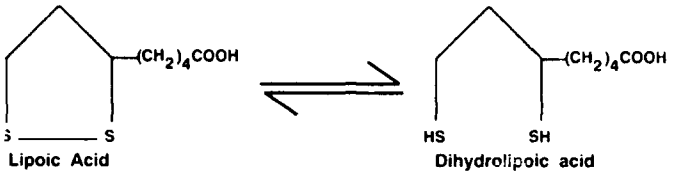


FIG. 1. Structure of lipoic and dihydrolipoic acids.

a final concentration of 1 m*M*. At varying times, aliquots of blood were hemolyzed in 0.01 *M* phosphate buffer (pH 7.5) containing 0.05 *M* EDTA, and the sulfhydryl concentration was determined using a modification of Ellman's technique (*23, 24*). Ten microliters of 10 m*M* 5,5'-dithiobis 2-nitrobenzoic acid was added to 1 ml of liver homogenate or hemolyzed blood and optical density was measured at 412 nm.

## RESULTS

The structure of lipoic and dihydrolipoic acids is shown in Fig 1. These two forms are readily interconvertible in oxidation–reduction reactions. Studies were carried out to determine the nonlethal doses of lipoic and dihydrolipoic acids in $CD2F_1$ mice. Results shown in Table I indicate that intraperitoneal administration of lipoic acid at concentrations below 275 mg/kg body wt was not lethal to $CD2F_1$ mice. Probit analysis of the data suggests that the $LD_{95}$ dose of lipoic acid was 319 mg/kg (95% confidence limits: 306 and 409 mg/kg) in mice. Intraperitoneal administration of dihydrolipoic acid at doses below 150 mg/kg was not lethal to the animals. The $LD_{95}$ of dihydrolipoic acid was 217 mg/kg (95% confidence limits: 210 and 230 mg/kg).

TABLE I
**Toxicities of Lipoic and Dihydrolipoic Acids in Male $CD2F_1$ Mice**

| Concentration (mg/kg)[a] | Percentage survival[b] | $LD_{95}$ (mg/kg)[c] |
|---|---|---|
| Lipoic acid | | |
| 250 | 100 | 319 (306, 409) |
| 275 | 100 | |
| 290 | 70 | |
| 300 | 60 | |
| 325 | 0 | |
| Dihydrolipoic acid | | |
| 100 | 100 | 217 (210, 230) |
| 150 | 100 | |
| 200 | 60 | |
| 250 | 0 | |

[a] Lipoic and dihydrolipoic acids were administered intraperitoneally to mice.

[b] Survival was monitored daily for 2 weeks. Each treatment group consisted of 20 mice.

[c] $LD_{95}$ dose was calculated from probit analysis. The numbers in the parentheses indicate 95% confidence limits.

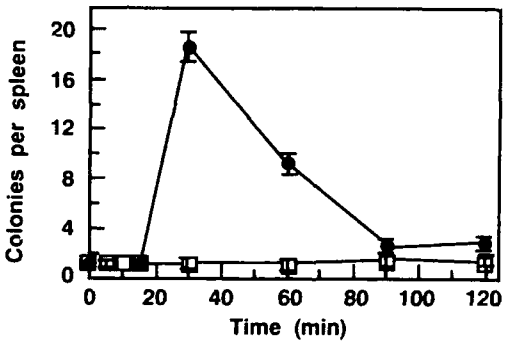


FIG. 2. Effect of time of lipoic and dihydrolipoic acid administration on endogenous spleen colony survival following 8 Gy irradiation. Lipoic (200 mg/kg) or dihydrolipoic (100 mg/kg) acid was administered intraperitoneally at indicated times before 8 Gy total-body irradiation. Endogenous spleen colonies were counted 12 days later as described under Materials and Methods. The 0-min data point represents the number of spleen colonies in control (saline-treated) animals. The results are mean ± SE for 16 mice. (●) Lipoic acid, (□) dihydrolipoic acid.

We further investigated the optimum time of lipoic and dihydrolipoic acid treatment to get the maximum protective effect against radiation injury. The animals were treated with either 200 mg/kg lipoic acid or 100 mg/kg dihydrolipoic acid at different times before irradiation, and the protective effect of these compounds against radiation injury to hematopoietic tissues was assessed by the endogenous spleen colony and survival assays. Results indicate that lipoic acid gave maximum protection to hematopoietic tissues (Fig. 2) and increased survival in mice (Fig. 3) when it was administered 30 min before irradiation. Dihydrolipoic acid had no radioprotective effect because it increased neither the number of spleen colonies (Fig. 2) nor survival (Fig. 3) in irradiated mice. The number of endogenous spleen colonies increased with increasing concentration of lipoic acid (Fig. 4). As shown in Table II, all animals treated with 75–200 mg/kg lipoic acid survived following 9 Gy irradiation. The number of surviving animals increased with increasing doses of lipoic acid following 10 Gy irradiation. We used 200 mg/kg lipoic acid in the following studies, because it was a nonlethal dose of the compound that gave maximum protection against radiation injury (Fig. 4 and Table II).

The radiation dose–response curve for mice treated with 200 mg/kg lipoic acid 30 min before irradiation is shown in Fig. 5. The $LD_{50/30}$s for mice treated with saline and lipoic acid were 8.67 Gy (8.62, 8.72 Gy) and 10.93 Gy (10.74, 11.16 Gy), respectively. The numbers in the parentheses indicate 95% confidence limits. Compared to saline, lipoic acid treatment produced a dose reduction factor (DRF) of 1.26 at the $LD_{50/30}$. Lipoic acid increased neither the survival nor the number of spleen colonies when administered to animals following irradiation (results not shown). Figure 6 shows the dependence of the number of spleen colonies

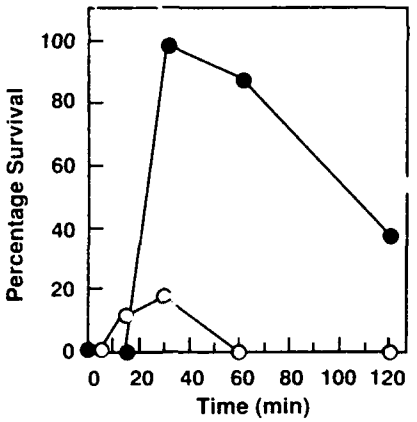


FIG. 3. Effect of time of lipoic and dihydrolipoic acid administration on survival following 9 Gy irradiation. Lipoic (200 mg/kg) or dihydrolipoic (100 mg/kg) acid was administered intraperitoneally at indicated times before 9 Gy total-body irradiation. Survival was monitored for 30 days following irradiation. The 0-min data point represents the survival in control (saline-treated) animals. The results are mean ± SE for 16 mice. (●) Lipoic acid, (○) dihydrolipoic acid.

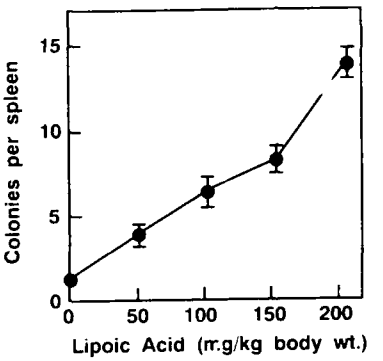


FIG. 4. Effect of varying concentrations of lipoic acid on endogenous spleen colony survival following 8 Gy irradiation. Lipoic acid was administered intraperitoneally at indicated concentrations 30 min before 8 Gy irradiation. The other details of the experiment are given in Fig. 2.

on radiation dose in control and lipoic acid-treated mice. Lipoic acid treatment significantly increased the number of spleen colonies ranging from >40 (confluent colony formation) at 7 Gy to 8.9 ± 1.6 at 9 Gy. Lipoic acid treatment resulted in a DRF of 1.5 at 10 colonies per spleen dose level.

The radioprotective effect of lipoic acid on hematopoietic stem cells was studied using the exogenous spleen colony assay. Figure 7 shows radiation dose–response curves for colony-forming units from mice treated with saline and lipoic acid. Both saline and lipoic acid treatment resulted in linear survival curves with no shoulder. The $D_0$ value for control cells was 0.81 ± 0.01, which is consistent with those reported earlier for $CD2F_1$ mice (*21*). Lipoic acid treatment increased the $D_0$ to 1.09 ± 0.01 ($P < 0.001$, $t$ test), yielding a dose reduction factor of 1.34 ± 0.01.

TABLE 2

**Effect of Varying Concentrations of Lipoic Acid on Survival following Whole-Body Irradiation**

| Lipoic acid[a] (mg/kg) | Survival (%)[b] | |
|---|---|---|
| | 9 Gy | 10 Gy |
| 50 | 83.3 | 0.0 |
| 75 | 95.8 | 0.0 |
| 100 | 95.8 | 12.5 |
| 150 | 95.8 | 20.8 |
| 200 | 100.0 | 91.7 |

[a] Lipoic acid was administered intraperitoneally to mice 30 min before irradiation.

[b] Survival was monitored for 30 days following irradiation. Each treatment group consisted of 24 mice.

The results of the present study provide evidence that intraperitoneal administration of lipoic acid but not dihydrolipoic acid protected mice from the lethal effects of γ irradiation. In contrast, dihydrolipoic acid but not lipoic acid increased the clonogenic cell survival in Chinese hamster V79 cells *in vitro* (*25*). It is likely that dihydrolipoic acid might be metabolized rapidly before it could reach the target cells *in vivo*. To find out why dihydrolipoic acid was not radioprotective in mice, we measured the sulfhydryl concentration in blood *in vitro* at different times following addition of 1 m*M* dihydrolipoic acid. Since lipoic acid was shown to accumulate in liver following *in vivo* administration (*26*), we compared the rate of decrease of sulfhydryl concentration in blood to that of liver homogenate. The results shown in Fig. 8 indicate that sulfhydryl concentra-

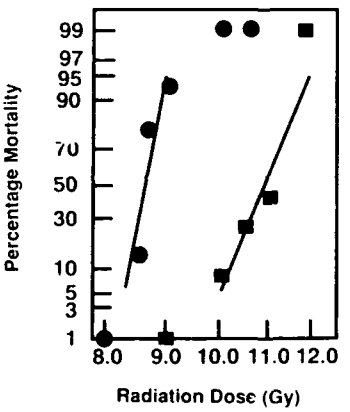


FIG. 5. Effect of lipoic acid on survival of mice following total-body irradiation. Lipoic acid (200 mg/kg) was administered to mice intraperitoneally 30 min before irradiation. Control group of mice received saline. Survival was monitored for 30 days and percentage mortality was probit-plotted against radiation dose. Experiments were repeated three to five times and each treatment group in each experiment consisted of 8–16 mice. (●) Saline, (■) lipoic acid.

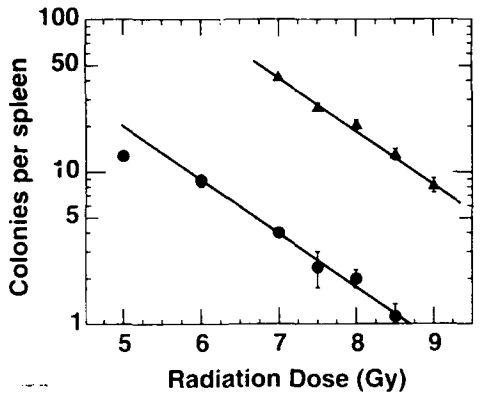


FIG. 6. Effect of lipoic acid on endogenous spleen colony survival following total-body irradiation. Lipoic acid (200 mg/kg) was administered to mice intraperitoneally 30 min before irradiation. The other details of the experiment are given in Fig. 2. Spleen colony numbers more than 40 represent confluent colony formation. (●) Saline, (▲) lipoic acid.

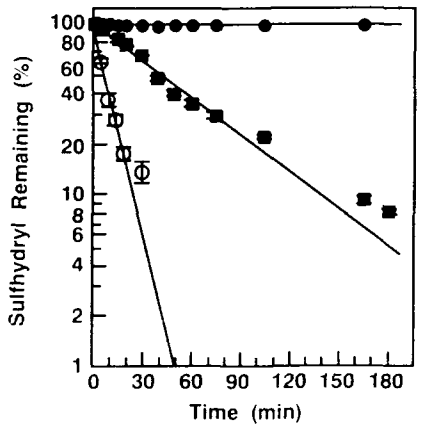


FIG. 8. Sulfhydryl measurement in blood and liver homogenate in the presence of 1 m*M* dihydrolipoic acid. Dihydrolipoic acid was added to heparinized mouse blood or liver homogenate to a final concentration of 1 m*M*. At the indicated times the sulfhydryl concentration was determined using a modification of Ellman's technique as described under Materials and Methods. The curves were fitted by least-squares regression analysis. The $t_{1/2}$ for dihydrolipoic acid in blood was 7.90 ± 0.23 min and in liver homogenate was 41.3 ± 2.2 min. The results are mean ± SE of nine measurements from three experiments. (●) Phosphate-buffered saline, (■) liver homogenate, (○) blood.

tion decreased more rapidly in blood, with a $t_{1/2}$ of 7.90 ± 0.23 min, than in liver homogenate. It is quite possible that dihydrolipoic acid might be metabolized rapidly or might combine with serum proteins as soon as it is administered to the animals, and hence not be protective in animals.

## DISCUSSION

The results presented in this paper indicate that lipoic acid protects hematopoietic tissues in mice from the lethal effects of ionizing radiation. The radioprotective effect of lipoic acid was demonstrated by determining the $LD_{50/30}$ (DRF = 1.26) and using the endogenous (DRF = 1.5) and exogenous spleen colony assays (DRF = 1.34). Significant radioprotection was achieved when lipoic acid was given before but not after irradiation. The extent of protection, however, depended greatly on the experimental conditions such as timing of treatment with lipoic acid in relation to irradiation and concentration of lipoic acid. It was reported that administration of WR-2721 (400 mg/kg) to $CB6F_1$ female mice 30 min before $\gamma$ irradiation yielded a DRF of 2.4 for stem cell survival, as determined by the exogenous spleen colony assay (27). Compared to WR-2721, lipoic acid is less effective in protecting the stem cells from radiation damage. Lipoic acid is a lipophilic compound whereas WR-2721 is a hydrophilic compound. Hence the subcellular sites at which these two compounds act may be different.

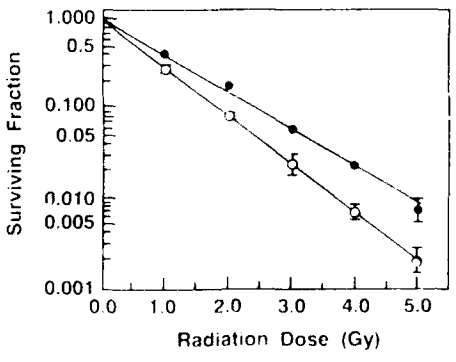


FIG. 7. Effect of lipoic acid on radioresponse of bone marrow stem cells. Donor mice were treated with saline or lipoic acid (200 mg/kg) 30 min before irradiation (0–5 Gy). Bone marrow cells were collected from femurs 18 h later and injected into the vein of lethally irradiated (9.4 Gy) recipient mice ($n = 15$–20). After 10 days the number of spleen colonies was determined. Dose–response curves were fitted using least-squares regression analysis. (○) Saline, (●) lipoic acid.

The results of the present study indicate that dihydrolipoic acid was not radioprotective in mice because it did not increase either the survival or the number of spleen colonies in irradiated mice. The rapid rate of the decrease of the sulfhydryl concentration in blood following treatment with dihydrolipoic acid (Fig. 8) suggests that when dihydrolipoic acid was administered to animals it might be metabolized rapidly or combine with serum proteins before it could reach the cellular sites, and hence not be protective.

Studies with Chinese hamster V79 cells *in vitro* indicate that dihydrolipoic acid but not lipoic acid increased the clonogenic cell survival following increasing doses of 50-kVp X irradiation (25). Studies with V79 cells *in vitro* suggest that dihydrolipoic acid may be the active cellular radioprotective agent. Studies on the antioxidant effects of lipoic acid *in vivo* indicate that the liver and heart homogenates prepared from rats treated with lipoic acid were significantly resistant to peroxyl radical-induced lipid peroxida-

tion. In contrast, dihydrolipoic acid but not lipoic acid inhibited peroxyl radical-induced lipid peroxidation *in vitro* in liver homogenate or microsomes (*13*). These studies suggest that, at the cellular level, lipoic acid has to be reduced to dihydrolipoic acid to provide protection against radiation damage and oxidant stress. It is possible that after lipoic acid was administered to animals it might be reduced to dihydrolipoic acid close to the cells being effectively protected.

Many publications have described the protective and curative effects of lipoic acid in heavy-metal poisoning (*8–12*). Studies on the influence of lipoic acid on the chemotherapeutic efficacy of vincristine sulfate indicate that the toxic side effects of vincristine sulfate can be reduced following adjuvant treatment with lipoic acid (*28*). Its therapeutical application is found in treatment of intoxication with the mushroom *Amanita Phalloides* (*5–7, 29, 30*), peripheral polyneuropathies (*31, 32*), and liver cirrhosis (*33*). It is suggested that free radical-mediated peroxidation of the cell membrane is a common pathway in the etiology of these pathologies (*34*). These studies suggest that lipoic acid exerts its therapeutic effect in pathologies in which free radicals are involved. Lipoic and dihydrolipoic acid inhibited lipid peroxidation *in vivo* and *in vitro*, respectively (*13, 16, 17*). Dihydrolipoic acid inhibited skin inflammation in mice induced by UVB radiation, xanthine/hypoxanthine, adriamycin, and phorbol myristate acetate (*14, 35*). These studies provide evidence that lipoic and dihydrolipoic acids provide protection against free radical-mediated injury. It is well known that free radicals generated during radiolysis of water play the most significant role in the indirect biological damage ind[illegible]by ionizing radiation (*36*). The results of the present study suggest that hemopoietic stem cells can be protected from radiation-induced free radical damage by lipoic acid.

## ACKNOWLEDGMENTS

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ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
SCIENTIFIC REPORT
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# Pathological Evaluation of WR-151327 Administered Orally in Irradiated and Non-Irradiated Male Mice

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## ABSTRACT

Studies were made on the radioprotective and toxic effects of orally administered WR-151327 in male CD2F1 mice. The lowest dose of orally administered drug permitting probit analysis of data was 450 mg per kg. The calculated radioprotective dose reduction factors (DRF) at 450 mg per kg and 900 mg per kg of body weight (BW) WR-151327 were 1.2 and 1.3, respectfully. Pathological examination at 8, 30 or 90 days post administration of 100, 450, or 900 mg per kg of the drug demonstrated that the major target organ for orally dosed mice was the testes. There was a decrease in the number of cells in the germinal cell layers of testes from animals administered 450 mg per kg WR-151327 or 10 Gy whole body irradiation after eight days. Moreover, there was a dramatic reduction in the germinal cells in mice seminiferous tubules treated with a combination of 450 mg per kg WR-151327 plus 10 Gy radiation after eight days.

## Introduction

Chemical radioprotection is exhibited by certain sulfhydryl (-SH) containing compounds if they are administered prior to ionizing radiation exposure.[5] WR-151327, S-3-(3-methylaminopropylamino)-propylphosphorothioic acid, is a water soluble phosphoroaminothiol compound which contains a -SH group upon dephosphorylation.[1] It has been identified as an effective radioprotector when administered orally[8] and has been demonstrated to provide protection against neutron radiation effects in the gastrointestinal tract[6] and bone marrow[7] when administered intraperitoneally (ip). A recent report by van Beek et al[9] indicated adverse pathological effects upon ip injection of WR-151327 to non-irradiated male mice. The purpose of this study was twofold: to determine an oral dose of WR-151327 providing radioprotection

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and to examine pathological changes in male mice following oral administration of the drug.

## Materials and Methods

Male CD2F1 mice were obtained from the National Cancer Institute, Frederick, MD. All mice were between eight and 12 weeks of age at the time of experimental use. Animals were provided with acidified water (pH 2.7) to prevent growth of Pseudomonas. They were fed mouse chow,* housed eight to a cage, and maintained on 12-hour light/dark cycle to prevent circadian rhythms that could influence responses.

Drugs were administered to mice between 8:30 A.M. and 9:00 A.M. about one hour prior to irradiation. Mice were placed in plastic containers approximately 15 minutes before total-body irradiation. They were irradiated in a bilateral $^{60}$BCo gamma-ray field at a dose rate of 1.0 Gy per min, receiving total doses of 7, 8, 9, 10, 11, 12, or 13 Gy. The control group was also irradiated at doses of 7.5, 7.75, 8.25, 8.5, or 8.75 Gy.

The radioprotector WR-151327 was obtained† and was administered to mice using stainless steel oral animal feeders.‡ Mice were divided into groups of at least 10 animals and given the following concentrations of the drug dissolved in sterile saline pH 7.0: 100, 450, and 900 mg per kg body weight. The 900 mg per kg drug dosage represented approximately one half the $LD_{10}$ of orally administered WR-151327. The drug concentration was adjusted so that 0.1 ml was administered for each 0.01 kg body weight. Approximately one hour prior to exposure, irradiated mice were given the WR-151327. Control mice received a similar 0.1 ml volume of sterile saline pH 7.0 for each 0.01 kg of body weight. Survival was monitored for 30 days. Data were analyzed by probit using the Finney method.[2] Briefly, this method is used to fit a dose-response curve to the observed proportion of animals surviving at each concentration of WR-151327, assuming an underlining normal distribution of tolerance to WR-151327.

The pathological effects of orally administered WR-151327 were determined by histological comparisons between tissues from treated mice and controls. Surviving animals were euthanised on days 8, 30, or 90 by lethal inhalation of methoxyflurane.§ Gross necropsy was performed, and tissue samples of trachea, lung, heart, skeletal muscle, pancreas, salivary gland, duodenum, ileum, jejunum, colon, kidney, liver, brain, eye, spleen, and testes were removed immediately and placed in saline formalin fixative. All tissues were embedded in paraffin wax, cut into four micron sections, and stained with hematoxylin and eosin. Light microscopy measurements were made of the circumference and the number of cells in the germinal cell layers of five seminiferous vesicles per testis for groups of eight treated mice euthanised at day 8. All slides were analyzed using a Bioquant Hipad Digitiser attached to a microscope (Leitz Laborlux D). Slides were decoded into experimental groups and raw data were statistically analyzed using two-way analysis of variance to compare the means of the number of cells and the circumferences of the testes in each group.

## Results

A modest enhancement in the length of time of survival of irradiated mice compared to controls was observed in groups

* Wayne Rodent Lab Blox, Wayne Pet Food Division, Continental Grain Company, Chicago, IL.
† US Bioscience, West Conshohocken, PA.
‡ Popper and Sons, Inc., New Hyde Park, NY.
§ Trademark Metofane, Pitman-Moore, USA.

previously administered WR-151327 at 100 mg per kg of body weight. However, the lowest practical dose of drug resulting in survival of sufficient numbers of animals permitting probit analysis of data was 450 mg per kg. In figure 1 are shown probit plots of percent mortality as a function of irradiation dose for controls and animals administered either 450 mg per kg or 900 mg per kg. Each data point is an average of at least eight animals. The dose reduction factor for WR-151327 at 450 mg per kg and 900 mg per kg was 1.206 (95 percent confidence limits 1.17 through 1.248) and 1.305 (95 percent confidence limits 1.263 through 1.355), respectively. This was calculated from probit analysis using the equation $LD_{50/30}$ = ($LD_{50/30}$ WR-151327 group)/($LD_{50/30}$ control group). The slope of the probit of the control was significantly different from the slopes of the lines at each dose of WR-151327. For this reason the reported dose reduction factor is valid only for $LD_{50/30}$.

The combined pathological results observed in 72 mice dosed with either 100, 450, or 900 mg per kg of BW WR-151327 and examined 8, 30, and 90 days following exposure indicated that 85 percent of the animals had focal atrophy of the testes. There were minor changes in other organs which were not correlated with dosage. Approximately 35 percent of the livers presented subacute microgranuloma, 21 percent of the lungs showed acute hemorrhage and congestion, eight percent had epithelial sloughing of the gastrointestinal tract, 10 percent presented acute interstitial nephritis, 10 percent presented subacute pancreatitis, one percent of the spleens showed acute congestion, three percent of the thyroid glands had multifocal necrosis, three percent of the lymph nodes had hyperplasia, and four percent of the sebaceous glands had adenoma. There were no pathological changes observed in brains, skeletal muscles, salivary glands, or the eyes.

Oral administration of the drug at 900 mg per kg caused diarrhea within 24 hours in 77 percent (n = 88) of the mice treated. This observation was confirmed at higher concentrations of WR-151327 (results not shown). Twenty-four control

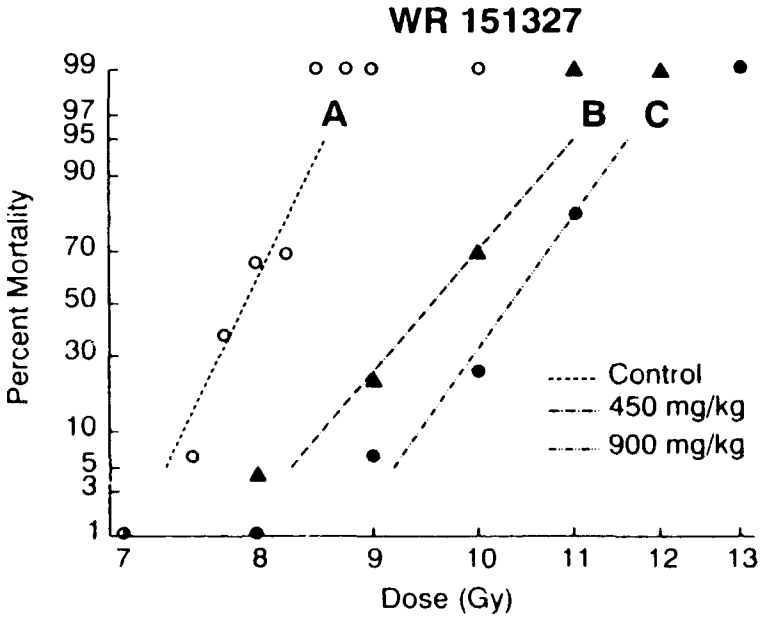


FIGURE 1. Percentage mortality after 30 days of groups of at least eight mice following oral administration of (A) saline (B) 450 mg per kg of WR151327, or (C) 900 mg per kg of WR151327 orally one hour before irradiation in a $^{60}Co$ gamma field at a rate of 1.0 Gy per min over the radiation dose range of 7 to 13 Gy. The data were analysed by probit, and the calculated dose reduction factor was 1.206 for B and 1.305 for C.

mice examined at days 8, 30, and 90 had one animal with a cyst in the kidney. No other pathological changes were observed. Following this broad survey of pathological results, the testes of the mice were studied in more detail.

In table I is shown the percent of testicular involvement at each dose of WR-151327 on days 8, 30, and 90. There is an increase in the percentage of animals presenting testicular damage when the dose was increased. In addition, there was an increase in the numbers of animals presenting testicular damage in groups administered either 100 or 450 mg per kg of BW of the drug with the passage of time. The 900 mg per kg dosed animals remained at 100 percent involvement on days 8, 30, and 90.

Pathological examination of groups of eight mice orally administered 0, 100, 450, or 900 mg per kg of BW WR-151327, subjected to 10 Gy total body gamma irradiation, and examined eight days later indicated focal atrophy of the testes in every animal. Control animals that were neither administered the drug nor subjected to irradiation presented normal testicular histology.

A transverse section of several seminiferous tubules of the testis from a mouse given saline orally and killed eight days later is shown in figure 2A. Each seminiferous tubule is surrounded by an outer compact connective tissue and an inner basement membrane. Enclosed in the basement membrane is the specialized germinal epithelium and Sertoli's cells. Sertoli's cells are slender, elongated cells with very irregular outlines extending from the basement membrane to the free luminal surface. The distinctive Sertoli's cell nucleus is ovoid or angular in shape, facilitating its discrimination from the spermatogenic cell that divides mitotically to produce several generations of cells.

TABLE I

Percentage of Groups of Eight Mice Presenting Testicular Pathological Changes

| *Concentration WR151327 mg/kg body weight* | *Percent of Mice Presenting Testicular Pathological Changes* | | |
|---|---|---|---|
| | *Day 8* | *Day 30* | *Day 90* |
| 100 | 50 | 75 | 75 |
| 450 | 75 | 88 | 100 |
| 900 | 100 | 100 | 100 |

Oral administration of WR-151327 at a concentration of 450 mg per kg caused degeneration of the germinal cell layer. In figure 2B is shown a transverse section of the seminiferous tubules from a mouse treated with WR-151327. Both Sertoli's cells and the spermatogenic cells were affected in these mice.

A transverse section of the seminiferous tubules from a mouse administered saline and irradiated one hour later with 10 Gy is shown in figure 2C. This photomicrograph indicated absence of spermatozoa and a reduction of cells in the germinal layer. The cells present had necrosis with cytoplasmic swelling and vacuolization. Mice treated with WR-151327 had a loss of cells but no cytoplasmic swelling. The presence of cells in the testes of irradiated mice eight days later indicates a degree of regeneration of cells after radiation injury. Following a single 10-Gy exposure, radiosensitive proliferating cells could be replaced within eight days by previously resting stem cells.

Combining treatment with WR-151327 at 450 mg per kg with exposure to 10-Gy total-body irradiation one hour later produced a pattern similar to treatment with WR-151327 alone. The photomicrograph of the seminiferous tubules of mice from this group (figure 2D) showed loss of cells with no cytoplasmic swelling. There was a greater reduction in cell number in mice treated with WR-151327 and irradiated than in mice treated with WR-151327 alone.

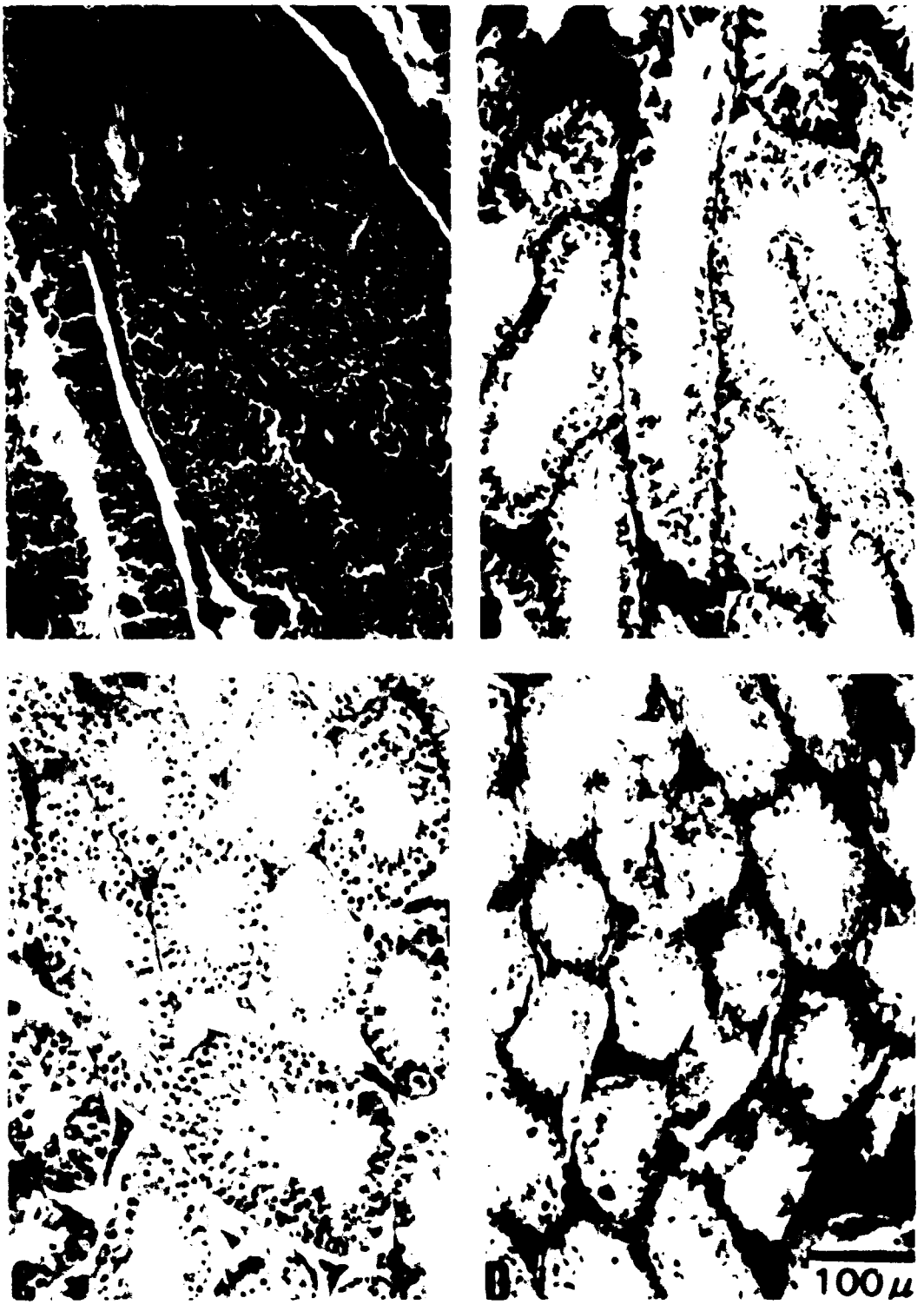


FIGURE 2. Photomicrographs of testes stained with hematoxylin and eosin eight days after oral administration of (A) saline, (B) 450 mg per kg of WR-151327, (C) saline and one hour later 10 Gy whole body irradiation from a $^{60}$Co gamma-ray source, and (D) 450 mg per kg of WR-151327 and one hour later whole body irradiation from a $^{60}$Co gamma-ray source.

In table II are shown the mean cell numbers in the germinal cell layer and the circumference size of the seminiferous tubules of the mice in each treatment group described for figure 2 A through D. There were eight animals used to collect data for each treatment group listed. Statistical analysis of the data revealed that the effect of irradiation alone and the administration of WR-151327 alone produced similar effects: 47 percent decline in cell number ($P < 0.001$). There is a dramatic decrease in the number of cells in the germinal layers of mice given the combined treatments of 450 mg per kg of WR-151327 plus 10 Gy irradiation ($P < 0.001$). The circumference size of the seminiferous tubules showed a 14 percent decrease in the group treated with WR-151327 only ($P < 0.0038$).

## Discussion

WR-151327 was studied as an oral radioprotector because it was identified as one of the six most effective compounds to emerge from the National Cancer Institute-sponsored radioprotector screening program conducted from 1980 to 1983.[1] WR-151327 also showed protective capability against neutron radiation effects in gastrointestinal tract[6] and bone marrow.[7] Sweeney[8] identified WR-151327 as the most effective orally administered radioprotector. In the present experiments, oral administration of WR-151327 provided radioprotection resulting in increased 30 day survival of mice after whole-body irradiation. The pathology results indicated that there were definite changes in the testes of mice orally administered 100 mg per kg of WR-151327 at eight days post exposure. In addition, 450 mg per kg elicited diffuse changes in other organs, such as the liver, lungs, pancreas, and kidney. Moreover, diarrhea observed in mice orally administered WR-151327 at 900 mg per kg indicated a significant pathophysiological response in the gastrointestinal tract. The results of this study are supported by the data obtained by van Beek et al[9] when they administered WR-151327 ip to mice at a dose of 540 mg per kg. The organs in which they found changes were the testes, salivary glands, and pancreas. The present experiments did not identify any changes in the salivary glands and this may reflect differences due to the route of drug administration.

Oral administration of 450 mg per kg of WR-151327 to mice provided modest radioprotection as evidenced by a dose reduction factor (DRF) of 1.206. However, oral administration of this concentration of drug produced significant testicular damage. Frequency of testicular damage increased with time following oral administration of 450 mg per kg of WR-151327; by 90 days, 100 percent of the mice showed damage to these organs.

TABLE II

Histometric Data of Mice in Each Treatment Group *

| | *Control* | *WR151327 450 mg/kg b w* | *Irradiation 10 Gy* | *WR151327 + Irradiation* |
|---|---|---|---|---|
| Germinal cell layer counts | 177 ± 20 | 126 ± 21 | 127 ± 4 | 35 ± 9 |
| Circumference of seminiferous tubule (mm) | 0.82 ± 0.04 | 0.74 ± 0.04 | 0.81 ± 0.03 | 0.67 ± 0.02 |

* Mean ± S.D

Both figure 2A (control) and figure 2B (drug treated) pictorially demonstrate the effects of this drug dose on the testes.

Oral administration of 450 mg per kg of WR-151327 to mice one hour prior to 10 Gy whole body gamma-irradiation exposure did not seem to afford protection to the germinal cell layer of the testes observed eight days after treatment. Ten Gy whole body irradiation alone reduced the germinal cell numbers in the testes, and the combination of 450 mg per kg of WR-151327 plus 10 Gy irradiation dramatically reduced the numbers of germinal cells. Figure 2A (controls), figure 2C (irradiated), and figure 2D (WR-151327 + irradiation) provide pictorial evidence of this testicular damage.

Another sulfhydryl containing compound, WR-2721 (S-2-[3-aminopropylamino] ethylphosphorothioic acid), has been shown to be cytotoxic to mice testes stem spermatogonia following ip injection.[3] A subsequent paper by the same authors noted spermatogonial survival enhancement by WR-2721 when animals were exposed to local high and low doses of irradiation of the testes.[4] However, the authors carefully pointed out that drug toxicity may have enhanced spermatogonial stem cell toxicity at low radiation doses.

Intraperitoneal injection of 540 mg per kg of WR-151327 resulted in mouse testicular damage.[9] Results presented in this paper did not demonstrate enhancement of spermatogonial stem cell survival when 450 mg per kg of WR-151327 was introduced prior to 10 Gy whole body exposure to radiation. The lack of enhanced spermatogonial stem cell survival using WR-151327 prior to irradiation (10 Gy) may reflect differences between this compound and WR-2721. In addition, the experimental protocols differed because WR-2721 was administered ip and in the present study WR-151327 was given orally. Also, mice in the present study received whole body irradiation by a $^{60}$Co gamma-ray source not local irradiation of the testes by $^{137}$Cs as previously described.[4] Since WR-151327 has been shown to be a testicular toxin when introduced orally or by intraperitoneal injection in mice, further examination of the other sulfhydryl containing WR-compounds would appear to be efficacious.

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