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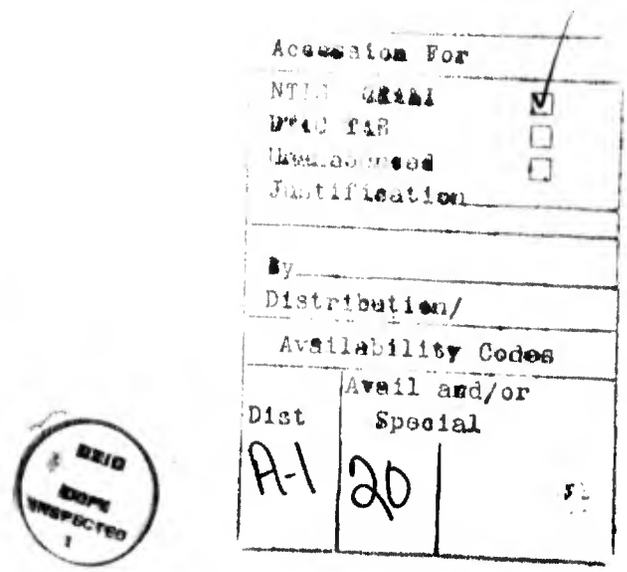

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Therapies for Radiation Injuries: Research Perspectives

I. Brook
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G.S. Madonna
R. M. DeBell
R. I. Walker

Exposure to radiation damages the immune, hematopoietic, and gastrointestinal components of the host defense system. This may lead to serious endogenous or exogenous infections. When radiation injury is combined with other physical trauma, e.g., burn or wound, the resulting damage to these systems is synergistic, and treatment for infection requires multiple approaches. This paper reviews successful single and combined therapeutic modalities for infections in irradiated mice and irradiated mice inflicted with trauma that are currently conducted at the Armed Forces Radiobiology Research Institute. The models of endogenous and exogenous infection and combined injury are described. The management of wounds infected with bacteria, exogenous systemic infection due to gram-negative enteric bacteria, and the chemoprophylaxis of enteric-derived systemic infection with quinolones is described. Infections can be treated successfully with proper antimicrobial therapy. In gamma- and neutron-irradiated mice, the immunomodulator trehalose dimycolate (TDM) was effective in treating endogenous infection. TDM with the antimicrobial ceftriaxone was effective in treating exogenous infection due to *Klebsiella pneumoniae*. Improvement in managing infection in irradiated and injured hosts will require further research using these diagnostic and therapeutic modalities. Accurate biological dosimetry is critical in determining if victims are at risk of developing infection. We found that radiation induced changes

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in plasma diamine oxidase activity; monitoring these changes was a useful indicator of the severity of radiation injury.

Introduction

Advances in surgical methods and other medical achievements have increased survival after trauma, but little improvement has been made in preventing later morbidity and mortality due to infectious complications. Several hundred thousand cases of microbial sepsis occur in the United States annually in patients who are immunosuppressed because of malignancies, therapeutic interventions, and trauma.[1]

Radiation injury is an immunocompromising event that opportunistic pathogens can exploit. This was evident in the people who died after the atomic blasts at Hiroshima and Nagasaki and more recently in accident victims at Chernobyl, U.S.S.R.,[2] and Goiania, Brazil. In these incidents, management of fatal infections was complicated by a lack of understanding of the interaction between conventional traumatic injury and radiation injury (combined injury). Further, in uncontrolled nuclear events, the radiation dose, the dose rate, and the quality of radiation are often unknown, and the uniformity of the victim's exposure cannot be determined easily. Nonuniform exposures that spare marrow make bone marrow transplants risky, even after high exposures to much of the body, because of surviving immunocompetent stem cells.[3] However, surviving stem cells allow the use of practical therapeutic approaches.

The nuclear accident at Chernobyl revealed the importance of biological dosimetry (the number of peripheral white blood cells was used as a measure of radiation damage in the victims) in estimating the radiation dose.[2] Biological dosimetry can assist in providing proper triage and expeditious medical care to injured individuals.

The therapeutic approaches used to treat patients from the recent accidents at Chernobyl[2] and Goiania were based on the initial administration of oral antibiotics to selectively suppress potential pathogenic populations of intestinal bacteria (i.e., selective decontamination). If fever developed in neutropenic patients, empiric treatment with multiple antibiotics was started;[2] if fever persisted, immunoglobulin was added (in Goiania). In some cases, it was necessary to treat fungal infections with amphotericin-B and herpes infections with topical acyclovir. This approach was satisfactory unless the radiation injury was complicated by burns, graft-versus-host reactivity, or radiation-induced enteritis.

The responses of the irradiated host to subsequent skin tissue trauma and infection are important because, as was seen after the Hiroshima and Nagasaki nuclear detonations and the Chernobyl accident, most of the short-term radiation victims suffered various degrees of physical trauma. Thus, developing effective therapies for treatment of combined injury victims is important.

The ability to manage radiation injuries has advanced with the commercial development and availability of cytokines, growth factors, and purified nontoxic immunomodulators. In one example, a lethal dose of gamma radiation in canines was reduced to a sublethal dose by administration of recombinant granulocyte colony-stimulating factor (T. MacVittie, unpublished data). In canine studies, animals given fluids, platelets, and antibiotics were significantly more resistant to radiation-induced mortality than those that were not treated.[4]

New therapies, combined with conventional management, such as that used at Chernobyl, are now being evaluated in various models of radiation and combined injury at the Armed Forces Radiobiology Research Institute (AFRRI).

Current research at AFRRI examines treatments for the infectious complications associated with radiation injury alone. In this paper, we describe research using some of these therapeutic approaches to control infections in animal models of radiation and radiation followed by trauma (combined injury). We describe animal models used to investigate radiation injury, our research on antimicrobials and immunomodulators in the therapy of radiation injury, and the use of biological dosimetry in determining the degree of radiation injury.

Animal Models of Combined Injury

In our work, antimicrobial therapies for bacterial infections accompanying radiation and tissue injury is evaluated in several animal models. Animal models of radiation injury best represent the complex interactions between the immune system and other systems, such as the gastrointestinal tract. Mouse models were previously used to evaluate agents prior to clinical use.[5] Mice are useful because of their documented response to bacterial challenges following irradiation,[6] they are easy to use, they can be used in large numbers to allow statistical validation of end points, and they cost less than other animal models. In our studies, we used 10- to 20-week-old B6D2F1/J hybrid female mice free of common mouse pathogens, particularly viruses, that interfere with research in immunocompromised animals.

We evaluated responses to irradiation, skin and soft tissue trauma, and irradiation followed by trauma and bacterial challenges. The models are classified as endogenous or exogenous bacterial infections (Table I). Responses to the quality of radiation, type of injury, and combined injury were evaluated in both models.

The types of radiation used in mouse models were ^{60}Co photons and mixed fission neutrons and photons produced by a Training Research Isotope General Atomic (TRIGA) reactor.[7] All mice were given midline tissue doses of 40 cGy per minute. We used $LD_{80/30}$ radiation doses to induce infections caused by endogenous microflora because at this dose effective therapy can activate undamaged cell populations and induce hematopoietic recovery. We used sublethal radiation doses to induce neutropenia, and then challenged the host with bacteria to evaluate therapeutic modalities (Table I).

Studies were performed in the following way. Mice were sublethally irradiated with the desired radiation dose and quality. Within 1 hour after exposure, the animals were fully anesthetized by inhalation of methoxyflurane, and a 1-inch x 1.5-inch skin wound or full-thickness skin burn was inflicted on the dorsal skin surface.[8,9] Saline (0.5 ml) was administered intraperitoneally (i.p.) immediately after injury. The injury covered approximately 15% of the body skin surface. In normal mice, the skin injuries were nonlethal and closed completely within 2 weeks. In irradiated mice (7.0 Gy, ^{60}Co), the skin injuries were

TABLE I

RADIATION QUALITIES AND DOSES IN MOUSE MODELS OF ENDOGENOUS AND EXOGENOUS INFECTIONS

Type of Infection	Radiation Source	Radiation Dose (cGy)[a]	Radiation plus Burns (cGy)[b]	Radiation plus Wounds (cGy)[b]
Endogenous	^{60}Co	1,025[c]	895	830
	TRIGA[d]	520[c]	465	415
Exogenous[e]	^{60}Co	700[f]	700	700
	TRIGA	350[f]	350	350

[a]All doses given at 40 cGy/minute.

[b]Dorsal skin injuries measuring 1 inch × 1.5 inch were inflicted on mice anesthetized by methoxyflurane inhalation within 1 hour after irradiation. Saline (0.5 ml) was administered i.p. immediately after injury.

[c]Indicated radiation doses are calculated $LD_{80/30}$ for the B6D2F1/J female mouse strain.

[d]TRIGA reactor was operated at 45 kW to produce a fission neutron to gamma ratio of 1:1. Mice were exposed in the radiation field as described elsewhere.[6]

[e]Exogenous infections were induced by challenging mice with *K. pneumoniae* (K5) at various times after irradiation and/or injury.

[f]Indicated radiation doses are nonlethal to B6D2F1/J female mice. A relative biological effectiveness of 2 was used to select the ratio of neutron-gamma radiation (1:1) provided by the TRIGA reactor.

usually nonlethal but wound closure was prolonged by 1–3 weeks.

The organisms that cause endogenous infection after exposure to lethal doses of radiation are derived from the host's gastrointestinal, skin, and mucous membrane flora. Skin injuries disrupt natural barriers to infection and provide another portal of bacterial entry. The organisms that cause most of these infections are aerobic gram-positive bacteria, such as *Staphylococcus* spp. and *Streptococcus* spp. Infections caused by gram-negative bacteria were reduced by acidifying the drinking water and by using the Micro-Isolator cage system.

Exogenous infections in irradiated mice were induced either by subcutaneous (s.c.) injection or by oral feeding of *Klebsiella pneumoniae*.[10–12] Following injection, a systemic infection developed rapidly (3–5 days), while systemic infection following oral feeding took longer (5–7 days). Exogenous infections in mice with combined injury were induced by injecting *K. pneumoniae* under the skin of the dorsal anterior area at desired intervals after irradiation. Mice with skin injury were challenged with a known dose of the organism under the burn site or on the wound site, 1 or 4 days after the trauma.[8,13]

These radiation models were also used to evaluate biological dosimeters and therapeutic interventions of infections, as described in the following sections.

Managing Bacterial Infection after Radiation Injury

Bacterial Infection after Irradiation

High doses of radiation were associated with increased mortality in animals,[14,15] largely due to increased susceptibility to various endogenous or exogenous pathogens.[15] Following irradiation, enteric organisms were recovered from lymphatic organs and the bloodstream.[16]

Organisms were detected more often in the spleen, liver, or blood of mice exposed to high doses of ^{60}Co gamma radiation than of mice exposed to lower doses. Bacteria were recovered in 3 of 100 mice given 7.0 Gy, in 13 of 100 mice given 8.0 Gy, in 23 of 90 given 9.0 Gy, and in 34 of 87 given 10.0 Gy. We also found a relationship between the dose of radiation and the type of bacteria causing sepsis.[16] The predominant organisms recovered in the tissues of mice given 10.0 Gy gamma radiation were *Escherichia coli*, anaerobic cocci, and *Bacteroides* spp. *Staphylococcus aureus* predominated in mice given 9.0 Gy gamma radiation. Most bacteria were isolated between days 9 and 13 after irradiation. This tendency was probably due to the additive effects of leukopenia, immunosuppression, and failure of mucosal barriers to prevent infections during this time.[15,17–19]

Mice given 6.5 Gy of gamma radiation showed increased susceptibility within the wound to challenge with bacteria commonly found in wound and soft tissue infections.[20] Organisms used for challenge included *S. aureus*, *E. coli*, *Streptococcus pyogenes*, and *K. pneumoniae*. Quantitative cultures of the infected wounds showed 10^3 to 10^4 more viable organisms in irradiated wounded mice, compared to nonirradiated and wounded mice.

The timing of wounding and bacterial challenge after irradiation had a significant effect on the severity of wound infection.[20] Challenging the mice with bacteria on and after the third day after irradiation caused the most severe infections, coinciding with the development of postirradiation leukopenia and thrombocytopenia. Schechmeister et al.[21] and Kaplan et al.[22] also observed that leukopenic mice were more susceptible to generalized infection 3–7 days after irradiation.

Severity of and susceptibility to local infections were directly correlated with the radiation dose.[6] When mice were infected with *S. aureus* after ^{60}Co irradiation and wounding, infections were observed only when the animals were exposed to a radiation dose higher than 7.0 Gy (T.B. Elliott and I. Brook, unpublished data).

Antimicrobial Therapy of Postirradiation Systemic Infection

Managing infections with antimicrobials in severely immunocompromised subjects is difficult. The efficacy of streptomycin therapy in reducing the mortality of irradiated animals was demonstrated in the 1950s.[23,24] Although antimicrobial therapy controlled the bacteremia, it did not prevent death in lethally irradiated animals.[23–25]

Brook et al.[26] administered gentamicin to lethally irradiated mice and reduced the systemic spread of Enterobacteriaceae, but not that of anaerobes. However, even the co-administration of metronidazole with gentamicin (effective only against anaerobic bacteria) did not prevent bacteremia and mortality.

Administration of penicillin and gentamicin to mice with wound infections prevented bacteremia, but was only partially effective in eliminating local infection with *S. aureus*, *E. coli*, and *K. pneumoniae*.[20]

Antimicrobials are essential in controlling local and systemic bacterial infections in irradiated hosts, although they are more effective in nonirradiated hosts. Irradiation impairs the immune system, which is needed to eradicate the organisms.

Large-scale clinical studies have not established principles for managing patients exposed to radiation. However, the expe-

rience gained by caring for patients who were accidentally exposed to radiation indicates that managing their infections is similar to managing infections in patients who are granulocytopenic because of chemotherapy, immunotherapy, or therapeutic irradiation. Early empirical broad-spectrum antibiotic therapy has become standard practice in the management of these patients and has contributed to their improved survival rate.[27] As soon as accurate microbiological identification is available, specific antimicrobial therapy is instituted.

Preliminary reports demonstrate that most patients exposed to sublethal alpha, beta, and gamma radiation at Chernobyl eventually regained normal neutrophil counts.[2] Until neutrophil counts are normal, however, it is important to provide irradiated patients with antimicrobial therapy against invading endogenous or exogenous bacteria.

Microorganisms from different sources, e.g., endogenous flora of the oral tract, upper respiratory tract, gastrointestinal tract, and skin, can cause infections in the immunocompromised host. Some of the organisms are part of the normal flora; however, others tend to colonize the mucous surfaces after depletion of the immune system. Nosocomial infections can be acquired from the community, the environment, blood products, and catheters.

Chemoprophylaxis of Enteric-Derived Systemic Infection

Because the bacterial flora of the gastrointestinal tract is the major source of infection in immunocompromised patients,[27] one way to reduce infections in irradiated hosts is by empiric suppression of the endogenous gastrointestinal gram-negative flora[28–30] while preserving the normal anaerobic gut flora. This approach was illustrated in studies that demonstrated the adverse effects of antimicrobials, which suppress the anaerobic flora.[26–31] After irradiation, aerobic and anaerobic components of the gut flora decrease. This decrease is followed by a rapid increase in aerobic and facultative flora.[26]

Because exposure to high doses of radiation also induces severe changes in the gastrointestinal mucosa, Enterobacteriaceae may easily penetrate the damaged mucosa. Antimicrobials that suppress anaerobes have deleterious effects on irradiated hosts. Brook et al.[26] demonstrated that mortality increased in lethally irradiated mice treated with metronidazole. Mice treated with metronidazole succumbed to infection by the 9th day after irradiation, while untreated irradiated mice died by the 17th day. The recovery of Enterobacteriaceae from tissues of mice treated with metronidazole showed a decrease in gastrointestinal anaerobic flora followed by a rapid increase in the number of aerobic and facultative organisms, compared to untreated irradiated mice.

These data show that antimicrobial agents effective against a wide spectrum of microorganisms may be needed to treat severe infections, especially those associated with intra-abdominal trauma. More studies are needed to determine the appropriate use of these agents in irradiated hosts, and to devise therapeutic modalities that will overcome their deleterious effects on the anaerobic gastrointestinal flora.

Selective decontamination (SD) of the gastrointestinal tract,[32] i.e., attempts to eradicate only the aerobic gram-negative bacilli using trimethoprim/sulfamethoxazole (TMP/SMZ) or quinolones, has been studied extensively. The systemic absorption of these agents also eradicates gram-negative organisms that might reach the bloodstream.

Several studies[33–36] showed conflicting data on the efficacy of TMP/SMZ to reduce the rate of infection in granulocytopenic patients. The recently introduced quinolones have potential for use in SD. These agents exhibit broad antimicrobial activity against aerobic gram-positive and gram-negative bacteria, especially against Enterobacteriaceae and *Pseudomonas aeruginosa*,[37] and have limited activity against anaerobic bacteria.

We found that oral administration of the quinolones ciprofloxacin, pefloxacin, and ofloxacin prolonged the survival of irradiated mice[38] and was synergistic with the immunomodulator glucan (M. Patchen, I. Brook, and T.B. Elliott, unpublished data). Additional studies are needed to compare the efficacy of quinolones with other modalities in achieving SD in the immunocompromised host. The quinolones may also be used to treat systemic infection caused by aerobic gram-negative or gram-positive bacteria in the immunocompromised host.[39]

The Role of Immunomodulators in Irradiated Mice

Severe impairment of the immune system, brought about by radiation-induced depletion of hematopoietic stem cells, generally leads to death from sepsis of either endogenous or exogenous origin. Mortality is higher if there is an additional wound or burn injury, i.e., combined injury. However, when host defenses are augmented before or after exposure to radiation, the chance of survival increases. Immunomodulators can prevent or reduce life-threatening sepsis and stimulate hematopoiesis to augment natural resistance in irradiated hosts.

The immunomodulator we investigated in the glycolipid trehalose dimycolate (TDM), which is found in the cell walls of mycobacteria, Nocardiae, and corynebacteria.[40,41] It consists of 6,6′-diesters of α, α-D-trehalose, and is an active component of Freund's complete adjuvant.[42] TDM was chosen because it is nontoxic, it stimulates macrophage activity,[43,44] and it enhances resistance to a variety of bacterial and parasitic infection.[43,45–47]

We found that B6D2F1 mice given 50 μg of TDM in 1% squalene (oil)-in-water emulsion (TDM/o) 1 hour after irradiation (10.25 Gy, ^{60}Co) had an increased 30-day survival rate of 70%, compared to 0% for irradiated mice given saline only (controls). However, TDM did not enhance survival in irradiated mice that received a 15% total-body surface area wound.

Cultures of liver homogenates obtained from mice treated with TDM/o on successive days after irradiation showed that translocated endogenous bacteria were reduced in these mice, compared to control mice (Table II). Furthermore, TDM/o, given before or after irradiation, increased the number of splenic endogenous colony-forming units (E-CFU).[11] This assay showed that immunomodulators can stimulate hematopoiesis and/or redistribute hematopoietic cells to the spleen.

TDM also increased survival after exogenous bacterial challenge. Mice were sublethally irradiated (7.0 Gy, ^{60}Co) and given TDM/o 1 hour after irradiation, i.p. On the fourth day after irradiation, mice received *K. pneumoniae* (10 x $LD_{50/30}$), s.c. Mice treated with saline died within 3 days, manifesting severe

TABLE II

BACTERIA FOUND IN LIVERS OF IRRADIATED MICE TREATED WITH TDM[a]

Type of Infection (day 0)	Day of Culture					
	1	3	5	7	9	11
Saline	0	0	0	45 ± 51[b]	245 ± 207	2751 ± 1917
TDM/o	0	0	0	34 ± 39	45 ± 50	20 ± 19[c]

[a]Saline (0.5 ml) or TDM/o (100 μg in 2% squalene oil emulsion) was injected i.p. into 30 B6D2F1 female mice, 1 hour postirradiation (10.25 Gy, ^{60}Co). On the indicated days, mice were euthanized, and homogenized livers were cultured on sheep blood agar and MacConkey's agar for the total number of bacteria (CFU/liver). Each value represents the mean ± SD of data obtained from five mice.

[b]Number of bacteria.

[c]The $p < 0.5$ when the TDM/o value is compared to the saline value on day 11.

neutropenia and infection. Most mice given TDM/o, however, survived (70% survival at 30 days after irradiation).[48] The protective effect of TDM was seen in surviving mice before significant marrow regeneration occurred. Increased survival was also observed in mice treated with TDM/o and challenged on day 14 with *K. pneumoniae*.[48]

We used a combined therapy of TDM/o with ceftriaxone, a cephalosporin antibiotic, to further increase resistance to lethal bacterial challenge after irradiation. Mice were sublethally irradiated (7.0 Gy) and given either saline or TDM/o, i.p., 1 hour after irradiation. Four days later, mice from each group were challenged with a supralethal dose (5000 x $LD_{50/30}$) of *K. pneumoniae*. On day 5, mice from each group were injected intramuscularly with ceftriaxone (75 mg/kg, daily) or water (control). Therapy was continued until day 14. Combined therapy resulted in 100% survival during the 30-day monitoring period, while ceftriaxone alone increased survival to 70%. All mice given TDM/o alone died. Thus, the combined therapy of an immunomodulator and an antibiotic increased survival synergistically, compared to either treatment alone.

TDM preparations were tested alone in a phase I cancer study[49] and with other stimulatory bacterial products and were shown to be safe and nontoxic. TDM preparations may be used in the future to increase nonspecific resistance to infection in immunosuppressed individuals and, thus, to reduce chances of sepsis. In addition, TDM may be used with antibiotics, cytokines, and cell growth factors to combat infection and to restore the function or increase survival of cell populations essential for normal host resistance.

Biological Dosimetry

The importance of accurate dosimetry for measuring exposure to radiation was demonstrated at the Chernobyl nuclear accident. Practical improvement in dosimetric determinations would help the treating physician provide appropriate treatment with limited resources. A biological dosimeter is a measurable biological response used to estimate the quantity of radiation to which a host is exposed. An ideal biological dosimeter meets the following requirements:

1. determines reliably and accurately the radiation dose linearly to the $LD_{100/30}$ range;
2. detects relatively small changes in the exposure dose (i.e., in the range <0.1 Gy);
3. uses easily obtained clinical samples or measurements that are noninvasive;
4. yields meaningful information in a relatively short time following exposure;
5. performs consistently, regardless of physiological interferences, such as wounds, burns, medications, diseases, or therapies;
6. operates easily, especially in field conditions;
7. operates for several years after exposure to radiation.

If a biological dosimeter meets these requirements, researchers could study long-term effects and clinical complications associated with radiation exposure.

At present, no single biological dosimeter fulfills all of these requirements. It is important to develop measurements to monitor radiation exposure and to detect various qualities of radiation, because exposures from accidents or weapons often consist of multiple radiation qualities. Development and testing of biological dosimeters usually relies on a single, relatively pure radiation quality.

Unlike physical or chemical radiation dosimetry, biological dosimetry is inherently much less accurate. The biochemical or physiological response is more important than the actual exposure dose. A reliable biological dosimeter would predict the actual survivability of an individual.

Biological dosimeters could be classified according to the physiological processes that they monitor: blood cell changes, serum factors, and chromosomal aberrations. One category could include chemical and cellular changes in other body fluids, such as urine and saliva.[50,51] Each type of dosimeter currently available has documented problems and deficiencies that interfere with its use or interpretation of data.[50,51]

In the event of a nuclear accident or detonation, many individuals would sustain combined injury. To date, no known biological dosimeter can measure radiation doses in models of combined injury; only the enzyme diamine oxidase (DAO) has been tested with relevant models.

We investigated DAO, which is the enzyme that follows ornithine decarboxylase in the polyamine pathway, for use as a biological dosimeter. We found it in almost all tissues, but it is found in especially high concentrations in the small intestine,[52] a prime target for radiation damage.[53] Changes in plasma DAO activity are indicative of the integrity of the small intestine. Our studies were performed to answer the following questions:

1. Are changes in DAO activity proportional to the radiation dose to which the organism was exposed?
2. Do these changes vary with radiation quality?
3. Can DAO be used to predict the radiation dose in a patient with combined injury?
4. Can DAO determine whether certain radiation therapies or protectants are effective?

Mouse plasma DAO activity increased on day 2 and decreased on day 4 after irradiation. The difference in DAO activity plotted against radiation doses of ^{60}Co of 7.0–11.5 Gy gave a correlation coefficient of 0.95. A similar response was found for 1–4 Gy fission neutrons.[54,55]

As the radiation dose increased from 0 to 16.1 Gy of ^{60}Co and from 0 to 7 Gy of fission neutrons, intestinal DAO activity decreased proportionally starting on day 4 after irradiation.[56] Animals with combined injury showed an identical decrease in DAO activity. However, plasma DAO activity did not increase by day 2 after irradiation in mice with combined injury, although on days 4 and 6 after irradiation, the responses of mice with combined injury were similar to those of the control mice, which included both unirradiated and irradiated animals. The reason the burn or wound injury depresses the plasma DAO activity in mice with combined injury on day 2 is unknown.

The usefulness of DAO in predicting the dose of radiation was evaluated in six groups of mice that were irradiated with various fission neutron radiation doses.[51] The predictions had a mean accuracy of approximately 88% of the actual values.

These are the only known studies of biological dosimetry to use animals with combined injury. The difficulties in using biological dosimeters to monitor simple radiation exposure are many, and with combined injury the situation is more complicated. Currently, it is necessary to rely on several biological dosimeters to measure the actual physiological damage caused either by radiation or combined injury. Because of the extent of the combined injury in any type of nuclear disaster, it is imperative that potential biological dosimeters be tested with combined injury models.

Managing Bacterial Infection in Irradiated and Injured Hosts

Our studies show that the synergy between antimicrobial and immunomodulator therapies holds promise for increasing the survival of irradiated victims and for managing the effects of radiation. The judicious use of biological dosimetry may allow for the optimal use of these agents. However, further studies are needed to determine the safety and toxicity of these agents in irradiated hosts.

Antibiotic therapy is only one component of the complex task of managing victims of irradiation or combined injury. Because antimicrobial agents alone have a limited role in managing infections in immunocompromised hosts, the immune system should be augmented by using immunomodulators. However, the use of immunomodulators is still experimental, and more research is needed to establish their potential use in irradiated hosts.

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References

1. Finland M: Changing ecology of bacterial infections as related to antimicrobial therapy. J Infect Dis 122:419–443, 1970
2. Gale RP: Immediate medical consequences of nuclear accidents: lessons from Chernobyl. JAMA 285:625–628, 1987
3. Champlin R: Treatment of victims of nuclear accidents: the role of bone marrow transplantation. Radiat Res 113:205–210, 1987
4. MacVittie TJ, Monroy RL, Vigneulle R, et al: Treatment of the radiation induced hemopoietic syndrome in canines. Lyon, France, NATO Research Study Group II, October 20–23, 1986
5. Steritz DD, Bondi A, McDermott D, et al: A burned mouse model to evaluate anti-Pseudomonas activity of topical agents. J Antimicrob Chemother 9:133–140, 1982
6. Miller CD, Hammond CW, Anderle SK: Studies on susceptibility to infection following ionizing radiation. V. Comparison of intraperitoneal and intravenous challenge at intervals following different doses of x-irradiation. J Exp Med 111:773–784, 1960.
7. Stewart DA, Ledney GD, Baker WH, et al: Bone marrow transplantation of mice exposed to a modified fission neutron (N/G 30:1) field. Radiat Res 92:268–279, 1982
8. Ledney GD, Stewart DA, Gruber DF, et al: Hematopoietic colon-forming cells from mice after wound trauma. J Surg Res 38:55–65, 1985
9. Ledney GD, Exum ED, Jackson WE III: Wound-induced alterations in survival of 60-Co irradiated mice: importance of wound timing. Experentia 41:614–616, 1985
10. Brook I, Elliott TB, Ledney GD: Quinoline therapy of Klebsiella pneumoniae sepsis following irradiation: comparison of pefloxacin, ciprofloxacin and ofloxacin. Radiat Res 122:215–217, 1990
11. Madonna GS, Ledney GD, Brook I, et al: Trehalose dimycolate enhances resistance to infection in neutropenic animals. Infect Immun 57:2495–2501, 1989
12. McChesney DG, Ledney GD, Madonna GS: Trehalose dimycolate enhances survival of fission neutron irradiated mice and Klebsiella pneumoniae-challenged irradiated mice. Radiat Res 121:71–75, 1990
13. Ledney GD, Madonna GS, MCChesney DG, et al: Complications of combined injury: radiation damage and skin wound trauma in mouse models in Treatment of Radiation Injuries, Edited by Browne D, Weiss JF, MacVittie TJ, et al, New York, Plenum Press, 1990, pp 153–163
14. Bennett LR, Rekers, PE, Howland J: Influence of infection on hematological effects and mortality following mid-lethal roentgen irradiation. Radiology 57:99–105, 1951
15. Benacerraf B: Influence of irradiation on resistance to infection. Bacteriol 24:35–40, 1960
16. Brook I, Walker RI, MacVittie TJ: Effects of radiation dose on the recovery of aerobic and anaerobic bacteria from mice. Can J Microbiol 37:719–722, 1986
17. Carter PB, Collins FM: The route of enteric infection in normal mice. J Exp Med 139:1189–1203, 1974
18. Miller CP: The effect of irradiation on natural resistance to infection. Ann NY Acad Sci 66:250–291, 1956
19. Collins FM: Mucosal defenses against Salmonella infection in the mouse. J Infect Dis 129:503–510, 1956
20. Brook I, Elliott TB: Treatment of wound sepsis in irradiated mice. Int J Radiat Biol 56:75–82, 1989
21. Schechmeister IL, Bond VP, Swift MN: The susceptibility of irradiated mice to infection as a function of post-irradiation time. J Immunol 68:87–95, 1952
22. Kaplan HW, Speck RS, Jawetz F: Impairment of antimicrobial defenses following total body irradiation of mice. J Lab Clin Med 40:682–691, 1958
23. Hammond CW: The treatment of post-irradiation infection. Radiat Res 1:448–458, 1954
24. Hammond CW, Vogel HH, Clark HW, et al: The effect of streptomycin therapy in mice irradiated with fast neutrons. Radiat Res 2:359–360, 1953
25. Miller CP, Hammond CW, Tompkins MJ, et al: The treatment of post irradiation infection with antibiotics, an experimental study on mice. J Lab Clin Med 39:462–479, 1952
26. Brook I, Walker RI, MacVittie TJ: Effect of antimicrobial therapy on the gut flora and bacteria infection in irradiated mice. Int J Radiat Biol 5:709–716, 1988
27. Bodey GP: Infection in cancer patients. A continuing association. Am J Med 81(Suppl 1A):11–26, 1986
28. EORTC Gnotobiotic Group: Protective isolation and antimicrobial decontamination in patients with high susceptibility to infection. Infection 6:175–191, 1978
29. Reiter B, Gee T, Young L, et al: Use of oral antibiotics during remission induction in adult patients with acute nonlymphoblastic leukemias (ANLL). (abstract) Clin Res 21:652, 1973
30. Storring RA, Jameson B, McElwain TJ, et al: Oral nonabsorbable antibiotics prevent infection in acute nonlymphoblastic leukemia. Lancet 2:837–840, 1977
31. Berg RD: Promotion of the translocation of enteric bacteria from the gastrointestinal tracts of mice by oral treatment with penicillin, clindamycin, or metronidazole. Infect Immun 33:854–861, 1981
32. Van Der Waaij D, Hofstra IT, Wiegersma N: Effects of beta-lactam antibiotics on the resistance of the digestive tract of mice to colonization. J Infect Dis 146:417–422, 1982
33. Bender JF, Schimpff SC, Young VM, et al: A comparative trial of tobramycin vs gentamicin in combination with vancomycin and nystatin for alimentary tract suppression in leukemia patients. Eur J Cancer 15:35–44, 1979
34. Gurwith MJ, Brunton HL, Lank BA, et al: A prospective controlled investigation of prophylactic trimethoprim-sulfamethoxazole in hospitalized granulocytopenic patients. Am J Med 66:248–256, 1979

35. Dekker AW, Rozenberg-Arska M, Sixma JJ, et al: Prevention of infection by trimethoprim-sulfamethoxazole plus amphotericin B in patients with acute nonlymphoblastic leukemia. Ann Intern Med 95:555–559, 1981
36. Pizzo PA, Robichaud KJ, Brenda KE, et al: Oral antibiotic prophylaxis in patients with cancer. A double-blind randomized placebo-controlled trial. J Pediatr 102:125–133, 1983
37. Bauernfeind A, Petermiller C: In vitro activity of ciprofloxacin, norfloxacin and nalidixic acid. Eur J Clin Microbiol 2:111–115, 1983
38. Brook I, Elliott TB: Pefloxacin therapy in the prevention of mortality after irradiation, in Proceedings of the 16th International Congress of Chemotherapy, Jerusalem, Israel, 1989, p 162
39. Hathorn JW, Rubin M, Pizzo PA: Empiric antibiotic therapy in febrile neutropenic cancer patient: clinical efficacy and impact of monotherapy. Antimicrob Agents Chemother 31:971–977, 1987
40. Lederer E: An update on natural and synthetic trehalose diesters, in Immunomodulators and Nonspecific Host Defense Mechanisms against Microbial Infections. Edited by Masihi K, Lange W, Oxford, England, Pergamon Press, 1988, pp 73–83
41. Lemaire G, Tenu JP, Petit JF, et al: Natural and synthetic trehalose diesters as immunomodulators. Med Res Rev 6:243–274, 1986
42. Azuma I, Ribi EE, Meyer TJ, et al: Biologically active components from mycobacterial cell walls. 1. Isolation and composition of cell wall skeleton and component P3. J Natl Cancer Inst 52:95–101, 1974
43. Kierszenbaum F, Zonia A, Wirth JJ: Macrophage activation by cord factor (trehalose 6,6'-dimycolate): enhanced association with and intracellular killing of Trypanosoma cruzi. Infect Immun 45:531–535, 1984
44. Yarkoni E, Wang L, Bekierkunst A: Stimulation of macrophages by cord factor and by heat killed and living BCG. Infect Immun 16:1–7, 1977
45. Masihi KN, Lange W, Brehmer W, et al: Immunological activities of nontoxic lipid A: enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. Int J Immunopharmacol 8:339–345, 1986
46. Olds GR, Chedid L, Lederer E, et al: Induction of resistance to Schistosoma mansoni by natural cord factor and synthetic lower homologues. J Infect Dis 141:473–478, 1980
47. Yarkoni E, Bekierkunst A: Nonspecific resistance against infection with Salmonella typhi and S. typhimurium induced in mice by cord factor (trehalose 6,6'-dimycolate) and its analogues. Infect Immun 14:1125–1129, 1976
48. Madonna GS, Ledney GD, Funckes, DC, et al: Monophosphoryl lipid A and trehalose dimycolate therapy enhances survival in sublethally irradiated mice challenged with Klebsiella pneumoniae, in Immunomodulators and Nonspecific Host Defense Mechanisms against Microbial Infections. Edited by Masihi K, Lange W, Oxford, England, Pergamon Press, 1988, pp 351–356
49. Vosika GJ, Gray GR: Phase I study of IV mycobacteria cell wall skeleton and cell wall skeleton combined with trehalose dimycolate. Cancer Treat Rev 67:785–790, 1983
50. Altman KI: Criteria for the evaluation and selection of radiation-induced metabolic changes as biochemical indicators of radiation damage, in Biochemical Indicators of Radiation Injury. Edited by Altman K, Vienna, International Atomic Energy Agency, 1971, pp 1–10
51. Walden TL Jr, Farzaneh NK: Biological assessment of radiation damage, in Textbook of Military Medicine, Part 2, Vol 2, Medical Consequences of Nuclear Warfare. Edited by Walker RI, Cerveny TJ, Washington, Office of the Surgeon General, U.S. Army, 1989, pp 85–103
52. Shakir KMM, Margolis S, Baylin S: Localization of histaminase (diamine oxidase) in rat small intestinal mucosa: site of release of heparin. Biochem Pharmacol 26:2343–2347, 1977
53. Conklin JJ, Walker RI, Hirsch EF: Current concepts in the management of radiation injuries and associated trauma. Surgery 156:809–829, 1983
54. DeBell RM, Ledney GD, Snyder SL: Quantification of gut injury with diamine oxidase activity: development of a fission neutron RBE and measurements with combined injury in mouse models. Radiat Res 112:508–516, 1987
55. DeBell RM, Ledney GD: Correlations of diamine oxidase activity with radiation dose in 60Cobalt and fission neutron irradiated mice, in Proceedings of the 8th International Congress of Radiation Research. Edited by Fielden EM, Fowler JF, Hendry JH, et al, London, Taylor & Francis, 1987, p 316
56. DeBell RM, Patchen ML, Holobaugh P: Diamine oxidase activity with glucan-F and WR2721 in irradiated mouse models, in Abstracts of Papers for the Thirty-Sixth Annual Meeting of the Radiation Research Society, 1988, p 73

Stress at Three Mile Island: Altered Perceptions, Behaviors, and Neuroendocrine Measures

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INTRODUCTION

More than a decade has passed since the first major nuclear-power-plant accident in the United States occurred at Three Mile Island (TMI), Goldsboro, Pennsylvania. Understanding the psychological aftereffects requires a thorough knowledge of the temporal antecedents because they are an essential internal component of the TMI accident. Therefore, this paper describes the TMI accident; reviews the events preceeding the psychological studies; and summarizes the major psychological, behavioral, and neuroendocrine findings of field studies of the TMI residents.

THE ACCIDENT AT TMI-2

The TMI nuclear power plant is located on the Susquehanna River, ten miles southeast of Harrisburg and 2 miles south of Middleton, Pennsylvania. Construction began on TMI-1 in 1968 and on TMI-2 in 1970. TMI-1 achieved criticality on June 5, 1974, with commercial operations beginning on September 30, 1974. TMI-2 achieved criticality on March 28, 1978, with commercial operations beginning on December 30, 1978, a mere 3 months before the accident occurred.[1] The populations within 5 and 15 miles of the TMI plant at the time of the accident were approximately 30,000 and 370,000, respectively.[2]

The TMI plant is a light-water reactor. This design uses ordinary pressurized water that is circulated through special pipes in the fuel assemblies. As the water circulates through the hot fuel assemblies, it cools the reactor by transferring the heat to the water, which produces the steam required to turn the turbines. The TMI design yields up to 880 MW of electricity.

However, on March 28, 1979, the flow of coolant water was interrupted. A demineralization system, which was designed to remove silt and chemicals from the Susquehanna River water, became clogged. A backup system should have automatically started feeding water to the fuel assemblies to prevent them from overheating, but the valves controlling the auxiliary feedwater pumps had been left closed during a routine inspection several days earlier.

The Medical Basis for Radiation-Accident Preparedness, III

Consequently, an absence of cooling water caused the fuel assembly rods inside the reactor core to overheat. When the plant operators correctly diagnosed the situation, the coolant-water valves were manually opened. However, the electromagnetic pressurizer relief valve failed to close, and the coolant water was diverted into the TMI-2 containment building. That water was radioactive, and it would remain in the containment building for more than a decade.

During the interruption in supply of cooling water to the fuel assemblies, some of the nearby residents living downwind from the plant reported a loud noise as the steam escaped from the damaged nuclear reactor through its cooling towers. Other residents reported simultaneous ground vibrations and believed that an earthquake was in progress.[3] As the new day dawned, along with it came curiosity, information seeking, and a deluge of news-media personnel. The first two days following the accident saw relatively little alarm,[4] probably because the residents of TMI still believed the situation was under control as late as Thursday, March, 29, 1979. At that point, however, they were faced with uncertainty, concern, and increasing anxiety as conflicting information from numerous sources transformed their once tranquil, rural existence into one of frustrating uncertainty.

The rollercoaster ride of emotions was heightened during the morning of March 30, 1979, when Pennsylvania Governor Thornburgh, after consultation with the chairman of the Nuclear Regulatory Commission (NRC), advised residents living within ten miles of TMI to remain indoors.[5] This announcement was followed by a noontime press release that recommended all pregnant women and preschool children living within 5 miles of TMI leave the area until the crisis was over, announced the schools were being closed, and announced state government workers were to leave their offices early. The local news that evening began, "The world has never seen a day quite like today ... and the biggest fear is that the worst is yet to come ... ." That statement typifies how concerned America in general, and the TMI residents in particular, were about TMI. That evening, TMI took 19 min of one regular 30-min network nightly news broadcast, more time than was devoted to the Kennedy assassination in 1963 or the fall of Saigon in 1975.[6]

Saturday, March 31, was even more troubling to the TMI residents. They were informed, albeit by well-meaning individuals at the NRC, that a hydrogen explosion could occur within the TMI-2 reactor containment. The President's Commission stated later that the report of a potential hydrogen explosion was incorrect. The Special Inquiry Group confirmed their findings by noting that fear was generated by "reports ... of a possible hydrogen bubble explosion [which] turned out to have been vastly exaggerated by the NRC's disorganized response to the emergency. The communication breakdown and information crises that ensued may have caused more alarm than the accident itself."[7] During the two-week emergency period, approximately 60% of the people living within 5 miles of the plant evacuated the area, and approximately 45% of those living within 10 miles of the plant also left.[8]

These inaccurate reports would render future releases suspect in the minds of the TMI-residents who experienced the rapid reversal of perception,

from one of safety to one of extreme uncertainty and vulnerability. Consequently, even though the NRC stated on April 4 that the schools would be reopening because the threat of an explosion from a hydrogen bubble had passed and the Governor on April 9 lifted the voluntary evacuation of pregnant women and children of preschool age,[9] the credibility of the officials had suffered irreparable harm during this 7 to 10 days.

AFTERMATH

Even after the announcement had been made, for the second time, that the situation was under control, the number of ventings that were necessary over the next several years and the 150 tons of debris that were collected from the accident at TMI[10] served as a continual reminder of what had occurred and what still remained.

Events Preceeding Psychological Studies

In general, society does not have a clear understanding of the physical properties associated with ionizing radiations or nuclear energy. The risk-perception research suggests that society sees radiation and nuclear facilities as more dangerous than knowledgeable experts do.[11] Slovic says that, despite the fact that the probable effects of a radiologic accident are small, "normal modes of thought, coupled with the special qualities of nuclear hazards that make them more memorable and imaginable, ... blur the distinction between the possible and the probable."[12]

Thus, the stressor at TMI did not terminate with any announcements, *per se*, but remained as an episodic stressor for the TMI residents. Several of those residents have filed litigation and subsequently settled out of court with the proviso that they no longer discuss the TMI situation. The Kemeny Commission[5] and other documents[7,15] concluded that, in spite of the serious damage to the TMI plant, the actual release of radioactivity would have a negligible effect on individuals and that mental stress would be the main effect.[13,14,15]

PSYCHOLOGICAL STUDIES AT TMI

The human response to stress is a process. The process can be characterized as the direct effects of the stressor, adaptation to the stressor, and the aftereffects from the stressor. How a person will be affected by a stressor depends on the magnitude and duration of the stressor, the individual's perception of how dangerous the stressor is or might become, and the person's (perceived) ability to deal with the stressor to minimize the threat of harm or loss. Therefore, differences in individual perceptions, which include different coping strategies to a stressful situation, could be expected to influence the ensuing behaviors and neuroendocrine measures (e.g., catecholamine values) of those experiencing the stressor. Coping usually refers to overt and intrapsychic behavior that is directed towards preventing,

avoiding, or controlling the source of stress.[16] The use of catecholamine values has been used in both acute[17,18] and chronic[13,19,20] stress studies because the byproducts of sympathetic nervous system activation are easily measured with radioenzymatic catechol-O-methyltransferase (COMT) procedures.[21]

During or after a stressful event, information seeking occurs to determine how dangerous the stressor is or may become.[16,22] If accurate and reliable information is not available, then further anticipatory stress results. As a result, people presented with the same information may react differently, with some exhibiting cognitive, behavioral, and neuroendocrine stress responses while others seem relatively unaffected. How this information is perceived and internalized can dramatically influence the stress process.

An example of variable reaction to stress occurred with the announcement of the hydrogen bubble, which increased the perceived magnitude of the stressor and yielded increased feelings of vulnerability among the people in the area. Some evacuated the area while others stayed. This diversity demonstrates how people cope differently with the same information.

Field Studies at TMI

Field studies were conducted at TMI[13,14] to investigate how different coping strategies influenced the psychological, behavioral, and neuroendocrine stress processes of the TMI residents. This study was unique because it examined the TMI residents during the protracted aftermath of TMI. Coping usually refers to overt and intrapsychic behavior directed at preventing, minimizing, avoiding, or controlling the source of stress.[16] Coping was measured with the Ways of Coping Inventory,[23,24] a recognized 68-item checklist that reflects a wide range of ways of coping with problems. Examples of the items range from "I make plans of action and follow them" to "I go on as if nothing has happened." The coping process can be conceptualized as consisting of two basic approaches: to alter or address the situation itself or to manage one's own emotional response to the situation. With a situation like TMI, one can do little to directly alter the problem. The coping strategy used by each TMI resident influenced his or her cognitive abilities, neuroendocrine measures, and symptom reporting.

Methods and Results

Five years after the accident, subjects from TMI and a control group were selected and interviewed in their homes. The control group was matched for age, education, socioeconomic status, etc. Several measures of stress were studied. Cognitive abilities were measured with an embedded-figures task similar to that used by Witkin et al.[25] This task consisted of eight complex geometric figures in which the subjects were to find a simpler geometric shape. Neuroendocrine measures were studied, including urinary catecholamines,[13,26] which are a well-recognized means of determining the relationship among sympathetic-adrenomedullary activity, behavior, and the

psychosocial environment.[27] Symptom reporting was obtained with the Symptom Checklist-90 (SCL-90),[28] which is a multidimensional inventory that assesses symptom distress, such as somatic distress, anxiety, depression, and contains a global symptom distress scale (i.e., total numbers of symptoms that are bothersome).

As a group, TMI residents exhibited greater symptoms of stress overall than did the control group (Fig. 1). Among TMI residents, emotional-focussed or reappraisal coping strategies were employed more frequently than was denial as a coping style. The emotional-focussed or reappraisal coping strategies resulted in fewer symptoms of stress, such as somatic distress (e.g., body aches), anxiety, total number of symptoms reported, and norepinephrine levels.[13] Conversely, use of denial as a coping style was associated with more symptoms, greater distress, and poorer performance on the embedded-figures task.[13]

In another field study,[14] we found that residents at TMI exhibited a different type of performance decrement. Because the TMI residents were exposed to the chance happening of an accident (i.e., the accident at TMI could have occurred at any nuclear power plant), it was postulated that their perceptions of other chance events could have been affected. So their reaction to repeated chance events was tested.

The subjects were asked to predict what the last digit of the next number would be in a series of randomly generated numbers. The series was produced with a random-number-generating program on an HP-41CV calculator or with a pair of dice. The action for these tasks was accomplished by either the subject (high-involvement condition) or the experimenter (low-involvement condition).

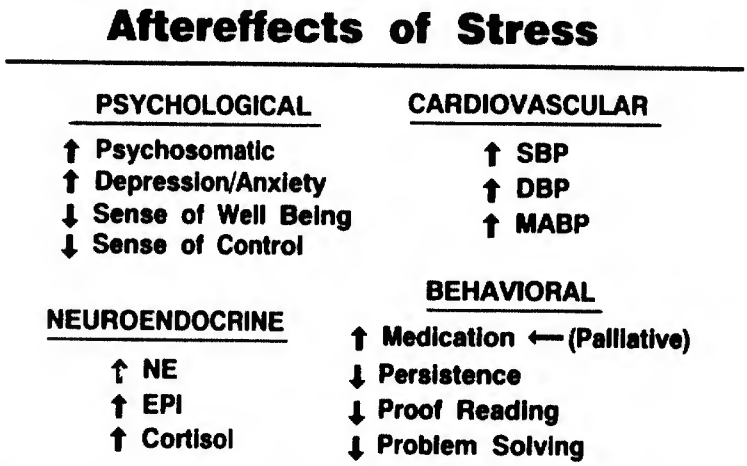

Figure 1. As a Group, TMI Residents Exhibited Greater Symptoms of Stress Overall Than Did a Control Group Matched for Age, Education, Socioeconomic Status, etc.; the Arrows Indicate the Indices of the TMI Residents Compared to Those of the Control Group.

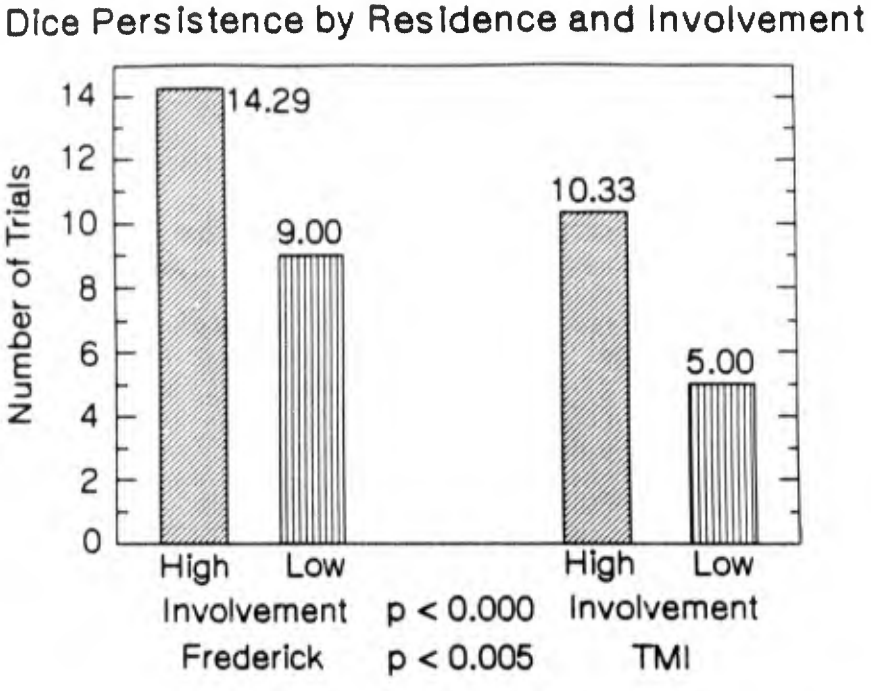

Figure 2. In a Study to See If TMI Residents' Perceptions of Chance Events Other Than a Nuclear Accident May Have Been Affected, Their Persistence on Chance Tasks Was Compared with That of Demographic Peers in a Control Group.

The TMI subjects were found to exhibit less persistence on these chance tasks compared with their demographic peers in a control group. Furthermore, TMI residents exhibited and expressed a high need to optimize their control over the chance tasks (e.g., by initiating the chance event themselves instead of the experimenter). The level of involvement by the subject (i.e., pushing a button on the calculator or rolling the dice) and the subject's location (TMI vs control group) dramatically influenced the subject's persistence on these chance tasks (Fig. 2). As a group, the TMI residents gave up sooner than did residents at the control group. However, the persistence of TMI residents who continually exhibited a high-control-oriented style of coping persisted longer on chance tasks than individuals using a low-control-oriented coping style.

Discussion

The differences observed between the coping styles are important for two reasons. The first demonstrates the long-lasting effects associated with a chronic and episodic stressor. The second demonstrates that when individuals

were involved in tasks with uncertain outcomes, greater persistence existed when subjects were highly involved in trying to solve the situation than when they were less involved. Subjects in the high-involvement condition reported that they felt they had more control over the outcome when they initiated the chance event than when the experimenter did. Another interpretation is that lack of involvement or participation in tasks with an uncertain outcome resulted in increased feelings of helplessness and an increased rate of giving up.

SUMMARY

This field research was conducted to investigate the possibility that the chronically stressful aftermath of the TMI accident would result in detectable stress effects that would be exhibited as decreased persistence on chance tasks. The findings confirmed that chronic-stress effects existed five years after the accident. The subjects' level of personal participation with their situation and their style of coping were shown to influence their persistence on challenging tasks.

Increasing attention is being paid to the evaluation of coping processes and to developing stress management procedures designed to enhance coping ability. The validity of the method and interpretation of results is strengthened by the results of a study of Federal Bureau of Investigation personnel. That study examined whether an emotional-focused or problem-focused coping strategy is more beneficial in reducing the adverse effects of the stress response associated with a hostage-captivity situation.[29] The study's findings were similar to those of our coping studies. It showed that, when emotional-focused coping is taught prior to an adverse situation, less stress symptomatology results.

The studies on coping strategy imply that people can be educated to use the technique of emotional-focused coping with such stressors as nuclear accidents or terrorist situations. Such education could reduce stress and, more critically, increase problem resolution through improved clarity of thought and increased thought-processing ability. In fact, this type of education could be beneficial for any individual involved in decision-making processes. Moreover, because the perceptions people have of the stressor are so important in determining their actions, this educational process could be inculcated in the preparation of the press releases associated with an incident to help the population cope with the event.

REFERENCES

1. Nuclear Regulatory Commission: *Investigation into the March 1979 Three Mile Island-2 Accident by the Office of Inspection and Enforcement*, NUREG-0600, Nuclear Regulatory Commission, Washington, D.C. (1979).

2. Rogovin, M., Frampton, G. V.: *Three Mile Island: A Report to the Commissioners and to the Public, Vol. 2, Part 1*, Nuclear Regulatory Commission Special Group, Washington, D.C. (1980).

3. Collins, D. L.: Coping strategies and their effects on the Three Mile Island area residents, District of Columbia Psychological Association, Washington, D.C. (Dec. 4, 1981).

4. Flynn, C. B.: *Three Mile Island Telephone Survey*, NUREG/CR-1093, U.S. Nuclear Regulatory Commission, Washington, D.C. (1979).

5. Kemeny, J. G., et al.: *Report of The President's Commission on the Accident at Three Mile Island. The Need for Change: The Legacy of TMI*, Washington, D.C. (1979).

6. Stephens, M.: *Three Mile Island*, Random House, New York (1979).

7. Dohrenwend, B.P., et al.: *Report of the Task Group on Behavioral Effects to the President's Commission on the Accident at Three Mile Island*, U.S. Government Printing Office, Washington, D.C. (1979).

8. Flynn, C. B., Chalmers, J. A.: *The Social and Economic Effects of the Accident at Three Mile Island*, NUREG/CR-1215, Mountain West Research, Tempe, Ariz. (1979).

9. Anon.: Nuclear Regulatory Commission aide Harold Denton says crisis at Three Mile Island is over; Governor Thornburgh says it is safe for pregnant women and young children to return to their homes, p. A1., *The New York Times* (April 10, 1979).

10. Wald, M. L.: After the meltdown: Lessons from a cleanup, p. 85, C1, *The New York Times* (April 24, 1990).

11. Slovic, P., Fischoff, B., Lichtenstein, S.: Facts and fears, *Environment* 21, 14-39 (1979).

12. Slovic, P.: *Images of Disaster: Perception and Acceptance of Risks from Nuclear Power*, National Council of Radiation Protection and Measurements, Washingtion, D.C. (1979).

13. Collins, D. L., Baum, A., Singer, J. E.: Coping with chronic stress at Three Mile Island: Psychological and biochemical evidence, *Health Psychol.* 2, 149-166 (1983).

14. Collins, D. L.: *Persistence Differences Between Three Mile Island Residents and a Control Group*, AD-A145567, National Technical Information Service, Springfield, Va. (1984).

15. Bell, M. C., Riechert, B. P.: *Possible Effects of Nuclear Radiation Accidents on Agriculture*, Bull. 655, The University of Tennessee Agricultural Experiment Station, Knoxville, Tenn. (1987), pp. 10-11.

16. Lazarus, R. S.: *Psychological Stress and the Coping Process*, McGraw Hill, New York (1966).

17. Krahenbuhl, G. S., Marett, J. R., King, N. W.: Catecholamine excretion in T-37 flight training, *Aviat. Space Environ. Med.* 48, 1405-1408 (1977).

18. Collins, D. L., Baum, A., Singer, J. E., Aftereffects associated with one or two stressors across conditions of complete, partial, or no control, pp. 318-322 in *Proceedings, Psychology in the Department of Defense*, United States Air Force Academy Technical Report 84-2, Colorado Springs, Colo. (1984).

19. Mason, J. W., et al.: Elevation of urinary norepinephrine/cortisol ratio in posttraumatic stress disorder, *J. Nerv. Ment. Dis.* 176, 498-502 (1988).

20. Davidson, L. M., Baum, A., Collins, D. L.: Stress and control related problems at Three Mile Island, *J. Appl. Psychol.* 12, 349-359 (1982).

21. Durrett L. R., Ziegler M. G.: A sensitive radioenzymatic assay for catechol drugs, *J. Neurosci. Res.* 5, 587-598 (1980).

22. Lazarus, R. S., Launier, R.: Stress-related transactions between person and environment, pp. 287-327 in Pervin, L. A., Lewis, M. (Eds.): *Perspectives in Interactional Psychology*, Plenum, New York (1978).

23. Aldwin, C., et al.: The ways of coping: A process measure, American Psychological Association Meeting, Montreal (September 1980).

24. Folkman, S., Lazarus, R. S.: An analysis of coping in a middle-aged community sample, *J. Health Soc. Behav.* 21, 219-239 (1980).

25. Witkin, H. A., Goodenough, D. R., Oltman, D. K.: Psychological differentiation: Current status, *J. Pers. Soc. Psychol.* 37, 1127-1145 (1979).

26. Baum, A., et al.: Psychological and endocrinological correlates of chronic stress at Three Mile Island, pp. 201-217 in Williams, R. B. (Ed.): *Perspectives on Behavioral Medicine, Vol. 2*, Academic Press, San Diego, Calif. (1985).

27. Frankenhaeuser M.: Sympathetic-adrenomedullary activity, behavior and the psychosocial environment, pp. 71-94 in Venables, P. H., Christie, M. J. (Eds.): *Research in Psychophysiology*, John Wiley, New York (1975).

28. Derogatis, L. R.: *SCL-90-R: Administration, Scoring, and Procedures Manual-II for the Revised Version*, Clinical Psychometric Research, Towson, Md. (1977).

29. Strentz, T., Auerbach, S. M.: Adjustment to the stress of simulated captivity: Effects of emotion-focused versus problem-focused preparation on hostages differing in locus of control, *J. Pers. Soc. Psychol.* 55, 652-660 (1988).

In Vivo Interleukin-1 (IL-1) Administration Indirectly Promotes Type II IL-1 Receptor Expression on Hematopoietic Bone Marrow Cells: Novel Mechanism for the Hematopoietic Effects of IL-1

By Claire M. Dubois, Francis W. Ruscetti, Jonathan R. Keller, Joost J. Oppenheim, Kjetil Hestdal, Richard Chizzonite, and Ruth Neta

Interleukin-1 (IL-1) has profound stimulatory effects on hematopoiesis but the mechanism(s) of action remain unknown. The direct action of IL-1 on hematopoietic progenitor cells requires the presence of a specific IL-1 receptor (IL-1R). In this report, we tested the effect of in vivo IL-1 treatment on the expression of IL-1R on bone marrow (BM) cells. Injection of mice with IL-1 results in a marked upregulation of IL-1R on light-density BM cells as on a subpopulation enriched for myeloid precursors. Pretreatment of mice with anti-type I IL-1R antibody (35F5), which has been shown to prevent the radioprotective effect of IL-1, also blocked IL-1–induced IL-1R expression on BM cells. This antibody did not directly bind and block IL-1 binding to the type II IL-1R expressed on hematopoietic cells, suggesting that IL-1R upregulation by IL-1 is indirect. It is therefore possible that IL-1 acts on type I IL-1R–expressing accessory cells such as stromal cells or T cells to induce production of hematopoietic growth factors (HGFs). In support of this, granulocyte colony-stimulating factor administration can induce the increase of IL-1R on BM cells. Thus, the increased expression of IL-1R on hematopoietic BM cells by IL-1 is indirect, probably mediated in part through endogenous HGF production. These results also suggest that the restorative hematopoietic effect of IL-1 occurs through both indirect and direct mechanisms.

IN VIVO INJECTION of interleukin-1 (IL-1) has profound hematopoietic effects.[1] These effects include initial peripheral neutrophilia due to mobilization of neutrophils from bone marrow (BM), followed by an expansion of this population in the marrow, cycling of hematopoietic progenitor cells, and eventual increase in their numbers.[2-6] In addition, IL-1, both in vivo and in vitro, induced increased levels of messenger RNA (mRNA) transcripts and proteins possessing hematopoietic growth factor (HGF) activities such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and IL-6.[7-12] Furthermore, IL-1 is biochemically identical to hematopoietin-1, which synergizes with CSF to promote the growth of primitive high proliferative potential colony-forming cells.[13-15] These effects presumably are the basis for the capacity of IL-1 to promote hematopoietic recovery and survival of mice treated with myelosuppressive drugs or exposed to lethal radiation.[15-19]

Despite extensive evidence for the stimulatory effects of IL-1 on hematopoiesis, it remains to be established whether IL-1 can act directly on hematopoietic BM cells. Because demonstration of such a direct effect would require the presence of specific IL-1 receptors (IL-1R), we initiated studies to examine the presence and regulation of IL-1R on murine BM cells. In this report, data are presented showing that the injection of mice with IL-1 results in considerable upregulation of IL-1R on BM cells. However, this effect appears to be indirect, because IL-1 effects can be blocked in vivo by antibody to the type I IL-1R, which is not expressed on BM cells but reduces the circulating HGF level. In addition, G-CSF administration can induce the increase of IL-1R on BM cells. These data suggest the involvement of IL-1–induced HGF in the IL-1–mediated effects on the marrow.

MATERIALS AND METHODS

Mice. CD2F1 male mice were purchased from the Animal Genetics and Production Branch, National Cancer Institute, National Institutes of Health (Frederick, MD). Animals were handled as previously described.[12]

Reagents. Human recombinant IL-1α (rIL-1α) and a neutralizing anti–IL-1R antibody (35F5, a rat monoclonal Ig [IgG_1]) were prepared at Hoffmann-LaRoche (Nutley, NJ). Control rat IgG was purchased from Cappel (Organon Teknika Corp, West Chester, PA). The hybridoma cell line producing the monoclonal antibody (MoAb) RB6-8C5 was provided by Dr R.L. Coffman (DNAX Corp, Palo Alto, CA) and the antibody was produced and purified as previously described.[20,21] Murine IL-4 was provided by Sterling (Malvern, PA); murine interferon (IFN)-γ was provided by Genentech (San Francisco, CA); and G-CSF was provided by Amgen (Thousand Oaks, CA).

Cell line. The murine cell line EL-4 6.1, a variant subline of EL-4 thymoma cells,[22] was provided by H.R. MacDonald (Ludwig Institute for Cancer Research, Basel, Switzerland).

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In vivo procedure. The antibody and cytokines were diluted in pyrogen-free saline on the day of injection. The antibody (35F5) was administered intraperitoneally (IP) 6 to 8 hours before IP injection of IL-1. BM cells were tested for the presence of IL-1R 16 to 18 hours after saline or IL-1 injection or after different periods of time, as indicated in the figure legends.

Preparation of BM cells. Murine BM cells were aspirated from femurs and light-density mononuclear cells were isolated by separation on Lymphocyte Separation Medium (Organon Teknika Corp, Durham, NC).

Purification of myeloid precursors. BM cells from saline- or IL-1–treated mice were labeled with either MoAb RB6-8C5 or control IgG in an indirect immunofluorescence assay. Briefly, BM cells were resuspended in Hanks' Balanced Salt Solution with 10% fetal bovine serum and incubated with the appropriate MoAb in a 1:100 dilution for 30 minutes at 4°C. The cells were washed and then incubated with fluorescein-labeled goat-antirat antibody (Kirkegaard-Perry Laboratories, Gaithersburg, MD) for 30 minutes at 4°C. The cells were then washed two times before cell sorting using FACStar (Becton Dickinson, Mountain View, CA).

Preparation of iodinated IL-1. Human rIL-1α was labeled with ^{125}I using chloramine-T reagent as described previously.[23] The radiolabeled IL-1α had a specific activity that ranged from 1 to 3 × 10^{15} cpm/mmol. There was no significant loss of biologic activity of radiolabeled IL-1α using the thymocyte comitogenic activity assay. The 35F5 antibody was labeled with Iodogen reagent (Pierce, Rockford, IL) according to the manufacture's method.

Receptor binding assay. Fractionated BM cell suspensions were washed once with cold medium and the cell pellet was treated for 1 minute on ice with 50 mmol/L glycine-HCl (pH 3) to remove potentially bound IL-1. Subsequently, the cells were washed twice with binding medium (RPMI 1% bovine serum albumin supplemented with 0.1% sodium azide and 10 mmol/L HEPES) and incubated at 4°C with 500 pmol/L ^{125}I-labeled IL-1α in a final volume of 0.2 mL. After 1 to 2 hours of incubation at 4°C, cell-bound radioactivity was separated from unbound ^{125}I–IL-1α by centrifugation of the sample through a mixture of 1.5:1 (vol/vol) dibutyl phthalate/bis(2-ethylhexyl)phthalate (Eastman Kodak Co, Rochester, NY). Nonspecific binding was determined by incubating the BM cells with labeled IL-1α in the presence of 50-fold excess of unlabeled ligand.

RESULTS

Effect of IL-1 on IL-1R expression on BM cells. To test the effect of IL-1 on the expression of IL-1R on BM cells, 300 ng of IL-1 was injected IP to mice and BM cells were harvested after different times and examined for IL-1 binding. As shown in Fig 1, the specific binding of IL-1 to light-density BM cells was significantly increased by 6 hours with maximum effect observed 13 to 18 hours posttreatment and returned to baseline levels after 48 hours. Treatment of mice with increasing doses of IL-1 resulted in a gradual augmentation in IL-1–specific binding to BM cells (Fig 2). Whereas 10 ng of IL-1 per mouse induced a slight increase in IL-1–specific binding, doses from 50 to 5,000 ng per mouse resulted in a sixfold to 12-fold augmentation with an ED_{50} of 80 ng per mouse. Scatchard analysis of BM cells from control mice showed one class of IL-1R with 33 to 114 binding sites per cell and an affinity of 2.4 ± 1.7 × 10^{-10} mol/L (Fig 3). Treatment with IL-1 increased the number of IL-1R to 235 to 1,080 per BM cell without any significant effect on affinity (kd = 2.2 ± 1.6 × 10^{-10} mol/L). Thus, in vivo IL-1 treatment resulted in increased IL-1R numbers on BM cells in a dose- and time-dependent fashion.

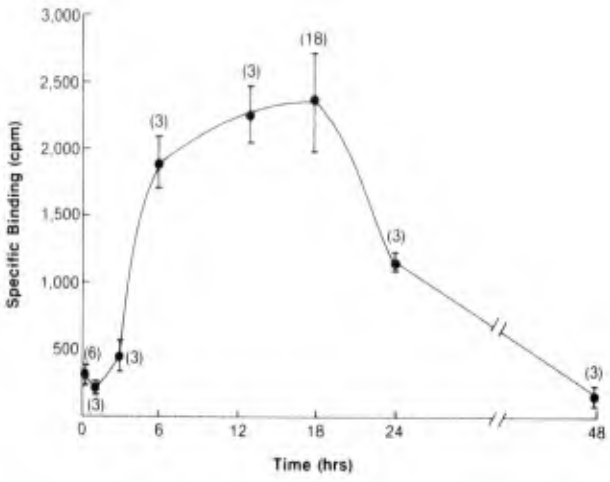

Fig 1. Time course of effect of in vivo IL-1 on IL-1R expression on BM cells. Mice were injected IP with 300 ng of IL-1. After different periods of time, BM cells were harvested. The cells were tested for the expression of IL-1R as described in Materials and Methods. Each point represents the mean (±SEM) of duplicate determinations using pooled cells from 3 to 18 animals as indicated in parentheses. The level of background binding was 222 ± 50 cpm, which was subtracted from the data shown here.

Increased IL-1R expression on an enriched myeloid precursor population. We next evaluated whether IL-1 treatment directly upregulated the IL-1R on myeloid precursors. For this purpose, cells were separated by fluorescence-activated cell sorting according to the differential expression of RB6-8C5 antigen on myeloid progenitor cells.[24] Mice received a single injection of saline or IL-1 and 12 to 16 hours after injection, BM cells were stained with MoAb RB6-8C5 or control antibody. As previously documented[24] and shown here for comparison, fluorescence-activated cell sorting (FACS) analysis of normal BM cells stained with the MoAb

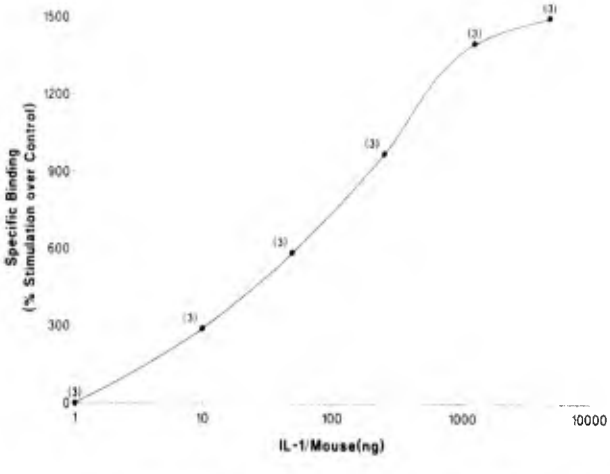

Fig 2. Dose-dependent effect of IL-1 on IL-1R expression. Mice were injected IP with increasing doses of IL-1. Sixteen to 18 hours after treatment, BM cells were harvested and tested for IL-1R expression as described in Materials and Methods. Each point represents the mean (SEM < 10%) of duplicate determinations of pooled cells from three animals.

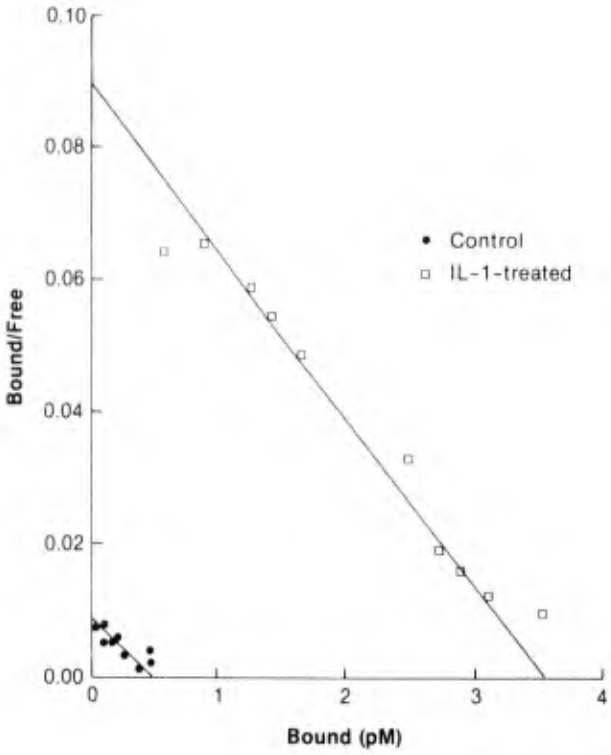

Fig 3. Scatchard analysis of ^{125}I–IL-1α binding to (●) saline and (□) IL-1–treated mice. Mice were injected IP with 300 ng IL-1 and BM cells were processed as described in Materials and Methods. Specific ^{125}I–IL-1α binding was determined by adding various concentrations of labeled IL-1 alone or in the presence of 50-fold excess of unlabeled IL-1α. Each point is the mean of duplicate determinations from which nonspecific binding (< 300 cpm) was subtracted. Scatchard data were analyzed using the LIGAND program.

RB6-8C5 showed three distinct populations: RB6-8C5^{neg}, RB6-8C5^{lo}, and RB6-8C5^{hi} (Fig 4A). The RB6-8C5^{hi} cells were enriched for the end-stage (segmented) neutrophils (>75%) and the RB6-8C5^{lo} cells contained greater than 80% of immature myeloid cells (myeloblasts, promyelocytes, myelocytes).[24] Administration of 1 μg IL-1 to mice resulted in a twofold increase in the RB6-8C5^{lo} population (41.1% for IL-1–treated compared with 20.7% for saline-treated mice) and a concomitant loss of RB6-8C5^{hi} cells (3.6% for IL-1–treated compared with 19.7% for saline-treated mice) (Fig 4B). Comparison of IL-1–specific binding on BM cells from saline- and IL-1–treated mice showed that the expression of IL-1R was induced on the RB6-8C5^{lo} population from IL-1–treated mice, whereas control RB6-8C5^{lo} BM cells did not express significant levels of IL-1–specific binding. In contrast, the RB6-8C5^{neg} populations of either treated or nontreated animals did not express significant levels of IL-1–specific binding (Table 1). We were not able to assess the expression of IL-1R on the IL-1–treated RB6-8C5^{hi} population because a very low number of these cells remains after IL-1 administration. Morphologic analysis of the FACS-sorted RB6-8C5^{lo} population from IL-1–treated mice showed that greater than 90% of the population were immature myelocytes with 2% neutrophils, 5% eosinophils and eosinophils precursors, and 2% lymphocytes, macrophages, and erythroid precursors. It is unlikely that lymphocytes (<2% of the population) contribute to the enhanced IL-1 binding because the removal (using magnetic beads) of BM cells expressing L3T4 (T helper), Lyt-2 (T suppressor/cytotoxic), or B220 (B cells) antigen did not change the magnitude of IL-1–specific binding (data not shown).

These results suggest that the treatment of mice with IL-1 induces the expression of IL-1R on an RB6-8C5^{lo} population of BM cells enriched for immature myeloid cells.

Effect of anti–IL-1R antibody on the upregulation of IL-1R on BM cells. Previous studies showed that the administration of an anti–IL-1R antibody, 35F5, almost completely blocked the radioprotective effect of IL-1, whereas equivalent concentration of control Ig had no effect.[12] Therefore, we examined whether the administration of 35F5 antibody, at a dose that blocks the radioprotective effect of IL-1,

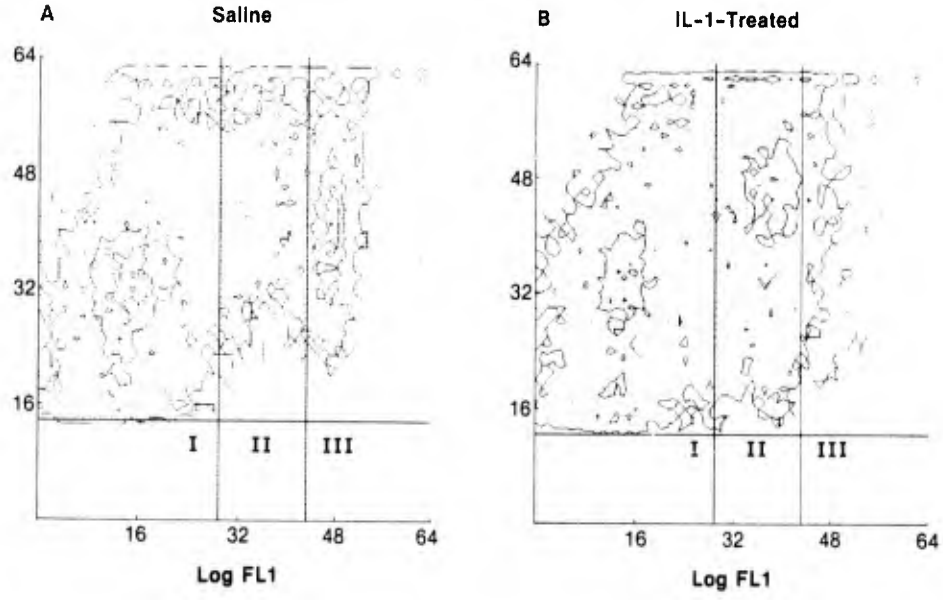

Fig 4. Flow cytometry analysis of BM cells from (A) saline or (B) IL-1–treated mice using RB6-8C5 antibody. Mice were injected with either saline or 1 μg IL-1α. Twelve hours after treatment, BM cells were harvested and labeled with RB6-8C5 as outlined in Materials and Methods. The background staining of the isotype-matched control antibody was less than 3% for both the saline- and IL-1–treated populations. Cells were gated according to fluorescent intensity into RB6-8C5^{neg} (sector I), RB6-8C5^{lo} (sector II), and RB6-8C5^{hi} (sector III) populations. A contour map of increasing fluorescence intensity on the X-axis versus cell size on the Y-axis, and the number of cells on the Z-axis was generated.

Table 1. Upregulation of IL-1R on RB6-8C5^{lo} Population of BM Cells

Cell Phenotype†	^{125}I–IL-1 Binding* (cpm)	
	Control Mice‡	IL-1–Treated Mice§
Unseparated BM	106 ± 9.2	2,145 ± 39
RB6-8C5^{neg}	38 ± 8.5	34 ± 1
RB6-8C5^{lo}	24 ± 11.3	2,602 ± 137
RB6-8C5^{hi}	556 ± 2.8	ND

Abbreviation: ND, nondetermined.

*The expression of IL-1R on BM cells was monitored as described in Materials and Methods. Each point represents the mean ± SEM of duplicate determination of pooled cells from five to 20 animals. The level of background binding was 274 ± 83, which was subtracted from the data shown here.

†BM cells were sorted by FACS into three different populations using the MoAb RB6-8C5 as described in Materials and Methods and shown in Fig 4.

‡The data extracted from Hestdal et al[24] are shown here for comparison.

§Mice received a single injection of 1 μg IL-1 and the BM cells were processed 12 hours after injection.

could also inhibit the upregulation of IL-1R on BM cells. Mice were injected with 100 μg of 35F5 antibody or saline 6 to 8 hours before injection of 300 ng of IL-1. Radioreceptor assays for IL-1R were performed 16 to 18 hours after IL-1 treatment. The administration of 35F5 antibody had no significant effect on the constitutive expression of IL-1R on BM cells, but blocked 80% of the IL-1–induced upregulation of IL-1R (Fig 5).

Binding of anti–IL-1R antibody to IL-1–induced IL-1R on BM cells. The 35F5 antibody is selective in that it blocks IL-1 binding to type I IL-1R expressed on a thymoma cell line (EL-4), fibroblasts, and epithelial cells but not to the type II IL-1R expressed on macrophages, granulocytes, and B cells.[25] The type of IL-1R expressed on IL-1–treated BM progenitor cells has not been reported. To assess the nature of this receptor, in vitro binding experiments using radiolabeled 35F5 antibody were performed. Our results indicate that radiolabeled 35F5 antibody did not bind to the IL-1R induced by IL-1 treatment on BM cells, but, as previously reported,[25] this antibody binds to the type I IL-1R expressed on EL-4 cells (Fig 6). Furthermore, in a competitive binding assay, the 35F5 antibody competed in a dose-dependent fashion with radiolabeled IL-1 for the IL-1R expressed on T cells, but failed to compete for the IL-1R expressed on IL-1–treated BM cells (Table 2). Collectively, these data strongly indicate that the 35F5 epitope is not detectable on the cell surface of BM cells, constitutively or after upregulation with IL-1.

Effect of G-CSF, IL-4, and IFN-γ on IL-1R expression on BM cells. Because the 35F5 antibody that blocks the in vivo marrow protective effects of IL-1[12] fails to block the in vitro binding of IL-1 to BM cells IL-1R, the effects of IL-1 on IL-1R expression by BM cells may be, at least in part, indirect. IL-1 effects may be dependent on an interaction of IL-1 with type I IL-1R present on cells other than BM cells. The 35F5 antibody was previously shown to block circulating IL-1–induced HGF production.[12] It is therefore possible that IL-1–induced cytokines play a role in the upregulation of IL-1R. To test this hypothesis, mice were injected with G-CSF, IL-4, or IFN-γ, and BM cells were harvested and assessed for IL-1R expression. The doses of these cytokines were chosen based on previously observed effects of their protection of mice from radiation-induced death.[26] Results presented in Fig 7 indicate that G-CSF but not IL-4 and IFN-γ can significantly increase IL-1R expression on BM cells.

DISCUSSION

Previous reports have shown that several types of mature cells, such as neutrophils, macrophages, B cells, and T cells, express low levels of functional high-affinity receptors for

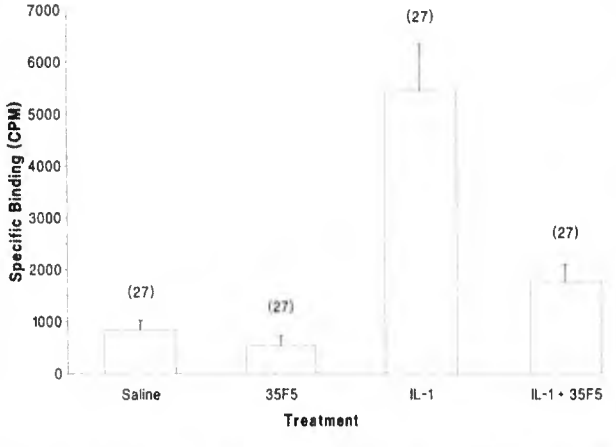

Fig 5. Block of IL-R expression induced by IL-1 by anti–IL-1R antibody. Mice were injected with 100 μg of anti–IL-1R antibody (35F5) or saline 6 to 8 hours before injection of 300 ng IL-1. Radioreceptor assays for IL-1R were performed 16 to 18 hours after IL-1 treatment. Each point represents the mean (±SEM) of nine experiments from which the level of background binding (286 ± 60) was subtracted. The numbers in parentheses represent the number of mice receiving each treatment.

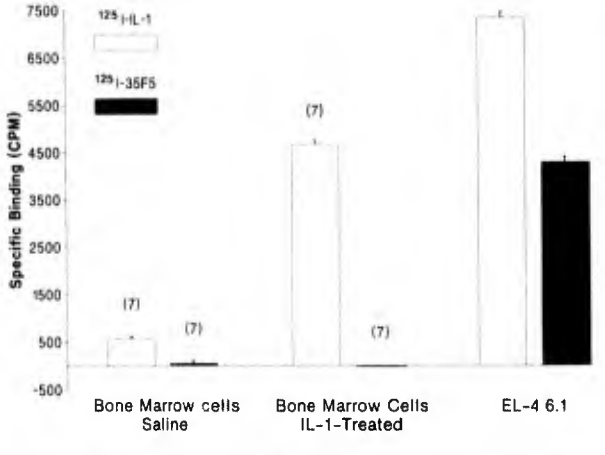

Fig 6. Binding of anti–IL-1R antibody to IL-1–treated and nontreated murine cells. Mice were injected IP with 300 ng of IL-1 and 16 to 18 hours after treatment, BM cells were harvested and tested for (□) radiolabeled IL-1 binding or (■) radiolabeled 35F5 binding as described in Materials and Methods. The data represent the mean (±SEM) of duplicate determinations of a representative experiment of two experiments performed. The level of background binding was 304 ± 38 cpm for IL-1 binding and 354 ± 76 cpm for 35F5 binding, which were subtracted from the data shown here.

Table 2. Effect of Anti–IL-1R Antibody (35F5) on ^{125}I–IL-1 Binding to Murine Cells

Cells	Antibody (ng/mL)	% Inhibition ^{125}I–IL-1 Binding*	
		35F5	Control Antibody
BM (IL-1–treated)†	50	6	2
	500	6	3
	5,000	1	9
EL4 6.1	5	20	—
	50	49	9
	500	84	7
	5,000	97	2

*The data are expressed as percent inhibition of ^{125}I–IL-1α specific binding in the presence of antibody when compared with the total specific binding in the absence of antibody.

†Mice received IP injection of 300 ng IL-1. Sixteen hours later, BM cells were harvested and processed as described in Materials and Methods.

IL-1.[27-30] Constitutive expression of IL-1R has also been shown on normal BM neutrophils.[24,25] Despite the profound hematopoietic effect of IL-1, we could detect low levels of IL-1R expressed on hematopoietic BM cells from normal mice (see Table 1). Previous reports indicated that IL-1 upregulates in vitro IL-1R expression on B lymphocytes and fibroblasts.[31,32] Therefore, we have attempted to identify physiologic agents that may regulate the expression of IL-1R on hematopoietic cells. Our report shows in vivo upregulation of IL-1R on hematopoietic BM cells by IL-1

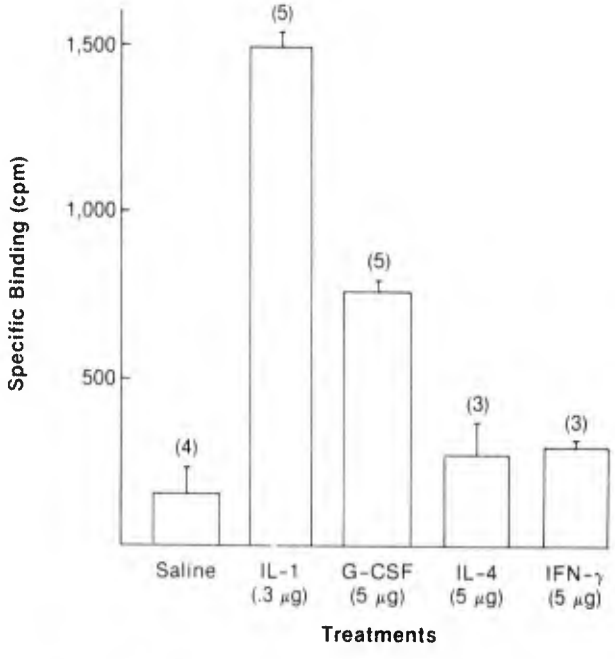

Fig 7. Upregulation of IL-1R expression by G-CSF. Mice were injected IP with either saline, human IL-1α (0.3 μg), human G-CSF (5 μg), murine IL-4 (5 μg), or murine IFN-γ (5 μg). Sixteen to 18 hours after treatment, BM cells were harvested and tested for IL-1R expression as described in Materials and Methods. The data represents the mean (±SEM) of duplicate determinations of a representative experiment of three experiments performed. The level of background binding was 234 ± 7 cpm, which was subtracted from the data shown here.

and G-CSF. This enhanced IL-1–specific binding was observed on enriched IL-1–treated BM cells expressing low levels of RB6-8C5 antigen. The RB6-8C5^{lo} population contains greater than 90% immature myeloid cells (myeloblasts, promyelocytes, and myelocytes) by morphology, and is 1.9-fold enriched for immature myeloid cells compared with total IL-1–treated BM cells (49% immature myeloid cells). These data suggest that the IL-1R is upregulated on an immature myeloid population of BM cells. However, because the RB6-8C5^{lo} population also contains other cell types (lymphocytes, segmented neutrophils, eosinophils, and erythroid cells), we cannot rule out the possibility that IL-1R is also upregulated on more mature populations of hematopoietic cells. Indeed, IL-1 binding was reported to be increased on peripheral blood neutrophils from patients infused with IL-1.[33] Further characterization of IL-1R–positive BM cells is currently under investigation.

Recent reports have shown the existence of two distinct types of IL-1R.[25,34] The type I IL-1R expressed on T lymphocytes, fibroblasts, and epithelial cells is an 80- to 90-Kd binding protein, whereas the type II IL-1R present on B cells, macrophages, and granulocytes is in the range of 60 to 70 Kd. This selective distribution was confirmed using MoAbs and polyclonal antibodies raised against the type I IL-1R that block IL-1 binding to a T-cell line (EL-4), fibroblasts, and epithelial cells, but not IL-1 binding to B cells, macrophages, and granulocytes.[25] Despite this distribution, the type I IL-1R antibody (35F5) inhibited most of the IL-1–enhanced expression of IL-1R on BM cells. This inhibition could not be related to direct binding and/or blocking of IL-1R on BM cells, because in culture these cells did not bind radiolabeled 35F5 and this antibody does not compete for IL-1 binding on marrow cells. These results, together with our previous observation that the IL-1R expressed on BM cells and hematopoietic cell lines is a 65-Kd binding protein,[35] suggest that these cells may predominantly express type II IL-1R.

The mechanism by which anti-type I IL-1R antibody inhibits the enhanced IL-1R expression presumably involves IL-1 stimulation of T cells, fibroblasts, and epithelial cells that are known to respond to IL-1 by production of multiple products including cytokines with hematopoietic activities such as G-CSF, GM-CSF, and IL-6. We have previously shown that G-CSF, GM-CSF, IL-3, and IL-6, but not IL-1 itself, induce in culture the expression of IL-1R on a mononuclear fraction of BM cells depleted of stromal cells.[35,36] In this study, upregulation of the IL-1R was achieved by in vivo injection of G-CSF. Therefore, G-CSF induced by stimulation of cells expressing type I IL-1R may directly upregulate type II IL-1R on BM cells. This hypothesis is further supported by the finding that injection of 35F5 antibody to mice that blocked IL-1R induction by IL-1 also reduced the titer of circulating HGFs and blocked HGF production by stromal cells.[12] In contrast, injection of IL-4, which was shown to promote the growth in vitro of early hematopoietic progenitor cells in synergy with other growth factors,[37] or injection of IFN-γ did not mimic IL-1 effects on receptor upregulation. Because the above cytokines failed to induce circulating HGFs[26] (unpublished

observation), these results strengthen our contention that G-CSF is directly involved in IL-1R upregulation on BM hematopoietic cells and may therefore represent a link between IL-1 and the upregulation of its receptor on hematopoietic BM cells. The possibility that other HGFs such as GM-CSF, IL-3, and IL-6 also participate in the in vivo upregulation of IL-1R is currently under investigation.

Administration of IL-1 to mice also results in a rapid elevation of glucocorticoids (GCs).[38] Because in vitro treatment of human monocytes with dexamethasone and B cells with prednisolone upregulates IL-1R expression,[32,39] it is possible that endogenous GC production in conjuncture with HGFs mediate the upregulation of IL-1R on hematopoietic BM cells. Indeed, we recently observed partial inhibition of the ability of IL-1 to upregulate its receptor on BM cells from adrenalectomized mice. In addition, treatment of mice with GCs and G-CSF resulted in a synergistic effect on IL-1R expression on BM cells[40] (manuscript in preparation). These results suggest that IL-1–induced endogenous GC production also participates in the upregulation of IL-1R expressed on hematopoietic BM cells.

The upregulation of IL-1R with IL-1 pretreatment may be involved in the myelorestorative and radioprotective effects of IL-1. Indeed, administration of anti–IL-1R antibody blocks in parallel the upregulation of IL-1R and its radioprotective effects.[12] Such an increase in IL-1R expression on hematopoietic BM cells would serve to amplify the observed in vivo hematopoietic recovery after IL-1 treatment.[2,4,6,15,16] Because irradiation induces production of endogenous IL-1,[12,41] it is likely that the greater availability of the receptor resulting from IL-1 pretreatment contributes to more effective use of endogenous IL-1. Therefore, radiation-induced low doses of endogenous IL-1 would become sufficient to promote hematopoietic recovery similar to that observed with high doses of IL-1 administered after irradiation.[42]

In contrast to the murine model in which high doses of IL-1 are relatively well tolerated, in humans, repeated IL-1 administration is more toxic, causing hypotension and fever.[43] However, there is now evidence that, when administered to people at its maximum tolerated dose, IL-1 has potent hematopoietic effects, including the capacity to ameliorate hematopoietic toxicity from myelotoxic chemotherapy (D.L. Longo, personal communication, March 1991). It is possible that, administered in an appropriate schedule (when the IL-1R is highly expressed) perhaps together with an IL-1R–inducing cytokine such as G-CSF, much lower doses of IL-1 could be effective. Thus, monitoring the expression of IL-1R may be useful in establishing a schedule of low-dose IL-1 treatment with reduced toxicity and maximal hematopoietic effects.

ACKNOWLEDGMENT

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REFERENCES

1. Moore MAS: Role of interleukin-1 in hematopoiesis. Immunol Res 8:165, 1989

2. Newton RC, Sandlin G, Pezzella K, Huang J, McKearn J: Flow cytometric analysis of the effect of interleukin-1 administration on bone marrow populations in mice. J Biol Response Mod 8:155, 1989

3. Kampschmidt RF, Upchurch, HF: Possible involvement of leukocytic endogenous mediator in granulopoiesis. Proc Soc Exp Biol Med 155:89, 1977

4. Stork LC, Peterson VM, Rundus CH, Robinson WA: Interleukin-1 enhances murine granulopoiesis in vivo. Exp Hematol 16:163, 1988

5. Neta R, Sztein MB, Oppenheim JJ, Gillis S, Douches SD: The in vivo effect of interleukin 1: 1. Bone marrow cells are induced to cycle after administration of interleukin 1. J Immunol 139:1861, 1987

6. Johnson CJ, Douglas JK, Topper MI, Braunschweiger PG, Furmanski P: In vivo hematopoietic effects of recombinant interleukin-1α in mice: Stimulation of granulocytic, monocytic, megakaryocytic, and early erythroid progenitors, suppression of late-stage erythropoiesis, and reversal of erythroid suppression with erythropoietin. Blood 73:678, 1989

7. Vogel SN, Douches SD, Kaufman EN, Neta R: Induction of colony stimulating factor in vivo by recombinant interleukin 1α and recombinant tumor necrosis factor. J Immunol 138:2143, 1987

8. Zucali JR, Dinarello CA, Oblon DJ, Gross MA, Anderson L, Weiner RS: Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony stimulating activity and prostaglandin E_2. J Clin Invest 77:1857, 1986

9. Bagby GC, Dinarello CA, Wallace P, Wagner C, Hefeneider S, McCall E: Interleukin 1 stimulates granulocyte-macrophage colony-stimulating activity release by vascular endothelial cells. J Clin Invest 78:1316, 1986

10. Fibbe WE, Van Damme J, Billiau A, Voogt PJ, Duinkerken N, Kluck PMC, Falkenburg JHF: Interleukin 1 (22 K factor) induces release of granulocyte-macrophage colony-stimulating activity from human mononuclear phagocytes. Blood 68:1316, 1986

11. Kaushanski K, Lin N, Adamson JW: Interleukin 1 stimulates fibroblast to synthesize granulocyte-macrophage and granulocyte colony-stimulating factors. J Clin Invest 81:92, 1988

12. Neta R, Vogel SN, Plocinski JM, Tare NS, Benjamin W, Chizzonite R, Pilcher M: In vivo modulation with anti–interleukin-1 (IL-1) receptor (p80) antibody 35F5 of the response to IL-1. The relationship of radioprotection, colony-stimulating factor, and IL-6. Blood 76:57, 1990

13. Mochizuki DY, Eisenman JR, Conlon PJ, Larsen AD, Tushinski RT: Interleukin 1 regulates hematopoietic activity, a role previously ascribed to hemopoietin 1. Proc Natl Acad Sci USA 84:5267, 1987

14. Zsebo KM, Wypych J, Yuschenkoff VN, Lu H, Hunt P, Dukes PP, Langley KE: Effects of hematopoietin-1 and interleukin 1 activities on early hematopoietic cells of the bone marrow. Blood 71:962, 1988

15. Moore MAS, Warren DJ: Synergy of interleukin 1 and granulocyte colony-stimulating factor: In vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc Natl Acad Sci USA 84:7134, 1987

16. Benjamin WR, Tare NS, Hayes TJ, Becker JM, Anderson TD: Regulation of hemopoiesis in myelosuppressed mice by human recombinant IL-1α. J Immunol 142:792, 1989

17. Moreb J, Zucali JR, Gross MA, Weiner RS: Protective effects of IL-1 on human hematopoietic progenitor cells treated in

vitro with 4-hydroperoxycyclophosphamide. J Immunol 142:1937, 1989

18. Futami H, Jansen R, MacPhee MJ, Keller J, McCormick K, Longo DL, Oppenheim JJ, Ruscetti FW, Wiltrout RH: Chemoprotective effects of rHIL 1α in cyclophosphamide-treated normal and tumor-bearing mice: Protection from acute toxicity, hematological effects, development of late mortality and enhanced therapeutic efficacity. J Immunol 145:4121, 1990

19. Neta R, Douches S, Oppenheim JJ: Interleukin 1 is a radioprotector. J Immunol 136:2483, 1986

20. Jutila MA, Kroese FGM, Jutila KL, Stall AM, Fiering S, Herzenberg LA, Berg EL, Butcher EC: LY-6C is a monocyte/macrophage and endothelial cell differentiation antigen regulated by interferon-γ. Eur J Immunol 18:1819, 1988

21. Lewinsohn DM, Bargatze RF, Butcher EC: Leukocyte-endothelial cell recognition: Evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. J Immunol 138:4313, 1987

22. Zubler RH, Erard F, Lees RK, Van Lear M, Mingari C, Moretta L, MacDonald HR: Mutant EL-4 thymoma cells polyclonally activate murine and human B cells via direct cell interaction. J Immunol 134:3662, 1985

23. Palaszynski EW, Ihle JN: Evidence for specific receptors for interleukin 3 on lymphokine-dependent cell lines established from long-term bone marrow cultures. J Immunol 132:1872, 1984

24. Hestdal K, Ruscetti FW, Ihle N, Jacobsen SEW, Dubois CM, Kopp WC, Longo DL, Keller JR: Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. J Immunol 147:22, 1991

25. Chizzonite R, Truitt T, Kilian PL, Stern AS, Nunes P, Parker KP, Kaffka KL, Chua AO, Lugg DK, Gubler U: Two high-affinity interleukin 1 receptors represent separate gene product. Proc Natl Acad Sci USA 86:8029, 1989

26. Neta R, Vogel SN, Sipe JD, Wong GG, Nordan RP: Comparison of in vivo effects of human recombinant IL-1 and human recombinant IL-6 in mice. Lymphokine Res 7:403, 1988

27. Paganelli Parker K, Benjamin WR, Kaffka KL, Kilian PL: Presence of IL-1 receptors on human and murine neutrophils: Relevance to IL-1–mediated effect in inflammation. J Immunol 142:537, 1989

28. Uhl J, Newton RC, Giri JG, Sandlin G, Horuk R: Identification of IL-1 receptors on human monocytes. J Immunol 142:1576, 1988

29. Matsushima K, Akahosi T, Yamada M, Furutani Y, Oppenheim JJ: Properties of a specific interleukin-1 receptor on human Epstein-Barr virus-transformed B lymphocytes: Identity of the receptors for IL-1α and IL-1β. J Immunol 136:449, 1986

30. Dower SK, Kronheim SR, March CJ, Conlon PJ, Hopp TP, Gillis SG, Urdal DL: Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. J Exp Med 162:501, 1985

31. Akahoshi T, Oppenheim JJ, Matsushima K: Interleukin-1 stimulates its own receptor on human fibroblasts through the endogenous production of prostaglandin(s). J Clin Invest 82:1219, 1988

32. Akahoshi T, Oppenheim JJ, Matsushima K: Induction of high affinity interleukin 1 receptor on human peripheral blood lymphocytes by glucocorticoid hormones. J Exp Med 167:924, 1988

33. Shieh JH, Gordon MS, Peterson RHF, Jakubowski AA, Gabrilove JL, Moore MAS: Modulation of cytokine receptors and superoxyde production in neutrophils treated with IL-1 in vitro and in vivo. Blood 76:165a, 1990 (abstr)

34. Bomsztyk K, Sims JE, Stanton TH, Slack J, McMahan CJ, Valentine MA, Dower SK: Evidence for different interleukin 1 receptors in murine B- and T-cell lines. Proc Natl Acad Sci USA 86:8034, 1989

35. Dubois CM, Ruscetti FR, Palaszynski EW, Falk LA, Oppenheim JJ, Keller JR: Transforming growth factor β is a potent inhibitor of interleukin 1 (IL-1) receptor expression: Proposed mechanism of inhibition of IL-1 action. J Exp Med 172:737, 1990

36. Dubois CM, Ruscetti FW, Oppenheim JJ, Keller JR: Induction of interleukin-1 receptor (IL-1R) on normal hematopoietic progenitor cells by colony-stimulating factor. Exp Hematol 18:615, 1990 (abstr)

37. Peschel C, Paul WE, Ohara J, Green I: Effects of B cell stimulatory factor-1/interleukin-4 on hematopoietic progenitor cells. Blood 70:254, 1987

38. Woloski BM, Smith EM, Meyer WJ, Fuller GM, Blalock JE: Corticotropin-releasing activity of monokines. Science 230:1035, 1985

39. Spriggs MK, Lioubin PJ, Slack J, Dower SK, Jonas U, Cosman D, Sims JE, Bauer J: Induction of an interleukin-1 receptor (IL-1R) on monocytic cells: Evidence that the receptor is not encode by a T cell-type IL-1R mRNA. J Biol Chem 265:22499, 1990

40. Dubois CM, Neta R, Ruscetti FW, Jacobsen SEW, Oppenheim JJ, Keller JK: Up-regulation of p65 IL-1 receptor (IL-1R) on bone marrow cells by G-CSF and glucocorticoids. J Cell Biochem Suppl 15F:109, 1990 (abstr)

41. Woloschak GE, Chang-liu CM, Jones PS, Jones CA: Modulation of gene expression in syrian hamster embryo cells following ionizing radiation. Cancer Res 50:339, 1990

42. Neta R, Oppenheim JJ: Cytokines in therapy of radiation injury. Blood 72:1093, 1988

43. Smith J, Urba W, Steis R, Janik J, Fenton B, Sharfman W, Colon K, Sznol M, Creekmore S, Wells N, Elwood L, Keller J, Hestdal K, Ewel C, Rossio J, Kopp W, Shimuzu M, Oppenheim J, Longo D: A phase 1 trial of interleukin-1 alpha (IL-1α) alone and in combination with indomethacin. Lymphokine Res 9:568, 1990 (abstr)

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COMPARATIVE EFFECTS OF PARTICULATE AND SOLUBLE GLUCAN ON MACROPHAGES OF C3H/HeN AND C3H/HeJ MICE

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Abstract — In order to compare both the actions of soluble glucan (glucan-F) and particulate glucan (glucan-P) on macrophages and the responsiveness of macrophages from C3H/HeJ and C3H/HeN mice to these immunomodulators, interleukin-1 (IL-1) levels, phagocytosis and superoxide production were monitored after an *in vitro* exposure to glucan-F or glucan-P. A 2 or 20 h exposure to either glucan preparation decreased the ability of both C2H/HeJ and C3H/HeN macrophages to ingest zymosan. In contrast, glucan-P, but not glucan-F, decreased (after a 20 h exposure) the uptake of both IgG opsonized erythrocytes and latex beads. Furthermore, glucan-P, but not glucan-F was as effective as zymosan (after a 1 h exposure) in inducing superoxide release by macrophages isolated from both C3H/HeN and C3H/HeJ mice. While the effects of glucan-P on PMA-induced superoxide release and IL-1 levels were similar in macrophages from C3H/HeJ and C3H/HeN mice, glucan-F was ineffective at enhancing PMA-induced superoxide release or increasing IL-1 levels in C3H/HeJ mice. Thus (1) the effects of glucan-P on phagocytosis of opsonized erythrocytes and latex beads are not mimicked by glucan-F and (2) while macrophages from C3H/HeJ mice respond normally (as compared with C3H/HeN macrophages) to glucan-P, they are hyporesponders to glucan-F. These findings indicate that the activation of macrophages by glucan-P involves different (or additional) pathways from those activated by glucan-F.

In contrast to C3H/HeN mice, C3H/HeJ mice are hyporesponsive to lipopolysaccharide (LPS) and in particular to the lipid A moiety of LPS (Watson, Riblet & Taylor, 1977; Hogan & Vogel, 1987). C3H/HeJ mice differ from other C3H substrains at a specific gene locus (lps^d) located on chromosome 4 which confers sensitivity to LPS (Watson, Kelly, Largen & Taylor, 1978). *In vitro*, this hyporesponsiveness manifests itself in a failure of macrophages to secrete IL-1 (Rosenstreich, Vogel, Jacques, Wahl & Oppenheim, 1978) and a failure of B-lymphocytes to proliferate (Rosenstreich & Glode, 1975) in response to LPS. The hyporesponsiveness appears to be due to a defect in some subsequent step in the LPS signalling pathway, since cells from C3H/HeJ mice contain normal LPS receptors (Gregory, Zimmerman & Kern, 1980; Haeffner-Cavaillon, Cavaillon & Szabo, 1985). This view is supported by the observation that macrophages from C3H/HeJ mice also respond abnormally to the calcium ionophore A23187 (Shinomya & Nakano, 1987). To further explore the differences between C3H/HeN and C3H/HeJ mice we have examined the *in vitro* responses of peritoneal macrophages isolated from both strains of mice to soluble and insoluble forms of the β-1,3-D-polyglucose immunomodulator, glucan.

Glucan isolated from the inner cell wall of the yeast *Saccharomyces cerevisiae*, exists in both particulate (glucan-P) and water soluble forms (glucan-F). Most studies have utilized particulate glucan which, when administered to normal rats or mice, (1) induces a hyperphagocytic state in the reticuloendothelial system (Riggi & DiLuzio, 1961; Wooles & DiLuzio, 1963), (2) enhances primary and secondary antibody responses (Wooles & DiLuzio, 1963), (3) potentiates cell-mediated immune responses (DiLuzio, 1967), (4) stimulates hematopoietic stem cell and progenitor cell proliferation (Patchen & MacVittie, 1986) and (5) enhances resistance to life-threatening bacterial, viral, fungal and parasitic microorganisms (DiLuzio, 1983). However, glucan-P also induces severe hepatosplenomegaly and granuloma formation (DiLuzio, 1983). These detrimental side-effects can be avoided, often without sacrificing much biological effectiveness, by

using soluble glucan (Seljelid, 1986). In fact, a recent study in trauma patients has demonstrated that the same glucan-F preparation as that examined in this study decreased both septic morbidity and mortality (Browder, Williams, Pretus & Olivero, 1990).

Macrophages possess specific β-1,3-glucan receptors (Czop & Austen, 1985a; Czop & Kay, 1991) and are one of the primary targets of glucan. The *in vitro* exposure of macrophages to glucan leads to an increased cytotoxicity (Bogwald, Johnson & Seljelid, 1982; Seljelid, Bogwald, Hoffmann & Larm, 1984) and increased production and/or release of lysosomal enzymes (Bogwald, Johnson, Hoffmann & Seljelid, 1984; Lew, Leslie, Riches & Henson, 1986), tumor necrosis factor, interleukin-1 (IL-1) and prostaglandins (Sherwood, Williams & DiLuzio, 1986; Rasmussen & Seljelid, 1989; Doita, Rasmussen, Seljelid & Lipsky, 1991) and leukotrienes (Czop & Austen, 1985b). While studies have indicated that the effects of soluble β1,3-glucan and either aminated β-1,3-glucan derivatized microbeads or particulate glucan on inhibiting the ingestion of heat-killed yeast (Goldman, 1988) and increasing the release of IL-1 (Rasmussen & Seljelid, 1989) by macrophages were comparable, no studies have compared their effects on inducing or priming macrophages for superoxide release, nor have studies investigated their actions on macrophages from C3H/HeJ mice.

By examining the effects of both soluble and particulate glucans on IL-1 production, phagocytosis and oxidative burst potential in macrophages from C3H/HeJ and C3H/HeN mice, we demonstrate that glucan-P and glucan-F can have different effects on the phagocytic ability and superoxide production of macrophages from C3H/HeN mice. Furthermore, we show that while glucan-P affects superoxide and IL-1 production similarly in macrophages from both strains of mice, macrophages isolated from C3H/HeJ mice are hyporesponders to glucan-F, but normal responders to glucan-P. Thus, some of the effects of glucan-P on macrophages must be mediated by different activation pathways than those of glucan-F, and the signalling pathways involved in glucan-F activation are defective in macrophages from C3H/HeJ mice.

EXPERIMENTAL PROCEDURES

Materials

The preparation of both of these glucans has been described previously by DiLuzio, Williams, McNamee, Edwards & Kilahama (1979). Endotoxin-free particulate glucan (glucan-P) was purchased from Accurate Chemical and Scientific Co. (Hicksville, NY). The particulate form was aspirated through a 26 gauge needle four or five times to disperse aggregated particles before use. Soluble glucan (glucan-F) was a DiLuzio preparation purchased from Tulane University School of Medicine (New Orleans, LA). It was produced by partially phosphorylating particulate glucan, and, except for a phosphate group substitution on every seventh glucose, glucan-F is chemically identical to glucan-P (Williams, McNamee, Jones, Pretus, Ensley, Browder & DiLuzio, in press). This form of soluble glucan has been shown to be therapeutically effective in trauma patients at doses that have no toxicity (Browder *et al.*, 1990). The concentration of bacterial endotoxin (lipopolysaccharide [LPS]) in the glucan preparations was <1 ng endotoxin/10 mg glucan as assessed in a *Limulus* lysate assay. LPS extracted from *Escherichia coli* or *Salmonella typhosa* (Difco, Detroit, MI) was used at the stated concentration in interleukin-1 (IL-1) assays. Human recombinant IL-1 was obtained from Hoffman LaRoche (Nuttley, NJ). A polyclonal rabbit anti-mouse IL-1-α was purchased from Genzyme (Boston, MA) and a control polyclonal rabbit anti-human albumin was purchased from Sigma (St. Louis, MO).

Macrophage isolation

Resident peritoneal macrophages were isolated from mice euthanized by cervical dislocation. Five milliliters of Hank's balanced salt solution without Ca^{2+} or Mg^{2+} (HBSS/wo) and containing 2 units/ml heparin was injected into the peritoneal cavity and media with cells withdrawn. The peritoneal cells were washed twice in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS/wo; Gibco, Grand Island, NY). Cells were resuspended in tissue culture media consisting of RPMI-1640 (Gibco) with 0.03% L-glutamine (Sigma, St. Louis, MO), 10 units/ml penicillin, 10 ng/ml streptomycin (Difco, Detroit, MI), and 5% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT). The fetal bovine serum had endotoxin levels of less than 0.08 ng/ml as assessed by the *Limulus* amebocyte lysate method. To ensure that cells were cultured under endotoxin-"free" conditions, endotoxin-free H_2O was used in making all solutions, and disposable plasticware was used during cell isolation and culturing. Cells were then plated onto tissue culture plates (35 mm^2 well cluster 6 plates) at $1-3 \times 10^6$ cells/well, allowed to

adhere for 2 h, and washed twice with media or PBS to remove nonadherent cells. Greater than 95% of the cells isolated in this manner were viable and 94% of the adherent cells stained positive with nonspecific esterase stain used to identify macrophages.

Macrophage treatments and preparation of cell lysates

Following isolation by adherence, macrophages were placed in RPMI-1640 containing glucan-P, glucan-F or LPS (positive control) and were incubated for 2 or 20 h at 37°C in a 5% CO_2 – 95% air atmosphere. Macrophage cultures were washed three times with PBS, counted and then freeze-thawed three times to lyse cells. Cell lysates were harvested and centrifuged for 10 min at 300 *g* to remove cellular debris before use.

Interleukin-1 assay

IL-1 production by macrophages incubated with glucan-P, glucan-F, or LPS was measured using the thymocyte proliferation assay (Oppenheim, Togawa, Chedid & Mizel, 1980). Thymocytes ($2-3 \times 10^6$) from 5 to 8-week-old C3H/HeJ mice were suspended in 0.1 ml of RPMI-1640 supplemented with 0.03% L-glutamine, 10 units/ml penicillin, 10 ng/ml streptomycin, 10% heat-inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, and 1 μg/ml concanavalin-A (Con A; Calbiochem, San Diego, CA) and then plated on 96 well tissue culture plates. A tenth of a milliliter of appropriate macrophage lysate diluted 8:1 was added to each dish. Cultures were set up in triplicate and incubated at 37°C in a 5% CO_2 – 95% air atmosphere. After 3 days, ^3H thymidine (1 μCi/well) was added to each well, and the plates were incubated for a final 18 h. Cells were harvested on fiber filters (Scantron, Vienna, VA), using a cell harvester; filters were air dried and counted for radioactivity. Glucan-P and glucan-F added directly to thymocytes at 100 μg/ml did not increase thymidine incorporation. Each experiment also included thymocytes treated with varying concentrations of recombinant IL-1.

Superoxide measurement

Superoxide was assayed by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C (Johnston, Godzik & Cohn, 1978). Cells were washed twice with Krebs – Ringer, phosphate containing 10 mM glucose (KRPG), and then 1 ml of reaction mixture (KRPG containing 50 μM/ml ferricytochrome C (Sigma, St. Louis, MO) with or without 0.1 mg/ml SOD) was added to each dish along with 500 ng/ml of phorbol myristate acetate (PMA; Consolidated Midland, Brewster, NY). Dishes were incubated at 37°C for 1 h, at which time the supernatant was harvested and the absorbance at 550 nm was measured spectrophotometrically. The value of the absorbance with SOD was subtracted from the value of the absorbance without SOD to determine superoxide levels. Superoxide levels were computed by using an extinction coefficient of 21,000.

Protein measurement

Macrophages were washed three times with PBS and lysed overnight in 0.2% Triton X-100. The cell lysate was measured for protein using the Bio-Rad method (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. The Bio-Rad assay utilizes the fact that when protein binds to an acidic solution of Coomassie Brilliant Blue G-250 the absorbance maximum shifts from 465 to 595. As noted by others, there is a linear relationship between the number of murine peritoneal macrophages and their protein content (Johnston *et al.*, 1978).

Fc-mediated phagocytosis

Fc-mediated phagocytosis was measured using ^{51}Cr labeled bovine erythrocytes (E). E were labeled by adding 200 μCi of $Na^{51}CrO_4$ (NEN, Boston, MA) to 4 ml of 5% (v/v) E in PBS and incubating for 1 h at 37°C with frequent mixing. ^{51}Cr-labeled E were washed three or four times in PBS and coated with anti-E(IgG) (Cordis, Miami, FL). Macrophage cultures were washed twice with PBS, and RPMI-1640 without serum or other additives was added along with ^{51}Cr-E(IgG) at an E:macrophage ratio of 50:1. Dishes were incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Cultured cells were then washed twice with PBS to remove noningested E(IgG), and 0.2% sodium chloride was added for 30 s to lyse any extracellular E(IgG). Dishes were again washed twice with PBS, and 1 ml of hypotonic buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES] pH = 7.4, 1.5 mM $MgCl_2$) was added to each well for 5 min to induce swelling. After swelling, 0.1 ml of lysis solution (3 ml of glacial acetic acid 5 g ethylhexadecyldimethylammonium bromide in 100 ml of H_2O) was added for 10 min. The cell nuclei which remained intact were counted to determine cell number while the ^{51}Cr in the supernatant was measured to quantitate ingestion of E(IgG).

Zymosan and latex bead ingestion

Immunological grade zymosan (ICN, St. Louis, MO) was prepared by boiling a 0.2% w/v solution of

zymosan in 0.9% saline for 15 min, followed by three washes in PBS/wo. Three hundred micrograms of zymosan were added to each well containing 2×10^5 macrophages in RPMI-1640 without serum. Macrophages were allowed to ingest zymosan for 1 h at 37°C, washed three or four times with PBS, fixed in formalin–acetone fixative, air dried, and counted microscopically to determine the number of macrophages that ingested two or more zymosan particles.

Fluorescently labeled latex beads 1.97 microns in diameter (Polysciences Inc., Warrington, PA) were added to dishes of macrophages at a concentration that resulted in 90% of the control macrophages ingesting three or more beads. Dishes were incubated for 60 min, washed three times with PBS to remove uningested beads, fixed in formalin–acetone fixative, and counted as above.

Statistical analysis

Unless indicated otherwise, data are represented as mean ± S.E.M. Statistical comparisons were made using the Mann–Whitney test. Where numerous (three to five) comparisons were made with a control, the Bonferroni inequality was used to distribute Type I error. All expressions of significance reflect this distribution of error.

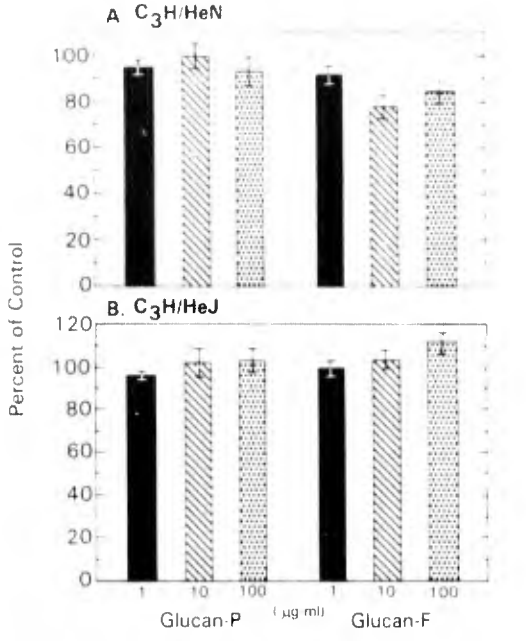

Fig. 1. Effect of glucan-P and glucan-F on adherent macrophage protein levels. Macrophages from either C3H/HeN or C3H/HeJ mice were isolated, allowed to adhere for 2 h, washed twice and refed media containing various concentrations of glucans. After 20 h cells were washed twice with PBS, adherent cells were lysed in 0.2% Triton-X and the protein content of the lysate assayed. Data are expressed as % nonglucan-treated dishes (control).

RESULTS

Adherent cell protein

To determine whether incubation with glucan affected the number of adherent macrophages in culture, the amount of adherent cell protein remaining after a 2 or 20 h incubation with varying concentrations of glucan-P or glucan-F was measured. There were no changes in the amount of adherent protein when macrophages from either C3H/HeN or C3H/HeJ mice were exposed for 2 h to concentrations of glucan-P or glucan-F ranging from 0.1 to 100 μg/ml (data not shown). However, after a 20 h incubation, macrophages from C3H/HeN mice (HeN-MØ) treated with either 10 or 100 μg/ml of glucan-F showed a small (12–15%) decrease in adherent cell protein [Fig. 1(A)]. Adherent protein from macrophages obtained from C3H/HeJ mice (HeJ-MØ) incubated for 20 h was not reduced by any concentration of glucan-P or glucan-F tested [Fig. 1(B)].

Superoxide release

When macrophages from HeN-MØ and HeJ-MØ were cultured continuously for 20 h in glucan-P or glucan-F and subsequently stimulated with PMA, a dose-dependent increase in superoxide (O_2^-) production was noted (Table 1). In the absence of pretreatment with glucan, PMA-induced O_2^- production was 40–60% lower in HeJ-MØ than in HeN-MØ. A 20-h incubation with glucan-P enhanced O_2^- release by HeJ-MØ in a dose-dependent manner so that O_2^- release by HeJ-MØ was comparable with that seen in HeN-MØ. In contrast to the effect of glucan-P on O_2^- production, glucan-F produced only a relatively small O_2^- stimulation in HeJ-MØ, compared with the response of HeN-MØ incubated with glucan-F (Table 1). The ability of glucan to alter O_2^- release occurred rapidly, since even a 2-h incubation with either glucan increased PMA-induced superoxide release, albeit to a lesser extent than the 20 h incubation.

It is well known that exposure to LPS primes macrophages so that they produce a large oxidative burst when exposed to subsequent stimuli (Pabst & Johnston, 1980). Figure 2 presents the PMA-induced

Table 1. Effect of preincubation with glucan-P and glucan-F on PMA-induced superoxide release†

Stimulus (μg/ml)	O_2^- (nM/mg protein/h) C3H/HeN 2 h	C3H/HeN 20 h	C3H/HeJ 2 h	C3H/HeJ 20 h
Control	70 ± 9	61 ± 10	50 ± 7	24 ± 3
Glucan-P				
(1)	81 ± 8	149 ± 17*	54 ± 4	90 ± 15*
(10)	133 ± 40*	181 ± 14*	82 ± 6*	204 ± 20*
(100)	200 ± 24*	216 ± 24*	140 ± 9*	268 ± 29*
Glucan-F				
(1)	88 ± 12	139 ± 21*	52 ± 8	32 ± 5
(10)	99 ± 11	188 ± 10*	49 ± 7	42 ± 9
(100)	161 ± 27*	285 ± 13*	59 ± 8	60 ± 11

*$P<0.01$ with respect to control values.

†C3H/HeN and C3H/HeJ macrophages were incubated for either 2 or 20 h in glucan-P or glucan-F. Cells were washed three times and superoxide release measured by the method of Johnston *et al.* (1978) using PMA (500 ng/ml) as the trigger. Control data are from untreated macrophages cultured for 2 or 20 h. Data represent mean ± S.E.M. of either 12 or 15 different determinations.

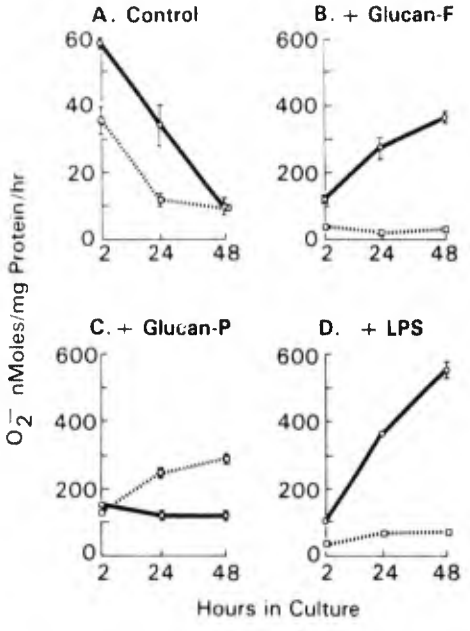

Fig. 2. Effect of LPS, glucan-P, and glucan-F on PMA-induced superoxide production in C3H/HeN (solid line) and C3H/HeJ (dashed line) macrophages. Cells were incubated for 2, 24 or 48 h in either tissue culture medium (control) or in tissue culture medium containing LPS (20 μg/ml), glucan-P (100 μg/ml), or glucan-F (100 μg/ml) as indicated. Note that the scale in A for the ordinate differs from the other graphs. Data, which are one representative experiment, are the mean ± S.E.M. of three measurements.

Table 2. Glucan-P, glucan-F, zymosan and PMA-stimulated superoxide release*

Stimulus	O_2^- (nM/mg protein/h) C3H/HeN	C3H/HeJ
Control	0	0
PMA (500 ng/ml)	90 ± 15	50 ± 3
Glucan-P (100 μg/ml)	58 ± 12	44 ± 8
Glucan-F (100 μg/ml)	0	0
Zymosan (100 μg/ml)	42 ± 6	27 ± 3

*Peritoneal macrophages from either C3H/HeN or C3H/HeJ mice were incubated with stimuli plus the reaction mixture for 1 h ($N = 9$).

superoxide production in macrophages that had been cultured for 2, 24 or 48 h in LPS, glucan-F or glucan-P. Figure 2(A) shows the O_2^- response of untreated HeJ-MØ and HeN-MØ to PMA. The O_2^- production of both HeJ-MØ and HeN-MØ decreased with time in culture so that, by 48 h in culture, little O_2^- was produced by either cell type. As noted in Table 1, HeJ-MØ that have been cultured for 2 or 24 h produce less O_2^- than

Table 3. Effect of preincubation with glucan-P and glucan-F on interleukin-1 production*

Macrophage lysate	(n)	[^{3}H] Thymidine uptake (counts/min × 10^{-3})	
		C3H/HeN	C3H/HeJ
Con A†	(27)	1.87 ± 0.22	1.87 ± 0.22
Control‡	(24)	2.77 ± 0.49	3.04 ± 0.68
Glucan-P (1 μg/ml)	(6)	3.99 ± 0.41	3.30 ± 0.43
Glucan-P (100 μg/ml)	(24)	15.87 ± 2.30	6.09 ± 0.92
Glucan-F (1 μg/ml)	(6)	5.74 ± 0.72¶	2.52 ± 0.15
Glucan-F (100 μg/ml)	(24)	6.89 ± 1.53¶	2.82 ± 0.44
LPS (20 μg/ml)	(24)	43.37 ± 5.52§	26.68 ± 4.82§
LPS§ (1 μg/ml)	(9)	28.68 ± 8.02	5.06 ± 0.98
IL-1† (2 ng/ml)	(16)	61.88 ± 6.40	61.88 ± 6.40¶

*Interleukin-1 production was assayed using the thymocyte proliferation assay. 0.1 ml of macrophage lysate diluted 8:1 from macrophages treated with indicated stimulus was added to wells containing thymocytes and Con A (1 μg/ml). After a 3 day incubation, [^{3}H] thymidine was added and dishes were incubated for 18 h before uptake of thymidine was assayed.

†Agents were added in the absence of lysate.

‡Control lysate was from untreated macrophages cultured for 20 h.

§Values for LPS-stimulated macrophage lysate were not corrected for the effects of LPS on the thymocyte proliferation. 5.26 ± 1.18 × 10^{-3} counts/min were obtained from wells containing LPS (20 μg/ml) in the absence of macrophage lysate.

§$P<0.001$ as compared with respective control values.

¶$P<0.05$ as compared with respective control values.

HeN-MØ. In this experiment glucan-P (100 μg/ml) was a significantly better stimulus for HeJ-MØ than for HeN-MØ [Fig. 2(C)]. (The same trend also is evident in Table 1 for treatment with 10 μg/ml and 100 μg/ml of glucan-P.) In HeJ-MØ, glucan-F (100 μg/ml) did not stimulate PMA-induced O_2^- production [Fig. 2(B)], and LPS [Fig. 2(D)] (20 μg/ml) was a poor stimulus. Incubating HeJ-MØ with both LPS and glucan-F did not further enhance superoxide production (data not shown). In contrast, in HeN-MØ both these agents produced a large increase in PMA-induced O_2^- production, which increased with time of exposure.

Related studies also examined the ability of glucan-P or glucan-F to directly trigger the oxidative burst by measuring O_2^- production in cells in which glucan-P, glucan-F, or zymosan (another well-known trigger of the oxidative burst in phagocytes) were substituted for the PMA in the ferricytochrome C reaction mixture. Both glucan-P and zymosan (particulate stimuli that are ingested by the macrophages) stimulated O_2^- release in HeN-MØ and HeJ-MØ in the absence of PMA, while glucan-F was ineffective (Table 2). However, neither glucan-P nor zymosan were as effective at triggering the release of O_2^- as was PMA.

Table 4. Effects of anti-interleukin-1-α on thymocyte proliferation assay*

		[^{3}H] Thymidine uptake (counts/min × 10^{-3})			
		C3H/HeN		C3H/HeJ	
		Anti-IL-1†		Anti-IL-1†	
Macrophage lysate	(n)	−	+	−	+
Con A (1 μg/ml)	(5)	1.9 ± 0.2		1.9 ± 0.2	
Control‡	(5)	2.2 ± 0.2		1.7 ± 0.4	
LPS (20 μg/ml)	(5)	52.3 ± 9.1	3.1 ± 0.9	4.5 ± 0.6	1.1 ± 0.3
Glucan-P (100 μg/ml)	(5)	10.0 ± 4.4	2.4 ± 1.6	3.6 ± 0.1	1.1 ± 0.7
Glucan-F (100 μg/ml)	(3)	12.0 ± 4.9	2.9 ± 4.9	1.9 ± 0.8	0.6 ± 0.1

*Experiments were done as in Table 1, except rabbit polyclonal anti-IL-1-α antibody was added to some of the samples as indicated. In control studies not shown rabbit polyclonal anti-human albumin did not reduce thymidine incorporation.
†Polyclonal rabbit anti-mouse IL-1-α (Genzyme Corp., Boston, MA) was added at a concentration of 0.4 units (40 μl) to a final volume of 200 μl.
‡Untreated HeN and HeJ macrophage lysates.

IL-1 production

As shown in Table 3, the incorporation of thymidine by thymocytes exposed to either Con A alone or to Con A plus lysate from untreated macrophages did not differ significantly, indicating that untreated macrophages had little or no IL-1 activity. In contrast, exposure of macrophages to a high concentration (20 μg/ml) of LPS (a well-known inducer of IL-1 production in macrophages; Dinarello, 1988) resulted in HeJ-MØ and HeN-MØ lysates that increased thymidine incorporation over the incorporation produced by control lysates by sixteen-fold and nine-fold, respectively. Lowering the concentration of LPS to 1 μg/ml decreased the lysate-induced stimulation of thymidine incorporation to ten-fold for HeN-MØ lysates and to only two-fold for HeJ-MØ lysates. These results are consistent with previous studies indicating that although macrophages from C3H/HeJ mice are less sensitive to LPS they are still capable of responding to high LPS concentrations (Vogel & Rosenstreich, 1981).

The data in Table 3 also demonstrate that although glucan-P did not stimulate IL-1 production as effectively as LPS, 100 μg/ml of glucan-P stimulated thymocyte proliferation by six-fold and two-fold in HeN-MØ and HeJ-MØ, respectively. A lower concentration of glucan-P (1 μg/ml) was ineffective. In contrast, 100 μg/ml of glucan-F had no effect on thymocyte proliferation activity of HeJ-MØ lysates, while it increased the proliferation activity of HeN-MØ lysates by two-fold. This difference was not a concentration effect because, in a subsequent experiment, 500 μg/ml of glucan-F also failed to stimulate production of the thymocyte proliferation factor in HeJ-MØ (data not shown).

To determine if the thymocyte proliferation induced by lysates from glucan-treated macrophages was due to the presence of IL-1, lysates that had been incubated with rabbit polyclonal antibody against mouse IL-1-α were tested in the proliferation assay. As a control, lysates also were incubated with rabbit polyclonal antibody against human albumin. Data from these experiments (Table 4) indicate that while anti-human albumin did not reduce thymocyte proliferation induced by lysates of macrophages treated with LPS, glucan-P, or glucan-F, anti-IL-1-α reduced thymocyte proliferation to control levels.

Phagocytosis

Opsonized erythrocytes. HeN-MØ and HeJ-MØ grown in tissue culture for 20 h ingested, during the 1 h incubation with E(IgG), an average of 3.4 ± 0.2 and 2.6 ± 0.3 E(IgG) per macrophage, respectively. Culturing C3H/HeN and C3H/HeJ

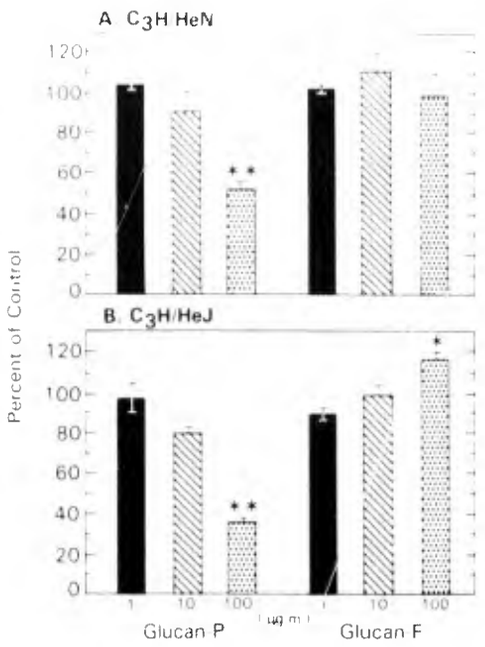

Fig. 3. Effect of glucan-P and glucan-F on the phagocytosis of IgG coated erythrocytes [E(IgG)]. Fc-mediated phagocytosis was measured using ^{51}Cr-labeled IgG coated bovine erythrocytes (described in Experimental Procedures). MØ were treated as in Fig. 1. Data are expressed as % control (nonglucan-treated dishes). $*P<0.05$, with respect to controls, $**P<0.001$, with respect to controls.

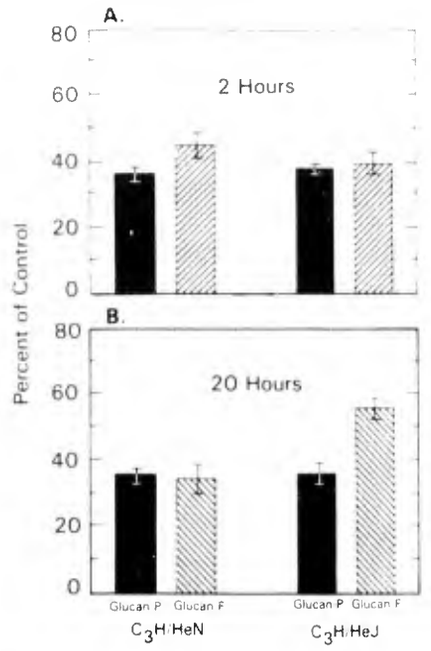

Fig. 4. Effect of glucan-P and glucan-F on ingestion of zymosan. Macrophages which had been exposed to 100 μg/ml of glucan-P or glucan-F for 2 or 20 h were incubated with zymosan particles at a ratio of 50 zymosan particles to 1 macrophage for 1 h. Data are expressed as % control (nonglucan-treated macrophages) phagocytosis. $*P<0.001$, with respect to controls

macrophages for 20 h in the presence of 100 μg/ml of glucan-P decreased the phagocytic index to 50 and 37% of control values, respectively (Fig. 3). In contrast, incubation with glucan-F (1 – 100 μg/ml) had no detrimental effect on ingestion of E(IgG) in either cell type, and actually enhanced phagocytosis at 100 μg/ml in HeJ-MØ [Fig. 3(B)].

In order to determine if a briefer exposure to glucans would produce similar effects on E(IgG) phagocytosis, C3H/HeN and C3H/HeJ macrophages that had been cultured for 19 h in control media were incubated with glucan-P or glucan-F (1 – 100 μg/ml) for only the 20th h of culture and then assayed for E(IgG) ingestion. There was no difference between the phagocytic index in these cells and control cells which had been cultured for 20 h in the absence of glucans (data not shown). These studies indicate that the inhibitory effect of glucan-P on E(IgG) phagocytosis develops with time after glucan exposure.

Latex beads. In order to determine if a 20 h exposure to high concentrations of glucan-P also produced a similar inhibition in phagocytosis when a different type of particle was ingested, HeN-MØ and HeJ-MØ were exposed to latex beads immediately after incubating cells with either 100 μg/ml of glucan-P or glucan-F for 20 h. While a short (1 h) exposure to glucan-P had no effect on the number of macrophages ingesting latex beads, a 20 h exposure to glucan-P decreased the percentage of macrophages ingesting two or more beads to 70 ± 3 from 95 ± 2 for HeJ-MØ and to 66 ± 3 from 98 ± 1 for HeN-MØ. In contrast, incubating either HeN-MØ or HeJ-MØ with glucan-F (100 μg/ml) for 20 h had no effect on the number of macrophages ingesting latex beads.

Zymosan. In human monocytes, the ingestion of zymosan, which is composed of β-glucan and α-mannan, is reduced by >50% following a 40 min pretreatment with soluble glucan (Czop & Austen, 1985a). To determine if glucan-P and glucan-F had a similar effect on HeN-MØ and HeJ-MØ, the ingestion of zymosan by macrophages previously exposed to glucan-P and glucan-F for either 2 or 20 h was monitored. In contrast to our previous results monitoring the ingestion of E(IgG), the ingestion of

zymosan was significantly decreased in both HeN-MØ and HeJ-MØ exposed to either glucan for 2 or 20 h (Fig. 4).

DISCUSSION

While soluble and particulate glucans have some of the same beneficial *in vivo* effects, particulate glucans can induce severe inflammatory responses such as granuloma formation (DiLuzio, 1983). Macrophages possess a specific β-glucan receptor whose stimulation during phagocytosis leads to the generation of leukotrienes (Czop & Austen, 1985b), the potentiation of cytostatic capacity (Seljelid *et al.*, 1984) and the release of acid hydrolases (Bogwald *et al.*, 1984). Therefore, it is likely that some of the detrimental effects of particulate glucan *in vitro* are likely to involve macrophages, and in particular the production of free radicals and cytokines by these cells. In order (1) to understand the difference between glucan-F and glucan-P and (2) to investigate the responsiveness of macrophages containing the lps^d gene to these immunomodulators, we have compared the affects of glucan-P and glucan-F on macrophages from both C3H/HeN and C3H/HeJ mice by assessing three different macrophage functions, phagocytosis, IL-1 production and oxidative burst potential.

Incubation for 2 or 20 h with either glucan-P or glucan-F diminished the ingestion of zymosan in both HeJ-MØ and HeN-MØ. These observations are in agreement with the findings in human macrophages that a 40 min exposure to β-glucan results in a large decrease in the subsequent ingestion of zymosan (Czop & Austen, 1985a,b), and suggest that zymosan (which is composed of both mannan and glucan) ingestion by macrophages may be mediated through a β-glucan receptor. They are also in agreement with the observation that ingestion of heat-killed yeast by mouse peritoneal macrophages is mediated by β-glucan receptors (Goldman, 1988). Furthermore, as reported in human macrophages (Czop & Austen, 1985a), we found that the ingestion of E(IgG) or latex beads was unaffected by short (1 – 2 h) incubations of HeN-MØ or HeJ-MØ with either glucan-F or glucan-P. On the other hand, a 20 h incubation with 100 μg/ml of glucan-P produced a 40 – 60% inhibition of the ingestion of both E(IgG) and latex beads in both HeJ-MØ and HeN-MØ. Since the ingestion of both latex beads and E(IgG) were similarly inhibited by prolonged incubation with high concentrations of glucan-P, it appears that this is a relatively nonspecific decrease in endocytic ability. It is possible that this inhibition may involve simply "filling the cells up" with glucan particles.

Enhancement of the oxidative burst is often an important part of macrophage activation and is associated with both enhanced cytotoxicity and chronic inflammation. The ability of glucan-P but not glucan-F, to induce superoxide release in the absence of additional stimuli (Table 2) is not surprising given the observation that zymosan ingestion, which is mediated predominantly by β-glucan receptors (Goldman, 1988; Czop & Austen, 1985a), stimulates superoxide production in macrophages. These observations suggest that the lack of *in vivo* inflammatory effects of glucan-F is related in part to its inability to elicit an oxidative burst in the absence of other stimuli.

Both glucan-F and glucan-P increase PMA-induced O_2^- production in HeN-MØ, but only glucan-P, not glucan-F, enhanced PMA-induced O_2^- production in HeJ-MØ. Since it is well known that macrophages exposed to LPS exhibit an enhanced oxidative burst and that macrophages from C3H/HeJ mice are defective in this response (Pabst & Johnston, 1980), it is important to note that the glucan preparations used in these studies had no detectable levels of LPS (see Experimental Procedures). Thus, the data in Table 1 and in Fig. 2(B), reflect real differences in the ability of glucan-F (not LPS) to activate macrophages from C3H/HeJ and C3H/HeN mice. Figure 2(A) and Table 1 demonstrate that HeJ-MØ cultured for 2 h release less O_2^- in response to PMA than HeN-MØ, and that PMA-induced superoxide production decreased with time in culture in macrophages from both substrains of mice. The stimulatory effect of glucan-F, like LPS, on the PMA-induced release of O_2^- by HeN-MØ increased with the length of exposure to these agents. Surprisingly, the priming effects of glucan-P on HeN-MØ were unchanged by the length of exposure to glucan-P [Fig. 2(C)]. In contrast, the priming action of glucan-P in HeJ-MØ was augmented by an increased length of exposure [Fig. 2(C)]. Thus, in terms of priming cells for increased PMA-induced O_2^- production HeN-MØ and HeJ-MØ respond differently to glucan-P, and HeJ-MØ fail to respond to glucan-F.

Our results indicating that glucan-P and glucan-F both produce significant increases in IL-1 production in HeN-MØ, are consistent with previous *in vitro* observations in mouse and human peritoneal macrophages that both soluble and particulate glucan increased IL-1 activity in cell supernatants (Sherwood *et al.*, 1987; Rasmussen & Seljelid, 1989).

Similarly, *in vivo* administration of soluble glucan in rats induces IL-1 production in splenic macrophages 12 h after administration (Sherwood *et al.*, 1987). However, in contrast to our findings in HeN-MΦ, only glucan-P, not glucan-F, induced IL-1 production in HeJ-MΦ. The failure of glucan-F to induce production of IL-1 in HeJ-MΦ was not due to a decrease in the number of macrophages, since, as shown in Fig 1, incubation with glucan-F actually produced a small increased in adherent cell protein. Thus, glucan-P activation of macrophages leading to an increase in IL-1 production as well as a priming of PMA-induced superoxide production involves either a different pathway or additional pathways (possibly related to endocytosis) from the signalling pathway(s) activated by glucan-F.

Previous studies have shown that both lymphocytes and macrophages from C3H/HeJ mice exhibit normal specific binding of LPS to their cell surface (Gregory *et al.*, 1980; Haeffner-Cavaillon *et al.*, 1983), suggesting that hyporesponsiveness to LPS is related to a defect in cell signalling rather than in receptor binding. This view is supported by several other observations, including the failure of HeJ-MΦ to produce IL-1 in response to A23187 (Shinomya & Nakano, 1987) and the ability of γ-interferon to reverse the hyporesponsiveness of HeJ-MΦ to LPS (Beutler, Tkacenko, Milsark, Krochin & Cerami, 1986). A recent study by Terada, Shinomya & Nakano (1989), which reported that HeJ-MΦ lack a calmodulin-binding protein, suggests the defect may involve a Ca signalling event. Our observations demonstrate that HeJ-MΦ respond abnormally (when compared with HeN-MΦ) to another substance, glucan-F. Thus, glucan-F provides an additional tool for elucidating the functional deficit in C3H/HeJ mice. While these findings do not elucidate the mechanism of glucan-F activation of macrophages, they suggest that glucan-F, but not glucan-P, relies on similar signalling pathways to activate macrophages as does LPS.

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REFERENCES

BEUTLER, B., TKACENKO, V., MILSARK, L., KROCHIN, N. & CERAMI, A. (1986). Effect of γ interferon on cachectin expression by mononuclear phagocytes. Reversal of lps^d (endotoxin resistance) phenotype. *J. exp. Med.*, **164**, 1791 – 1796.

BOGWALD, J., JOHNSON, E., HOFFMANN, J. & SELJELID, R. (1984). Lysosomal glycosidases in mouse peritoneal macrophages stimulated *in vitro* with soluble and insoluble glycans. *J. Leukocyte Biol.*, **35**, 357 – 371.

BOGWALD, J., JOHNSON, E. & SELJELID, R. (1982). The cytotoxic effect of mouse macrophages stimulated *in vitro* by a β-1,3-D-glucan from yeast cell walls. *Scand. J. Immun.*, **15**, 297 – 304.

BROWDER, W., WILLIAMS, D., PRETUS, H. & OLIVERO, G. (1990). Beneficial effect of enhanced macrophage function in the trauma patient. *Ann. Surg.*, **211**, 605 – 612.

CZOP, J. & AUSTEN, K. F. (1985a). A β-glucan inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway. *J. Immun.*, **134**, 2588 – 2593.

CZOP, J. & AUSTEN, K. F. (1985b). Generation of leukotrienes by human monocytes upon stimulation of their β-glucan receptor during phagocytosis. *Proc. natn. Acad. Sci. U.S.A.*, **82**, 2751 – 2755.

CZOP, J. & KAY, J. (1991). Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. *J. exp. Med.*, **173**, 1511 – 1520.

DILUZIO, N. R (1967). Evaluation of the graft vs host reaction of the immune competence of lymphoid cells of mice with altered reticuloendothelial function. *J. Reticuloendothel. Soc.*, **4**, 459 – 475.

DILUZIO, N. R. (1983). Immunopharmacology of glucan: a broad spectrum enhancer of host defense mechanisms. *Trends Pharmac. Sci.*, **4**, 344 – 347.

DILUZIO, N. R., WILLIAMS, D. L., MCNAMEE, R. B., EDWARDS, B. F. & KILAHAMA, A. (1979). Comparative tumor inhibitory and antibacterial activity of soluble and particulate glucan. *Int. J. Cancer*, **24**, 773 – 779.

DINARELLO, C. (1988). Cytokines: interleukin-1 and tumor necrosis factor. In *Inflammation: Basic Principles and Clinical Correlates* (eds Gallin, J., Goldstein, I. and Snyderman, R.) p. 195. Raven Press, New York.

DIOTA, M., RASMUSSEN, L., SELJELID, R. & LIPSKY, P. (1991). Effect of soluble animated β-1,3-D-polyglucose on human monocytes: stimulation of cytokine and prostaglandin E_2 production but not antigen-presenting function. *J. Leukocyte Biol.*, **49**, 342 – 351.

Goldman, R. (1988). Characteristics of the β-glucan receptor of murine macrophages. *Expl Cell Res.*, **174**, 481 – 490.

Gregory, S. H., Zimmerman, D. H. & Kern, M. (1980). The lipid A moiety of lipopolysaccharide is specifically bound to B cell subpopulations of responder and nonresponder animals. *J. Immun.*, **125**, 102 – 107.

Haeffner-Cavaillon, N., Cavaillon, J. & Szabo, L. (1985). Cellular receptor for endotoxin. In *Handbook of Endotoxin*, Vol. 3 (ed. Berry, L.). Elsevier North Holland Biomedical Press, New York.

Hogan, M. & Vogel, S. (1987). Lipid A-associated proteins provide an alternate "second signal" in the activation of recombinant interferon-γ-primed, C3H HeJ macrophages to a fully tumoricidal state. *J. Immun.*, **139**, 3697 – 3702.

Johnston, R., Godzik, C. & Cohn, Z. (1978). Increased superoxide anion production in immunologically activated and chemically elicited macrophages. *J. exp. Med.*, **148**, 115 – 127.

Liw, D., Leslie, C., Riches, D. & Henson, P. (1986). Induction of macrophage lysosomal hydrolase synthesis and secretion by β-1,3-glucan. *Cell. Immun.*, **100**, 340 – 350.

Oppenheim, J., Togawa, A., Chedid, L. & Mizel, S. (1980). Components of mycobacteria and muramyl dipeptide with adjuvant activity induce lymphocyte activating factor. *Cell. Immun.*, **50**, 71 – 80.

Pabst, M. & Johnston, R. (1980). Increased production of superoxide anion by macrophages exposed *in vitro* to muramyl dipeptide or lipopolysaccharide, *J. exp. Med.*, **151**, 101 – 113.

Patchen, M. L. & MacVittie, T. J. (1986). Comparative effects of soluble and particulate glucans on survival in irradiated mice. *J. biol. Resp. Modif.*, **5**, 45 – 60.

Rasmussen, L. T. & Seljelid, R. (1989). The modulatory effect of lipoproteins on the release of interleukin 1 by human peritoneal macrophages stimulated with β-1,3-D-polyglucose derivatives. *Scand. J. Immun.*, **29**, 477 – 484.

Riggi, S. & DiLuzio, N. R. (1961). Identification of an RE stimulating agent in zymosan. *Am. J. Physiol.*, **200**, 297 – 300.

Rosenstreich, D. L. & Glode, L. M. (1975). Difference in B cell mitogen responsiveness between closely related strains of mice. *J. Immun.*, **115**, 777 – 780.

Rosenstreich, D. L., Vogel, S. N., Jacques, A. R., Wahl, L. & Oppenheim, J. (1978). Macrophage sensitivity to endotoxin: genetic control by a single codominant gene. *J. Immun.*, **121**, 1664 – 1670.

Seljelid, R. (1986). A water soluble aminated β-1,3-D-glucose derivative caused regression of solid tumors in mice. *Biosci. Rep.*, **6**, 845 – 852.

Seljelid, R., Bogwald, J., Hoffmann, J. & Larm, O. (1984). A soluble β-1,3-*D*-glucan derivative potentiates the cytostatic and cytolytic capacity of mouse peritoneal macrophages *in vitro*. *Immunopharmacology*, **7**, 69 – 73.

Sherwood, E., Williams, D. & DiLuzio, N. (1986). Glucan stimulates production of antitumor cytolytic/cytostatic factor(s) by macrophages. *J. biol. Resp. Modif.*, **5**, 504 – 526.

Sherwood, E. R., Williams, D. L., McNamee, R., Jones, E., Browder, I. W. & DiLuzio, N. (1987). Enhancement of interleukin-1 and interleukin-2 production by soluble glucan. *Int. J. Immunopharmac.*, **9**, 261 – 267.

Shinomiya, H. & Nakano, M. (1987). Calcium ionophore A23187 does not stimulate lipopolysaccharide nonresponsive C3H/HeJ peritoneal macrophages to produce interleukin-1. *J. Immun.*, **139**, 2730 – 2736.

Terada, Y., Shinomiya, H. & Nakano, M. (1989). Defect of calmodulin-binding protein in expression of interleukin-1β gene by LPS-nonresponder C3H/HeJ mouse macrophages. *Biochem. biophys. Res. Commun.*, **158**, 723 – 729.

Vogel, S. & Rosenstreich, D. (1981). LPS-unresponsive mice as a model for analyzing lymphokine-induced macrophages differentiation *in vitro*. In *Lymphokines*, p. 149. Academic Press, New York.

Watson, J. K., Kelly, M., Largen, M. & Taylor, B. (1978). The genetic mapping of a defective LPS response gene in C3H/HeJ mice. *J. Immun.*, **120**, 422 – 427.

Watson, J., Riblet, R. & Taylor, B. (1977). The response of recombinant inbred strains of mice to bacterial lipopolysaccharides. *J. Immun.*, **118**, 2088.

Williams, D., McNamee, R., Jones, E., Pretus, H., Ensley, H., Browder, W. & DiLuzio, N. R. A method for the solubilization of β-1,3-glucan isolated from *Saccharomyces cerevisiae*. *Carbohydrate Res.* (in press).

Wooles, W. R. & DiLuzio, N. R. (1963). Reticuloendothelial function and the immune system. *Science*, **142**, 1078 – 1080.

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Kinetics of superoxide scavenging by dismutase enzymes and manganese mimics determined by electron spin resonance

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This study presents an e.s.r. assay for superoxide dismutase (SOD). Enzymic reactions were studied in which Cu,Zn-SOD, Mn-SOD and Fe-SOD each competed with the spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) for superoxide anion (O_2^-) at pH 7.8. O_2^- from dissolved KO_2 (potassium superoxide) in dimethyl sulphoxide was added directly to the enzyme solutions containing DMPO. The results show that, in this competition reaction system, the kinetics of the reactions between the enzymes and O_2^- follow a function $y = f([SOD]^{0.5})$. The rate constant, $k_{SOD} = 6.4 \times 10^9$ M^{-1}·S^{-1}, determined for Cu,Zn-SOD is approximately an order of magnitude larger than those for Mn-SOD and Fe-SOD. A comparative study of reported SOD mimics, including Mn^{2+}, MnO_2–desferrioxamine mesylate (Desferal) and MnO_2–Desferal–ascorbate, was done. The results show that solutions of these complexes are approximately three orders of magnitude less active than Cu,Zn-SOD and approximately two orders of magnitude less active than Mn-SOD or Fe-SOD. The results also suggest that the reactivity toward O_2^- in solutions of these complexes originates from the Mn^{2+} present and not from the MnO_2–Desferal complexes.

INTRODUCTION

Enzymes catalysing the dismutation of superoxide anion (O_2^-) have been isolated, characterized and discussed in various papers and reviews [1–6]. These superoxide dismutase (SOD) enzymes contain metal ions which are cyclically reduced and oxidized during catalysis [7–9]. Although exact details of the catalytic mechanism are still under investigation, the cyclic redox reactions of the metals as shown in reactions (1) and (2) are generally accepted [10]:

$$M^{n+}\cdot SOD + O_2^- \rightleftharpoons [M^{n+}\cdot SOD - O_2^-] \rightleftharpoons M^{(n+1)+}\cdot SOD + O_2 \quad (1)$$

$$M^{(n+1)+}\cdot SOD + O_2^- + 2H^+ \rightleftharpoons [M^{(n+1)+}\cdot SOD - O_2H_2] \rightleftharpoons M^{n+}\cdot SOD + H_2O_2 \quad (2)$$

Two of the three types of metal-containing enzymes, Cu,Zn-SOD and Fe-SOD, have been studied by stopped-flow techniques, and the resultant data fit the Michaelis–Menten steady-state kinetic model [11–13]. Steady-state assumptions provide the rationale for many competition kinetic assays developed for reactions having transient free radicals as substrates [14,15]. The present paper reports results of competition-kinetic studies of SOD enzymes and of manganese-containing preparations that are reportedly mimics of SOD enzymes.

Competition assays for O_2^- dismutase activity in aqueous solutions have a background spontaneous dismutation reaction occurring (reaction 3):

$$2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \quad (3)$$

The rate constant for this reaction is approx. 2×10^5 M^{-1}·s^{-1} at pH 7.8 [16]. Any detector molecule must compete for O_2^- against this spontaneous dismutation background reaction plus dismutase enzymes or mimics present in reaction solutions. 5,5-Dimethyl-1-pyrroline 1-oxide (DMPO) reacts with O_2^- forming the DMPO–O_2^- spin adduct radical through the bimolecular mechanism shown in reaction (4) [17]:

$$O_2^- + DMPO \rightarrow DMPO\text{–}O_2^- \quad (4)$$

The calculated rate constant (k_{DMPO}) for reaction (4) is about 18 M^{-1}·s^{-1} at pH 7.8, and the spin adduct formed is sufficiently stable for quantification by e.s.r. [18,19]. It is known that O_2^- reacts with both of the spin adducts: DMPO–O_2^- and the decomposition product, DMPO–OH, formed from DMPO–O_2^- [20,21]. The products of these reactions with O_2^- are e.s.r.-silent. The rates of each reaction depend on the concentrations of DMPO–O_2^-, DMPO–OH and the O_2^- concentration. Therefore, competition experiments using DMPO as a detector should rely on an initial introduction of O_2^- rather than a steady-state flux of O_2^- generated continuously to minimize formation of e.s.r.-silent products. Also, it must be demonstrated that the combined DMPO–O_2^- and DMPO–OH e.s.r. signal varies with the logarithm of the SOD concentration. This insures that spontaneous dismutation plus scavenging by DMPO–O_2^- and DMPO–OH are not masking O_2^- removal by SOD, permitting the use of DMPO as a detector molecule in competition assays over a limited range centred around the equality shown in eqn. (A):

$$k_{DMPO}[DMPO][O_2^-] = k_{SOD}[SOD][O_2^-] \quad (A)$$

Reactions (1) and (2) combined have the overall rate constant k_{SOD}. The comparatively low k_{DMPO} value permits competition assays for relatively small SOD concentrations, since k_{SOD} values approach rate constants for diffusion-controlled reactions [7,22,23].

A dismutase assay based on competition between SOD and DMPO for O_2^- has been described [19]. The assay used a xanthine–xanthine oxidase O_2^--generating system rather than an initial introduction of potassium superoxide (KO_2) as described in the present study. The decrease in DMPO–O_2^--spin-adduct concentration was measured at various SOD concentrations and found to be a logarithmic function of the enzyme concentration. The concentration of SOD that reduced the DMPO–O_2^- spin adduct concentration by 50% (ID_{50}) was determined, and eqn. (A) was used to calculate k_{SOD} values for Cu,Zn-SOD, Fe-SOD, Mn-SOD, and additional O_2^- scavengers.

Abbreviations used: SOD, superoxide dismutase; O_2^-, superoxide (anion); DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; DMSO, dimethyl sulphoxide; ID_{50}, concentration of SOD that reduced the DMPO–O_2^--spin-adduct concentration by 50%; Desferal, desferrioxamine mesylate; KO_2, potassium superoxide.

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The search for low-molecular-mass SOD mimics has been extensive, because such compounds could augment endogenous cellular enzyme levels [24–28]. Recently, Mn complexes of desferrioxamine mesylate (Desferal) have been prepared and examined for SOD-enzyme-mimetic activity [29,30]. Two of these Mn–Desferal complexes appear to have O_2^--dismutation activities equal or superior to Mn^{2+} when assayed by the cytochrome *c* competition assay [31]. However, EDTA eliminated the O_2^- dismutase activity of each complex and that of Mn^{2+} [31,32]. In addition, there are numerous subtle artifacts that may undermine attempts to assay dismutase activities of metals or metal complexes by the cytochrome *c* assay method [33]. First, oxidized cytochrome *c* may be reduced directly by the metal or metal complexes [34,35]. Conversely, reduced cytochrome *c* may be oxidized by the metal or metal complexes [36,37]. Also, the test compound may inhibit the enzymic production of O_2^- by the xanthine–xanthine oxidase generating system. Finally, rapid reoxidation of the reduced metal intermediate by molecular oxygen (reversal of reaction 1) must be considered in assays generating a low steady-state concentration of O_2^- [33]. These problems have led to numerous attempts to develop an alternative method for determining SOD activity. The present paper reports the utilization of an e.s.r. method based on competition for O_2^- between DMPO and authentic SOD enzymes or suspected mimics.

EXPERIMENTAL

Bovine erythrocyte Cu,Zn-SOD, *Escherichia coli* Mn-SOD and Fe-SOD, Desferal, horse heart cytochrome *c*, xanthine, xanthine oxidase, L-ascorbic acid, potassium superoxide and Hepes buffer were obtained from Sigma (St. Louis, MO, U.S.A.). $MnSO_4$ and phosphates for buffers were obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) and manganese(IV) oxide (MnO_2) was from Alfa Products (Ward Hill, MA, U.S.A.). H.p.l.c.-grade dimethyl sulphoxide (DMSO) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Chelex 100 cation-exchange resin was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Reagents were used without further purification. All reagent preparations and transfers were done using polyethylene or polypropylene labware to avoid possible undesirable trace-metal-ion contamination from glassware. Metal-free water was used in all solutions. This water was obtained by stirring (4–6 h; room temperature) Chelex 100 resin (1%, w/v) with NANO-pure water obtained from a Sybron/Barnstead NANO-pure system. The water was then filtered using a 0.2 μm-pore-size disposable plastic filter and stored refrigerated for further use. Buffers were treated again with Chelex 100 resin and the pH was adjusted to 7.8 before use.

Solutions of SOD enzymes (0.01–100 nM) and manganese complexes (0.01–10 μM) were prepared in metal-free phosphate or Hepes buffers (5 mM, pH 7.8). The specific activity of SOD enzymes measured by the cytochrome *c* assay method was used to determine enzyme concentrations in solutions used in the kinetic experiments [1]. The green MnO_2–Desferal and pink MnO_2–Desferal ascorbate complexes were synthesized as described elsewhere [31] and assayed in kinetic experiments the same day. $MnSO_4$ solutions were prepared in metal-free water and then diluted to the required concentration (0.01–10 μM) in buffer.

The spin trap DMPO obtained from Aldrich was shown to be free of radical impurities by e.s.r. before its use in competition experiments. DMPO concentrations were determined spectrophotometrically (wavelength 227 nm; $\varepsilon = 8 \times 10^3$ M^{-1}·cm^{-1}) [38,39].

The kinetic experiments were done by adding O_2^- directly to buffered (pH 7.8) SOD, MnO_2–Desferal, MnO_2–Desferal–ascorbate or Mn^{2+} solutions containing DMPO (0.2 M). The final pH of reaction mixtures after completion of the experiments was pH 7.7±0.1. Saturated O_2^- stock solutions were prepared by dissolving KO_2 in dry DMSO in capped plastic test tubes. The test tubes were fitted with a Teflon-covered syringe needle through which dry nitrogen gas was continuously bubbled to eliminate air and humidity above the solutions. In kinetic experiments, combined e.s.r. spectra of DMPO–O_2^-/DMPO–OH spin adducts recorded 30–45 s after addition of sufficient KO_2 to yield 20 μM-O_2^- in reaction mixtures. Each point in the kinetic plots represents an average of at least five separate determinations made with different enzyme or manganese preparations. Since it is known that DMPO–O_2^- decomposes forming DMPO–OH [17,38,40], the spectrometer modulation amplitude was increased to 1.0 mT in order to combine the e.s.r. spectra of the DMPO–O_2^- and DMPO–OH into a single e.s.r. spectrum. This procedure permits a more accurate measurement of the O_2^- trapped by DMPO. The O_2^- concentration in the KO_2 stock solution was measured by double integration of the first-derivative DMPO–O_2^- e.s.r. spectrum after addition of a known volume of KO_2 solution to 1.0 ml of DMSO containing DMPO (0.2 M). The stable free radical 3-carboxyproxyl was the concentration standard used to estimate that the KO_2 stock solution was 2 mM, based on spectral integration and a 91% spin-trapping efficiency of O_2^- by DMPO in DMSO [41]. Spin-adduct concentrations in kinetic experiments were also determined by double integration of their first-derivative e.s.r. spectra. Furthermore, to avoid possible complications involving the presence of multiple spin adducts (DMPO–O_2^- and DMPO–OH) having different lifetimes in aqueous solutions, double integrations were carried out using the complete e.s.r. spectrum of the DMPO spin adducts. This was achieved by rapidly scanning the spectrum over a large scan range and using a high modulation amplitude. The e.s.r. spectrum obtained in this manner is virtually collapsed into a single peak.

After recording an e.s.r. spectrum for double integration, an e.s.r. spectrum of a separate identical reaction mixture was recorded with the spectrometer set for the kinetic experiments. This allows the peak-to-peak height of an individual e.s.r. line to be correlated with the concentration of DMPO spin adducts, since all e.s.r. spectra in the kinetic experiments were taken under identical conditions and no changes were observed in the e.s.r. linewidths. Hereafter, the low-field e.s.r. peak used to determine the total spin-adduct concentration present will be referred to as '[DMPO–O_2^-]'.

All e.s.r. spectra were recorded on a Varian E-109 X-band spectrometer at 100 kHz magnetic-field modulation. The magnetic field was set at 340.0 mT, microwave frequency at 9.510 GHz, microwave power at 10 mW and the time constant at 0.128 s. The instrument settings for kinetic experiments and for double integration respectively were: modulation amplitude: 1.0 mT and 4.0 mT; scan time: 2 min and 30 s; scan width: 10 mT and 50 mT. For Mn^{2+} e.s.r. spectra the magnetic field was set at 370.0 mT, microwave frequency at 9.510 GHz, microwave power at 10 mW, time constant at 0.25 s, scan time at 4 min and scan width at 200.0 mT.

The data generated by competition assays were analysed by assuming that a steady state was established during the reactions. The measured final concentration of DMPO–O_2^- spin adduct in the absence of dismutase, $[\text{DMPO–}O_2^-]_0$, is a function of the velocities of both the reaction of O_2^- with DMPO, spontaneous O_2^- dismutation at pH 7.8 (V_{SPON}; reactions 3 and 4), plus scavenging of O_2^- by DMPO–O_2^- and DMPO–OH spin adducts (V_{SA}):

$$[\text{DMPO–}O_2^-]_0 = f(V_{DMPO}, V_{SPON} \text{ and } V_{SA})$$

The measured final concentration of DMPO–O_2^- spin adduct in the presence of dismutase, [DMPO–O_2^-]$_f$, is a complex function:

$$[\text{DMPO–}O_2^-]_f = f(V_{\text{DMPO}}, V_{\text{SPON}}, V_{SA} \text{ and } V_{\text{SOD}})$$

Eqn. (B) can be derived from the steady-state model plus the fact that $[O_2^-] = K_m$ when the competition condition shown in eqn. (A) is satisfied [13–15]:

$$([\text{DMPO–}O_2^-]_0/[\text{DMPO–}O_2^-]_f) - 1 = k_{\text{SOD}}[\text{SOD}]/k_{\text{DMPO}}[\text{DMPO}] \quad \text{(B)}$$

The equation may be applied to the reaction mixtures used experimentally to determine the order with respect to SOD or dismutase [42].

Double-reciprocal plots of percentage inhibition as a function of dismutase concentration have been described [43–45]. Percentage inhibition for the reaction mixtures is:

$$100 \times \frac{[\text{DMPO–}O_2^-]_0 - [\text{DMPO–}O_2^-]_f}{[\text{DMPO–}O_2^-]_0}$$

[DMPO–O_2^-]$_0$ and [DMPO–O_2^-]$_f$ must be proportional to [O_2^-], which varies inversely with the logarithm of the SOD concentration.

RESULTS

Although the reaction between O_2^- and DMPO initially generates the DMPO–O_2^- spin adduct (reaction 4), it is well known that this spin adduct has a half-life of about 35 s at pH 7.8, decomposing into products including DMPO–OH [17,39,40]. Therefore accurate measurement of O_2^- in an aqueous solution by spin trapping with DMPO is difficult, and care must be taken to develop a method which accounts for all the O_2^- that has reacted with DMPO. In most cases when O_2^- reacts with DMPO in an aqueous environment, a superimposition of the e.s.r. spectra corresponding to DMPO–O_2^- and DMPO–OH is observed. The e.s.r. spectra of these spin adducts have been well characterized, with hyperfine coupling constants $a_N = 1.41$ mT, $a_H = 1.13$ mT and $a_H = 0.125$ mT for DMPO–O_2^-, and, for DMPO–OH, $a_N = a_H = 1.49$ mT [46]. Fig. 1(*a*) shows the twelve-line DMPO–O_2^- e.s.r. spectrum superimposed on the typical DMPO–OH 1:2:2:1 quartet (marked by arrows) generated after addition of KO_2. A similar spectrum can be generated in 5 mM-Hepes buffer, pH 7.8. Since the DMPO–OH originates from the DMPO–O_2^- in an aqueous solution, a more accurate representation and measurement of the total amount of O_2^- reacting with DMPO will be a combination of the DMPO–O_2^- and DMPO–OH e.s.r. spectral intensities. Fig. 1(*b*) shows the e.s.r. spectrum obtained when the contributions from the DMPO–O_2^- and DMPO–OH e.s.r. peaks in Fig. 1(*a*) are combined into single peaks. This spectrum was generated after an increase in the instrument modulation amplitude from 0.05 mT (Fig. 1*a*) to 1.0 mT (Fig. 1*b*). Since the intensity of the e.s.r. signal is directly proportional to the concentration of the species being measured, double integration of the first-derivative spectrum in Fig. 1(*b*) allows determination of the total concentration of spin adducts originating from the reaction between O_2^- and DMPO. This type of e.s.r. measurement is essential in studies involving competition-kinetic reactions using O_2^- and DMPO.

The spectrum in Fig. 1(*b*) represents a combination of the e.s.r. spectra of DMPO–O_2^- and DMPO–OH spin adducts, each of which has a different stability. The combined e.s.r. spectrum's decay rate was examined to determine whether kinetic measurements could be made after O_2^- addition. Fig. 2(*a*) shows that the decay with time of the low-field e.s.r. peak in Fig. 1(*b*) was relatively small over a 3 min time period; 30 s were required to start the e.s.r. measurement after O_2^- addition. All kinetic experiments measured the e.s.r. signal 30 to 45 s after O_2^- addition (arrows) to take advantage of the relatively modest change in signal intensity over that time span.

On the basis of preliminary empirical results, a 0.2 M-DMPO concentration was chosen for reaction mixtures. The optimal initial O_2^- concentration was then determined which satisfied two experimental criteria. First, the resultant e.s.r. signal from the chosen O_2^- concentration in 0.2 M-DMPO had to be large

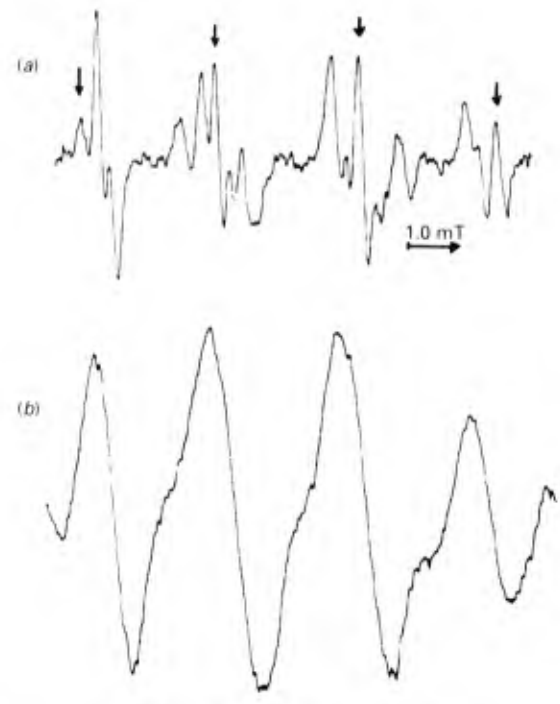

Fig. 1. E.s.r. spectra of DMPO adducts after O_2^- addition

(*a*) An e.s.r. spectrum of DMPO–O_2^- and DMPO–OH adducts at an instrument modulation amplitude of 0.05 mT. Other instrument settings are listed in the Experimental section. The DMPO–OH 1:2:2:1 quartet is marked by arrows in the combined DMPO–O_2^- and DMPO–OH spectrum. (*b*) An e.s.r. spectrum of a solution identical with the one used in (*a*) with the same instrument settings, except the instrument modulation amplitude was changed to 1.0 mT.

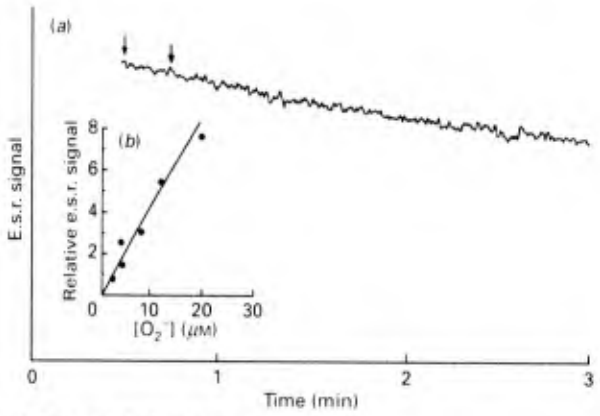

Fig. 2. E.s.r. spectral responses

(*a*) Reduction of the combined DMPO–O_2^- and DMPO–OH adducts with time. The instrument magnetic field was locked on the low-field e.s.r. peak in Fig. 1(*b*) and the scan range was set to zero. Measurement began 30 s after O_2^- addition to the reaction mixture. Inset (*b*): relative e.s.r. signal intensity as a function of O_2^- concentration. Instrument settings were those for kinetic experiments in the Experimental section.

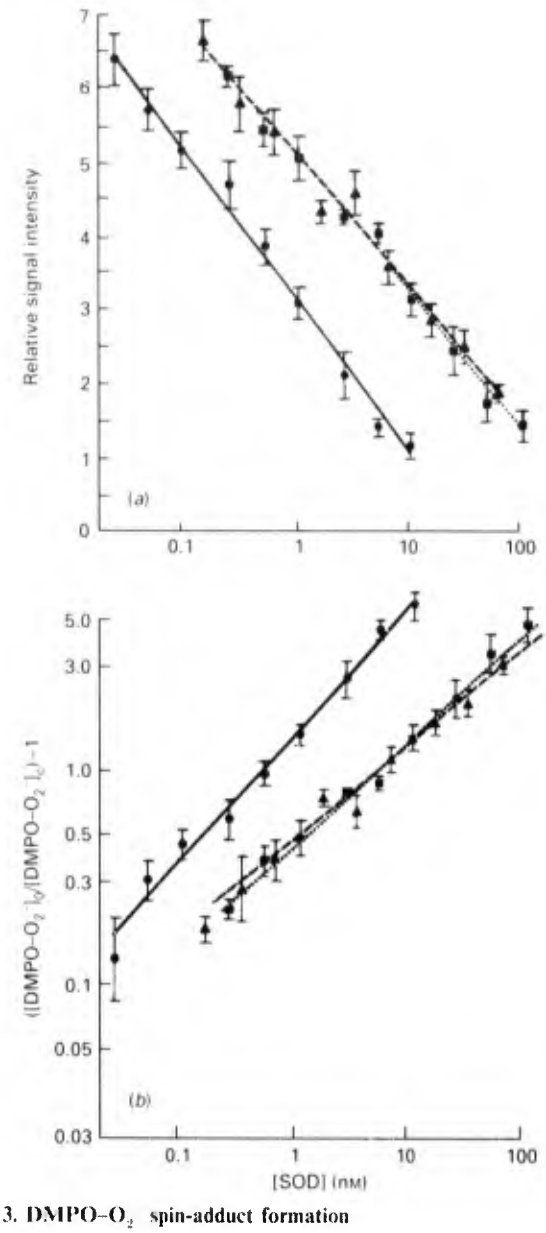

Fig. 3. DMPO–O_2^- spin-adduct formation

(*a*): ●, Spin-adduct formation at increasing [Cu,Zn-SOD]; ▲, at increasing [Mn-SOD]; ■, at increasing [Fe-SOD]. Bars represent the S.E.M. for each value. The straight-line correlation coefficients are −0.95 for Cu,Zn-SOD, −0.95 for Fe-SOD and −0.93 for Mn-SOD data. (*b*) Graphical representations of the logarithm of the overall reaction velocities, $([DMPO\text{–}O_2^-]_0/[DMPO\text{–}O_2^-]_c)-1$, as a function of log[SOD]. ●, Cu,Zn-SOD; ▲, Mn-SOD; ■, Fe-SOD. Bars represent the S.E.M. at each value. Cu,Zn-SOD data have a straight-line correlation coefficient of 0.95, Fe-SOD has a value of 0.93, and Mn-SOD has a value of 0.95.

Table 1. ID_{50} values for SOD species, Mn^{2+} standard and Mn^{2+} complexes

The ID_{50} values for either SOD enzyme or Mn^{2+} were determined by the analytical methods shown; 95% confidence intervals are shown in brackets. The Mn^{2+} standard was prepared from $MnSO_4$. Mn^{2+} in complexes was measured by e.s.r. and adjusted to concentrations equal to those of the standards.

	ID_{50} (M)	
Graphical method of determination ... Compound	$[DMPO\text{–}O_2^-]_t$ versus log (concn.)	1/Percentage inhibition versus $1/[SOD]^{0.5}$ or $1/[Mn^{2+}]$
Cu,Zn-SOD	$0.53\ [0.44, 0.64]\times10^{-9}$	$0.59\ [0.35, 0.83]\times10^{-9}$
Fe-SOD	$5.5\ [4.6, 6.6]\times10^{-9}$	$5.4\ [4.0, 6.8]\times10^{-9}$
Mn-SOD	$5.9\ [4.7, 7.6]\times10^{-9}$	$4.7\ [3.0, 6.4]\times10^{-9}$
Mn^{2+} standard	$1.4\ [1.1, 1.5]\times10^{-6}$	$1.7\ [-0.70, 4.1]\times10^{-6}$
Mn^{2+} complexes	$1.2\ [1.0, 2.0]\times10^{-6}$	$1.3\ [0.71, 1.9]\times10^{-6}$

enough to be conveniently measured. Secondly, the initial O_2^- concentration selected had to be in the linear response range for e.s.r. signal intensity as a function of O_2^- concentration. Fig. 2(*b*) shows results obtained after the addition of increasing O_2^- concentrations to 0.2 M-DMPO in 5 mM-phosphate buffer, pH 7.8. There is an apparently linear relationship between the e.s.r. relative signal intensity and $[O_2^-]$ up to 20 μM under the assay conditions described. There was a loss of linear response above 20 μM-O_2^- in our reaction mixtures. About 50% of the 20 μM initial O_2^- concentration was trapped by 0.2 M-DMPO in reaction mixtures, with the remainder being lost through spontaneous dismutation (reaction 3). Therefore, a 20 μM-O_2^- initial concentration was selected for kinetic assays because it best satisfied both the above experimental criteria.

The relative e.s.r. signal was measured at increasing SOD concentrations in reaction mixtures with 5 mM-phosphate buffer, pH 7.8, 0.2 M-DMPO, and initial O_2^- concentrations of 20 μM. Fig. 3(*a*) shows the linear correlation between DMPO spin adducts formed and log[SOD] for each enzyme. The linear relationship is maintained over two logarithmic cycles of SOD concentration. At enzyme concentrations above or below those shown, the linear relationship is not followed. There is no significant difference between Fe-SOD and Mn-SOD ($P > 0.1$), but the slope of the Cu,Zn-SOD line differs from the slopes of lines for each of the other enzymes ($P < 0.05$). The ID values are shown in Table 1.

The data generated in the reaction mixtures can be analysed to determine the order of SOD in the overall reaction [42]. Plots of the $\log([DMPO\text{–}O_2^-]_0/[DMPO\text{–}O_2^-]_c)-1$ versus log[SOD] (eqn. B) are shown in Fig. 3(*b*). The Cu,Zn-SOD line has a slope of 0.59, Fe-SOD data have a slope of 0.48, and Mn-SOD has a slope of 0.44. The average of the slopes for all three enzymes is 0.5. This unexpected result indicates that, in these competition assays, SOD acts as if it were a half-order molecular species.

The slope of approx. 0.5 for enzymes in Fig. 3(*b*) indicates that the data would be curvilinear on a linear scale, increasing rapidly at low enzyme concentrations and levelling off at higher enzyme concentrations. In addition to an apparent half-order reaction to explain the data, there are two other possible reasons for the curvilinear direct plot. It is possible that H_2O_2 causes reversibility of reaction (2) at higher enzyme concentrations. More O_2^- could be generated and trapped by DMPO, resulting in a smaller value for $[DMPO\text{–}O_2^-]_0/[DMPO\text{–}O_2^-]_c$. Furthermore, it has recently been proposed that H_2O_2 reacts with SOD yielding hydroxyl radicals, which could also be trapped by DMPO, yielding a lower-than-expected $[DMPO\text{–}O_2^-]_0/[DMPO\text{–}O_2^-]_c$ ratio [47]. In order to determine the effect of H_2O_2 on the DMPO-spin-adduct e.s.r. spectra, the H_2O_2 formed in reaction mixtures was removed by addition of 500 units of catalase/ml before O_2^- addition. A second series of control experiments incorporated a fivefold excess of H_2O_2 (50 μM) in reaction mixtures before O_2^- addition. Neither removal of H_2O_2 by catalase nor prior enhancement of the H_2O_2 concentration had an effect on the DMPO-spin-adduct e.s.r. spectra. Another possible factor at low enzyme concentrations could be reaction of O_2^- with DMPO–O_2^-

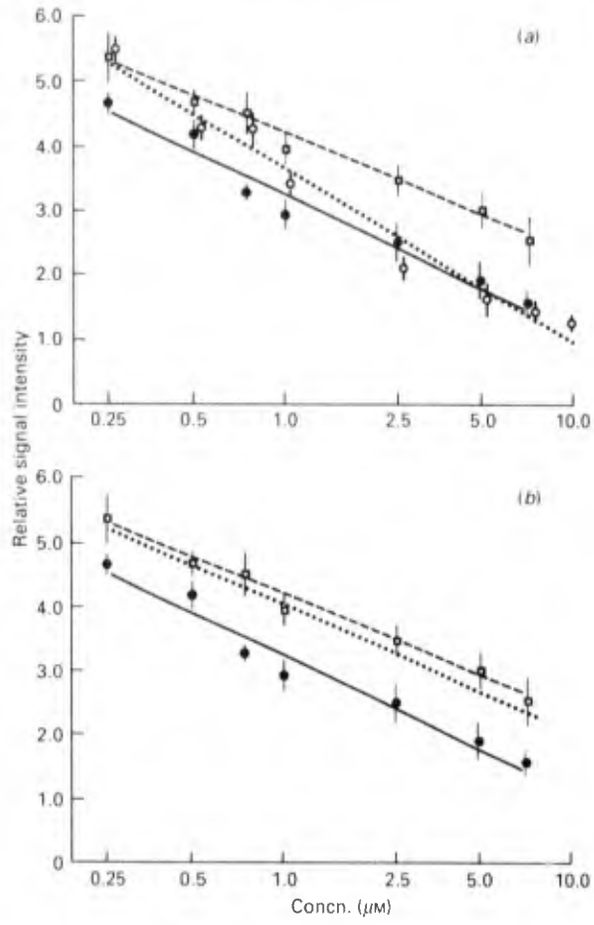

Fig. 4. DMPO–O_2^- spin-adduct formation

(*a*) Spin adduct formation: ●, at increasing [$MnSO_4$]; □, at increasing MnO_2 Desferal; ○, at increasing [MnO_2 Desferal – ascorbate]. The straight-line correlation coefficients are −0.91 for $MnSO_4$, −0.84 for MnO_2 Desferal and −0.95 for MnO_2 Desferal–ascorbate. (*b*) Spin-adduct formation for $MnSO_4$ (●), MnO_2 Desferal (□) and MnO_2 Desferal ascorbate corrected for ascorbate (·····).

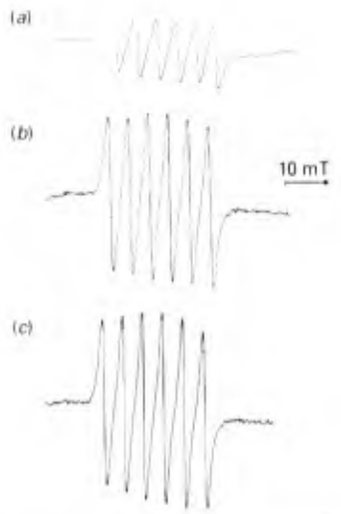

Fig. 5. E.s.r. spectra of 0.1 mM-$MnSO_4$ in three solvents

(*a*) 5 mM-Phosphate buffer, pH 7.8; (*b*) solution in (*a*) acidified below pH 5.5; (*c*) 5 mM-Hepes buffer, pH 7.8.

or DMPO–OH spin adducts [20,21]. However, at low enzyme concentrations the relative e.s.r. signal intensity decreased linearly, indicating that scavenging of O_2^- by the spin adducts is unimportant. Since H_2O_2 or reaction of O_2^- with DMPO–O_2^- and DMPO–OH appear to have no effect on the results, the most likely explanation is an apparent half-order process.

The data in Fig. 3(*a*) were analysed by double-reciprocal plots of percentage inhibition versus [SOD]. These data only approximate straight lines as the SOD concentrations varied. Double-reciprocal plots of percentage inhibition as a function of $[SOD]^{0.5}$ more closely fit a straight line for each enzyme. The straight-line correlation coefficients and ordinate intercepts for Cu,Zn-SOD, Mn-SOD and Fe-SOD were, respectively, 0.82, 0.89 and 0.88, and 1.11 and 0.93 and 1.03.

The relative e.s.r. signal was measured at increasing manganese, MnO_2–Desferal or MnO_2–Desferal–ascorbate concentrations in 5 mM-phosphate buffer, pH 7.8, as described for SOD above. Fig. 4(*a*) indicates the linear relationship between DMPO spin adducts formed and the logarithm of the concentration for each preparation. The slope of the MnO_2–Desferal–ascorbate line indicated a possible contribution to reduction of the e.s.r. signal by increasing ascorbate at higher concentrations. Ascorbate was measured at the concentrations used to generate data in Fig. 4(*a*), and the point-by-point correction resulted in the data shown in Fig. 4(*b*). The MnO_2–Desferal–ascorbate preparation corrected for ascorbate yields results similar to the MnO_2–Desferal preparation. Moreover, both Desferal preparations were different from the Mn^{2+} standard, which appeared more active by a factor of 3 as an O_2^- scavenger. ID_{50} values were 0.58×10^{-6} M for Mn^{2+}, 1.7×10^{-6} M for MnO_2–Desferal and 1.4×10^{-6} M for MnO_2–Desferal–ascorbate.

A 5 mM-potassium phosphate buffer, pH 7.8, was used for SOD enzyme experiments described above. However, it is known that Mn^{2+} forms hydroxides or phosphates at basic pH. The e.s.r. spectra were obtained of 0.1 mM-$MnSO_4$ samples in different solvents, and the results are shown in Fig. 5. The Mn^{2+} e.s.r. spectrum was present in each solution, but 5 mM-phosphate buffer, pH 7.8, clearly decreased the relative signal intensity. The relative e.s.r. signal intensity was equal for Mn^{2+} in acidified phosphate or 5 mM-Hepes, pH 7.8. The e.s.r. signal intensity observed in Fig. 5(*b*) represents total Mn^{2+} present in solution. Since the intensity of e.s.r. signals shown in Figs. 5(*b*) and 5(*c*) are equal, the results suggest that Hepes buffer stabilizes total free Mn^{2+} sufficiently to allow quantification. Results similar to those in Fig. 5 were obtained for each complex in Hepes and phosphate buffer at pH 7.8.

Kinetic experiments were done with $MnSO_4$ standards, MnO_2–Desferal complex and MnO_2–Desferal–ascorbate complexes in 5 mM-Hepes, pH 7.8. The concentrations of Mn^{2+} in each of the complexes were adjusted to equal the Mn^{2+} concentration measured by e.s.r. in standards prepared from $MnSO_4$. This procedure ensures different concentrations of $MnSO_4$, MnO_2–Desferal complex and MnO_2–Desferal–ascorbate complex. However, the free Mn^{2+} concentration is nearly equal in each preparation. Fig. 6(*a*) shows the results of these kinetic determinations. There appears to be a correlation between the relative e.s.r. signal and the logarithm of the Mn^{2+} concentration over nearly two logarithmic cycles. There was a decrease in the e.s.r. relative signal intensity in the absence of dismutase in 5 mM-Hepes, pH 7.8, compared with 5 mM-phosphate, pH 7.8, from 7.6 to 5.4 units. The decrease may reflect a reaction of O_2^- with 5 mM-Hepes and was considered equal in each solution. There appears to be a slight difference in the slopes of MnO_2–Desferal–ascorbate lines compared with either Mn^{2+} or MnO_2–Desferal. The presence of ascorbate probably accounts for this difference. However, statistically there is no difference ($P > 0.1$) between

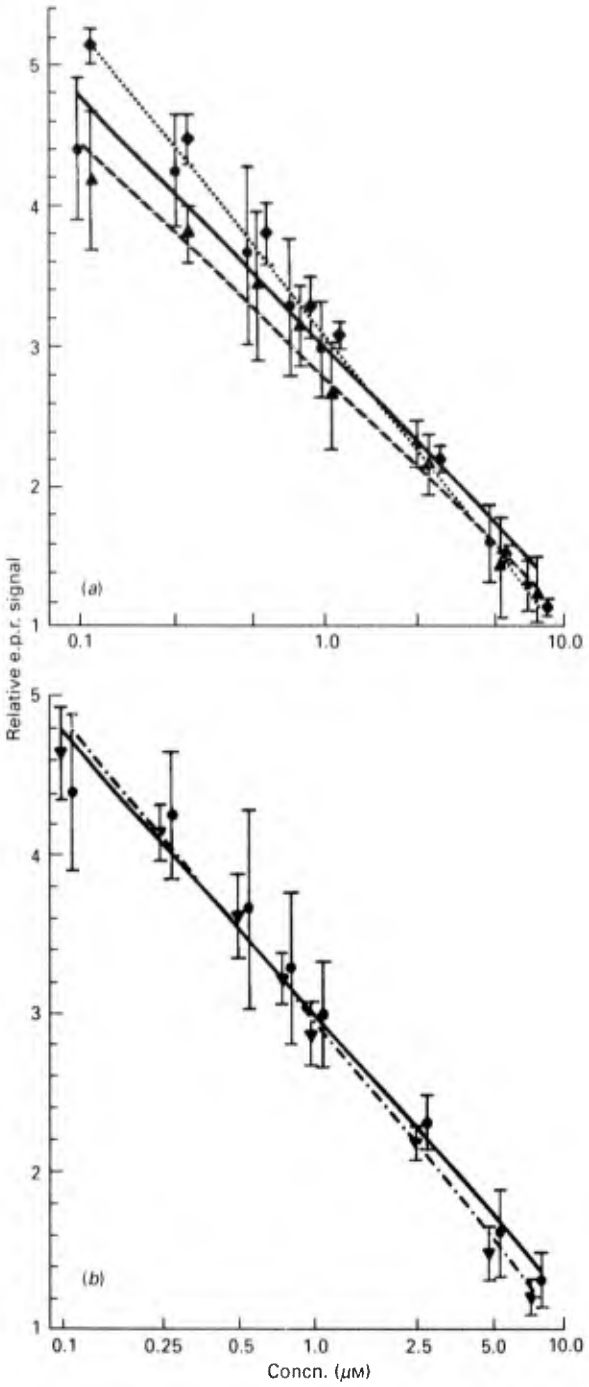

Fig. 6. DMPO spin-adduct formation for Mn^{2+} and complexes

(*a*) Spin-adduct formation: ●, at increasing $[Mn^{2+}]$; ▲, at increasing [MnO_2–Desferal complex]; ◆, at increasing [MnO_2–Desferal–ascorbate]. (*b*) Spin-adduct formation: ●, at increasing $[Mn^{2+}]$; ▼, data for the MnO_2–Desferal and MnO_2–Desferal–ascorbate were combined. The straight-line correlation coefficients are −0.91 for $MnSO_4$ standards and −0.83 for the combined-complex data.

each of the slopes of the three lines, suggesting that dismutase activity in solutions of the two Mn^{2+} complexes resides in Mn^{2+}. Fig. 6(*b*) shows results from kinetic experiments comparing combined MnO_2–Desferal and MnO_2–Desferal–ascorbate-complex data with data for the $MnSO_4$ standard. The probability that the lines differ is $P > 0.1$. The calculated ID_{50} for each of the lines is listed in Table 1.

The data for Mn^{2+} in $MnSO_4$ standards and Mn^{2+} for the combined complexes were analysed by double-reciprocal plots of percentage inhibition versus $[Mn^{2+}]$. Linear plots were generated and ID_{50} values estimated at the 50%-inhibition point (Table 1). Further, data from Table 1 were used to calculate velocity constants shown in Table 2.

Table 2. Second-order rate constants for SOD species and Mn^{2+}

Rate constants for O_2^- reaction with SOD enzymes and Mn^{2+} were estimated. Determinations were made from average ID_{50} values from Table 1 on the basis of the equality expression (A) (see the text). The 95% confidence intervals are shown in square brackets.

Compound	Rate constant ($M \cdot s^{-1}$)
Cu,Zn-SOD	$6.4\,[4.9, 7.9] \times 10^9$
Fe-SOD	$6.6\,[5.5, 7.7] \times 10^8$
Mn-SOD	$6.8\,[5.4, 8.2] \times 10^8$
Mn^{2+} standard	$2.3\,[0.46, 4.1] \times 10^6$
Mn^{2+} complexes	$2.9\,[2.2, 3.6] \times 10^6$

DISCUSSION

The observation that the DMPO–O_2^- spin adduct e.s.r. signal is inversely proportional to log[SOD] for each of the three enzymes tested supports the validity of this competition assay. A similar result was reported for Cu,Zn-SOD using DMPO as a spin trap and a xanthine–xanthine oxidase O_2^- generator [19]. A number of studies based on competition assays for SOD activity indicate that inhibition of the reaction of O_2^- with detector molecule is a function of log[SOD] [43–45]. Therefore it is reasonable to conclude that the measured DMPO–O_2^- spin adduct e.s.r. signal is a function of the O_2^- concentration, which in turn depends on log[SOD]. In the above experiments, the rate of spontaneous O_2^- dismutation or reaction of O_2^- with the DMPO–O_2^- and DMPO–OH adducts formed was ineffective in masking the enzymic contribution to O_2^- removal over the linear ranges of the assays.

The unexpected finding that competition reaction mixtures are apparently half-order with respect to SOD may be explained if steady-state conditions are present. Redox cycling of SOD occurs when $[O_2^-] \gg [SOD]$, establishing conditions where both the M^{n+}–SOD and $M^{(n-1)+}$–SOD forms of the enzyme compete against DMPO for O_2^- (reactions 1 and 2). Also, fast-kinetic results have shown that, under less-than-saturating O_2^- concentrations, the rate-limiting step for the Cu,Zn-SOD and Fe-SOD enzymes is reaction with O_2^- [11,12]. Reactions (1) and (2) may then be represented as the following steady-state expression:

$$M^{n+}\text{–SOD} + O_2^- \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} O_2 + M^{(n-1)+}\text{–SOD}$$

$$+ O_2^- + 2H^+ \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} H_2O_2 + M^{n+}\text{–SOD}$$

In this case, $k_{-2} \ll k_2$, M^{n+}–SOD–O_2^- and $M^{(n-1)+}$–SOD–O_2H_2 are comparatively small, and the steady-state intermediate is $M^{(n-1)+}$–SOD. Starting with the expression $k_{+1}[O_2^-][M^{n+}\text{–SOD}] = (k_{+2} + k_{-1})[M^{(n-1)+}\text{–SOD}]$, it can be shown that:

$$[M^{n+}\text{–SOD}][O_2^-]/[M^{(n-1)+}\text{–SOD}] = (k_{+2} + k_{-1})/k_{+1} = K_m$$

If SOD is defined as the total concentration of enzyme in all forms, then the following expression results:

$$(\text{SOD} - [M^{(n-1)+}\text{–SOD}])[O_2^-]/[M^{(n-1)+}\text{–SOD}] = K_m$$

Rearrangement results in an expression similar to the Briggs–Haldane equation [48]:

$$[M^{(n+1)+}\text{–SOD}] = \text{SOD}\,[O_2^{\cdot-}]/([O_2^{\cdot-}] + K_m)$$

At half-maximal velocity, by definition, $[O_2^{\cdot-}] = K_m$. This necessary condition for steady-state kinetics substituted into the equation analogous to the Briggs–Haldane expression yields the result $[M^{(n+1)+}\text{–SOD}] = 0.5\,\text{SOD}$ with $[M^{(n+1)+}\text{–SOD}] \approx [M^{n+}\text{–SOD}]$. Therefore competition reactions between DMPO and nearly equal concentrations of M^{n+}–SOD and $M^{(n+1)+}$–SOD for $O_2^{\cdot-}$ result in an apparent half-order with respect to SOD. It must be stressed that this apparent half-order applies specifically to competition reactions when $[O_2^{\cdot-}] \gg [\text{SOD}]$. Often a low steady state $[O_2^{\cdot-}]$ generated enzymically and higher SOD concentrations are employed to estimate dismutase levels [1–3,15,19].

There are limitations imposed when attempting to assay $O_2^{\cdot-}$ dismutation using a competition method. One important factor is accuracy of the value of k_{DMPO} for estimates of k_{SOD}. The value $k_{\text{DMPO}} = 18\ \text{M}^{-1}\cdot\text{s}^{-1}$ was used in the present study, but there is considerable variation reported for this rate constant [18,49]. The accuracy of values for k_{SOD} reported above will be directly related to the accuracy of the k_{DMPO} value chosen for calculation. In addition, second-order rate constants determined from average ID_{50} values are higher than many previous determinations [7,11,12,22,50]. Elevated k_{SOD} values in Table 2 are probably based on median ID_{50} values that are too low. Spontaneous dismutation plus $O_2^{\cdot-}$ reaction with DMPO–$O_2^{\cdot-}$ or DMPO–OH spin adducts may be causing underestimation of ID_{50} values and overestimation of velocity constants in the present study.

The competition between DMPO and Mn^{2+} for $O_2^{\cdot-}$ occurs in the micromolar Mn^{2+} concentration range. Relatively high Mn^{2+} concentrations are required because the reaction rate constant of $O_2^{\cdot-}$ with Mn^{2+} is much lower than k_{SOD} in eqn. (A). The increased Mn^{2+} concentration required approaches the 20 μM-$O_2^{\cdot-}$ concentration initially present in reaction mixtures. This relative reduction in the ratio of $O_2^{\cdot-}$ to Mn^{2+} compared with the higher analogous ratio with the SOD enzymes may result in a simple chemical reaction between Mn^{2+} and $O_2^{\cdot-}$ rather than a dismutation reaction. Mn^{2+} competition with DMPO using these limiting $O_2^{\cdot-}$ concentrations would not permit steady-state redox cycling of Mn^{2+} in competition assays.

An additional major finding of the present study is that the SOD activity of the MnO_2–Desferal and MnO_2–Desferal–ascorbate complexes is correlated with the Mn^{2+} concentration present in the preparations. Since Mn^{2+} is always present in either the MnO_2–Desferal or the MnO_2–Desferal–ascorbate complexes, it is not possible to demonstrate SOD activity uniquely attributable to either complex or Mn^{2+} in any of the preparations. The possibility exists that SOD activity in complex preparations is due to Mn^{2+}, complex, a combination of both free Mn^{2+} and complex, or additional manganese species acting alone or in combination with any or all of the above materials [31]. Nevertheless, the observation of equivalent SOD activity for $MnSO_4$ standards and manganese-complex preparations adjusted to concentrations of Mn^{2+} equal to those of standards strongly supports the contention that $O_2^{\cdot-}$ removal by complexes is due to Mn^{2+}.

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REFERENCES

1. McCord, J. M. & Fridovich, I. (1969) J. Biol. Chem. **244**, 6049–6055
2. Keele, B. B., Jr., McCord, J. M. & Fridovich, I. (1970) J. Biol. Chem. **245**, 6176–6181
3. Yost, F. J., Jr. & Fridovich, I. (1973) J. Biol. Chem. **248**, 4905–4908
4. Dougherty, H. W., Sadowski, S. J. & Baker, E. E. (1978) J. Biol. Chem. **253**, 5220–5223
5. Steinman, H. M. (1982) in Superoxide Dismutase (Oberley, L. W., ed.), vol 1, pp. 11–68, CRC Press, Boca Raton, FL
6. Fridovich, I. (1986) Ad. Enzymol. Relat. Areas Mol. Biol. **58**, 61–97
7. Klug, D., Rabani, J. & Fridovich, I. (1972) J. Biol. Chem. **247**, 4839–4842
8. Pick, M., Rabani, J., Yost, F. & Fridovich, I. (1974) J. Am. Chem. Soc. **96**, 7329–7333
9. Lavelle, F., McAdam, M. E., Fielden, E. M., Roberts, P. B., Puget, K. & Michelson, M. (1977) Biochem. J. **161**, 3–11
10. Osman, R. & Basch, H. (1984) J. Am. Chem. Soc. **106**, 5710–5714
11. Bull, C. & Fee, J. A. (1985) J. Am. Chem. Soc. **107**, 3295–3304
12. Fee, J. A. & Bull, C. (1986) J. Biol. Chem. **261**, 13000–13005
13. Michaelis, L. & Menten, M. L. (1913) Biochem. Z. **49**, 333–369
14. Adams, G. E., Boag, J. W., Currant, J. & Michael, B. D. (1965) in Pulse Radiolysis (Ebert, M., Keene, J. P., Swallow, A. J. & Baxendale, J. H., eds.), pp. 131–143, Academic Press, New York
15. Bors, W., Michel, C. & Saran, M. (1985) in CRC Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., ed.), pp. 181–188, CRC Press, Boca Raton, FL
16. Behar, D., Czapski, G., Rabani, J., Dorfman, L. M. & Schwarz, H. A. (1970) J. Phys. Chem. **74**, 3209–3213
17. Harbour, J. R., Chow, V. & Bolton, J. R. (1974) Can. J. Chem. **52**, 3549–3553
18. Finkelstein, E., Rosen, G. M. & Rauckman, E. J. (1980) J. Am. Chem. Soc. **102**, 4994–4999
19. Mitsuta, K., Mizuta, Y., Kohno, M., Hiramatsu, M. & Mori, A. (1990) Bull. Chem. Soc. Jpn. **63**, 187–191
20. Samuni, A., Murali Krisna, C., Riesz, P., Finkelstein, E. & Russo, A. (1989) Free Radicals Biol. Med. **6**, 141–148
21. Buettner, G. R. (1990) Free Radicals Res. Commun. **10**, 11–15
22. Forman, H. J. & Fridovich, I. (1973) Arch. Biochem. Biophys. **158**, 396–400
23. Rabani, J., Klug, D. & Lilie, J. (1973) J. Phys. Chem. **77**, 1169–1175
24. Weinstein, J. & Bielski, B. H. J. (1980) J. Am. Chem. Soc. **102**, 4916–4919
25. Lengfelder, E. & Weser, U. (1981) Bull. Eur. Physiopathol. Respir. **17**, 73–80
26. Darr, D., Zarilla, K. A. & Fridovich, I. (1987) Arch. Biochem. Biophys. **258**, 351–355
27. Kubota, S. & Yang, J. T. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3283–3286
28. Samuni, A., Krishna, C. M., Riesz, P., Finkelstein, E. & Russo, A. (1988) J. Biol. Chem. **263**, 17921–17924
29. Rabinowitch, H. D., Privalle, C. T. & Fridovich, I. (1987) Free Radicals Biol. Med. **3**, 125–131
30. Rabinowitch, H. D., Rosen, G. M. & Fridovich, I. (1989) Free Radicals Biol. Med. **6**, 45–48
31. Beyer, W. F., Jr. & Fridovich, I. (1989) Arch. Biochem. Biophys. **271**, 149–156
32. Archibald, F. S. & Fridovich, I. (1982) Arch. Biochem. Biophys. **214**, 452–463
33. Goldstein, S., Michel, C., Bors, W., Saran, M. & Czapski, G. (1988) Free Radicals Biol. Med. **4**, 295–303
34. Ilan, Y., Ilan, Y. A. & Czapski, G. (1978) Biochem. Biophys. Acta **503**, 399–401
35. Marcus, R. A. & Sutin, N. (1985) Biochem. Biophys. Acta **811**, 2265–2322
36. Augustin, M. A. & Yandell, J. K. (1979) Inorg. Chem. **18**, 577–583
37. Koppenol, W. H., Levine, F., Hatmaker, T. L., Epp, J. & Rush, D. L. (1986) Arch. Biochem. Biophys. **251**, 594–599
38. Buettner, G. R. & Oberley, I. W. (1978) Biochem. Biophys. Res. Commun. **83**, 69–74
39. Kalyanaraman, B., Felix, C. C. & Sealy, R. C. (1982) Photochem. Photobiol. **36**, 5–12
40. Finkelstein, E., Rosen, G. M. & Rauchkman, E. J. (1979) J. Mol. Pharmacol. **16**, 676–685

41. Carmichael, A. J., Samuni, A. & Riesz, P. (1985) Photochem. Photobiol. **41**, 635–642
42. Cornish-Bowden, A. (1976) Principles of Enzyme Kinetics, p. 4, Butterworth and Co., London
43. Kobayashi, Y., Okahata, S., Tanabe, K. & Usui, T. (1978) J. Immunol. Methods **24**, 75–78
44. Eldred, G. E. & Hoffert, J. R. (1981) Anal. Biochem. **110**, 137–143
45. Sun, Y., Oberley, L. & Li, Y. (1988) Clin. Chem. **34**, 497–500
46. Buettner, G. R. (1987) Free Radicals Biol. Med. **3**, 259–303
47. Yim, M. B., Chock, P. B. & Stadtman, E. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5006–5010
48. Briggs, G. E. & Haldane, J. B. S. (1925) Biochem. J. **19**, 338–339
49. Yamazaki, I., Piette, L. H. & Grover, T. A. (1990) J. Biol. Chem. **265**, 652–659
50. Rabani, J., Klug, D. & Fridovich, I. (1972) Isr. J. Chem. **10**, 1095–1106

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Ionizing Radiation and Calcium Channels

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INTRODUCTION

Ionizing radiation alters many neuronal systems that are regulated by calcium ions. Because ionizing radiation reduces voltage-sensitive (VS) calcium uptake in rat brain synaptosomes, experiments were conducted to elucidate the mechanisms of reduced calcium uptake. This paper reviews the work being done in this laboratory.[1-5]

METHODS

A crude, synaptosomal fraction preparation from the brains of male Sprague-Dawley rats and gamma irradiation from a ^{60}Co source at a dose rate of 10 Gy/min were used as described by Mullin *et al.*[6] KCl-stimulated $^{45}Ca^{2+}$ uptake into rat brain synaptosomes was measured as described by Leslie *et al.*[7] [^{3}H]nimodipine binding assays were based on the methods reported by Skattebol and Triggle.[8]

RESULTS

The fastest and highest rate of VS calcium uptake occurred at 3 s with 65 mM KCl. Irradiation (3 Gy and 10 Gy/min) reduced calcium uptake at all incubation times (3, 10, 30, and 60 s) and KCl concentrations (15–65 mM). Irradiation (1–30 Gy) reduced 65 mM KCl-stimulated calcium uptake after 3 s (FIG. 1). Enhancement of 15 mM KCl-stimulated calcium uptake by Bay K 8644 was reduced by irradiation. Nimodipine binding to dihydropyridine (DHP) L-type calcium channel receptors was not altered following irradiation. Prostaglandin $F_2\alpha$ ($PGF_2\alpha$), inositol 1,4,5-trisphosphate (IP3), phorbol esters (PE), Bay K 8644, and interleukin 1α (IL-1α) alone did not reverse the decrease in calcium influx caused by irradiation, but in combination they did reverse it. For example, pretreatment with 1 nM to 100 nM of PE and 1 nM of IP3, 1 nM of Bay K 8644, 10 nM $PGF_2\alpha$, or 20-hr pretreatment with 2,000 units of IL-1α significantly reversed decreases in calcium uptake induced by irradiation (TABLE 1).

DISCUSSION

Ionizing radiation significantly reduced KCl-stimulated, VS calcium uptake in rat brain synaptosomes. The inhibitory effect of irradiation on the VS calcium channels

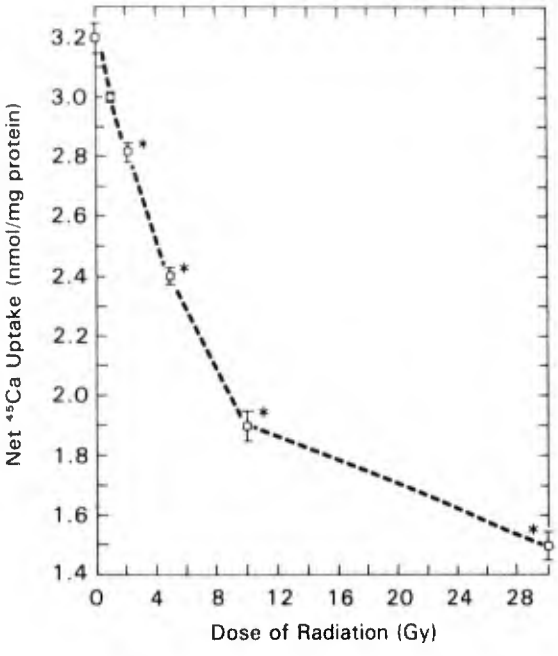

FIGURE 1. Effect of gamma irradiation on 65 mM KCl-stimulated calcium uptake by rat whole-brain synaptosomes, measured during 3-s periods. Points and bars represent mean ±SEM values from three separate experiments, each using triplicate samples. *Significantly different from control value: $p < 0.05$.

was not mediated by DHP receptors because irradiation did not alter nimodipine binding to DHP L-type calcium channel receptors.

If pretreatment with IP3, PE, or $PGF_2\alpha$ reversed the radiation-induced reduction in calcium influx, it would indicate that the formation of IP3, diacylglycerol, and $PGF_2\alpha$ is necessary for calcium influx and that radiation interferes with their

TABLE 1. Effect of PE in Combination with IP3, Bay K 8644, $PGF_2\alpha$, or 2,000 Units of IL-1α on Calcium Uptake (nmol/mg protein) in Irradiated (3 Gy and 10 Gy/min gamma rays) and Nonirradiated Rat Whole-Brain Synaptosomes

Concentration of Drug	Concentration of PE (nM)[a]			
	0	1	10	100
1 nM IP3				
Irradiated	1.9 ± 0.05	3.2 ± 0.05[b]	4.1 ± 0.15[b]	4.6 ± 0.10[b]
Nonirradiated	2.6 ± 0.05	3.6 ± 0.10[c]	4.3 ± 0.10[c]	4.8 ± 0.05[c]
1 nM Bay K 8644				
Irradiated	[illegible] ± 0.10	3.3 ± 0.10[b]	4.0 ± 0.15[b]	4.4 ± 0.15[b]
Nonirradiated	2.8 ± 0.10	3.5 ± 0.10[c]	3.9 ± 0.10[c]	4.6 ± 0.10[c]
10 nM $PGF_2\alpha$				
Irradiated	2.0 ± 0.10	3.0 ± 0.10[b]	3.8 ± 0.15[b]	4.7 ± 0.10[b]
Nonirradiated	2.7 ± 0.10	3.5 ± 0.10[c]	3.9 ± 0.10[c]	4.8 ± 0.15[c]
2,000 units IL-1α				
Irradiated	1.8 ± 0.15	3.3 ± 0.10[b]	4.2 ± 0.15[b]	4.5 ± 0.10[b]
Nonirradiated	2.4 ± 0.10	3.5 ± 0.05[c]	4.2 ± 0.05[c]	4.6 ± 0.10[c]

[a]Values are mean ± SEM of three separate experiments, each using triplicate samples.
[b]Significantly different from irradiated 0 PE values: $p < 0.05$.
[c]Significantly different from nonirradiated 0 PE values: $p < 0.05$.

formation. So, IP3, PE, Bay K 8644, $PGF_2\alpha$, and IL-1α were tested to determine if they could reverse the radiation-induced decrease in calcium influx. None of the compounds alone reversed the decrease in calcium uptake induced by irradiation, but in combination they did reverse it, suggesting that ionizing radiation might affect protein kinase C activity, which is linked to the opening and closing of ion channels[9] and the mobilization of calcium. These experiments demonstrate that the stimulation of protein kinase C by PE together with the mobilization/influx of calcium by calcium ionophores is necessary to attenuate radiation-induced decreases in calcium influx. Currently, we are comparing protein kinase C activity and G protein receptor binding of irradiated and nonirradiated synaptosomes to determine whether decreases in calcium uptake induced by irradiation are due to impairment of protein kinase C, alterations in G protein receptors, or both.

REFERENCES

1. KANDASAMY, S. B. & W. A. HUNT. 1988. Effect of ionizing radiation on calcium channels in rat brain synaptosomes. Soc. Neurosci. (Abstr.) **54:** 13.
2. KANDASAMY, S. B., W. A. HUNT & A. DUBOIS. 1989. Effect of ionizing radiation on calcium channels in rat ileum myenteric plexus synaptosomes. Soc. Neurosci. (Abstr.) **147:** 19.
3. KANDASAMY, S. B., T. C. HOWERTON & W. A. HUNT. 1991. Reductions in calcium uptake induced in rat brain synaptosomes by ionizing radiation. Radiat. Res. **125:** 158–162.
4. KANDASAMY, S. B. & W. A. HUNT. 1990. Arachidonic acid and prostaglandins enhance potassium-stimulated calcium influx into rat brain synaptosomes. Neuropharmacol. **29:** 825–829.
5. KANDASAMY, S. B. & W. A. HUNT. 1990. Role of protein kinase C in radiation-induced decreased calcium-uptake in rat brain synaptosomes. The Toxicologist. (Abstr.) 422.
6. MULLIN, M. J., W. A. HUNT & R. A. HARRIS. 1986. Ionizing radiation alters the properties of sodium channels in rat brain synaptosomes. J. Neurochem. **47:** 489–495.
7. LESLIE, S. W., M. B. FRIEDMAN, R. E. WILCOX & S. Y. ELROD. 1980. Acute and chronic effects of barbiturates on depolarization-induced calcium influx into rat synaptosomes. Brain Res. **185:** 409–417.
8. SKATTEBOL, A. & D. J. TRIGGLE. 1987. Regional distribution of calcium channel ligand (1,4-dihydropyridine) binding sites and $^{45}Ca^{2+}$ uptake processes in rat brain. Biochem. Pharmacol. **36:** 4163–4166.
9. MARX, J. L. 1987. Polyphosphoinositide research updated. Science **235:** 974–976.

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BEHAVIORAL TOXICITY OF SELECTED RADIOPROTECTORS

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ABSTRACT

Effective radioprotection with minimal behavioral disruption is essential for the selection of protective agents to be used in manned spaceflight. This overview summarizes the studies on the behavioral toxicity of selected radioprotectors classified as phosphorothioates (WR-2721, WR-3689), bioactive lipids (16, 16 dimethylprostaglandin E_2($DiPGE_2$), platelet activating factor (PAF), leukotriene C_4), and immunomodulators (glucan, synthetic trehalose dicorynomycolate, and interleukin-1). Behavioral toxicity was examined in laboratory mice using a locomotor activity test. For all compounds tested, there was a dose-dependent decrease in locomotor behavior that paralleled the dose-dependent increase in radioprotection. While combinations of radioprotective compounds ($DiPGE_2$ plus WR-2721) increased radioprotection, they also decreased locomotor activity. The central nervous system stimulant, caffeine, was able to mitigate the locomotor decrement produced by WR-3689 or PAF.

INTRODUCTION

Chemical compounds that effectively protect mammals from the lethal effects of radiation have been studied for more than four decades /1,2/. Many of these compounds are effective only at toxic doses /3/. To perform extravehicular activities (EVA) during space station missions, individuals must remain alert and behaviorally active. Radiation hazards are significantly increased during an EVA because of decreased shielding, the possibility of solar particle events, or exposure to nuclear material associated with the space station /4/. The study of radioprotectors has focused on obtaining the maximal dose reduction factor (DRF). However, doses that give the greatest protection from radiation, often produce side effects that prevent the normal behavioral functioning of an organism. We have begun a systematic examination of the behavioral toxicity of a variety of radioprotectors. The goal of this research is to obtain a compound that offers maximal radioprotection with minimal behavioral toxicity.

Our behavioral screening program involves the quantification of spontaneous locomotor behavior in the laboratory mouse. The locomotor activity test has been recommended as an initial screening test for chemical compounds by both the U.S. Environmental Protection Agency /5/ and the World Health Organization /6/. Spontaneous locomotor activity was selected as the initial screening protocol because (1) it is a naturally occurring behavior that does not require laborious training procedures: (2) it can be assessed using automated devices that eliminate observer recording bias; and (3) it has been determined to be a sensitive measure for assessing the behavioral toxicity of a variety of agents, including pesticides /7/, stimulants /8,9/, neuroleptics /9/, radioprotectors /10-17/, and ionizing radiation /18-20/.

This review examines the behavioral toxicity (as measured by decrements in locomotor activity) of promising phosphorothioate radioprotectors, bioactive lipids, and immunomodulators. The behavioral effects described in this paper were observed in nonirradiated animals.

METHODOLOGY

Subjects

Male outbred mice (BALB/c x DBA/2)F1, also known as CD2F1 mice (Charles River Breeding Laboratory, Raleigh, NC) were used for most of the behavioral studies reported in this paper. This mouse strain was selected because of the large data base available at our Institute and the National Cancer Institute. All mice were

quarantined on arrival and representative animals were screened for evidence of disease. Mice were tested when they were 11-17 weeks old. They were housed in groups of 8-10 in Microisolator cages (Lab Products, Maywood, NJ) on hardwood chip contact bedding in an AAALAC- (American Association for Accreditation of Laboratory Animal Care) accredited facility. Rooms were maintained at 21°C ± 1°C with 50% ± 10% relative humidity on a 12-hour light/dark cycle. Commercial rodent chow and acidified water (pH 2.5) were freely available. Prior to testing, all animal experiments were approved by our Institutional Animal Care and Use Committee.

Locomotor Activity Measurement

A computerized Digiscan Animal Activity Monitor (Omnitech Electronics, Columbus, OH) was used to quantify locomotor behavior /9/. The apparatus used an array of infrared photodetectors spaced 2.5 cm apart to determine horizontal locomotor activity (ambulation) expressed as the total distance traveled. Immediately following injection of the compound(s), mice were placed into individual activity monitors (20 x 20 x 30 cm) where ambulation was recorded every 5 min for 1 hr to ascertain the onset of behavioral effects of the drug. Thereafter, activity was recorded at 1-hr intervals until the behavior of all groups returned to control-level activity. Each animal was tested only once. All testing took place during the dark portion of the light-dark cycle when the mice were most active. There were 8-16 animals tested with each dose of each drug evaluated. One-way analysis of variance was used to determine significance levels for the effects of each compound on locomotor activity. Post hoc comparisons were made using Dunnett's test /21/.

RESULTS AND DISCUSSION

Phosphorothioates

The phosphorothioate drugs S-2(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) and S-2-(3-methylaminopropylamino)ethylphosphorothioic acid, (WR-3689) are two of the leading radioprotectors currently available. These compounds were first synthesized as part of the Antiradiation Drug Development Program sponsored by the Walter Reed Army Institute of Research, and are still often referred to by their Walter Reed (WR) designation number /22/. WR-2721 has been used during clinical trials for radiotherapy /23/, and also protects against the cytotoxic effects of some chemotherapeutic agents /24/. It produces side effects such as hypotension, hypocalcemia, vomiting, sneezing, and somnolence in humans /23/. In rodents, WR-2721 decreases motor function /10,11,12,16,25/. Although the side effects reported in humans may be tolerable in a hospital setting, they are likely to present problems in situations where performance must be maintained (e.g., spacecraft).

WR-3689 has recently received increased attention as an alternative to WR-2721 /26,27/. This compound differs from WR-2721 by the addition of a single terminal N-methyl group. WR-3689 has similar levels of radioprotective efficacy when compared to WR-2721 /26/. Both phosphorothioates protect against fast neutrons and gamma radiation, although they are more effective against gamma rays /27,28/. WR-3689, however, appears to have a lower toxicity than WR-2721 when given systemically /29/ or when administered directly into the brain by intraventricular injection /30/. Both WR-2721 and WR-3689 are highly water soluble; therefore, little of these drugs or their metabolites crosses the blood-brain barrier. When placed directly into the brain, mice injected with WR-2721 or WR-3689 were lethargic; higher doses resulted in ataxia /30/.

We quantified the effects of WR-3689 on spontaneous locomotor behavior. Mice were administered intraperitoneal (IP) doses ranging from 50 to 400 mg/kg WR-3689 (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) or the saline vehicle. A dose of 50 mg/kg did not significantly affect locomotor activity. Doses of 100 and 200 mg/kg produced significant decrements 25-30 min postinjection, while the higher dose (400 mg/kg) resulted in significant decreases by 15 min postinjection. Figure 1 shows that doses of 100 and 200 mg/kg decreased activity for 4-5 hrs. A dose of 400 mg/kg, disrupted motor activity for 7 hrs. These results were similar to those found with WR-2721 administered IP /31/.

We then attempted to mitigate the locomotor decrements produced by WR-3689 using a central nervous system (CNS) stimulant. We chose the methylxanthine, caffeine, because of its wide use in the pharmacological industry and relatively low toxicity. To select a dose of caffeine to be combined with WR-3689, we first determined the dose-response curve for the duration of the stimulatory effect of anhydrous caffeine (Sigma, St. Louis, MO). Caffeine was administered in a saline vehicle (control) in doses of 2.5 to 40 mg/kg by oral gavage (PO). Doses of 5 mg/kg or more significantly increased locomotor activity within 5 min of administration. Figure 2 illustrates that doses of 5, 10, 20, and 40 mg/kg caffeine produced significant locomotor increases that persisted for up to 3, 4, 4, and 6 hrs, respectively.

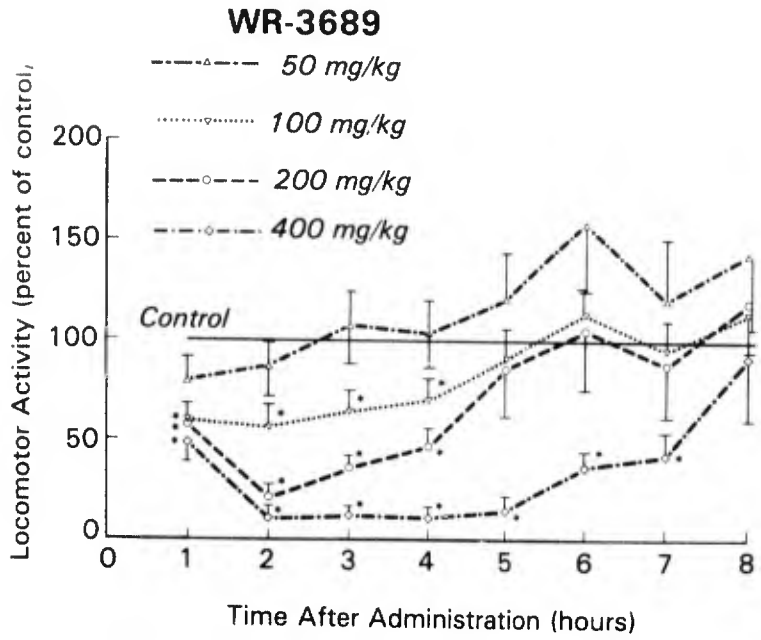

Fig. 1. Time course of the effects of WR-3689 on locomotor activity (ambulation) measured in hourly intervals. WR-3689 was administered IP immediately before testing. The solid line at 100% represents the saline vehicle control group. Note that a dose of 200 mg/kg produced locomotor decrement for 4-5 hrs postadministration. Vertical bars represent the SEM. * $p < 0.05$ from vehicle control group.

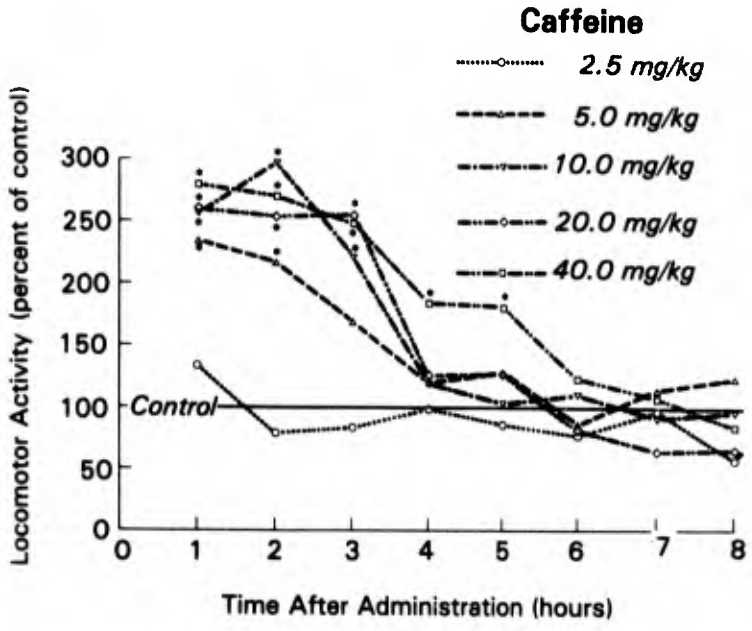

Fig. 2. Time course of the behavioral effects (locomotor activity) of central nervous system stimulant, caffeine. Caffeine was administered by gavage (PO) immediately before testing. There was a dose-dependent increase in the magnitude and duration of the locomotor effect. The solid line at 100% represents the saline vehicle control group. Note that a dose of 40 mg/kg produced a locomotor increase for 5-6 hrs after administration. * $p < 0.05$ from vehicle control group.

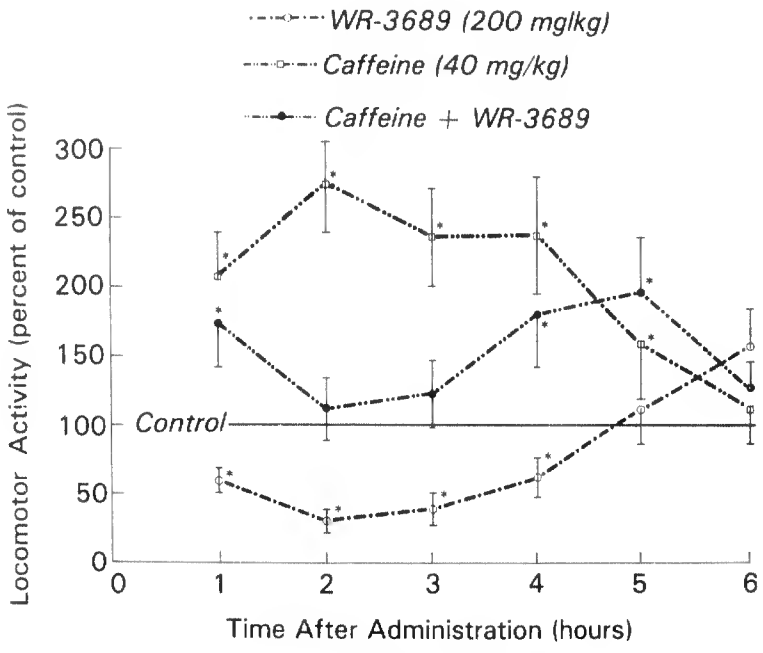

Fig. 3. Effects of combining WR-3689 (200 mg/kg, IP) and caffeine (40 mg/kg, PO) on locomotor activity. Caffeine completely mitigated the behavioral decrement produced by WR-3689. The solid line at 100% represents the saline control group. Vertical bars represent the SEM. * $p < 0.05$ from vehicle control group.

Based on the results obtained from the locomotor activity dose-response curves generated for WR-3689 and caffeine, a combination study was performed to determine whether caffeine could mitigate the locomotor performance decrement induced by WR-3689. In this experiment, a dose of 40 mg/kg caffeine was administered orally, followed immediately by an IP injection of 200 mg/kg WR-3689. These doses and routes of administration were selected because the two drugs exhibited a similar time course using these parameters. Figure 3 shows that an IP dose of 200 mg/kg of WR-3689 resulted in locomotor activity decrements for 4-5 hrs postinjection. However, 40 mg/kg PO of caffeine in combination with WR-3689 maintained locomotor activity at or above control levels for up to 6 hrs after injection. Lower doses of caffeine reversed the locomotor decrement for shorter periods of time. For example, doses of 10 or 20 mg/kg caffeine in combination with 200 mg/kg WR-3689 elevated locomotor activity to control levels for up to 1 hr and 4 hrs, respectively.

When we evaluated the radioprotective effect of WR-3689 alone compared to caffeine and WR-3689 combined, we found that animal survival rates were similar /32/. Thus, using the locomotor activity test model, we were able to mitigate performance decrement without compromising radioprotective efficacy. The reversal of the behavioral toxicity of WR-3689 by caffeine has only been confirmed in the locomotor activity test paradigm. Other behavioral tests should be performed to verify these findings. The mechanism of action of caffeine in mitigating the locomotor decrement produced by WR-3689 is unknown at this time. It may be mediated by blockade of adenosine receptors in the CNS or through accumulation of the cyclic nucleotide, cyclic AMP, by inhibition of phosphodiesterase /33/.

Bioactive Lipids

This class of radioprotective compounds includes the metabolites of arachidonic acid (e.g., prostaglandins and leukotrienes) as well as other phospholipids, (e.g., platelet activating factor (PAF)) /2/. The most promising radioprotective compounds in this class include the synthetic methylated derivative of the naturally occurring prostaglandin E_2, 16,16-dimethyl prostaglandin E_2 ($DiPGE_2$), leukotriene C_4 (LTC_4), and PAF. Radioprotective efficacy of these compounds has been clearly demonstrated, e.g., $DiPGE_2$ /34-40/, LTC_4 /41-43/, and PAF /44/. Each of these compounds has been shown to be radioprotective for animal survival (CD2F1 male mice) when administered before ^{60}Co irradiation. $DiPGE_2$ at 0.4 mg/kg yields a DRF of 1.45 /14/; LTC_4 at 0.4 mg/kg yields a DRF of 1.9 /43/, and PAF at 0.3 mg/kg yields a DRF of 1.7 /44/.

We evaluated the effects of the radioprotective bioactive lipids on locomotor behavior /13,14,15/. All compounds were administered subcutaneously (SC). Figure 4 shows that there were dose-dependent decreases in locomotor

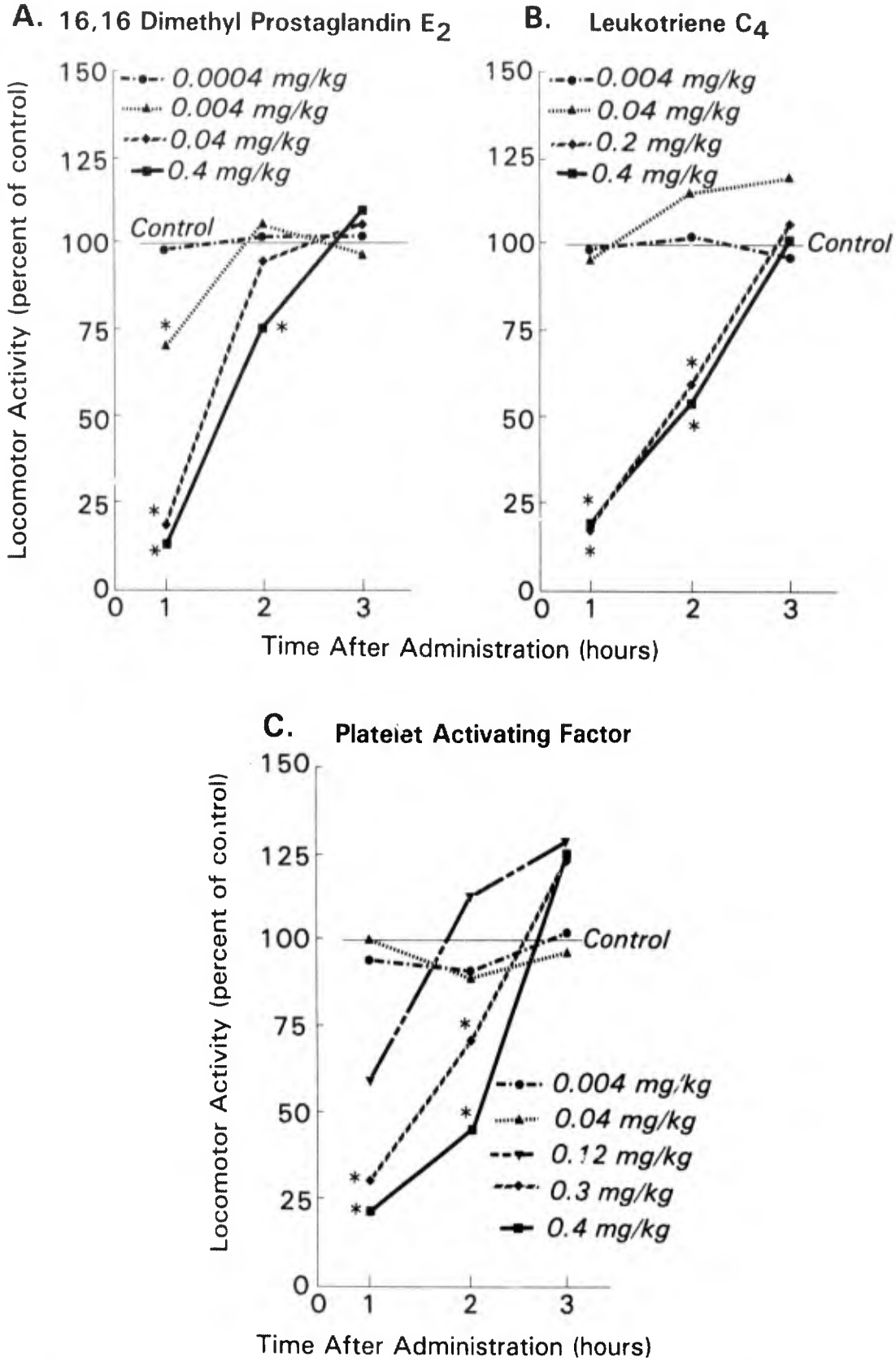

Fig 4. Time course of locomotor behavior following administration of radioprotective bioactive lipids. (A) 16, 16 dimethyl prostaglandin E_2, (B) leukotriene C_4 and (C) platelet activating factor. Compounds were administered SC immediately prior to behavioral testing. Data are presented as a percent of the vehicle (4% ethanol in saline) control group. * $p < 0.05$ from vehicle control group.

activity for $DiPGE_2$, LTC_4, and PAF. At the highest dose test, each compound produced locomotor decrements within 5-10 min of administration (data not shown) followed by recovery to control levels approximately 3 hrs after injection.

The methylxanthines, theophylline /45/ and isobutyl methylxanthine /46/, protect mice from the lethal toxicity of PAF. We found that the methylxanthine, caffeine (40 mg/kg IP), mitigated the locomotor decrements produced by 0.12 and 0.3 mg/kg PAF SC. This dose of caffeine, however, was not effective in alleviating the locomotor decrements produced by 0.4 mg/kg $DiPGE_2$ SC (data not shown).

The bioactive lipids produce a number of side effects. Many of the prostaglandins, including $DiPGE_2$, induce diarrhea and sedation /47/. LTC_4 increases hematocrit and mean arterial blood pressure that may be followed by prolonged hypotension /13,48/, and PAF administration may lead to a severe hypotension, reflecting peripheral vasodilation and decreased cardiac output /49/. However, the mechanism(s) by which these compounds exert their effects on locomotor behavior are unknown.

From the experiments performed, it is not possible to determine whether the decreases in locomotor activity produced by $DiPGE_2$, LTC_4, or PAF are the result of a peripheral or central mechanism. Prostaglandins readily cross the blood-brain barrier /50/, and LTC_4 does not /51/. PAF can influence blood-brain barrier permeability, but does not appear to penetrate it /52/. In addition, many bioactive lipids are synthesized by brain tissue /52,53/. The decrease in locomotor activity observed following administration of the radioprotective compounds may, therefore, be due to a direct physiological response or to intermediate messengers produced by the bioactive lipids.

Immunomodulators

Many immunomodulators show moderate radioprotection (DRF's < 1.4) by stimulating host immune responses. To reduce radiation injury, these compounds act principally on the macrophage. The immunomodulator glucan, a beta 1,3 linked polysaccharide isolated from the cell wall of the yeast *Saccharomyces cervisiae*, is capable of enhancing a variety of hematopoietic responses that results in protection against gamma radiation /54,55/. When soluble glucan (250 mg/kg) was injected intravenously postirradiation, a DRF of 1.07 was obtained /54/. We found that this dose administered to C_3H/HeN female mice resulted in severe locomotor decrements, with significant decreases in activity beginning within 20-25 min after injection and lasting for 7-8 hrs /56/.

Synthetic trehalose dicorynomycolate (S-TDCM) is a chemically prepared form of a naturally occurring glycolipid secreted on the surface of the cell walls of several strains of bacteria, including mycobacteria /57/. The immunomodulatory effects of S-TDCM have since been shown to provide many beneficial properties. For example, when administered 20-24 hrs before fission neutron radiation (neutron to gamma dose ratio, N:G=1), it offers modest (DRF=1.07) radioprotection /58/. We recently found that a dose of 100 µg/mouse S-TDCM increased survival from 15% (saline-treated) to 80% for B6D2F1 female mice exposed to 5.6 Gy fission neutrons (N:G=1). Mice receiving this dose of S-TDCM exhibited only minimal locomotor decrement and no reduction in food intake, water consumption, or body weight. A dose of 50 µg was less radioprotective, while 200 µg produced greater toxicity without increased radioprotection /59/.

The immunomodulators, glucan and S-TDCM, may modulate the peptide cytokine, interleukin-1 (IL-1), which may be indirectly responsible for their radioprotective efficacy /58,60/. IL-1, in turn, is likely to release other mediators, such as products of arachidonic acid metabolism. IL-1 itself is radioprotective /61/ and when combined with WR-2721, provided greater than additive protection /2/. However, we recently found that this combination also produced a greater than additive behavioral toxicity (data not shown).

Combinations of Radioprotectors

It has been determined that combinations of radioprotective agents can provide increased protection /2,3/. For example, the combination of WR-2721 and $DiPGE_2$ has been shown to enhance radioprotection /14,36/. The behavioral toxicity as measured by decreases in locomotor activity, however, is more pronounced /14/. Similarly, the addition of the metals selenium and copper to WR-2721 increased the survival of mice exposed to gamma radiation, compared to WR-2721 alone. Behavioral toxicity also increased as radioprotection improved /2/.

GENERAL DISCUSSION

The studies reported in this paper indicate that all radioprotective compounds investigated were behaviorally toxic at protective doses. As radioprotection increases in a dose-dependent manner, so does behavioral decrement. This concept is illustrated in Figure 5A. Thus, as is the case with many drugs, the use of radioprotectors is limited by

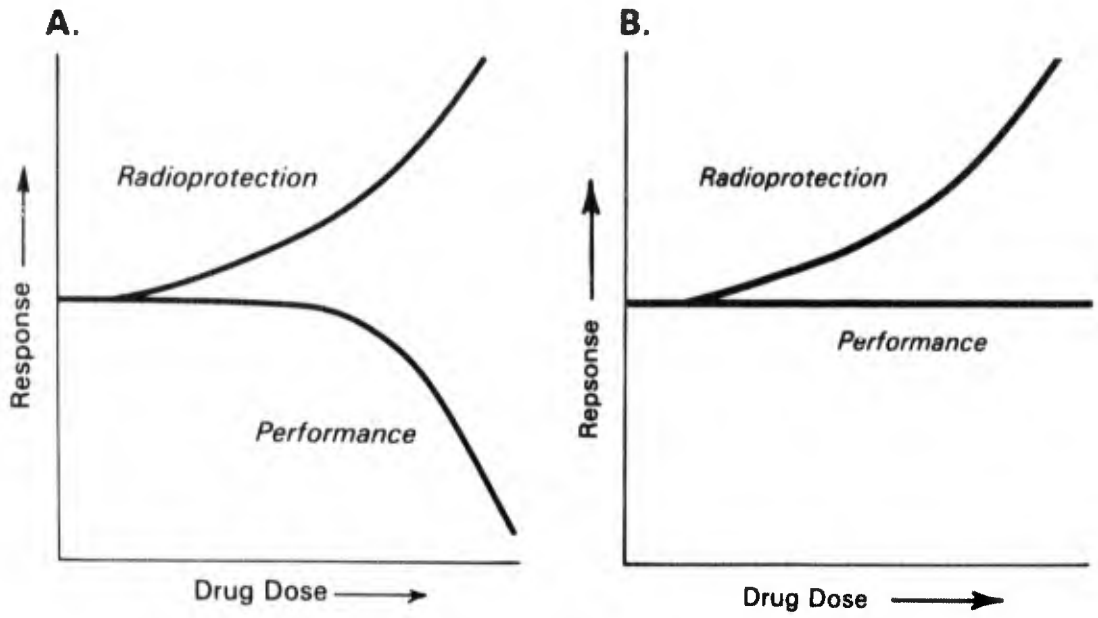

Fig. 5. (A) Schematic representation of the relationship between radioprotection and behavioral toxicity. As the level of radioprotection increases with increasing doses of the protectant compound, the amount of behavioral toxicity (performance decrement) also increases. Behavioral toxicity was measured by decreases in locomotor activity. (B) Schematic representation of how the ideal radioprotector combination would operate. Radioprotection is maintained, while performance is not compromised.

their toxicity. The toxicity of many radioprotectors may be related to secondary effects and not a direct action of the radioprotectve qualities of the compound /3/. It is hoped that future studies will lead to a formulation of a radioprotective cocktail that will allow maximum radioprotection without compromising performance (Figure 5B). Approaches to this end have included the combination of low doses of two or more compounds that operate by different mechanisms /2/. Further, we demonstrated that by combining caffeine with some radioprotectors, we were able to mitigate the locomotor decrement induced by these protectors. This research serves as a basis for developing effective countermeasures against the behavioral toxicity of these compounds. Additional tests are necessary to further characterize the behavioral toxicity of radioprotectors. Because radioprotectors, such as the phosphorothioate WR-2721, are known to produce nausea and vomiting in patients, it may be useful to include an antiemetic compound in the radioprotective cocktail. Recent evidence suggests that the $5HT_3$ antagonists, a new class of antiemetic compounds, are effective at non-toxic doses at alleviating nausea and vomiting induced by radiation /62,63/.

The radiation hazards in space include both acute effects (e.g., performance decrement, prodroal syndrome, and lethality) and late or chronic effects (e.g., cancer, mutation, and cataracts). A solar particle event occurring during a space mission is likely to result in the need for continuous radioprotection for 12-48 hrs /64/. Most of the radioprotection data available, however, is based on a single administration of the protector before a brief exposure (several minutes) to ionizing radiation. It is clear that there is a need to determine the effectiveness of radioprotectors over a sustained period of radiation exposure. Moreover, the development of radioprotectors that minimize performance decrement will greatly assist us in conquering the depths of space.

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REFERENCES

1. L.I. Giambarresi and R.I. Walker, Prospects for radioprotection, in: *Textbook of Military Medicine, Part I, Vol 2, Medical Consequences of Nuclear Warfare*, ed. R.I. Walker and T.J. Cerveney, Office of the Surgeon General, Department of the Army, Falls Church, VA 1989, pp. 245-273.

2. J.F. Weiss, K.S. Kumar, T.L. Walden, Jr., R. Neta, M.R. Landauer and E.P. Clark, Advances in radioprotection through the use of combined agent regimens, *Int. J. Radiat. Biol.* 57, 709-722 (1990).

3. J.R. Maisin, Protection against ionizing radiation by combinations of radioprotectors, *Pharmacol. Ther.* 39, 189-1^3 (1988).

4. J.J. Conklin and M.P. Hagan, Research issues for radiation protection for man during prolonged spaceflight, *Adv. Radiat. Biol.* 13, 215-284 (1987).

5. W.F. Sette and T.E. Levine, Behavior as a regulatory endpoint. in: *Neurobehavioral Toxicology*, ed. Z. Annau, Johns Hopkins University Press, Baltimore, MD 1986, pp. 391-403.

6. World Health Organization, Environmental Health Criteria, *Principles and Methods for the Assessment of Neurotoxicity Associate with Exposure to Chemicals*, Geneva 1986.

7. M.R. Landauer, W.T. Tomlinson, R.L. Balster and R.C. MacPhail, Some effects of the formamidine pesticide chlordimeform on the behavior of mice, *Neurotoxicology* 5, 91-100 (1984).

8. N.R. Swerdlow, F.J. Vaccarino, M. Amalric and G.F. Koob, The neural substrates for the motor-activating properties of psychostimulants: A review of recent findings, *Pharmacol. Biochem. Behav.* 25, 233-248 (1986).

9. R.E. Fitzgerald, M. Berres and U. Schaeppi, Validation of a photobeam system for assessment of motor activity in rats, *Toxicology* 49, 433-339 (1988).

10. M.R. Landauer, H.D. Davis, J.A. Dominitz and J.F. Weiss, Dose and time relationships of the radioprotector WR-2721 on locomotor activity in mice, *Pharmacol. Biochem. Behav.* 27, 573-576 (1987).

11. M.R. Landauer, H.D. Davis, J.A. Dominitz and J.F. Weiss, Long-term effects of radioprotector WR-2721 on locomotor activity and body weight of mice following exposure to ionizing radiation, *Toxicology* 49, 315-323 (1988).

12. M.R. Landauer, H.D. Davis, J.A. Dominitz and J.F. Weiss, Comparative behavioral toxicity of four sulfhydryl radioprotective compounds in mice: WR-2721, cysteamine, diethyldithiocarbamate and N-acetylcysteine, *Pharmacol. Ther.* 39, 97-100 (1988).

13. M.R. Landauer, H.D. Davis, and T.L. Walden. Behavioral and physiological effects of leukotriene C_4, *Prostaglandins Leukot. Essent. Fatty Acids* 39, 247-252 (1990).

14. M.R. Landauer, T.L. Walden, Jr. and H.D. Davis, Behavioral effects of radioprotective agents in mice: Combination of WR-2721 and 16,16 Dimethyl Prostaglandin E_2, in: *Frontiers in Radiation Biology*, ed. E. Riklis, VCH Publishers, New York 1990, pp. 199-208.

15. M.R. Landauer, H.D. Davis and T.L. Walden, Jr. Behavioral toxicity of radioprotective bioactive lipids, in: *Eicosanoids and Other Bioactive Lipids in Cancer and Radiation Injury*, eds. K.V. Hohn, L.J. Marnett, S. Nigam, and T.L. Walden, Jr., Vol 1, Kluwer Publishers, Boston, in press.

16. W.F. Liu, J.H. Shih, R.F. Lin, C. Ma, C.H. Lin, C.Y. Liu, C.C. Chang and M.T. Wu, Relationship between radioprotective and neuromotor effects of S-2(3-aminopropyl-amino)ethylphosphorothioate (WR-2721) in mice, *Neurotoxicol. Teratol.* 9, 333-337 (1987).

17. K. Tikal, J. Plevova and J. Hvizdosva, Behavioural effects of cystamine and mexamine in rats, *Act. Nerv. Super. (Praha)* 21, 59-62 (1979).

18. D. Kimeldorf and E. Hunt, *Ionizing Radiation: Neural Function and Behavior,* Academic Press, New York 1965.

19. M.R. Landauer, G.D. Ledney and H.D. Davis, Locomotor behavior in mice following exposure to fission-neutron irradiation and trauma, *Aviat. Space Environ. Med.* 58, 1205-1210 (1987).

20. D.M. Maier and M.R. Landauer, Onset of behavioral effects in mice exposed to 10 Gy ^{60}Co radiation, *Aviat. Space Environ. Med.* 61, 893-898 (1990).

21. B.J. Winer, *Statistical Principles in Experimental Design,* McGraw-Hill, New York 1962.

22. D.E. Davidson, M.M. Grenan and T.R. Sweeney, Biological characteristics of some improved radioprotectors, in: *Radiation Sensitizers: Their Use in the Clinical Management of Cancer,* ed. L.W. Brady, Masson, New York 1980, pp. 309-320.

23. M.M. Kligerman, A.T. Turrisi, R.C. Urtasun, A.L. Norfleet, T.L. Phillips, T. Barkley and T. Rubin, Final report on phase I trial of WR-2721 before protracted radiation therapy, *Int. J. Radiat. Oncol. Biol. Phys.* 14, 1119-1122 (1988).

24. D. Glover, S. Grabelsky, K. Fox, C. Weiler, L. Cannon and J. Glick, Clinical trials of WR-2721 and cis-platinum, *Int. J. Rad. Oncol. Biol. Phys.* 16, 1201-1204 (1989).

25. V. Bogo, A.J. Jacobs and J.F. Weiss, Behavioral toxicity and efficacy of WR-2721 as a radioprotectant, *Radiat. Res.* 104, 182-190 (1985).

26. D.Q. Brown, W.J. Graham, III, L.J. MacKenzie, J.W. Pittock, III and L. M. Shaw, Can WR-2721 be improved upon? *Pharmacol. Ther.* 39, 157-168 (1988).

27. J.S. Rasey, S. Magee, N. Nelson, L. Chin and K.A. Krohn, Response of mouse tissues to neutron and gamma radiation: Protection by WR-3689 and WR-77913, *Radiother. Oncol.* 17, 167-173 (1990).

28. C.P. Sigdestad, D.J. Grdina, A.M. Conner and W.R. Hanson. A comparison of radioprotection from three neutron sources and ^{60}Co by WR-2721 and WR-151327, *Radiat. Res.* 106, 224-233 (1986).

29. D. Murray, E.M. Altschuler, N. Hunter and L. Milas, Protection by WR-3689 against gamma-ray-induced intestinal damage: Comparative effect of clonogenic cell survival, mouse survival, and DNA damage, *Radiat. Res.* 120, 339-351 (1989).

30. A.M. Spence, S.W. Edmondson, K.A. Krohn, Z. Grunbaum, J.S. Rasey and J.E. Steele, Toxicity and biodistribution of radioprotectors, WR2721, WR77913, and WR3689, in the CNS following intraventricular or intracisternal administration, *Int. J. Raaiat. Oncol. Biol. Phys.* 12, 1653-1660 (1986).

31. J.F. Weiss, K.S. Kumar, V. Srinivasan and M.R. Landauer, Comparison of toxicity and radioprotective efficacy of WR-2721 and WR-3689 in mice, *Proceedings of the American Association for Cancer Research* 31, 433 (1990).

32. K.S. Kumar, J.F. Weiss, M.R. Landauer and E.P. Clark, Radioprotection by combinations of WR-3689 and caffeine, *Abstracts of the 38th Annual Meeting of the Radiation Research Society,* New Orleans, LA, p. 193 (1990).

33. T.W. Rall, Central nervous system stimulants: The methylxanthines, in: *The Pharmacological Basis of Therapeutics,* eds. A. Goodman Gilman, L.S. Goodman, T.W. Rall and F. Murad, Macmillan, New York 1985, pp. 589-603.

34. W.R. Hanson and C. Thomas, 16,16-Dimethyl prostaglandin E_2 increases survival of murine intestinal stem cells when given before photon radiation, *Radiat. Res.* 96, 393-398 (1983).

35. W.R. Hanson and E.J. Ainsworth, 16,16-Dimethyl prostaglandin E_2 induced radioprotection in murine intestinal and hematopoietic stem cells, *Radiat. Res.* 103, 196-203 (1985).

36. W.R. Hanson, Radioprotection of murine intestine by WR-2721, 16,16 dimethyl prostaglandin E_2 and the combination of both agents, *Radiat. Res.* 111, 361-373 (1987).

37. W.R. Hanson, Radiation protection by exogenous arachidonic acid and several metabolites, in: *Prostaglandin and Lipid Metabolism in Radiation Injury,* eds. T.L. Walden, Jr. and H.N. Hughes, Plenum Press, New York 1987, pp. 233-243.

38. T.L. Walden, Jr., M. Patchen and S.L. Snyder, 16, 16-dimethyl prostaglandin E_2 increases survival in mice following irradiation, *Radiat. Res.* 109, 440-448 (1987).

39. L.K. Steel, T.L. Walden, H.N. Hughes and W.E. Jackson III, Protection of mice against mixed fission neutron-gamma (n:gamma = 1.1) irradiation by WR-2721, 16, 16 dimethyl PGE_2, and the combination of both agents, *Radiat. Res.* 115, 605-608 (1988).

40. L.K. Steel and G.N. Catravas, Protection against ionizing radiation with eicosanoids, in: *Eicosanoids and Radiation,* ed. P. Polgar, Kluwer Publishers, Boston 1988, pp. 79-87.

41. W.R. Hanson, K.A. Houseman and P.W. Collins, Radiation protection *in vivo* by prostaglandins and related compounds of the arachidonic acid cascade, *Pharmacol. Ther.* 39, 347-356 (1988).

42. T.L. Walden, Jr., Pretreatment with leukotriene C_4 enhances the whole-animal survival of mice exposed to ionizing radiation, *Ann. NY Acad. Sci.* 524, 431-433 (1988).

43. T.L. Walden, Jr., Radioprotective efficacy of LTC_4 in mice, *Abstracts of the 37th Annual Meeting of the Radiation Research Society,* Seattle, WA, p. 185 (1989).

44. H.N. Hughes, T.L. Walden, Jr. and L.K. Steel, Radioprotective efficacy of PAF, *Abstracts of the 37th Annual Meeting of the Radiation Research Society,* Seattle, WA, p. 186 (1989).

45. A. Subissi, M. Del Monte and M. Crisuoli, Effects of anti-asthma drugs on PAF-induced death in mice, *Prostaglandins* 36, 797-805 (1988).

46. J.M. Young, P.J. Maloney, S.N. Jubb and J.S. Clark, Pharmacological investigation of the mechanisms of platelet-activating factor induced mortality in the mouse, *Prostaglandins* 30, 545-551 (1985).

47. E.K.Y. Chiu and J.S. Richardson, Behavioral and neurochemical aspects of prostaglandins in brain function, *Gen. Pharmacol.* 16, 163-175 (1985).

48. S. Moncada, R.J. Flower and J.R. Vane, Prostaglandins, prostacyclin, thromboxane A_2, and leukotrienes, in: *The Pharmacological Basis of Therapeutics,* eds. A. Goodman Gilman, L.S. Goodman, T.W. Rall and F. Murad, Macmillan, New York 1985, pp. 660-673.

49. A. Myers, A.P. Tores Durate and P. Ramwell, Pharmacological manipulation of platelet-activating factor toxicity in rodents, *Adv. Prostaglandin Thromboxane Leukotriene Res.* 17, 833-837 (1987).

50. L.Z. Bito, H. Davson and J.R. Hollingsworth, Facilitated transport of prostaglandins across the blood-cerebrospinal fluid and blood-brain barrier, *J. Physiol.* 253, 273-285 (1976).

51. R. Spector, E.J. Goetzl, The role of concentrative leukotriene transport systems in the central nervous system, *Biochem. Pharmacol.* 35, 2849-2853 (1986).

52. R. Kumar, S.A.K. Harvey, M. Kester, D.J. Hanahan and M.S. Olson, Production and effects of platelet-activating factor in the rat brain, *Biochem. Biophys. Acta* 963, 375-383 (1988).

53. S. Murphy and B. Pearce, Eicosanoids in the CNS: Sources and effects, *Prostaglandins Leukot. Essent. Fatty Acids* 31, 165-170 (1988).

54. M.L. Patchen, M.M. D'Alesandro, M.A. Chirigos and J.F. Weiss, Radioprotection by biological response modifiers alone and in combination with WR-2721, *Pharmacol. Ther.* 39, 247-254 (1988).

55. J.R. Maisin, Chemical protection against ionizing radiation, *Adv. Space Res.* 9, # 10, 205-212 (1989).

56. M.R. Landauer, M.L. Patchen and H.D. Davis, Time course of behavioral effects in mice treated with soluble glucan, *Abstracts of the 4th International Conference on Immunopharmacology,* Osaka, Japan, p. 91 (1988).

57. E. Lederer, An update on natural and synthetic trehalose diesters, in: *Immunomodulators and Nonspecific Host Defense Mechanisms Against Microbial Infections,* eds. K. Masihi and W. Lange, Pergamon Press, Oxford, England 1988, pp 73-83.

58. D.G. McChesney, G.D. Ledney and G.S. Madonna, Trehalose dimycolate enhances survival of fission neutron-irradiated mice and *Klebsiella pneumoniae*-challenged irradiated mice, *Radiat. Res.* 121, 71-75 (1990).

59. M.R Landauer, G.D. Ledney, D.G. McChesney, and H.D. Davis, Synthetic trehalose dicorynomycolate: Relationship between toxicity and radioprotective efficacy against fission neutron radiation. *Abstracts of the International Colloquium on Neutron Radiation Biology,* Rockville, MD (1990).

60. M.A. Chirigos and M.L. Patchen, Survey of newer biological response modifiers alone and in combination with WR-2721, *Pharmacol. Ther.* 39, 243-246 (1988).

61. R. Neta, Role of cytokines in radioprotection, *Pharmacol. Ther.* 39, 261-266 (1988).

62. G.L. King and M.R. Landauer, Effects of zacopride and BMY-25801 (Batanopride) on radiation-induced emesis and locomotor behavior in the ferret, *J. Pharmacol. Exp. Ther.* 253, 1026-1033 (1990).

63. T.J. Priestman, Clinical studies with ondansetron in the control of radiation-induced emesis, *Eur. J. Cancer Clin. Oncol.* 25, Suppl 1, S29-S33 (1989).

64. E.J. Ainsworth, S.M.J. Afzal, D.A. Crouse, W.R. Hanson and R.J.M. Fry, Tissue responses to low protracted doses of high LET radiations or photons: Early and late damage relevant to radioprotective countermeasures, *Adv. Space. Res.* 9, # 10, 299-313 (1989).

In: *Eicosanoids and Other Bioactive Lipids in Cancer and Radiation Injury.* K. V. Honn, L. J. Marnett, S. Nigam, and T. L. Walden, Jr., eds. Kluwer Academic Publishers, Boston, 1991.

BEHAVIORAL TOXICITY OF RADIOPROTECTIVE BIOACTIVE LIPIDS

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INTRODUCTION

The ideal radioprotective agent for use in radiotherapy and civil defense should provide effective protection with minimal behavioral disruption. The bioactive lipids are among the compounds that have demonstrated radioprotective efficacy in cellular assays or animal survival studies. Members of this group that have been determined to be radioprotective include prostaglandins, leukotrienes, and platelet-activating factor (PAF). While protective agents have been identified in both the cyclooxygenase and lipoxygenase pathways, as well as for the phospholipid derived PAF, not all bioactive lipids provide radioprotection (1). The most promising radioprotective compounds include the synthetic methylated derivative of the naturally occurring prostaglandin E_2, 16,16-dimethyl prostaglandin E_2 ($DiPGE_2$), leukotriene C_4 (LTC_4), and PAF. In our laboratory, they are maximally effective for enhancing animal survival when administered 5-10 minutes before irradiation, and provide dose reduction factors (DRF) ranging from 1.45 to 1.9 (2-4). The radioprotective mechanisms of the bioactive lipids have not been clearly elucidated, but they are believed to act through different receptor systems. Mechanisms ranging from alterations of biological mediators, such as cyclic AMP, to hypoxia and cardiovascular effects have been postulated (5,6).

Because of the important biological roles and the significant radioprotection afforded by $DiPGE_2$ (2, 7-11), LTC_4 (3, 12, 13), and PAF (4), we investigated the behavioral toxicity of these compounds. In addition, we evaluated the behavioral effects of mice exposed to the sulfhydryl radioprotector S-2 (3-aminopropylamino)ethylphosphorothioic acid (WR-2721) (14) alone and in combination with $DiPGE_2$, because previous research established that combining these agents increases the degree of radioprotection (15, 16). An ideal combination of agents should provide enhanced radioprotection without an increase in behavioral side effects. Behavioral toxicity was evaluated using a test for spontaneous locomotor activity, a paradigm recommended by the World Health Organization (17). The locomotor activity test has been determined to be a sensitive measure for assessing the behavioral toxicity of radioprotectors (18-21).

MATERIALS AND METHODS

Subjects.

Male CD2F1 male mice, 10 to 12 weeks old were obtained from Charles River Breeding Laboratory

(Raleigh, NC). They were quarantined on arrival, and representative animals were screened for evidence of disease. Mice were housed in groups of 8-10 in Micro-Isolator cages on hardwood chip contact bedding in an AAALAC accredited facility. Rooms were maintained at 21° +/- 1°C with 50% relative humidity on a 12-12 hr light-dark cycle. Commercial rodent chow (Wayne Rodent Blox) and acidified water (pH, 2.5) were freely available. All mice were euthanized by inhalation of carbon dioxide at the end of the experiment.

Drugs

A range of doses of $DiPGE_2$, LTC_4 and PAF were administered to mice. Each mouse was tested only once. Compounds were dissolved in 4% ethanol in saline, and administered to mice subcutaneously (SC) in the nape of the neck in a volume of 100 µl. Behavioral evaluation began immediately following drug administration. In the WR-2721 and $DiPGE_2$ combination study, all mice received two injections. The first injection was either WR-2721 (200 mg/kg) or the saline vehicle administered intraperitoneally (IP). The second injection, administered 5 min after the first, was either a SC dose of $DiPGE_2$ (0.4 mg/kg) or the vehicle, 4% ethanol in saline. Mice were placed in the test apparatus immediately after receiving the second injection.

Locomotor activity measurement.

A computerized Digiscan Animal Activity Monitor (Omnitech Electronics, Columbus, OH) was used to quantitate locomotor behavior. The apparatus used an array of infrared photodetectors spaced 2.5 cm apart to determine locomotor activity expressed as the total distance traveled. Immediately following injection of the compound(s) animals were placed into the activity monitor where ambulation was recorded every 5 min for 1 hr to ascertain the behavioral onset of the drug. Thereafter, activity was recorded at 1-hr intervals until all groups returned to control levels. All testing took place during the dark portion of the light-dark cycle.

One-way analysis of variance was used to determine significance levels for the effects of each compound on locomotor activity. Post hoc comparisons were made using Dunnett's test.

RESULTS AND DISCUSSION

Each of the three major compounds tested, $DiPGE_2$, LTC_4, and PAF, produced dose-dependent decrements in locomotor behavior (Figure 1). At the higher doses tested, the behavioral decrement induced by each compound was rapid in onset, occurring within 5-10 minutes postadministration. Locomotor activity returned to control levels by 3 hr postadministration at the highest doses evaluated.

Each of the three compounds tested has been shown to be radioprotective for animal survival when administered prior to cobalt-60 irradiation. $DiPGE_2$ at 0.4 mg/kg yields a DRF of 1.45 (16); LTC_4 at 0.4 mg/kg, a DRF of 1.9 (13); and PAF at 0.3 mg/kg, a DRF of 1.7 (4).

WR-2721 and $DiPGE_2$ alone and in combination induced almost complete cessation of locomotor activity (Figure 2). WR-2721 produced significant locomotor deficits within 15 min that lasted for 3 hr. Administration of 0.4 mg/kg $DiPGE_2$ resulted in significant decrements within 5 min of injection which

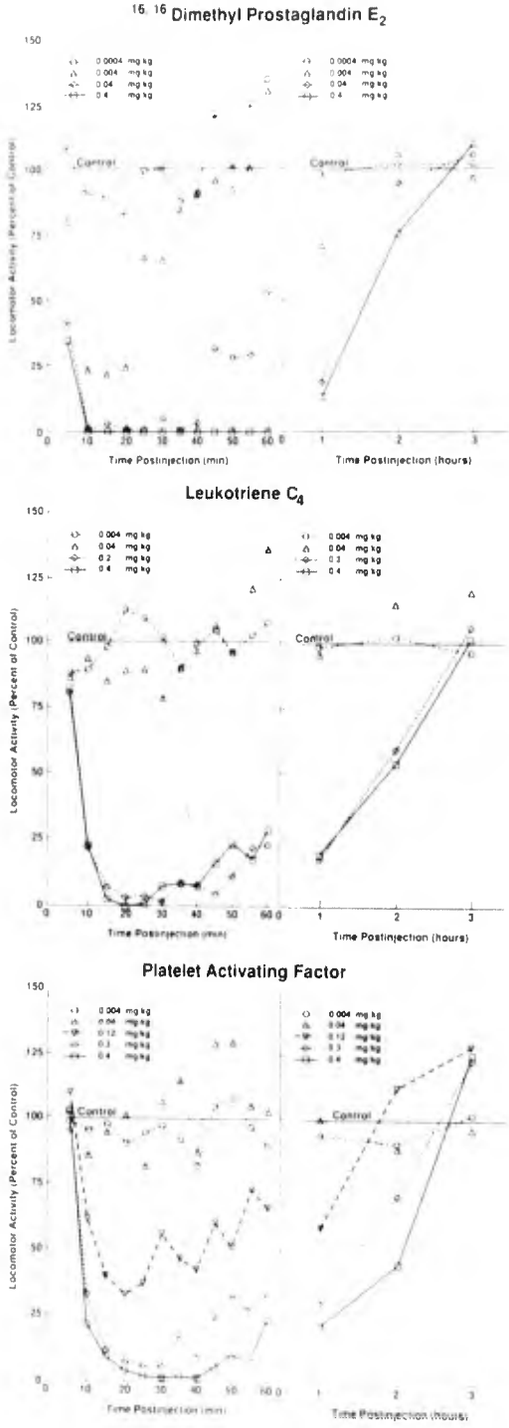

Figure 1. Onset (left) and duration (right) of locomotor deficit following administration of $DiPGE_2$, LTC_4 or PAF. Drugs were administered SC prior to testing as described in text. Data are expressed as a percent of the vehicle control group. (N = 9-13/dose).

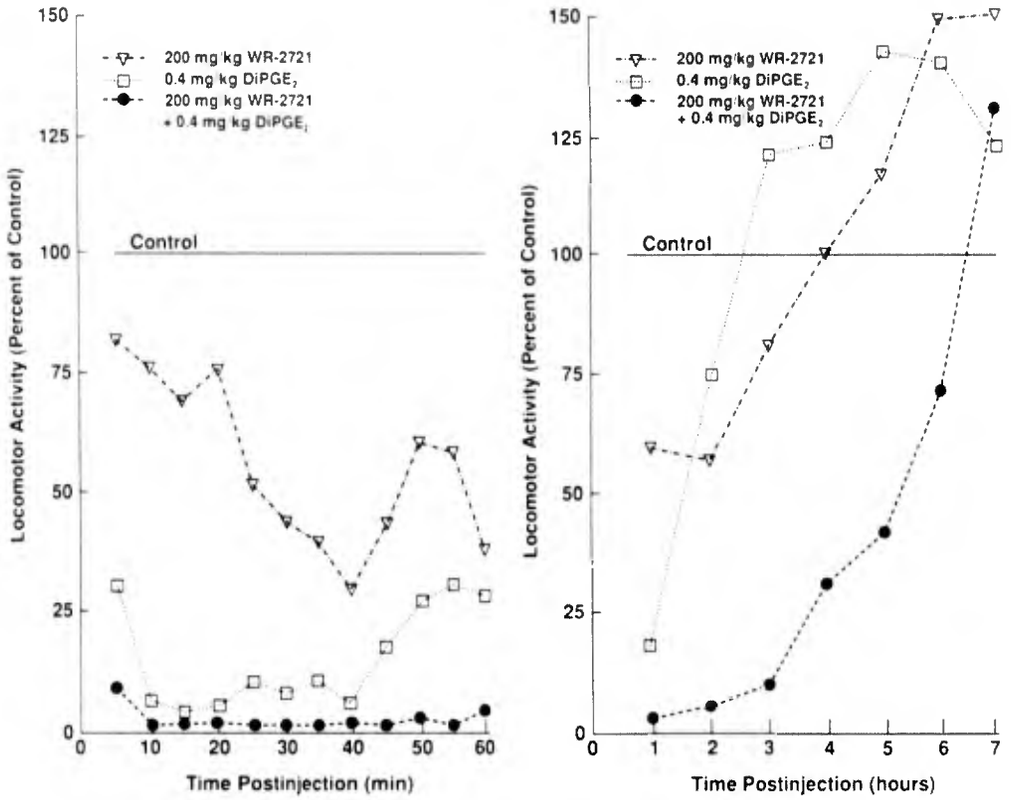

Figure 2. Effects of WR-2721 and $DiPGE_2$, alone or in combination, on onset (left) and duration (right) of behavioral decrement. WR-2721 (IP) $DiPGE_2$ (SC) or the combination was administered prior to assessment of locomotor behavior as described in text. Data are expressed as a percent of the vehicle control group (N = 8-12/group).

persisted for 2 hr. The combination of WR-2721 and $DiPGE_2$ resulted in deficits within 5 min of injection that remained below control levels for 6 hr following drug administration.

The combination of WR-2721 and $DiPGE_2$ administered to CD2F1 mice before cobalt-60 irradiation has been determined to provide a greater DRF than either compound alone (16). Pretreatment with WR-2721 provided a DRF of 1.90, while pretreatment with $DiPGE_2$ increased survival with a DRF of 1.45, and the combination yielded a DRF of 2.15. Although the combination of WR-2721 and $DiPGE_2$ enhanced radiation survival, it also produced the most severe and longest acting behavioral decrements. Therefore, increases in radioprotection also produced concomitant increases in behavioral toxicity.

The results of the research summarized in this report confirm related findings on the effects of bioactive lipids on behavior. Exogenously administered prostaglandins, including those of the E series, have been demonstrated to produce sedative or tranquilizing effects in a variety of species (22). In addition, intracerebroventricular administration of LTC_4 has been reported to decrease locomotor activity in the rat (23).

Bioactive lipids produce a variety of physiological responses. For example, $DiPGE_2$ induces diarrhea and sedative effects (18); LTC_4 produces increases in hematocrit and mean arterial blood pressure, which may be followed by prolonged hypotension (24, 25); and PAF results in profound hypotensive action, reflecting peripheral vasodilation and decreased cardiac output (26). In addition, the radioprotective phosphorothioate WR-2721 has produced hypotension, nausea, vomiting, and mild somnolence during clinical trials (27). The mechanism(s) by which these compounds exert their effects on locomotor activity, however, remain unknown.

It is not possible from these experiments to determine whether the adverse behavioral effects produced by the radioprotective compounds evaluated are mediated by a peripheral or central mechanism. Prostaglandins are capable of crossing the blood-brain barrier (28), while LTC_4 (29) and WR-2721 cannot (30). PAF can influence blood-brain barrier permeability, but does not appear to penetrate it (31). In addition, many bioactive lipids are synthesized by brain tissue (31, 32). The decrease in locomotor activity reported for these bioactive lipids may, therefore, be the result of intermediate messengers produced by the bioactive lipids administered or in response to their physiological action.

In general, the onset of behavioral decrement was highly correlated with the optimal pre-radiation administration time of the agent. The duration of the performance decrement, however, lasted considerably longer than the radioprotective effect, suggesting different mechanisms of action. Although bioactive lipids remain viable radioprotective and therapeutic agents, pharmacological concentrations of many of these compounds are behaviorally toxic, which may limit their usefulness.

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REFERENCES

1. Walden, T.L., Jr. J. Radiat. Res., 29: 255-260, 1988.
2. Walden, T.L., Jr., Patchen, M. and Snyder, S.L., Radiat. Res., 109: 440-448, 1987.
3. Walden, T.L., Jr. Ann. NY Acad. Sci. 524: 431-433, 1988.
4. Hughes, H.N., Walden, T.L., Jr. and Steel, L.K. Abstracts of the 37th Annual Meeting of the

Radiation Research Society, p. 186, 1989.
5. Halushka, P.V., Mais D.E., Mayeux, P.R. and Morinelli, T.A., Ann. Rev. Pharmacol. Toxicol., 10: 213-239, 1989.
6. Weiss, J.F., Kumar, K.S., Walden, T.L., Jr., Neta, R., Landauer, M.R. and Clark, E.P. Int. J. Radiat. Biol., 57:709-722, 1990
7. Hanson, W.R. and Thomas, C. Radiat. Res. 96: 393-398, 1983.
8. Hanson, W.R. and Ainsworth, E.J. Radiat. Res. 103: 196-203, 1985.
9. Hanson, W.R. In: Prostaglandin and Lipid Metabolism in Radiation Injury, (Eds. T.L. Walden, Jr. and H.N. Hughes), Plenum Press, New York, 1987, pp. 233-243.
10. Steel, L.K. and Catravas, G.N. In: Eicosanoids and Radiation, (Ed. P. Polgar), Kluwer Academic Publishers, Boston, 1988, pp. 79-87.
11. Steel, L.K., Walden, T.L., Jr., Hughes, H.N., and Jackson, W.E., III. Radiat. Res. 115: 605-608, 1988.
12. Walden, T.L, Jr., Patchen, M.L. and MacVittie, T.J. Radiat. Res. 113: 388-395, 1988.
13. Walden, T.L., Jr. Abstracts of the 37th Annual Meeting of the Radiation Research Society, p. 185, 1989.
14. Davidson, D.E., Grenan, M.M. and Sweeney, T.R. In: Radiation Sensitizers: Their Use in the Clinical Management of Cancer, (Ed. L.W. Brady), Masson, New York, 1980, pp. 309-320.
15. Hanson, W.R. Radiat. Res., 111: 361-373, 1987.
16. Landauer, M.R., Walden, T.L., Jr. and Davis, H.D. In: Frontiers in Radiation Biology, (Ed. E. Riklis), VCH Publishers, Weinheim, West Germany, in press.
17. World Health Organization, Environmental Health Criteria, Principles and Methods for the Assessment of Neurotoxicity Associated with Exposure to Chemicals. World Health Organization, Geneva, 1986.
18. Landauer, M.R., Walden, T.L., Jr., Davis, H.D. and Dominitz, J.A. In: Prostaglandin and Lipid Metabolism in Radiation Injury, (Eds. T.L. Walden, Jr. and H.N. Hughes), Plenum Press, New York, 1987, pp. 245-251.
19. Landauer, M.R., Davis, H.D., Dominitz, J.A. and Weiss, J.F. Pharmacol. Biochem. Behav. 27: 573-576. 1987.
20. Landauer, M.R., Davis, H.D., Dominitz, J.A. and Weiss, J.F. Pharmacol. Ther., 39: 97-100, 1988.
21. Landauer, M.R., Davis, H.D., Dominitz, J.A. and Weiss, J.F. Toxicology 49: 315-323, 1988.
22. Chiu, E.K.Y. and Richardson, J.S. Gen. Pharmacol. 16: 163-175, 1985.
23. Brus, R., Krzeminski, T., Juraszczyk, Z., Kurcok, A., Felinska, W. and Kozik, W. Biomed. Biochim. Acta 45: 1153-1158, 1986.
24. Landauer, M.R., Walden, T.L. Jr., Davis, H.D., Cranford, M.E. and Farzaneh, N.K. Abstracts of the 37th Annual Meeting of the Radiation Research Society, p. 185, 1989.
25. Moncada, S., Flower, R.J. and Vane, J.R. In: The Pharmacological Basis of Therapeutics, (Eds. A. Goodman Gilman, L.S. Goodman, T.W. Rall and F. Murad), Macmillan: New York, 1985, pp. 660-673.
26. Myers, A., Tores Durate, A. P. and Ramwell, P. Adv. Prostaglandin Thromboxane Leukotriene Res. 17: 833-837, 1987.
27. Kligerman, M.M., Turrisi, A.T., Urtasun, R.C., Norfleet, A.L., Phillips, T.L., Barkley, T. and Rubin, T. Int. J. Radiat. Oncol. Biol. Phys. 14: 1119-1122, 1988.
28. Bito, L.Z., Davson, H., and Hollingsworth, J.R. J. Physiol. 253: 273-285, 1976.
29. Spector, R. and Goetzl, E.J. Biochem. Pharmacol. 35: 2849-2853, 1986.
30. Utley, J.F., Marlowe, C. and Wadell, J.W. Radiat. Res. 68: 284-291, 1976.
31. Kumar, R., Harvey, S.A.K., Kester, M., Hanahan, D.J. and Olson, M.S. Biochim. Biophys. Acta 963: 375-383, 1988.
32. Murphy, S. and Pearce, B. Prostaglandins Leukotrienes Essential Fatty Acids 31: 165-170, 1988.

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Therapy of Infections in Mice Irradiated in Mixed Neutron/Photon Fields and Inflicted with Wound Trauma: A Review of Current Work

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LEDNEY, G. D., MADONNA, G. S., ELLIOTT, T. B., MOORE, M. M., AND JACKSON, W. E., III. Therapy of Infections in Mice Irradiated in Mixed Neutron/Photon Fields and Inflicted with Wound Trauma: A Review of Current Work. *Radiat. Res.* 128, S18–S28 (1991).

When host antimicrobial defenses are severely compromised by radiation or trauma in conjunction with radiation, death from sepsis results. To evaluate therapies for sepsis in radiation casualties, we developed models of acquired and induced bacterial infections in irradiated and irradiated–wounded mice. Animals were exposed to either a mixed radiation field of equal proportions of neutrons and γ rays ($n/\gamma = 1$) from a TRIGA reactor or pure γ rays from ^{60}Co sources. Skin wounds (15% of total body surface area) were inflicted under methoxyflurane anesthesia 1 h after irradiation. In all mice, wounding after irradiation decreased resistance to infection. Treatments with the immunomodulator synthetic trehalose dicorynomycolate (S-TDCM) before or after mixed neutron–γ irradiation or γ irradiation increased survival. Therapy with S-TDCM for mice irradiated with either a mixed field or γ rays increased resistance to *Klebsiella pneumoniae*-induced infections. Combined therapy with S-TDCM and ceftriaxone for *K. pneumoniae* infections in mice exposed to a mixed radiation field or to γ rays was more effective than single-agent therapy. In all irradiated–wounded mice, single therapy of acquired infections with an antibiotic or S-TDCM did not increase survival. Survival of irradiated–wounded mice after topical application of gentamicin sulfate cream suggested that bacteria colonizing the wound disseminated systemically in untreated irradiated mice, resulting in death from sepsis. In lethal models of acquired infections in irradiated–wounded mice, significant increases in survival were achieved when systemic treatments with S-TDCM or gentamicin were combined with topical treatments of gentamicin cream. Therapies for sepsis in all mice exposed to a mixed field were less effective than in mice exposed to γ rays. Nonetheless, the data show a principle by which successful therapy may be provided to individuals receiving tissue trauma in conjunction with radiation injury. © 1991 Academic Press, Inc.

INTRODUCTION

When a host's antimicrobial defenses are severely compromised by γ or neutron radiation, death from sepsis may occur (*1*). Therapy with antibiotics for infections that accompany hematopoietic system failure decreases morbidity and mortality of neutron-irradiated mice (*2*). Limited research has been done on antibiotic therapy of infection in neutron-irradiated hosts. Streptomycin therapy for infection in fast-neutron-irradiated mice increased survival times but not overall survival rates (*3*). Treating infections with antibiotics in neutron-irradiated mice may be more difficult because tissues involved in the defense against microbial infection undergo more damage than that seen after an equal dose of γ radiation (*4, 5*). Trauma inflicted on γ-irradiated hosts increases the difficulty of treating bacterial infections significantly compared to treating trauma or radiation exposure alone (*2, 6, 7*). Penicillin treatments contain but do not eradicate *Staphylococcus aureus* from infected muscle wounds in mice given sublethal doses of γ rays (*8*). Antibiotic treatments for wound sepsis may be more difficult to develop for γ-irradiated mice because the complications after wound trauma are more severe (*9*).

The disaster at Chernobyl (*10*) and the atomic detonations at Hiroshima and Nagasaki (*11*) underscore the need for effective therapies for infection in humans subjected to nuclear radiation and physical trauma. Newly synthesized antibiotics and immunomodulators may treat infections in severely immunocompromised hosts effectively. For example, we have shown that the immunomodulator synthetic trehalose dicorynomycolate (S-TDCM), when injected into γ-irradiated mice either alone or with a third-generation cephalosporin, ceftriaxone, increased survival from induced lethal *Klebsiella pneumoniae* infections (*12*). Using S-TDCM with the fluoroquinolone ofloxacin to treat acquired infections after γ irradiation and skin wound trauma also increased survival times of mice (*13*).

This paper reviews our findings on the survival of irradiated mice and of irradiated–wounded mice treated with S-TDCM and antibiotics for endogenously acquired nosocomial infections and exogenously induced *K. pneumoniae* infections. The survival of neutron-irradiated mice treated with S-TDCM and antibiotics is compared with the survival obtained for γ-irradiated mice receiving the same treatments.

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MATERIALS AND METHODS

Animals

B6D2F1/J female mice were obtained from Jackson Laboratory (Bar Harbor, ME). C3H/HeN female mice were obtained from the National Cancer Institute Animal Breeding Facility (Frederick, MD). The mice were maintained as described previously (*14*). 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.

Neutron Irradiations

Neutron irradiations were performed using the AFRRI TRIGA Mark-F reactor. This reactor is a movable-core pool-type facility with maximum operational steady-state power of 1 MW. The reactor was operated at 45 kW. The neutron-to-photon dose ratio of 1 was achieved by irradiating mice through a 15-cm lead shield, 255 cm from the tank wall and 120 cm above the exposure room floor. The mean energy for neutrons and photons in experiments was approximately 0.8 MeV (*15*). Thus mice received radiation doses with a spectrum of energies that contained an equal mixture of neutrons (*n*: 50%) and γ photons (γ: 50%). All reactor irradiations were performed at a total dose rate of 38 cGy/min. 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.

Irradiation with γ Photons

B6D2F1/J mice were irradiated bilaterally at 40 cGy/min in a ^{60}Co γ-ray 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 ratios were 0.988 for bilateral ^{60}Co γ irradiation and 0.98 for unilateral ^{60}Co γ irradiation. Dosimetric techniques for measuring TRIGA reactor and ^{60}Co-produced radiations have been described (*5*).

Skin Wounding

Mice were anesthetized by inhalation of methoxyflurane before wounding. Full-thickness, nonlethal skin injuries were inflicted after irradiation by removing a 1- by 1.5-in. section of dorsal skin and underlying panniculous carnosus muscle with a steel punch. Details for inflicting skin wounds were described previously (*14*).

Bacteria

The bacteria used in the majority of our studies were a clinical isolate of *K. pneumoniae* capsule type 5. The bacteria were harvested in the logarithmic phase of growth in brain–heart infusion broth (*8, 12*). Mice were given doses of viable bacteria based on colony-forming units (CFU) as described previously (*8, 12*). Endogenous bacteria found in nonirradiated normal mice or irradiated mice were identified by standard techniques using blood agar and MacConkey agar. Specimens were incubated in air and 5% CO_2 or in anaerobic jars where necessary for identification.

Therapy

S-TDCM was a gift of Pibi ImmunoChem Research, Inc. (Hamilton, MT). The chemical formulation of S-TDCM results in a molecular weight of about 1250 Da (*14*). Briefly, S-TDCM is prepared by the condensation of ^{32}C-labeled mycolic acid residues with the sugar trehalose. Prior to use, stock S-TDCM concentrations were prepared using pyrogen-free materials by dissolving in hexane and drying under nitrogen (*14*). The dried product was then homogenized in 0.2% Tween 80–0.9% NaCl and sonicated. Endotoxin levels in 1 mg/ml S-TDCM samples were <0.006 ng/ml as determined by the limulus lysate assay. Various concentrations of S-TDCM were injected intraperitoneally (ip) into mice in 0.5-ml volumes.

Oxacillin sodium, gentamicin sulfate, ofloxacin, and ceftriaxone sodium were used systemically. All antibiotics were prepared in pyrogen-free water, and 0.1-ml quantities were 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) and a generic 0.1% gentamicin sulfate cream were used topically. The creams were applied once daily for 10 consecutive days in 0.5–0.7 g amounts sufficient to cover the wounded site.

Statistical Analyses

Survival data for mice in experimental groups were obtained for 30 days after irradiation. Comparisons were made by the generalized Savage (Mantel–Cox) procedure (*16*). Probit analysis of numbers of mice surviving 30 days was made on log-transformed doses using the methods of Finney (*17, 18*).

RESULTS

Survival of Normal and Skin-Wounded Mice after Exposure to Mixed-Field Irradiation (n/γ = 1) or to γ Rays

We developed immunocompromised models for acquired and induced bacterial infections by irradiating or irradiating and wounding the two strains of mice. The aim was to establish n/γ-ray and ^{60}Co γ-ray doses that mice could survive after successful therapeutic interventions for sepsis. Further, to compare the efficacy of treatments for sepsis, we wanted to use mixed-field and photon-only exposures that produced the same degree of lethality in the absence of treatment. We performed 30-day survival probit analyses to calculate the relative biological effectiveness (RBE) of exposure to the mixed radiation field compared to γ irradiation in mice, based on $LD_{50/30}$ end points.

The two mouse strains were used because their intestinal bacterial microflora were different (Table I). While gram-positive organisms (presumptive lactobacilli) were found in both mouse strains, *Escherichia coli* and *Proteus mirabilis* were isolated only from the C3H/HeN strain. These *Enterobacteriaceae* are common opportunistic intestinal microflora in irradiated mice. Translocation of the intestinal microflora from the lumen of the gut in irradiated hosts results in endogenously derived septicemia (*2*). Thus groups of B6D2F1/J and C3H/HeN mice were given mixed-field doses ranging from 2.5 to 6.0 Gy. A comparative study was done with these mouse strains given ^{60}Co γ-ray photons in doses ranging from 5.5 to 11.0 Gy. In both sets of experiments, additional groups of mice received skin wounds 1 h after irradiation. Table II presents the $LD_{50/30}$, RBE, and slope values for all of these experiments. The $LD_{50/30}$ values for B6D2F1/J mice were higher than for C3H/HeN mice, indicating resistance to the lethal effects of radiation or radiation–wounding. Wounding after irradiation reduced the $LD_{50/30}$'s for both mouse strains. Slope values for mixed-field and γ-irradiated B6D2F1/J mice and for γ-irradiated C3H/HeN mice were decreased ($P < 0.009$) by wound

TABLE I
Differences in Intestinal Microflora in C3H/HeN Radiosensitive Mice and B6D2F1/J Radioresistant Mice[a]

		Mouse strain					
		C3H/HeN			B6D2F1/J		
Tissue	Gram's stain	Number positive/ tested	Mean CFU	±SE	Number positive/ tested	Mean CFU	±SE
Ileum	Positive	5/5	1.3×10^8	2.3×10^8 7.1×10^7	5/5	7.1×10^7	1.7×10^8 2.9×10^7
	Negative *E. coli*	3/5	1.1×10^5	2.0×10^5 6.4×10^4	0/5	—	—
	Negative anaerobic	0/5	—	—	—	—	—
Cecum	Positive	5/5	5.0×10^8	6.4×10^8 4.0×10^8	5/5	2.2×10^8	3.0×10^8 1.5×10^8
	Negative *E. coli*	5/5	1.6×10^5	3.4×10^5 7.4×10^4	0/5	—	—
	Negative anaerobic *B. ovatus*	5/5	1.7×10^7	3.2×10^7 9.3×10^6	3/5	1.4×10^7	8.1×10^7 2.4×10^6

[a] Nonirradiated normal mice were used in this analysis. The gram-positive bacteria are presumptive *Lactobacillus* sp. *Proteus mirabilis* is frequently found in C3H/HeN mice, but was not identified in these studies; only the major bacterial genera and species were identified.

trauma. The decreased slope values indicate additional contributions by wound trauma to biological variability. The slope for C3H/HeN mice exposed to the mixed field was not changed ($P = 0.22$) by adding wound trauma. Thus only curves representing the survival of C3H/HeN mice irradiated with the mixed field could be drawn parallel.

The significance of the intestinal microflora data in relation to survival may be explained as follows. In γ-photon-irradiated mice the intestinal microflora have an equal impact on survival in both strains when the animals are inflicted with skin wounds. The intestinal colonization of C3H/HeN mice with opportunistic bacteria did not change the mode of death after mixed-field irradiation and the infliction of skin wounds. Presumably, opportunistic bacteria of wounded or unwounded B6D2F1/J mice were not a factor in overall survival after mixed-field or γ irradiation. The RBE value of about 2.0 for both irradiated–wounded and irradiated B6D2F1/J mice supports this observation. It may be that colonization of the wound site by gram-positive bacteria from other sources (skin, bedding) contributed to mortality equally, independent of the quality of radiation.

TABLE II
Survival of Normal and Skin-Wounded Mice after Mixed-Field or γ-Photon Irradiation

		Radiation only			Radiation and wound		
Mouse strain	Radiation source	Slope[a]	$LD_{50/30}$ (cGy)	RBE[b]	Slope	$LD_{50/30}$ (cGy)	RBE
B6D2F1/J	^{60}Co[c]	35.8	964 ± 30	1.95	22.3	761 ± 25	2.0
	Reactor	40.3	494 ± 1	—	23.4	381 ± 8	—
C3H/HeN	^{60}Co[d]	37.6	732 ± 13	1.73	20.8	653 ± 21	2.11
	Reactor	29.1	423 ± 7	—	31.5	310 ± 7	—

[a] Wound trauma significantly ($P < 0.009$) decreased slope value for mixed-field and γ-irradiated B6D2F1/J mice and for γ-irradiated C3H/HeN mice. The slope for mixed-field-irradiated C3H/HeN mice was not changed ($P = 0.22$) by inflicting wound trauma. Slope values for B6D2F1/J and C3H/HeN mice were similar after γ irradiation and γ irradiation with subsequent wound trauma. Slope values for these strains of mice were significantly different after mixed-field irradiation ($P = 0.0002$). The P value was 0.096 between the strains given mixed-field radiation and wound trauma.

[b] Values were calculated at $LD_{50/30}$.

[c] Bilateral exposure.

[d] Unilateral exposure.

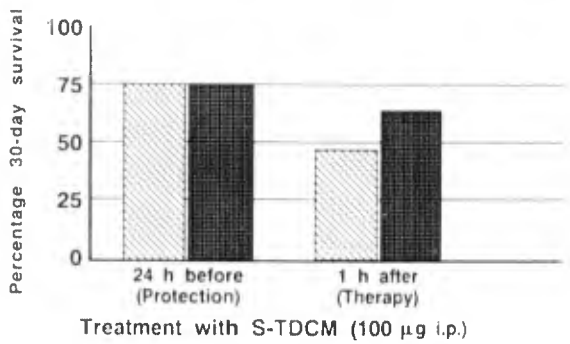

FIG. 1. Survival of mixed-field-irradiated (▩) and γ-irradiated (□) mice treated with S-TDCM. Groups of 20 B6D2F1/J mice were injected ip with 100 μg S-TDCM before or after irradiation with comparable $LD_{80/30}$'s, and survival was recorded for 30 days. Survival in saline-treated mice (0.2% Tween–0.9% NaCl or 0.9% NaCl alone) was 10–15% after irradiation. Survival in all S-TDCM-treated groups was increased significantly ($P < 0.05$), compared to survival for saline-treated groups.

In conclusion, in B6D2F1/J mice, 7.0 Gy γ rays and 3.5 Gy mixed-field radiation were used as sublethal immunocompromising doses while 10.25 Gy γ rays and 5.15 Gy mixed-field radiation were used as the $LD_{80/30}$'s. In C3H/HeN mice, 8.0 Gy γ rays and 4.0 Gy mixed-field radiation were used as minimum lethal radiation doses in irradiation–wounding studies.

Survival of Irradiated Mice after TDM Treatment

Substances used to treat casualties in nuclear radiation environments should have minimal effects on task performance (*19*). Locomotor and consumatory behaviors of normal nonirradiated mice are affected only slightly by 100–200 μg ip doses of S-TDCM (*20*). Therefore, we determined the 30-day survival of irradiated mice ($LD_{80/30}$) treated with S-TDCM (Fig. 1). S-TDCM (100 μg, ip) was equally effective (75% survival) when injected into B6D2F1/J mice 24 h before either 10.25 Gy γ irradiation or 5.15 Gy mixed-field irradiation. S-TDCM treatments 1 h after the respective $LD_{80/30}$'s resulted in 45% survival for γ rays and 65% survival for mixed-field radiation ($P > 0.05$).

We evaluated the protective and therapeutic values of natural TDM from *Mycobacterium phlei* in 2% squalene oil (TDM-O) in 0.2% Tween-80 saline for radiation-induced injuries by performing complete dose–response survival curves with B6D2F1/J mice. (Experiments with TDM-O were done before the synthetic preparation of TDM became available.) The dose reduction factors (DRFs) for mixed-field-irradiated and γ-irradiated mice were somewhat greater (Table III) in protection studies (50 or 100 μg TDM-O 24 h before irradiation) than in therapy studies (50 or 100 μg TDM-O 1 h after irradiation). While the DRFs for irradiated mice treated with TDM-O ranged between 1.1 and 1.3, the LD_{50}'s are significantly different ($P < 0.05$) for saline-treated, irradiated mice. Values for the DRF as high as 2.7 were reported for mice treated with *s*-2(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) (*21*) and 1.3 for mice treated with bacterial endotoxin (lipopolysaccharide; LPS) (*22*). While the DRFs reported here for γ-irradiated mice are lower than those for mice treated with WR-2721 and somewhat similar to those for mice treated with LPS, both agents have substantial toxicity compared to S-TDCM. Also, S-TDCM is the first immunomodulator reported to have beneficial effects in animals exposed to mixed field radiation (*23*). The DRF for S-TDCM indicates a modest efficacy in treating neutron-irradiated victims. In contrast, the DRF for WR-2721 in a high-neutron field ($n/\gamma = 20$) produced by the reactor was only 1.2 (*24*).

Endogenous Spleen Colonies in Irradiated Mice after S-TDCM Treatment

Bacterial endotoxins, when injected into heavily irradiated mice, enhance hematopoietic recovery as measured by increases in the number of peripheral blood leukocytes and spleen colony-forming units (*22*). We evaluated S-TDCM for its ability to accelerate hematopoietic recovery in irradiated mice by counting endogenous spleen colony-forming units (E-CFU-S) (Fig. 2). Treatments with S-TDCM either before or after mixed-field or γ irradiation increased E-CFU-S more ($P < 0.05$) than in untreated mice. Increasing the dose of S-TDCM to 400 μg for treatment after irradiation did not increase the colony numbers significantly from those recorded for 100-μg S-TDCM treatments before irradiation. While S-TDCM increased E-CFU-S, no quantitative relationship between survival and number of colonies is predicted. Exhaustive studies with bacterial en-

TABLE III
Survival of Irradiated Mice after TDM-O Treatment

	Radiation quality[a]			
	Mixed-field[b]		Gamma rays[c]	
Treatment	$LD_{50/30}$	DRF[d]	$LD_{50/30}$	DRF[d]
24 h before irradiation				
TDM-O	596	1.12	1178	1.27
0.9% NaCl	531	—	928	—
1 h after irradiation				
TDM-O	581	1.12	988	1.06
0.9% NaCl	518	—	928	—

[a] $LD_{50/30}$'s for B6D2F1/J mice were determined from log-transformed dose–response survival data prepared by giving groups of animals γ radiation in 50-cGy increments and mixed-field radiation in 25- or 50-cGy increments (10–20 mice were irradiated at each dose).

[b] 100 μg TDM-O and 0.2% Tween-80 saline.

[c] 50 μg TDM-O.

[d] DRFs were calculated by dividing $LD_{50/30}$ for TDM-O-treated mice by the $LD_{50/30}$ for control mice. All DRF values for TDM-O-treated mice are different from controls ($P < 0.05$).

dotoxin in γ-irradiated mice suggest a qualitative and not a quantitative relationship between survival and E-CFU-S (*22*).

Resistance of Irradiated Mice to Induced Bacterial Infections after S-TDCM Treatment

Several natural products derived from bacterial cell walls, chemically modified cell wall products, and synthetic analogues of natural cell wall materials enhance the resistance of normal mice to microbial challenge (*25, 26*). Because treating irradiated mice with S-TDCM increased survival and stimulated hematopoietic recovery, we tested S-TDCM for an ability to increase resistance against lethal infections induced with *K. pneumoniae*. To develop a model of infection in immunocompromised mice suitable for treatment with S-TDCM, we determined the range of susceptibility to *K. pneumoniae* after sublethal irradiation. Groups of 8–16 B6D2F1/J were challenged sc with 10-fold dilutions of *K. pneumoniae* on 1, 4, 7, 10, and 14 days after irradiation with 3.5 Gy mixed-field radiation or 7.0 Gy γ rays. Figure 3 shows the $LD_{50/30}$ survival data for these experiments. There was no significant difference in susceptibility ($P > 0.05$) to bacterial challenge 1, 4, and 7 days after exposure to either the mixed-field radiation or γ rays, but γ-irradiated mice were more susceptible to infection than mice exposed to mixed-field radiation at 10 and 14 days ($P < 0.05$). Maximum susceptibility to challenge with *K. pneumoniae* was at 4 days after irradiation with the calculated $LD_{50/30}$ CFU of 80 and 290 for the mixed-field exposures and γ-ray exposures, respectively. In normal nonirradiated mice the $LD_{50/30}$ for sc challenge with *K. pneumoniae* is approximately $5–7 \times 10^6$ CFU.

The susceptibility of irradiated mice to challenge with *K. pneumoniae* may be related to the low number of peripheral blood elements at the time of challenge and during the subsequent development of sepsis (Fig. 4). In mice exposed to the mixed field, peripheral blood leukocyte numbers were lower than those in γ-irradiated mice at the time of bacterial challenge, while platelet numbers returned to normal levels earlier in mice exposed to a mixed field than in γ-irradiated mice. Perhaps the higher platelet levels in mice exposed to a mixed field accounted for the decreased susceptibility to *K. pneumoniae* challenge the second week after irradiation.

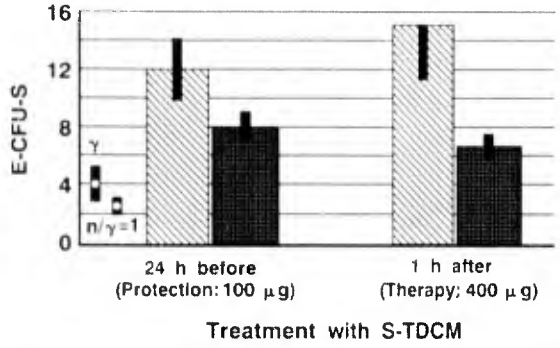

FIG. 2. Endogenous spleen colony-forming units (E-CFU-S) in mixed-field-irradiated (▩) and γ-irradiated (□) mice treated with S-TDCM. Twelve to fifteen mice were injected ip with 100 μg S-TDCM 24 h before irradiation or 400 μg S-TDCM after irradiation. Spleens were harvested at 10 days and fixed in Bouin's solution, and the nodules that formed were counted. The average number of nodules formed after control treatments (0.2% Tween–0.9% NaCl or 0.9% NaCl alone) were 2–3 for mixed-field-irradiated mice and 3–5 for γ-irradiated mice; the number of nodules formed after S-TDCM treatment was increased significantly ($P < 0.05$), compared to nodules formed in saline-treated groups.

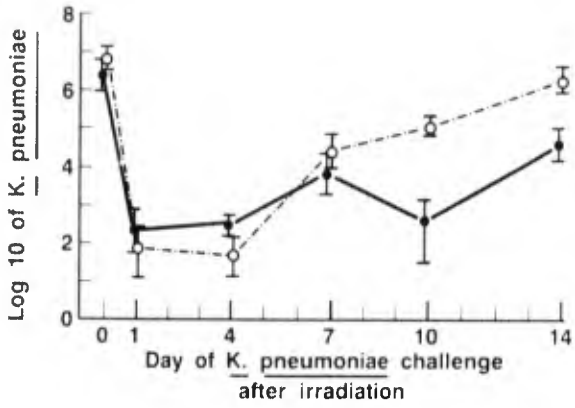

FIG. 3. $LD_{50/30}$ of *K. pneumoniae* for irradiated B6D2F1/J mice. Mice received comparable γ (●) and mixed-field (○) radiation doses of 7.0 and 3.5 Gy, respectively. *K. pneumoniae* was injected sc into groups of 8–16 mice on the indicated days. Each $LD_{50/30}$ is based on survival data from six groups of mice, each group receiving a 10-fold dilution of *K. pneumoniae* ranging between 10^1 and 10^8 viable CFU. The $LD_{50/30}$ was determined by probit analysis for each type of radiation and each time of challenge after irradiation. Vertical bars represent the upper and lower 95% confidence limits of each $LD_{50/30}$. Susceptibility to *K. pneumoniae* challenge was greater ($P < 0.05$) at Days 10 and 14 after γ irradiation than it was after mixed-field irradiation.

We used an end point of 80% survival to test the effectiveness of S-TDCM in treating *K. pneumoniae* sepsis induced in B6D2F1/J mice 4 days after exposure to sublethal levels of mixed-field or γ irradiation (Fig. 5). The resistance of the host was enhanced by S-TDCM more effectively in mice exposed to the mixed field than in those exposed to γ rays. Treatment with S-TDCM increased resistance to 10^3 $LD_{50/30}$'s of *K. pneumoniae* in mice exposed to the mixed field and to about 4×10^2 $LD_{50/30}$ in γ-irradiated mice. This is a significant increase in resistance in both radiation models, but normal mice are about 50 times more resistant to *K. pneumoniae* challenge than S-TDCM-treated, irradiated mice. Other studies evaluating S-TDCM for treatment of *K. pneumoniae* sepsis in irradiated mice have been reported (*12, 23*).

Survival from Lethal Infections in Irradiated Mice after Antibiotic Treatment

The use of antibiotics for the management of infections in irradiated hosts was reviewed in work published by one of our group (*2*). We recently evaluated two new antibiotics, ceftriaxone (a third-generation cephalosporin) and ofloxa-

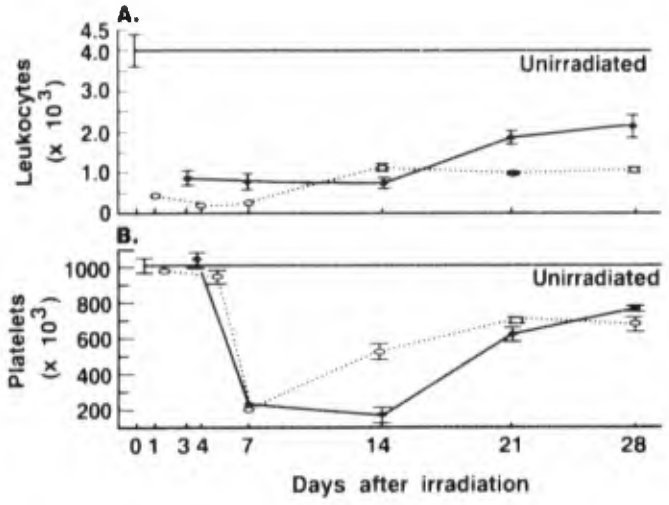

FIG. 4. Peripheral blood leukocytes and platelets in mice given comparable mixed-field (○) and γ (●) irradiations. Groups of three to eight B6D2F1/J mice were bled at the indicated times after irradiation. Normal cell values were obtained from groups of 10 mice. Leukocytes and platelets were significantly reduced ($P < 0.05$) over the 28-day period. (A) Mixed-field irradiation reduced ($P < 0.05$) the leukocyte count significantly compared to that for γ-ray photons in the first week of the experiment and during the last week of the experiment ($P < 0.05$). (B) Platelets were significantly reduced ($P < 0.05$) in γ-irradiated mice compared to those exposed to the mixed field at 14 days.

cin (a fluoroquinolone), for their efficacy in treating induced infections in γ-irradiated mice and mice exposed to the mixed field (Table IV). While different infection models in irradiated mice were used to evaluate these antibiotics, it is clear that they increased survival significantly. Ceftriaxone therapy for mice exposed to the mixed field and infected with *K. pneumoniae* had no specific efficacy: the mice received ≃90 $LD_{50/30}$'s of *K. pneumoniae*, while γ-irradiated mice received ≃1.7×10^4 $LD_{50/30}$'s of *K. pneumoniae*.

Compared to γ-irradiated animals, little research has been done on antibiotic treatments for endogenously acquired infections in neutron-irradiated hosts. Early work (*3*) showed that streptomycin therapy for acquired infections in mice given midlethal doses ($LD_{75/30}$) to near-lethal doses ($LD_{90/30}$) of fast neutrons decreased mortality by 25 and 15%, respectively. (Treating infections in neutron-irradiated hosts with new classes of antibiotics or new modalities of therapy (*12, 23, 27*) should be studied.) Along these lines, a synergistic increase in survival ($P < 0.05$) was achieved in mice exposed to the mixed field (3.5 Gy) systemically treated with S-TDCM and ceftriaxone for *K. pneumoniae* sepsis, compared to single-agent therapy with an immunomodulator or antibiotic (Fig. 6). A synergistic increase in survival from *K. pneumoniae* sepsis by combined treatments was not ($P > 0.05$) obtained in mice irradiated with 7.0 Gy γ rays. However, combined treatments with S-TDCM and ceftriaxone resulted in more survivors (95%) than did ceftriaxone alone (75%) ($P > 0.05$).

Survival Time from Sepsis of Irradiated Mice Inflicted with Wound Trauma after Systemic Combined-Modality Therapy with Antibiotics and S-TDCM

The therapeutic effectiveness of S-TDCM or antibiotics can be obviated by increasing the challenge dose of bacteria or may be decreased when wound or burn trauma is also present. Colonization of both the injured site and the intestinal tract by opportunistic bacteria leading to massive systemic bacterial translocation may overwhelm therapeutic efforts. Because S-TDCM and antibiotics effectively treated acquired and induced sepsis in irradiated mice, we performed experiments testing their efficacy in treating endogenously acquired infection in irradiated mice inflicted with wound trauma. Table V presents survival data for C3H/HeN mice given 8.0 Gy γ rays (an approximate $LD_{100/30}$) and a 15% skin wound followed by systemic antibacterial treatments. These studies showed that only antibiotics increased the survival time of irradiated–wounded mice. S-TDCM increased survival from a lethal dose of γ rays, but it had an apparent detrimental effect in irradiated mice inflicted with wounds by reducing the survival time from 5.9 ± 0.2 days for controls to 5.5 ± 0.1 days. These survival times were not significantly different from each other ($P > 0.05$). Ofloxacin and gentamicin therapy of irradiated-wounded mice increased ($P < 0.05$) survival times most. In experiments not reported here, S-TDCM also tended to reduce survival time when combined with ofloxacin (8.9 ± 0.2 days) compared to ofloxacin alone (9.2 ± 0.2 days). We did not test the effect of S-TDCM on survival in gentamicin-treated mice in this experimental series. We concluded from these experiments that single-agent therapy with S-TDCM or antibiotics increases survival of irradiated

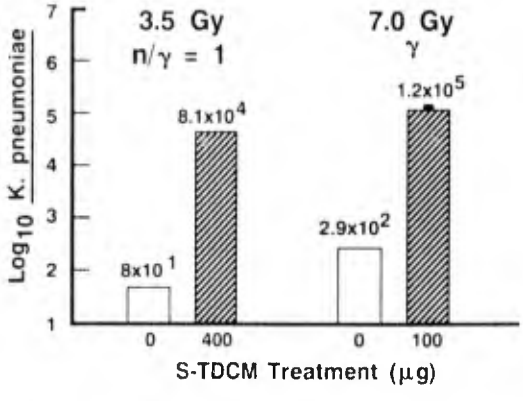

FIG. 5. Resistance of irradiated mice to bacterial challenge after therapy with S-TDCM. Groups of 16–20 B6D2F1/J mice were given comparable doses of γ rays and mixed-field radiation. At 2 h after irradiation, γ-irradiated mice received 100 μg S-TDCM while mice exposed to the mixed field received 400 μg S-TDCM. Mice were challenged sc with *K. pneumoniae* 4 days after irradiation and were observed for 30-day survival. The bars for irradiated mice (□) represent the number of *K. pneumoniae* CFU required for a 50% survival end point, while the bars for S-TDCM-treated irradiated mice (▨) represent an 80% survival end point.

TABLE IV
Survival of Irradiated Mice Given Antibiotic Therapy for Induced *Klebsiella pneumoniae* Infections

Radiation	Infection model	Treatment[a]	30-day survival, %[b]
7.0 Gy γ rays	5×10^6 CFU, sc Day 4	Ceftriaxone	11/16, 69%
	5×10^6 CFU, sc Day 4	0.9% NaCl	0/10, 0%
8.0 Gy γ rays	10^8 CFU, po Day 2	Ofloxacin	51/60, 85%
	10^8 CFU, po Day 2	0.9% NaCl	16/60, 25%
3.5 Gy mixed-field radiation	7.2×10^3 CFU, sc Day 4	Ceftriaxone	18/20, 90%
	7.2×10^3 CFU, sc Day 4	0.9% NaCl	0/20, 0%

[a] Ceftriaxone, 1.875 mg/mouse sc, was administered once daily for 10 consecutive days commencing 1 day after sc challenge with *K. pneumoniae;* ofloxacin, 1.0 mg/mouse po, once daily for 7 consecutive days starting 1 day after po challenge with *K. pneumoniae* Control treatment of irradiated–infected mice was 0.1 ml 0.9% NaCl.

[b] Therapy with antibiotics significantly ($P < 0.01$) increased survival when compared to respective saline-treated control mice.

hosts, but S-TDCM alone or with an antibiotic seemed to reduce survival time in irradiated–wounded mice.

Antibiotic-resistant organisms obviate the efficacy of antibacterial treatments. Thus, to determine why single-agent therapy with ofloxacin and combined modality therapy with S-TDCM and ofloxacin did not increase the number of survivors, we analyzed the bacteria colonizing the wound site and the bacteria that had been translocated to the liver to determine the antibacterial effectiveness of these agents. The liver is the principal organ involved in disseminated sepsis (*28, 29*). Table VI lists the bacteria associated with the wound site and liver of wounded, irradiated–wounded, or irradiated mice. Gram-negative and gram-positive bacteria were found on the wound site of irradiated–wounded mice treated with S-TDCM or treated with saline.

Gram-positive and gram-negative bacteria were 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

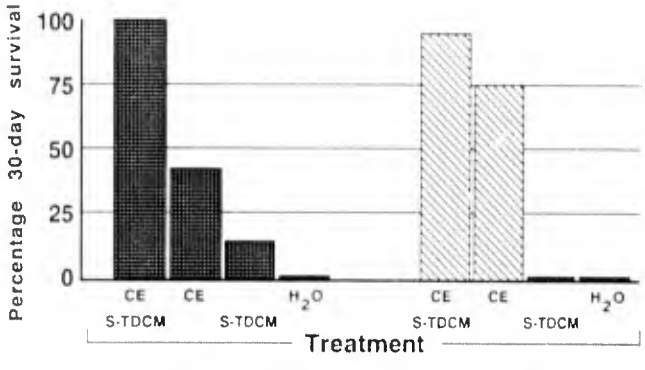

FIG. 6. Survival after therapy with S-TDCM and ceftriaxone for sepsis in irradiated mice. Groups of 20 B6D2F1/J mice were irradiated and treated as follows: S-TDCM = 200 μg ip 1 h after irradiation; CE = 75 mg/kg ceftriaxone sc once daily for 10 consecutive daily doses commencing 1 day after *K. pneumoniae* challenge (Day 4 after irradiation). *K. pneumoniae* doses were 3.3×10^5 CFU ($\simeq 3 \times 10^4$ $LD_{50/30}$) for mice exposed to the mixed field (▩) and 5.2×10^6 CFU ($\simeq 2.2 \times 10^4$ $LD_{50/30}$) for γ-irradiated mice (□). In mice exposed to the mixed field, survival was increased by combined modality therapy with S-TDCM and ceftriaxone which acted synergistically ($P = 0.0389$) compared to therapy with a single agent. In γ-irradiated mice, no synergistic increase in survival after combined therapy was found ($P = 0.149$).

TABLE V
Survival Time of Lethally Irradiated Mice Inflicted with Wound Trauma and Given Systemic Antibiotics[a]

Treatment	Survival time (days)	Survival fraction
Saline + ofloxacin	9.2 ± 0.2[b]	0/20
Saline + gentamicin	8.8 ± 0.4	0/20
Saline + oxacillin	6.6 ± 0.3	0/20
S-TDCM + H_2O	5.5 ± 0.1	0/20
Saline + H_2O	5.9 ± 0.2	0/40

[a] C3H/HeN mice were given 8.0 Gy γ radiation 1 h before inflicting skin wound trauma. All mice other than those treated with S-TDCM were treated with 0.5 ml 0.9% saline after wound trauma. The mean survival time for mice given 8.0 Gy radiation only and not wounded was 12.8 ± 0.4 days. Survival of 70% was recorded for mice given 200 μg S-TDCM ip 1 h after 8.0 Gy. Antibiotics or H_2O were provided once daily commencing 2 days after skin wound trauma and for 9 or 10 consecutive days thereafter. In three different experimental series the survival times for irradiated–wounded mice given no treatments ranged between 7 and 9 days.

[b] Ofloxacin and gentamicin significantly increased ($P < 0.05$) survival times of irradiated–wounded mice, compared to all other treatment groups. All antibiotics significantly increased ($P < 0.05$) survival times of irradiated-wounded mice, compared to survival times after S-TDCM treatment.

TABLE VI
Isolation of Bacteria from Wound and Liver of γ-Irradiated–Wounded Mice after S-TDCM and Ofloxacin Therapy[a]

Experimental group	Day of culture: 4–5 Wound	4–5 Liver	6–11 Wound	6–11 Liver
Treatment				
Saline or S-TDCM	*S. aureus* *S. faecium* *E. coli* *P. mirabilis*	*P. mirabilis*	[b]	[b]
Ofloxacin	*S. aureus* *S. faecium*	[c]	*S. aureus* *S. xylosus*	*S. aureus* *A. viridans* *S. faecium* *S. faecalis*
S-TDCM/ofloxacin	*S. aureus* *S. faecium*	*S. faecium* *S. aureus*	*S. aureus* *S. xylosus* *S. faecium*	*S. aureus* *S. faecium* *A. viridans* *S. faecalis*
Control				
Wounded	*S. aureus* *S. xylosus* *Streptococcus* spp. *S. epidermidis*	[c]	*S. aureus* *S. xylosus* *Streptococcus* spp.	[c]
Irradiated	[d]	[c]	[d]	*S. aureus* *E. coli* *K. oxytoca*

[a] C3H/HeN mice were wounded 1 h after 8.0 Gy γ irradiation, and antibiotic therapy began 4 h later. S-TDCM (200 μg) was given ip 1 h after irradiation, immediately after wounding. Mice in each group were euthanized 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.

with ofloxacin or ofloxacin and S-TDCM. 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 colonizing the wound site.

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

Bacteria colonizing the wound site were disseminated systemically in irradiated mice, as noted in Table VI. Because topical and systemic antibiotics applied together are more effective in treating wound infections (*30*), we evaluated several common topical antibiotic preparations for their efficacy in treating wound infections in mice injured after irradiation. Common disinfectants were also evaluated. The agents tested and the survival data obtained are listed in Table VII. In this experimental series, as well as in other tests, gentamicin cream increased survival time most ($P < 0.05$), and hence was used in later experiments with systemic antibiotics and S-TDCM alone or in combination.

Systemic antibiotic therapy included sc treatments with oxacillin, ofloxacin, and gentamicin in both γ-irradiated mice and those exposed to the mixed field. Systemic ceftriaxone was evaluated only in those exposed to the mixed field. The survival data for topical and systemic antibiotic treatments are presented in Fig. 7. In mice wounded after 8.0 Gy γ 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 $P < 0.05$ when compared to the survival obtained for topical gentamicin treatments and $P < 0.01$ for all other comparisons. In mice wounded after 3.8 Gy mixed-field radiation, topical gentamicin sulfate in combination with all the antibiotics tested systemically increased survival significantly ($P < 0.01$). There were no significant differences in survival between the antibiotic treatment

TABLE VII
Survival of Irradiated–Wounded Mice after Topical Application of Antibiotics[a,b]

Antibiotic/product and manufacturer	Survival	
	Mean ± SE (days)[c]	Fraction
0.1% gentamicin sulfate/ Garamycin cream, Schering	7.71 ± 0.57	0/7
1% silver sulfadiazine/ Silvadene cream, Marion	6.13 ± 0.35	0/8
2% mupirocin/ Bactroban ointment, Beecham	5.13 ± 0.23	0/8
Untreated control	4.38 ± 0.18	0/8

[a] Mice were wounded 1 h after 8.0 Gy γ irradiation. Treatment with each topically applied antibiotic commenced 4 h after wounding and was applied for 5 consecutive days. In other experimental series not indicated here, topical gentamicin increased the mean survival time and resulted in an irregular but small number of 30-day survivors.

[b] 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, Chlorox). Because wound colonization by bacteria was not altered by these disinfectants in normal mice, they were not evaluated in irradiated mice.

[c] Topical application of gentamicin sulfate and silver sulfadiazene significantly increased ($P < 0.05$) the survival of treated mice compared to untreated control mice. The mean survival time for gentamicin-treated mice differed significantly from silver sulfadiazene-treated mice ($P = 0.0213$).

groups ($P > 0.05$). 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 (*31*). *S. aureus* was frequently found colonizing the wound and disseminating to the liver in irradiated–wounded mice (Table VI).

To demonstrate the efficacy of S-TDCM treatments with combination antibiotic therapies (topical plus systemic), we used 0.1% topical gentamicin sulfate in combination with systemic gentamicin, oxacillin, ofloxacin, and ceftriaxone treatments. In models of lethal exposures to the mixed field and to γ rays followed by skin wound trauma, topical gentamicin and S-TDCM therapy with or without systemic gentamicin treatment resulted in approximately 60% survival (Fig. 8). S-TDCM effectively increased survival ($P < 0.05$) when combined with antibiotic treatments, compared to antibiotic treatments given without S-TDCM. Only S-TDCM combined with gentamicin produced 30-day survival approximating 50% in both mice exposed to the mixed field and those exposed to γ rays. 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 antibiotics without topical gentamicin sulfate in S-TDCM-treated, irradiated–wounded mice or the combination gentamicin treatments in mixed-field-irradiated–wounded mice (Table VI).

DISCUSSION

The data presented above support the idea that it is possible to treat overwhelming sepsis successfully in a mouse model of lethal irradiation and wounding. The success rate depends on the microflora colonizing the wound site and the susceptibility of the microflora to both systemic and topically applied antibiotics. It is significant that, in mice exposed to γ rays and in mice exposed to the mixed field, topical antibiotic applications are a prerequisite to achieving survival with either systemic antibiotic or S-TDCM treatment. Such treatment obviates the *earlier* mortality (compared to untreated irradiated–wounded controls) seen in mice given S-TDCM with and without systemic antibiotic therapy. Indeed, topical antibiotic therapy alone is capable of increasing survival time as well as increasing the survival from lethal irradiation–wounding events. While topically applied antibiotics kill bacteria colonizing the wound, it is important to note that application of the cream base in which the antibiotic is prepared may enhance mortality. In an experimental series testing the effect of a placebo cream on irradiated–wounded mice, the survival time was 5.7 ± 0.2 days compared to 8.1 ± 0.8 days for untreated controls and 11.7 ± 0.4 days for mice treated with cream

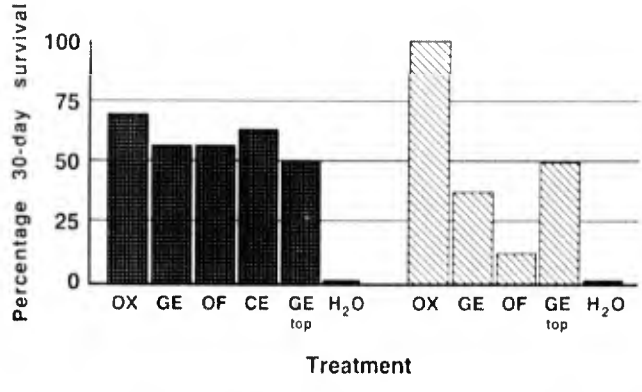

FIG. 7. Survival in irradiated mice inflicted with wound trauma after combined therapy with topical 0.1% gentamicin sulfate and systemic antibiotics. C3H/HeN mice were wounded 1 h after irradiation. Antibiotic therapies commenced 4 h after injury and were provided daily for 10 days. Groups of 20 mice exposed to the mixed field (▩) and groups of 16 mice exposed to γ rays (□) were treated topically with 0.1% gentamicin sulfate and systemically (sc) with 150 mg/kg/day oxacillin (OX), 7.5 mg/kg/day gentamicin (GE), 40 mg/kg/day ofloxacin (OF), or 75 mg/kg/day ceftriaxone (CE). Control groups were treated with 0.1 ml sterile H_2O plus gentamicin cream or were given no antibiotic therapy. In mice irradiated in the mixed field, all antibiotic treatments were equally effective ($P > 0.05$) and increased survival ($P < 0.05$) over H_2O-treated controls. In this experimental series, topical gentamicin alone or with systemic oxacillin and gentamicin increased survival significantly ($P < 0.05$) compared to all other treatments in γ-irradiated mice.

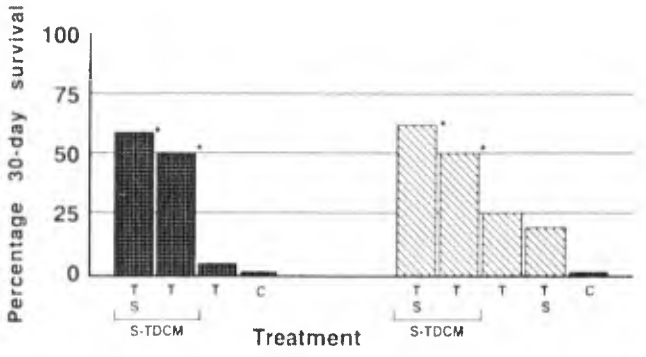

FIG. 8. Survival in irradiated mice inflicted with wound trauma and given combined therapy with topical 0.1% gentamicin sulfate and S-TDCM. C3H/HeN mice were wounded 1 h after irradiation. S-TDCM (200 μg ip) was given immediately after wound trauma. Gentamicin therapy was applied topically and/or given sc 4 h after injury and daily thereafter for 9 days. Groups of 20 mice exposed to the mixed field (▩) and groups of 16 mice exposed to γ rays (□) 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 mice exposed to the mixed field. Systemic gentamicin sulfate with S-TDCM but without topical gentamicin was not evaluated in mice exposed to γ rays or to the mixed field. In mice exposed to either type of radiation, gentamicin therapy with S-TDCM and with or without systemic gentamicin therapy increased survival significantly ($P < 0.05$), compared to all other treatment groups.

containing 0.1% gentamicin sulfate. All treatment groups contained 8–12 mice and were significantly different ($P < 0.05$) from each other. In Fig. 7 we show a significant increase in survival for animals treated with topical gentamicin and systemic oxacillin. This was not observed when irradiated–wounded mice were treated with S-TDCM in conjunction with topical gentamicin and systemic oxacillin (Fig. 5). These findings point to bacterial resistance to antibiotic-based treatments that is presumably related to changes in the microflora found in the environment at the time of the experiment.

In this paper, we document for the first time that an immunomodulator (S-TDCM) combined with antibiotics can successfully treat a variety of sepsis conditions in mice irradiated with a mixed field ($n/\gamma = 1$) and those irradiated with ^{60}Co γ rays. In particular, we demonstrated successful therapeutic interventions for disseminated sepsis in both mixed-field- and γ-irradiated mice inflicted with wound trauma. Our findings are significant because of the therapeutic principle involved. This principle could be considered for application in nuclear disasters where most casualties could receive multiple injuries.

Substances such as S-TDCM that enhance nonspecific host defenses may be useful in therapy for endogenously acquired and exogenously induced bacterial sepsis in hosts receiving "simple" injury due to neutron or photon irradiation. S-TDCM alone is effective as an antibacterial treatment; there is a probable synergistic antibacterial activity when it is combined with appropriate antibiotics.

When radiation injury is complicated by wound trauma, it may be possible to employ combined-modality therapy to achieve survival from disseminating bacterial sepsis. Treating the wound with effective topical antibiotics seems necessary if S-TDCM (and perhaps related immunomodulators) or systemic antibiotics are to enhance survival.

S-TDCM also enhances hematopoietic recovery in irradiated hosts, but no direct link between increased hematopoiesis and survival is known. Because of this hematopoietic effect and the capacity to stimulate host defenses against a number of microorganisms, it is interesting to consider the potential for using S-TDCM (or similar agents) in radiation accidents, such as that at Chernobyl (*32*). Successful application of such agents could reduce the clinical problems and complications of bone marrow transplantation in mass casualty situations.

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REFERENCES

1. H. H. Vogel, Jr., J. W. Clark, C. W. Hammond, D. B. Cooper, and C. P. Miller, Endogenous infection in mice irradiated with fast neutrons or gamma rays. *Proc. Soc. Exp. Biol. Med.* **87,** 114–119 (1954).
2. I. Brook, Use of antibiotics in management of postirradiation wound infection and sepsis. *Radiat. Res.* **115,** 1–25 (1988).
3. C. W. Hammond, H. H. Vogel, Jr., J. W. Clark, D. B. Cooper, and C. P. Miller, The effect of streptomycin therapy on mice irradiated with fast neutrons. *Radiat. Res.* **2,** 354–360 (1955).
4. H. H. Vogel, Jr., and D. L. Jordan, A comparison of damage to the small intestine by fission neutrons and gamma rays from ^{60}Co. In *Gastrointestinal Radiation Injury* (M. F. Sullivan, Ed.), pp. 327–330. Excerpta Medica, New York, 1968.
5. D. A. Stewart, G. D. Ledney, W. H. Baker, E. G. Daxon, and P. A. Sheehy, Bone marrow transplantation of mice exposed to a modified fission neutron (N/G - 30:1) field. *Radiat. Res.* **92,** 268–279 (1982).
6. T. B. Elliott, I. Brook, and S. M. Stiefel, Quantitative study of wound infection in irradiated mice. *Int. J. Radiat. Biol.* **58,** 341–350 (1990).
7. Conference Report: The First Consensus Development Conference on the Treatment of Radiation Injuries. *Int. J. Radiat. Biol.* **57,** 437–442 (1990).
8. I. Brook and T. B. Elliott, Treatment of wound sepsis in irradiated mice. *Int. J. Radiat. Biol.* **56,** 78–82 (1989).
9. G. D. Ledney, G. S. Madonna, D. G. McChesney, T. B. Elliott, and I. Brook, Complications of combined injury: Radiation damage

and skin wound trauma in mouse models. In *Treatment of Radiation Injuries* (D. Browne, J. F. Weiss, T. J. MacVittie, and M. V. Pillia, Eds.), pp. 153–164. Plenum, New York, 1990.

10. R. P. Gale, Immediate medical consequences of nuclear disasters: Lessons from Chernobyl. *J. Am. Med. Assoc.* **258,** 625–628 (1987).
11. G. V. Leroy, The medical sequelae of the atomic bomb explosion. *J. Am. Med. Assoc.* **134,** 1143–1148 (1947).
12. G. S. Madonna, G. D. Ledney, T. B. Elliott, I. Brook, J. T. Ulrich, K. B. Meyers, M. L. Patchen, and R. I. Walker, Trehalose dimycolate enhances resistance to infection in neutropenic animals. *Infect. Immun.* **57,** 2495–2501 (1989).
13. G. S. Madonna, M. M. Moore, G. D. Ledney, T. B. Elliott, and I. Brook, Combined therapy of septicemia with ofloxacin and/or synthetic trehalose dicorynomycolate (S-TDCM) in irradiated and wounded mice. *Microecol. Ther.* **19,** 135–139 (1989).
14. G. S. Madonna, G. D. Ledney, M. M. Moore, and T. B. Elliott, Treatment of mice with sepsis following irradiation and trauma with antibiotics and synthetic trehalose dicorynomycolate (S-TDCM). *J. Trauma* **31,** 316–325 (1991).
15. G. H. Zeman and K. P. Ferlic, *Paired Ion Chamber Constants for Fission Gamma-Neutron Fields,* Technical Report TR84-8. Armed Forces Radiobiology Research Institute, Bethesda, MD, 1984.
16. E. T. Lee, *Statistical Methods for Survival Data Analysis,* pp. 122–129. Lifetime Learning Publ., Belmont, CA, 1980.
17. D. J. Finney, *Probit Analysis,* 3rd ed., pp. 1–99. Cambridge Univ. Press, New York, 1971.
18. D. J. Finney, *Statistical Method in Biological Assay,* 3rd ed., pp. 370–379. Macmillan Co., New York, 1979.
19. R. I. Walker, Requirements of radioprotectors for military and emergency needs. *Pharmacol. Ther.* **39,** 13–20 (1988).
20. M. R. Landauer, H. D. Davis, K. S. Kumar, and J. F. Weiss, Behavioral toxicity of selected radioprotectors. *Adv. Space Res.,* in press.
21. J. M. Yuhas, Biological factors affecting the radioprotective efficiency of S-2-[3-aminopropylamino]ethylphosphorothioic acid (WR-2721): $LD_{50/30}$ doses. *Radiat. Res.* **47,** 526–529 (1971).
22. E. J. Ainsworth, From endotoxins to newer immunomodulators: Survival-promoting effects of microbial polysaccharide complexes in irradiated animals. *Pharmacol. Ther.* **39,** 223–241 (1988).
23. D. G. McChesney, G. D. Ledney, and G. S. Madonna, Trehalose dimycolate enhances survival of fission neutron-irradiated mice and *Klebsiella pneumoniae*-challenged irradiated mice. *Radiat. Res.* **121,** 71–75 (1990).
24. L. K. Steel, A. J. Jacobs, L. I. Giambarresi, and W. E. Jackson III, Protection of mice against fission neutron irradiation by WR-2721 or WR-151327. *Radiat. Res.* **109,** 469–478 (1987).
25. K. Masihi and W. Lange (Eds.), *Immunomodulators and Nonspecific Host Defense Mechanisms Against Microbial Infections.* Pergamon, Oxford, 1988.
26. J. A. Majde (Ed.), *Immunopharmacology of Infectious Diseases: Vaccine Adjuvants and Modulators of Non-Specific Resistance.* Liss, New York, 1987.
27. T. B. Elliott, G. S. Madonna, G. D. Ledney, and I. Brook, Combined therapy for postirradiation infection. *Microecol. Ther.* **19,** 105–108 (1989).
28. I. Brook and G. D. Ledney, Oral ofloxacin therapy of *Pseudomonas aeruginosa* sepsis in mice after irradiation. *Antimicrob. Agents Chemother.* **34,** 1387–1389 (1990).
29. I. Brook and G. D. Ledney, Quinolone therapy of *Klebsiella pneumoniae* sepsis following irradiation: Comparison of pefloxacin, ciprofloxacin, and ofloxacin. *Radiat. Res.* **122,** 215–217 (1990).
30. G. Stringel, R. Bawdon, M. Savrich, L. Guertin, and J. Horton, Topical and systemic antibiotics in the prevention of wound infection. *J. Pediat. Surg.* **24,** 1003–1006 (1989).
31. J. J. Rahal, Antibiotic combinations: The clinical relevance of synergy and antagonism. *Medicine* **54,** 179–195 (1978).
32. A. Baranov, R. P. Gale, A. Guskova, E. Piatkin, G. Selidovkin, L. Muravyova, R. Champlin, N. Danilova, L. Yevseeva, L. Petrosyan, S. Pushkareva, M. Konchalovsky, A. Gordeeva, T. Protasova, Y. Reisner, M. R. Mickey, and P. I. Terasaki, Bone marrow transplantation after the Chernobyl nuclear accident. *N. Engl. J. Med.* **321,** 205–212 (1989).

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Original Contribution

INHIBITION OF GLUTATHIONE REDUCTASE ACTIVITY BY A CARBAMOYLATING NITROSOUREA: EFFECT ON CELLULAR RADIOSENSITIVITY

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Abstract—Nitrosoureas inactivate cellular glutathione reductase. N,'N'1,3-bis(trans-4-hydroxycyclohexyl)-N'-nitrosoureas (BCyNU), a nitrosourea reported to selectively inhibit glutathione reductase (GR) activity, was examined to determine if it could be used as a means to inhibit cellular levels of this enzyme in radiobiology studies. Confirmation of drug-induced inhibition of GR activity was demonstrated using a cell-free model system employing purified GR. Cellular studies with Chinese hamster V79A03 showed that BCyNU decreased cellular glutathione content concomitant with an inhibition of specific GR activity. Under relatively nontoxic conditions, cellular exposure to BCyNU (25 μM, 0.25 h) either before or after radiation treatment, increased cellular radiosensitivity with the optimum time for drug addition being immediately following radiation. At a BCyNU dosage which produced $\leq$5% cell toxicity, a marked decrease in radioresistance was characterized as a reduction in both D_q (24 $\pm$ 1.5%) and D_o (8 $\pm$ 0.5%) concomitant with a 25 $\pm$ 2% decrease in cellular glutathione reductase (GR) activity. At cytotoxic drug dosages (25 μM, 1 h; cell survival 79 $\pm$ 7%), a marked radiosensitization manifested by a 1.25 $\pm$ .07-fold reduction in the D_q was observed concomitant with a 49 $\pm$ 4% decrease in GR activity. Using cells enriched in different stages of the cell cycle, BCyNU caused cell-age dependent cytotoxicity with preferential killing of cells in the radioresistant late-S-phase, a likely explanation for its radiosensitizing capabilities at high drug dosages. Data obtained at nontoxic drug dosages suggest that GR-inactivation may be an important component of cellular response to free-radical induced damage.

Keywords—Glutathione reductase, Glutathione, Nitrosourea, BCyNU, Carbamoylation, Radiosensitizer, Free radicals

INTRODUCTION

It is well documented that glutathione (GSH) and redox enzymes can function as modulators of cellular responses to free radical-induced damage via radiation exposure.[1-5] Previous studies have suggested that the maintenance of the cellular reductive environment, primarily measured as the ratio of GSH to glutathione disulfide (GSSG), is an important determinant of intrinsic cellular radioresistance.[6,7] A key enzyme in the maintenance of the GSH/GSSG ratio is glutathione reductase (GR) which catalyzes the conversion of GSSG to GSH;[8] there is little data however, concerning the importance of this enzyme in cellular resistance to radiation or chemical exposure. Research efforts in radiobiological studies have primarily focused on the reduction of endogenous GSH as a means to increase cellular radiation sensitivity.[2,4,5,6] While these studies, under certain experimental conditions, support a mechanistic relationship between cellular GSH content and radioresponse, the significance of the enzymes involved in the maintenance of a reductive cellular environment has not been fully addressed.

The use of inhibitors of GR activity is one approach to examine the role of this enzyme in cellular resistance to free-radical induced stress. Previous investigations of nitrosourea-radiation interactions have involved the use of nitrosoureas (i.e., 1,3-bis(2-chloroethyl)-1-nitrosourea BCNU) that physiologically decompose to yield two types of electrophilic intermediates, an alkylating and a carbamoylating moiety.[9,10,11] These intermediates can be, respectively, alkylate or carbamoylate nucleophilic targets.[12] The alkylating moiety reacts with DNA, causing the production of DNA-DNA and protein-DNA crosslinks[13] while the carbamoylating moiety is responsible for GR inactivation. The relatively specific action of car-

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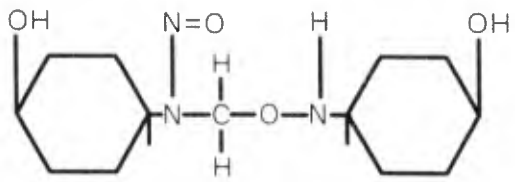

BCyNU

Fig. 1. Structure of BCyNU.

bamoylating nitrosourea on GR activity is substantiated by the observation that twenty other cytoplasmic enzymes were unaffected by BCNU.[14]

Alternatively, N,N′ 1,3 bis(trans-4-hydroxycyclohexyl)-N′-nitrosourea (BCyNU), an analog of BCNU, has also been reported to be an inhibitor of GR activity, yet without any concomitant induction of DNA strand breaks associated with nitrosoureas previously used in oxidative stress studies.[10,15] This drug, shown in Fig. 1, was reported to have no effect on alkaline phosphatase, serum glutamic pyruvic transaminase, and glutathione peroxidase (GPx).[15] BCyNU possesses negligible alkylating activity, forming a carbamoylating isocyanate which specifically carbamoylates the sulphydryl moiety of the 43rd amino acid in the active site of cellular GR, resulting in a decrease in enzyme activity.[16] The use of BCyNU may provide the most relatively specific inhibitor of GR activity available. Additionally, the use of BCyNU to inhibit GR activity is reportedly more advantageous than the use of BCNU based on BCyNU's higher water solubility and reduced cytotoxicity.[10,15]

In this paper we have attempted to focus on the relationship of GR specific activity and radiation sensitivity using a carbamoylating nitrosourea to inhibit GR specific activity and to determine the most effective sequence of time of drug exposure and radiation exposure. Additionally, although drug concentrations were used in previous nitrosourea-radiation studies resulting in significant cell killing, a possible nitrosourea-induced cell killing of a radioresistant fraction of the cell population was not addressed.[10] Using a cell free model system and cell cycle studies, data was obtained which addresses the relevance of using a carbamoylating nitrosourea to study the role of GR specific activity in cellular radiation response. Most importantly, this paper examines drug-induced radiosensitivity at nontoxic doses concomitant with GR inactivation.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise noted. BCyNU was generously provided by Dr. Kenneth Tew, (Fox Chase Cancer Institute, Philadelphia, PA). BCyNU was dissolved in 70% ethanol immediately prior to treatment and is 99% pure. The abbreviation, BCyNU, has been previously used by others to designate the nitrosourea, 1,3-biscyclohexyl-1-nitrosourea (ref. 10), which is a different nitrosourea from the one used in this report. We used 1,3-bis(hydroxycyclohexyl-nitrosourea) abbreviated as BCyNU in this study to conform to the abbreviation suggested by others.[15]

The Chinese hamster V79A03 cell line used in these experiments was previously characterized.[17] Cells were maintained as monolayers in tissue culture flasks (Corning, Corning, NY) in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), approximately 1.75 mM L-glutamine, and 25 mM HEPES buffer. All media contained antibiotics and a fungicide. The cultures were maintained at 37°C in a humidified 3% CO_2 and 97% air gassing environment. The population doubling time was approximately 9.5–10 h. Non-drug treated plating efficiencies ranged from 88%–99%. Exponentially growing cells in complete medium were used in all experiments except for the designated cell synchrony experiments.

For all drug exposures, exponentially growing cells (10^2 to 4×10^5) in a monolayer were exposed to BCyNU concentrations ranging from 10–100 μM. BCyNU was added to the complete medium for various times ranging from 15 min to 24 h. Following drug exposure, the medium was aspirated from the plates and the cells were rinsed twice with Dulbecco's phosphate buffered saline (PBS) containing 0.54 mM Na_2-EDTA. In the cell cycle studies, designated aliquots of cells collected via elutriation, were exposed to BCyNU (10, 50 or 150 μM) in suspension for 1 h at room temperature immediately following collection.

The enzyme assays were performed with both cell extracts and reagent enzymes (i.e., GR, catalase, GSH peroxidase (GPx), superoxide dismutase (SOD), and γ glutamyl transferase (γ-GT) in some cases.

Cells were prepared for GSH, enzyme, and protein assays as follows: Typically, 1×10^7 cells were collected from 25 100-mm petri dishes; each was maintained at a designated low cell density (3.5×10^5 cells/dish), using the trypsinization technique. The cells were washed with PBS, concentrated into microcentrifuge tubes, and stored in PBS at −40°C. Immediately before analysis, the samples were thawed and digitonin was added to a final concentration of 2.5 mg/ml. After vortexing, the cellular debris was pelleted by centrifugation. The supernatant was transferred to another microcentrifuge tube and main-

tained at 4°C for enzyme activity and protein analysis.

GR specific activity was determined using the method of Mize and Langdau[18] by monitoring the oxidation of NADPH at 340 nm. The reaction mixture contained 0.2 M KCl, 1 mM EDTA, and 1 mM oxidized GSH in 0.1 M potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of NADPH (final concentration, 0.1 mM). One unit of GR oxidizes 1 nmol of NADPH per minute at 25°C.[18] Cellular GSH content was determined using the method of Griffith as described elsewhere.[15]

Catalase activity was determined by using the method of Beers and Sizer[19] which is based on following the reduction of hydrogen peroxide at 240 nm at 25°C. One unit of catalase activity will decompose 1 μmol of H_2O_2 per minute at 25°C (pH 7.0).[19]

GPx activity was determined by using a modified Ellman's procedure.[20] The reaction of 0.1 mM GSH 0.1 with 0.2 mM dithiobisnitrobenzoic acid (DTNB) was monitored at 412 nm. Sodium azide (1 mM) was added to inhibit the activity of catalase.

The γ-glutamyl transferase activity assay is based on the kinetic method of Szasz in which γ-glutamyl p-nitroanilide and glycylglycine are used as donor and acceptor substrates, respectively.[21] The kinetic determination of γ-GT activity is made at 405 nm. One unit of γ-GT activity is defined as the amount of enzyme that will catalyze the formation of one μmol of p-nitroaniline per minute under the conditions of the assay.

The method of Marklund et al. was used to determine SOD activity.[22] One unit of SOD activity will cause a 50% inhibition in the reduction of ferricytochrome *c* by superoxide generated by a xanthine/xanthine oxidase system at 25°C and pH 7.8.

Cellular protein content was determined by the Coomassie brilliant blue dye binding method[23] using BioRad's standard protein reagents (BioRad, Richmond, CA). Bovine γ-globulin was used as the standard.

In both cell-free and cellular biochemical assays, data represent the mean ± standard error from two to nine experiments with a total of 3 samples per point per experiment. Statistical significance was determined using the Kruskal-Wallis test which is a nonparametric oneway analysis of variance.[24]

For radiation exposures, the media was aspirated and cells in a monolayer were irradiated with X-rays delivered by a 50-kVp irradiator using a tungsten target, and attenuated with a 0.012-mm Al filter at a dose rate of 19.0 Gy/min.[25] Following irradiation, cells were returned to the incubator for 7 days to allow for colony formation.

Cell survival was determined using the standard clonogenic cell survival assay. Briefly, colonies were washed with 0.9% saline, fixed with methanol, and stained with crystal violet. Colonies of ≥100 cells were counted to assess survival. Cell survival data, including those not shown, are presented as the mean ± standard error from two to fifteen experiments with three plates per point per experiment unless otherwise noted in the figure legends.

The X-ray dose response survival data were analyzed using a cell survival analysis computer program.[26] The survival data were fitted to a repair saturation model (Eq. 1) from which the radiation sensitivity parameters (i.e., D_q, D_o, and extrapolation number [n]) were determined. In Eq. (1), D = dose; S = survival; n and q are fitted parameters; c represents the plating efficiency. Statistical significance was determined using a *t*-test.

$$S = cn/((n - 1) + e^{qD}) \quad (1)$$

Cells enriched in different stages of the cell cycle were selected for experimental use by the centrifugal elutriation method.[27] This synchrony method relies on the increase in buoyant density that occurs as cells progress through the cell cycle. Separations were obtained by varying the flow rate as described by Meistrich.[27] The procedure involved use of a centrifuge (model J-21C fitted with a model JE-6 elutriator rotor; Beckman Instruments, Palo Alto, CA) operated at a constant rotor speed of 1,700 rpm. Approximately 1×10^8 cells in 10 ml of media, were introduced into the elutriation chamber at a flow rate of approximately 9–12 ml per min. The cells were separated by increasing the flow rate of medium (α-MEM containing 5% FBS and DNase, 20 μg/ml) through the centrifuge chamber in an incremental manner from 9–55 ml per min at room temperature. The 12–13 fractions collected in sterile culture tubes were placed immediately on ice and cell cycle parameter analysis were then performed.

Mitotic and labeling index assays were performed on cells obtained from the centrifugal elutriation protocol to characterize their enrichment in various stages of the cell cycle.[28] Typically, these cells were pretreated with 0.1 mM of bromodeoxyuridine (BrdUrd) and 0.01 mM of fluorodeoxyuridine (FdUrd) for 20 min immediately before collection by the elutriation procedure described earlier. Following the elutriation procedure, an aliquot of each of the 12–13 fractions was counted and evaluated using a particle counter (Coulter Diagnostics, Hialeah, FL). Coulter volume data are presented as mean coulter channel number. Aliquots for mitotic and labeling in-

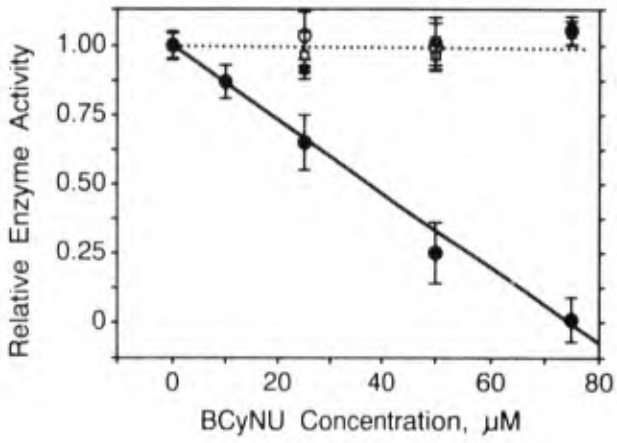

Fig. 2. Effect of BCyNU on the activity of various purified enzymes. In a cell free model system the effect of BCyNU exposure on catalase, SOD, γ-GT, GPx, and GR specific activity was examined. Parameters of this cell-free model system are specified in the Methods section. Relative absorbance was determined as follows: Equal units of each enzyme examined were used at each drug dose. Relative enzyme activity is a ratio of the units measured at each drug dose per units measured without drug addition. The control value would be 1 under these conditions. Points represent the mean ± S.E. of two to four experiments with three samples/point. ○, catalase; ■, SOD; ▲, γ GT; □, GPx; and ● GR. In some cases the symbols overlap such that some are not visible.

dices measurements were prepared as follows. Cells were washed with phosphate buffer solution (PBS), fixed with a solution of methanol and glacial acetic acid (3:1) containing 0.2% sodium citrate (weight/volume), and attached onto slides using a slow speed cytospin centrifuge (Shandon, Pittsburgh, PA). The cells on the slides were then dehydrated with 95% ethanol. All samples were treated with PBS containing heat-denatured (i.e., 90°C for 20 min) ribonuclease-B (1.0 mg/ml) for 5–10 min. Staining for the mitotic assay involved a 5 min exposure to PBS containing 1 μg/ml propidium iodide. The indirect immunofluorescence staining procedure was used to identify the fraction of cells that incorporated BrdUrd into their DNA.[28]

RESULTS

In an effort to address the specificity of the BCyNU-isocyanate induced inhibition of GR activity, the effect of BCyNU on the activity of several commercially available enzymes was examined. As illustrated in Fig. 2, the data show that a BCyNU-concentration-dependent inhibition of GR activity occurred without a concomitant effect on catalase, GPx, and SOD activities. While there is a suggestion of a slight effect on γ-GT activity, statistical analysis showed that BCyNU exposure did not have a significant effect on γ-GT specific activity. These data confirm the previously reported relative selectivity of BCyNU-induced inhibition of GR activity compared to the activity of these other enzymes.[14,15]

It was necessary to determine to what extent BCyNU exposure may also indirectly affect cellular GSH content. Figure 3A shows that a 1 h exposure to BCyNU resulted in a significant decrease in GR specific activity concomitant with a decrease in cellular GSH levels. Cellular exposure to progressively increasing concentrations of BCyNU for 1 h caused a depletion of GSH content. Following cessation of BCyNU treatment, the GSH content started to recover and had returned to control level at 24 h (Data not shown). The time course of drug-induced depletion of GR specific activity and GSH levels following continuous exposure to 25 μM BCyNU is illustrated in Fig. 3B. The initial reduction of GSH content is followed by a slight recovery of GSH levels within 24 h. It is unclear as to whether this GSH decrease is due to the decrease in GR specific activity affecting the γ-glutamyl cycle or a possible direct reaction of GSH with the BCyNU-isocyanate.

Modification of radiation sensitivity by BCyNU-induced GR specific activity inhibition was assessed by examining the influence of BCyNU exposure on radiation survival curves. Cells were treated with 25 μM BCyNU for 1 h immediately before (pre) or after (post) radiation exposure. Figure 4A demonstrates that at this drug concentration, BCyNU typically caused approximately a 21 ± 2% decrease in cell survival. The inset figure shows the same data normalized for drug-induced cytotoxicity. In this figure, the effect of GR specific activity inhibition on the radiation response clearly illustrates a drug-induced radiosensitization. BCyNU-induced radiosensitization was manifested as both a 26 ± 1% reduction in the D_q (D_q CTL = 3.85 ± 0.026, D_q $BCyNU_{(pre)}$ = 2.85 ± 0.02, and D_q $BCyNU_{(post)}$ = 3.01 ± 0.06) and a 25 ± 1% reduction in the D_o (D_o CTL = 1.45 ± 0.05, D_o Pre & Post = 1.10 ± 0.09). Under these conditions GR specific activity was decreased by 49 ± 7%, associated with a 22 ± 6% decrease in cellular GSH content. Since cellular exposure to BCyNU either before or after radiation resulted in radiosensitization, additional studies were warranted regarding the time frame of drug exposure relative to irradiation.

In 15 experiments under conditions of negligible toxicity (surviving fraction > 91 ± 4%), BCyNU exposure (25 μM, 15 min) sensitized cells to a 6 Gy dose of ionizing radiation (Figure 4B) resulting in a 2- to 5-fold decrease in cell survival. This drug-induced radiation modification was observed whether the cultures were exposed to drug before or after radiation treatment ($p < 0.05$ under all treatment conditions). The

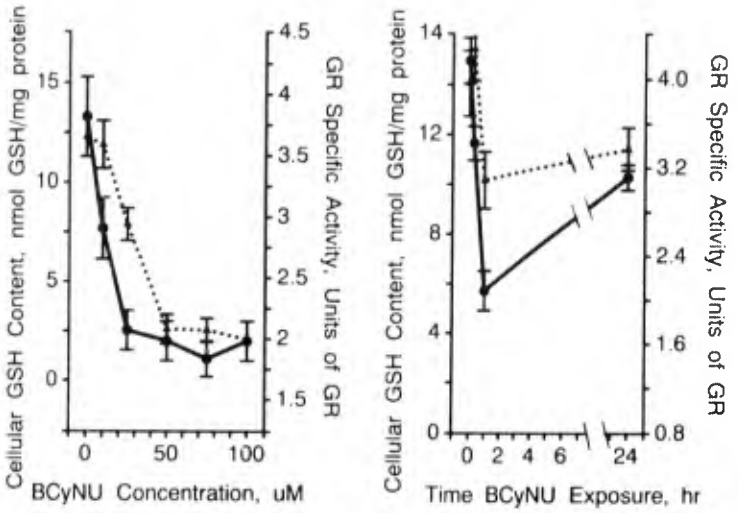

Fig. 3(A). Effect of BCyNU exposure (1 h) on GSH content and GR specific activity in V79 cells. GR specific activity was monitored via the consumption of NADPH, measured spectrophotometrically at 340 nm. GSH was measured by the method of Tietze. Points represent the mean ± S.E. of three samples/point from three to six experiments. Curves are identified as follows: ●, GR specific activity; and ▲, GSH content nm/mg protein.
Fig. 3(B). Effect of 25 μM BCyNU exposure on GR specific activity and GSH content. Measurements made as described above. Points represent the mean ± S.E. of three samples/point from two to three experiments; ●, GR specific activity; ▲, GSH content nm/mg protein.

greatest radiosensitization occurred when cells were exposed to BCyNU immediately after radiation treatment resulting in a fivefold-increase ($p < 0.05$) in cell killing by radiation. The magnitude of radiosensitization by BCyNU exposure is reduced if drug administration relative to radiation treatment is delayed 2 h. However, cellular exposure to BCyNU even at 1 or 2 h before or after radiation, still results in a twofold-increase in radiation-induced cell killing ($p < 0.05$). In subsequent experiments we chose to examine the effects of cellular BCyNU exposure after radiation treatment. In these experiments however, the marked radiosensitization associated with a 24 ± 5% (figure 2B) decrease in GR specific activity and a 7 ± 3% depletion of GSH (figure 2B), was concomitant with a 12 ± 2% decrease in cell survival due to BCyNU exposure alone.

Although GR specific activity inhibition via

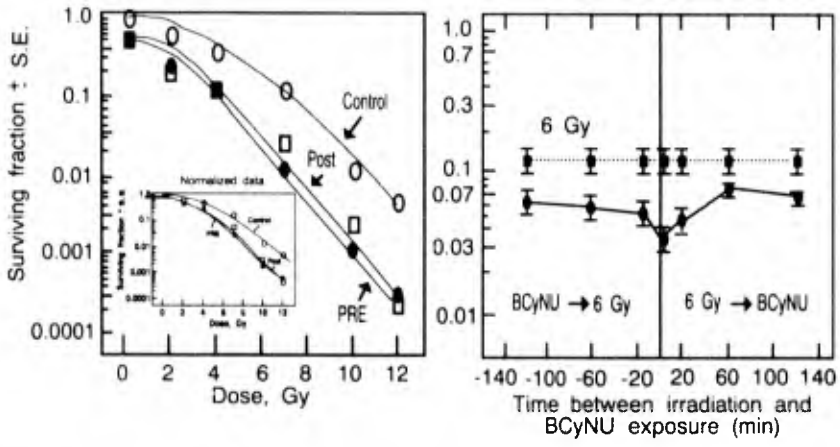

Fig. 4(A). Effect of cytotoxic BCyNU treatment on the radiosensitivity of V79A03 cells. Approximately equal numbers of exponentially growing cells were treated with BCyNU (25 μM, 1 h) and radiation (0–12 Gy) and then plated for colony formation. ○ control, no drug; □, BCyNU exposure before radiation treatment; ●, BCyNU exposure after radiation treatment. Data have not been normalized for BCyNU-induced cell killing. In the inset chart, the data have been normalized for the BCyNU-induced cell killing. Data are representative of two experiments with four replicates/point.
Fig. 4(B). Modification of radiation sensitivity by BCyNU: Time of drug exposure relative to radiation. Cells were exposed to BCyNU (25 μM, 15 min) at varying times either before or after radiation (Solid circles). Solid squares represent cells exposed to radiation but with no drug treatment. Data have been normalized for BCyNU-induced killing. Data represent the mean ± S.E. from five to fifteen experiments with three samples/point.

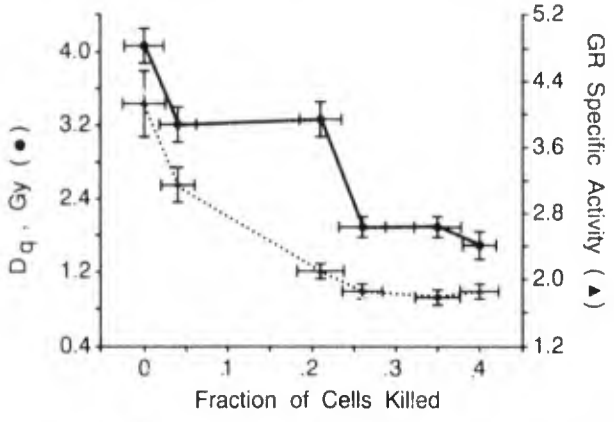

Fig. 5. Relationship between glutathione reductase specific activity, radiation sensitivity, and the fraction of cells killed following exposure of V79A03 cells to BCyNU. The D_q measurement was obtained from dose-response survival curves in which the GR specific activity had been inhibited by BCyNU. The symbols at the zero point represent the control samples.

BCyNU exposure resulted in cellular radiosensitization, factors such as drug cytotoxicity and concomitant GSH depletion restrict a complete evaluation of BCyNU as a useful drug to examine the relationship between GR activity and radioresistance.

To determine to what extent the radiosensitization could be correlated with GR specific activity with regard to the magnitude of the drug-induced cell killing, a range of BCyNU concentrations was used in additional radiation dose-response survival studies. These dose-response survival data are not shown, however, Fig. 5 illustrates both the pattern of GR specific activity (left axis) and the shoulder parameter data (right axis) obtained from these curves relative to the drug-induced cell killing (x axis). BCyNU concentrations of 10, 25, 50, 75, and 100 μM were used, which resulted in a reduction of cell survival to levels of 96 $\pm$ 5%, 79 $\pm$ 4.5%, 72 $\pm$ 6%, 64 $\pm$ 4%, and 59 $\pm$ 5% of control, respectively. The shoulder of the radiation survival curve were both significantly reduced in three to nine experiments using these BCyNU concentrations. While these data demonstrate a correlation with reduction in cellular GR specific activity by D_q's, the fact that a significant percentage (21–41%) of the cells are also killed by drug treatment alone makes it difficult to assess the relationship between GR specific activity and cellular radioresponse. For example, drug-induced cell killing of a radioresistant fraction of the cell population is one possibility that could explain the ability of BCyNU to radiosensitize cells in cases where drug cytotoxicity was significant.[29]

Previously reported data demonstrating that nitrosoureas including BCyNU are radiosensitizers at toxic drug dosages (cell killing by drug 50–70%), suggest that a cell age-dependent killing could be involved.[10] Since it is possible that the fraction of cells killed by BCyNU exposure was radioresistant, cell cycle studies were initiated. Cells enriched in different stages of the cell cycle were exposed to 10, 50, or 150 μM BCyNU for 1 h prior to radiation exposure. Cell age-dependent BCyNU cytotoxicity was demonstrated as shown in Fig. 6A. At high drug dosages, cells enriched in mid-S phase were most drug-resistant while cells enriched in late S-phase were most sensitive to BCyNU exposure. The cell cycle parameter evaluation (figure 6B) was constructed from an analysis of cell number per fraction, mean coulter number per fraction, labeling index, and mitotic index to monitor the enrichment of the cell fractions via the elutriation.

Additional experiments were designed to avoid two major problems associated with using nitrosoureas to investigate GR specific activity and radiation response, i.e., cell age dependent-cytotoxicity and possible direct or indirect GSH depletion by drug exposure; therefore: (a) the drug concentration used resulted in negligible drug toxicity; and (b) there was no significant decrease in the GSH content under these conditions. Figure 7 shows that BCyNU (10 μM, 1 h) exposure following radiation resulted in a significant radiosensitization primarily characterized by a decrease in the survival curve shoulder and a slight decrease in the D_o. The shoulder of the survival curve as measured by the D_q (3.85 $\pm$ 0.17 Gy; for controls) was reduced (2.94 $\pm$ 0.25 Gy; for drug treated) ($p < 0.05$). The D_o was reduced from 1.41 $\pm$ 0.05 (controls) to 1.31 $\pm$ 0.04 (drug treated). Therefore, under conditions of negligible toxicity and GSH modulation, BCyNU exposure results in a marked decrease in radioresistance manifested as a .91 $\pm$ 0.09 Gy decrease in the survival curve shoulder concomitant with a 25 $\pm$ 3% decrease in cellular GR activity.

DISCUSSION

The cellular reductive capacity is controlled to a large extent by several key enzymes which maintain cellular levels of GSH.[6–8] The particular importance of one of these enzymes, GR, in DNA damage repair and in cellular radioprotection is not fully understood. Since nitrosoureas have been reported to inhibit cellular GR specific activity,[9,11,14,15,30,31,32] it was the purpose of this study to determine whether a particular nitrosourea, BCyNU, could be used as an effective inhibitor of cellular GR specific activity and to assess the optimal experimental conditions needed to minimize problematic and artifactual results. Prior studies addressing nitrosourea-radiation interactions have focused primarily on (a) the role of alkylating

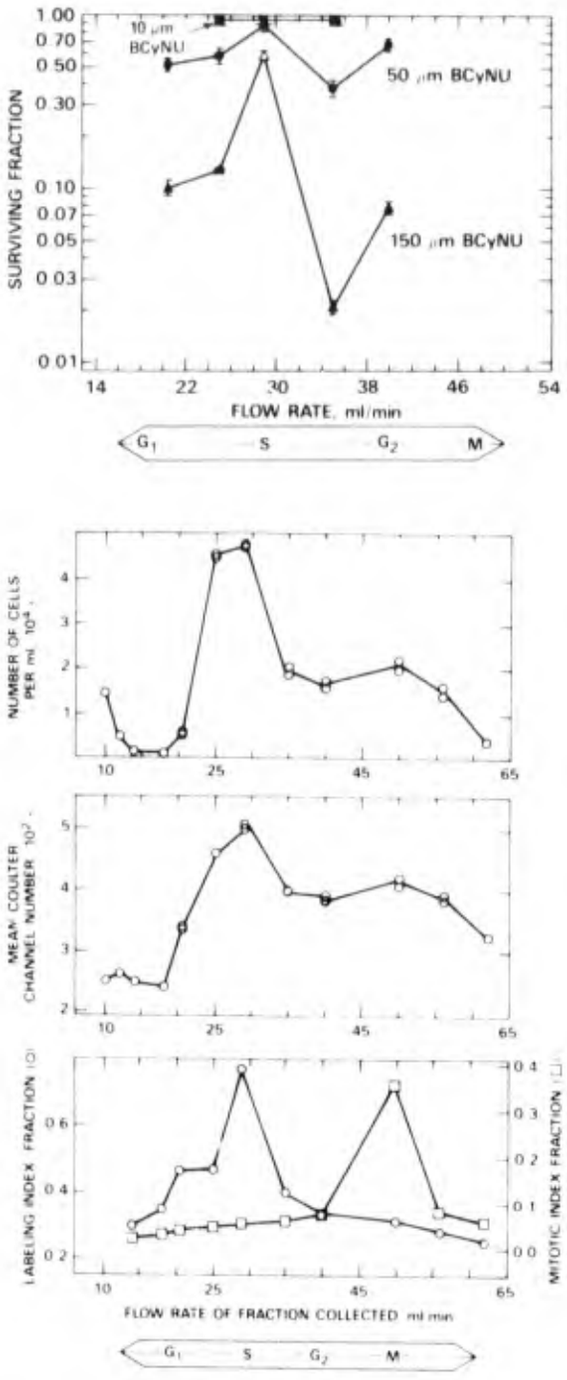

Fig. 6. *Top panel:* Effect of cell cycle on BCyNU-induced cell killing. Cells enriched in various phases of the cell cycle were exposed to BCyNU (10, 50, 150 μM) for 1 h at room temperature. ■, 10 μM; ●, 50 μM; ▲, 150 μM. These data are representative of three experiments with three to five plates/point. *Lower panel:* Cell cycle parameters. A sample of cells from each fraction collected from the elutriator was analyzed to determine cell number, mean coulter channel number, percent labeled mitosis, and labeling index.

nitrosoureas and radiation response; (b) the use of toxic doses of nitrosourea resulting in a 40–50% killing of the cell population; and (c) did not report any resultant effects on GSH content.[10,33–36] This report presents data concerning the nitrosourea-radiation interaction using a nonalkylating carbamoylating nitrosourea not previously used in reported radiation synergism studies with toxic nitrosourea dosages, and addresses the question of drug-induced cell cycle effects. Additionally, this report considers the possible contribution of GSH depletion via the inhibition of GR activity.

In general, nitrosoureas metabolize to form the electrophilic intermediates, alkylating carbonium ions and carbamoylating isocyanates, both of which can react with nucleophilic intracellular sites.[11,12,15] The physiological degradation of BCyNU does not result in the formation of the chloroethyl carbonium ion which is the alkylating moiety formed by degradation of most nitrosoureas.[30] Hence this drug has been suggested to be an effective means of inhibiting GR activity because it carbamoylates the active site of GR[16] yet possesses negligible alkylating activity typically found in many nitrosoureas, including those used in previous studies addressing nitrosourea-radiation interactions.[10,11,33–35]

Using a cell-free model system, the results presented here confirmed the drug-induced inactivation of GR specific activity by the isocyanate formed during the physiological degradation of BCyNU. The reactivity of nitrosoureas and isocyanates for GR has previously been demonstrated by both in vivo results which showed that only GR was inactivated from among 20 erythrocyte enzymes assayed by treatment with BCNU and in vitro results of others.[14,15] Our results, showing that BCyNU at the concentrations tested had no effect on GPx, SOD, γ-GT or catalase activity, support the idea that BCyNU may provide a relatively specific means to inhibit GR specific activ-

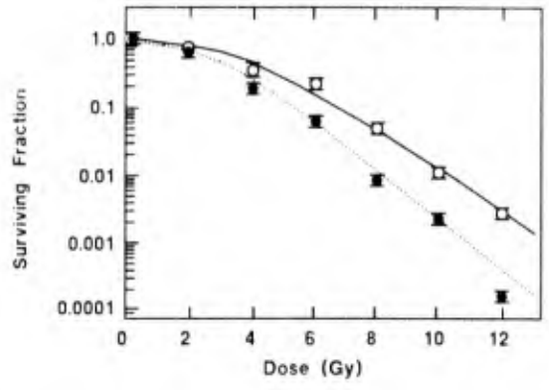

Fig. 7. Effect of nontoxic BCyNU treatment on the radiosensitivity of V79A03 cells. Cells were treated with a nontoxic dose of BCyNU (10 μM, 1 h) after radiation. Cellular GR activity levels were measured following exposure of V79A03 cells to BCyNU. No drug treatment, (○); BCyNU treatment (●) resulted in a 25% inhibition of GR activity.

ity, based on these enzyme targets. However, since the isocyanate has the potential to react with other cellular targets, the specificity of BCyNU is only relative to those enzyme targets determined to be unaffected by it. Previous in vivo reports have shown that at a nitrosourea concentration of 100 μg/ml, other cellular enzymes were slightly affected by nitrosourea exposure.[31] A BCyNU-induced depletion of cellular GSH content was observed in other studies[15] which was attributed to a change in the γ-glutamyl cycle and the GSH/GSSG ratio[7] however, GSH could be another cellular target of the BCyNU-isocyanate and that could partially explain a reduction in GSH levels.

The data presented here agree with previously reported studies which showed that the nitrosourea-induced radiosensitization was manifested primarily as a decrease in the shoulder or D_q and to a lesser degree in the D_o (ref. 33). In addition, we show that BCyNU treatment, whether before or after radiation exposure are both effective treatment protocol schedules to induce radiosensitization. The magnitude of radiosensitization was greatest when BCyNU was given immediately following radiation exposure confirming a distinct interaction effect between the effects of drug exposure and the radiation injury. The data presented here demonstrating that both nontoxic and toxic dosages of BCyNU radiosensitize V79 cells are consistent with similar findings in a previous report with a toxic dose of a structurally similar nitrosourea.[10] While our findings with BCyNU are in agreement with previous investigations demonstrating that some nitrosoureas are radiosensitizers, drug dosages used in those studies caused between 30 and 54% cell killing and the possible involvement of modification of the cellular oxidation-reduction potential by the drug was not discussed.[10,33,35,36] It was suggested that this radiation synergism was related to the reported ability of a carbamoylating nitrosourea to decrease the rate of repair of DNA damage.[10] While this is a possible explanation, selective killing by nitrosoureas of a radioresistant fraction of the cell population could also partially explain data showing that carbamoylating nitrosoureas at toxic drug doses are radiosynergists.[29]

The data indeed demonstrate that there is a cell cycle-dependent BCyNU cytotoxicity, with the greatest cell killing by drug occurring for cells in the mid to late S-phase with moderate cell killing for G_1-cells. Sinclair and Morton have shown that cells in mid- to late S-phase are the most radioresistant compared with other phases of the cell cycle.[29] Early S-phase cells are least sensitive to BCyNU exposure, and are more radiosensitive than the later S-phase cells. Others have shown that alkylating nitrosoureas cause a cell cycle-dependent drug toxicity with the maximum cytotoxicity seen in late G_1- and mid S-phase.[33–36] Since those previous studies and the data presented here indicate that preferential cell cycle killing is characteristic of some nitrosoureas, it would be necessary to consider this factor in a discussion of the mechanism of radiation-nitrosourea interaction. While this cell cycle-dependent killing can be exploited to benefit clinical use of nitrosoureas and radiation, a decrease in the most radioresistant fraction in the cell population must be considered as a possible explanation for the increase in cellular radiosensitivity seen in studies addressing nitrosourea-radiation interaction mechanisms. Indeed, the ability of BCyNU exposure to selectively kill a radioresistant subpopulation of cells suggests that it might be an attractive candidate for combined chemo and radiation therapy and could be used as a radiation synergist, like heat or thiol depletion, to enhance radiation therapy.[37–39]

Alternatively, enhancement of cellular radiation response in our studies could in part result from a modification of cellular GSH levels prior to radiation exposure via the inhibition of GR specific activity. GR specific activity is a fundamental part of the maintenance of normal cellular oxidation-reduction potential and may play a key role in cellular response to free radical induced damage.[1] Our data and results from Tew[15] showed that GR activity inhibition by certain BCyNU dosages results in a reduction in cellular GSH levels to approximately 25% of control within 1 h of BCyNU exposure. Although GSH depletion can increase radiosensitivity, the magnitude of GSH depletion needed to achieve this increase radiation, exceeds that magnitude of GSH depletion obtained via those BCyNU treatments that inactivate GR concomitant with GSH depletion.[2,4–7] While it has been demonstrated that GSH depletion can enhance the lifetime of oxygen-reactive radicals in mammalian cells, a further study showed that the radiation-induced free radicals are no longer present after 1 second;[40] therefore, the radiation sensitizing effect of BCyNU observed in cells treated with this drug after radiation exposure, is probably not due to GSH free radical scavenging capabilities. Since others have demonstrated that GSH and antioxidant enzymes are important effectors of oxidative DNA damage repair,[41,42] it appears likely that the radiation sensitizing effect of BCyNU may be due to an enhanced radiation-induced damage repair.

Using nontoxic doses of BCyNU that did not concomitantly decrease cellular GSH content, we attempted to address the question of GR inactivation and the resultant modification in radiation response. Under these experimental conditions, BCyNU cell-

cycle dependent cytotoxicity and/or GSH depletion were unlikely to influence the results. The data suggest that the 25 ± 2% decrease in GR specific activity is directly related to the increase in radiation sensitivity. A rapid drug-induced alteration in the cellular redox-status via inhibition of GR specific activity would be expected to have a profound disruption on many biochemical processes and cellular components. It has been suggested that a shift in the GSH/GSSG ratio can delay oxidative damage repair[7] and that a change in the ratio may inactivate other enzymes catalyzed by thioltransferases[43] such as adenylate cyclase and phosphofructokinase. A significant change in the cellular metabolic state can affect radiation response.[44] Other thiol-requiring enzymes that could affect radiation-induced damage repair include DNA polymerase α and ribonucleotide reductase.[45,46] Additionally, protein synthesis is affected by a change in GSH/GSSG.[43]

BCyNU is an effective radiosensitizer in vitro at both toxic and nontoxic doses causing an inactivation of GR. In the case of the former, the mechanism may involve a BCyNU-induced cell cycle killing of radioresistant cells; at nontoxic doses, BCyNU significantly decreases GR specific activity altering the intracellular redox state that has been shown to be important in cellular radioresponse possibly via the inhibition of some cellular process involved in repair of the radiation-induced lesions.[39-45] While the BCyNU-induced inhibition of specific GR activity suggests that the increase in cellular radiosensitivity may be due to inhibition of that enzyme activity, it may not be the most significant or only component of the BCyNU-induced radiosensitization. It is possible that the BCyNU-derived isocyanate may be reacting with alternative cellular targets (i.e., DNA repair enzymes, membrane receptors with –SH endgroups) which in a stressed cellular redox status, caused by a concomitant GSH depletion, ultimately affect cellular response to free radical-induced damage. While additional studies are necessary, the data indicate that this drug may provide a useful tool to investigate the role of GR specific activity in cellular radiosensitivity.

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REFERENCES

1. Arrick, B.A.; Nathan, C.F. Glutathione metabolism as a determinant of therapeutic efficacy: A Review. *Cancer Res.* **44**:4224–4232; 1984.
2. Biaglow, J.E.; Varnes, M.E.; Clark, E.P.; Epp, E.R. The role of thiols in cellular response to radiation and drugs. *Radiat. Res.* **95**:437–455; 1983.
3. Pryor, W.A. *Free radicals in biology. 1st edition.* New York: Academic Press; 1982.
4. Biaglow, J.E.; Varnes, M.E.; Tuttle, S.W.; Oleinick, N.O.; Glazier, G.; Clark, E.P.; Epp, E.R.; Dethlefsen, L.A. The effect of L-buthionine sulfoximine on the aerobic radiation response of A549 human lung cells. *Int. J. Radiat. Oncol. Biol. Phys.* **12**:1143–1147; 1986.
5. Bump, E.A.; Yu, N.Y.; Brown, M. Radiosensitization of hypoxic tumor cells by depletion of intracellular glutathione. *Science.* **217**:544–545; 1982.
6. Clark, E.P.; Epp, E.P.; Morse-Gaudio, M.; Biaglow, J.E. The role of glutathione in the aerobic radioresponse: I. Sensitization and recovery in the absence of intracellular glutathione. *Radiat. Res.* **108**:238–250; 1986.
7. Baker, M.A.; Taylor, Y.C.; Brown, J.M. Radiosensitization, thiol oxidation, and inhibition of DNA repair by SR 4077. *Radiat. Res.* **113**:346–355; 1988.
8. Meister, A. Selective modification of glutathione metabolism. *Science.* **220**:472–477; 1983.
9. Babson, J.R.; Reed, D.J. Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. *Biochem. Biophys. Res. Commun.* **83**:754–762; 1982.
10. Kann, H.E.; Blumenstein, B.A.; Petkas, A.; Schott, M.A. Radiation synergism by repair-inhibiting nitrosoureas in L1210 cells. *Cancer Res.* **40**:771–775; 1980.
11. Johnston, T.P.; Wheeler, G.P.; McCaleb, G.S.; Bowdon, B.J.; Montgomery, J.A. Nitrosoureas as potential radiosynergists. In: Serrou, B.; Schein, P.S.; Imbach, J.L., eds. *Nitrosoureas in cancer treatment.* Amsterdam, Netherlands: Elsevier/North Holland; 1984:139–151.
12. Montgomery, J.A.; James, R.; McCaleb, G.A.; Johnston, T.P. The modes of decomposition of 1,3-bis(2-chloroethyl)-1-nitrosourea and related compounds. *J. Amer. Chem. Soc.* **10**:668–674; 1967.
13. Kann, H.E.; Kohn, K.W.; Lyles, J.M. Inhibition of DNA repair by the 1,3-bis(2-chloroethyl)-1-nitrosourea breakdown product, 2-chloroethyl isocyanate. *Cancer Res.* **34**:398–402; 1974.
14. Frischer, H.; Ahmad, T. Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU (1,3 bis(chloroethyl)-1-nitrosourea). *J. Lab. Clin. Med.* **89**:1080–1091; 1977.
15. Tew, K.; Kyle, G.; Johnson, A.; Wang, A.L. Carbamoylation of glutathione reductase and changes in cellular and chromosome morphology in a rat cell line resistant to nitrogen mustards but collaterally sensitive to nitrosourea. *Cancer Res.* **45**:2326–2330; 1985.
16. Pai, E.F.; Schulz, G.E. The catalytic mechanism of glutathione reductase as derived from X-ray diffraction analyses of reaction intermediate. *J. Biol. Chem.* **258**:1752–1757; 1983.
17. Hagan, M.P.; Matsushita, T.; Bonura, T.; Shotola, A.; Alkali-labile lesion and uracil-DNA-glycosylase-sensitive site removal after BrdUrd and UVB treatment of Chinese hamster cells. *Photochemistry and Photobiology* **35**:371–377; 1982.
18. Mize, C.E.; Langdau, R.G. Hepatic glutathione reductase I. Purification and general kinetic properties. *J. Biol. Chem.* **237**:1589–1595; 1962.
19. Beers, R.F.; Sizer, I.W.A. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**:133–140; 1952.
20. Ellman, G.L. Tissue sulfhydryl groups. *Archives of Biochemistry.* **82**:70–77; 1959.
21. Szasz, G. A kinetic photometric method for serum γ-glutamyltranspeptidase. *Clin. Chem.* **15**:124–132; 1969.
22. Marklund, S.L. Spectrophotometric study of spontaneous disproportionation of superoxide anion radical and sensitive direct assay for superoxide dismutase. *J. Biol. Chem.* **251**:7504–7507; 1976.

23. Bradford, W.P. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254; 1976.
24. Kruskal, W.H. A nonparametric test for the several sample problem. *Ann. Math. Statist.* **23**:525–540; 1952.
25. Mohaupt, T.; Zeman, G.H.; Blakely, W.F.; Elkind, M.M. Laboratory x-ray irradiator for cellular radiobiology. In: AFRRI, eds. *AFRRI Technical Report 89-1.* Bethesda, MD: Defense Nuclear Agency, 1989.
26. Albright, N. Computer programs for the analysis of cellular survival data. *Radiat. Res.* **112**:331–340; 1987.
27. Meistrich, M.L. Experimental factors involved in separation by centrifugal elutriation. In: Pretlow, T.; Pretlow, T., eds. *Cell separations: Methods and selected applications.* New York: Academic Press; 1983:33–61.
28. Gratzner, H.G. Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science.* **218**:474–475; 1982.
29. Sinclair, W.K.; Morton, R.A. X-ray sensitivity during the cell generation cycle of cultured Chinese Hamster cells. *Radiat. Res.* **29**:450–47; 1966.
30. Montgomery, J.A. The chemistry of the nitrosoureas. In: Serrou, B.; Schein, P.S.; Imbach, J.L., eds. *Nitrosoureas in Cancer Treatment* Amsterdam, Netherlands: Elsevier/North-Holland; 1984:13–20.
31. Maker, H.S.; Weiss, C.; Brannan, T.S. The effects of BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) and CCNU (1-(20chloroethyl)-3-cyclohexyl-1-nitrosourea) on glutathione reductase and other enzymes in mouse tissue. *Res. Comm. Chem. Pathol. and Pharmacol.* **40**:355–366; 1983.
32. Ali-Osman, F.; Caughlin, J.; Gray, G.S. Decreased DNA interstrand cross-linking and cytotoxocity induced in human brain tumor cells by 1,3-bis(2-chloroethyl)-1-nitrosourea after in vitro reaction with glutathione. *Cancer Res.* **49**:5954–5958; 1989.
33. Wheeler, K.T.; Deen, D.F.; Wilson, C.B.; Williams, M.E.; Shepherd, B.S. BCNU-modification of the in vitro response in 9L brail cells of rats. *Int. J. Radiat. Oncol. Biol. Phys.* **2**:79–88; 1977.
34. Tobey, R.A.; Crissman, H.A. Comparative effects of three nitrosourea derivatives on mammalian cell progression. *Cancer Res.* **35**:460–465; 1975.
35. Hill, S.A.; Bauer, K.D.; Keng, P.C.; Siemann, D.W. The effect in the KHT sarcoma of CCNU and MISO on cell cycle progression evaluated by flow-cytometry. *Int. J. Radiat. Oncol. Biol. Phys.* **10**:1619–1625; 1984.
36. Murray, D.; Meyn, R.E. Cell-cycle dependent cytotoxicity of alkylating agents: Determination of nitrogen mustard-induced DNA cross-links and their repair in Chinese Hamster Ovary cells synchronized by centrifugal elutriation. *Cancer Res.* **46**:2324–2329; 1986.
37. Issels, R.D.; Biaglow, J.E.; Epstein, L.; Gerweck, L.E. Enhancement of cysteamine cytotoxicity by hyperthermia and its modification by catalase and superoxide dismutase in Chinese hamster ovary cells. *Cancer Res.* **44**:3911–3915; 1984.
38. Biaglow, J.E.; Issels, R.W.; Gerweck, L.E.; Varnes, M.E.; Jacobson, B.; Mitchell, J.B.; Russo, A. Factors influencing the oxidation of cysteamine and other thiols: Implications for hyperthermic sensitization and radiation protection. *Radiat. Res.* **100**:298–312; 1984.
39. Clark, E.P. Thiol-induced biochemical modification of chemo- and radioresponses. *Int. J. Radiat. Oncol. Biol. Phys* **12**:1121–1126; 1987.
40. Hodgkiss, R.J.; Vojnovic, B.; Woodcock, M.; Michael, B.D. A single-shot rapid-mixing device for radiobiological studies with mammalian cells. *Int. J. Radiat. Biol.* **55**:705–715; 1989.
41. Demple, B.; Halbrook, J. Inducible repair of oxidative DNA damage in *Escherichia coli. Nature.* **304**:466–468; 1983.
42. Revesz, J. GSH yield and repair of single strand breaks. *Br. J. Cancer* [*Suppl*] **6**:55–60; 1984.
43. Mannervik, B.; Axelsson, K.; Larson, K. Thioltransferase In: Kakoby, W.B., ed. *Methods in enzymology.* Vol. 77. Orlando FL: Academic Press; 1981:281–285.
44. Biaglow, J.E.; Tuttle, S.W.; Davis, M.E.; Montgomerie, D.W.; Donahue, J.J.; Epp, E.R. Altered metabolic states sensitize cultured human lung tumors to radiation and chemotherapy agents. *Abs. of Papers for the 37th Annual Mtg. of the Radiat. Res. Soc.* **37**:158 abstr. (1989).
45. Weissbach, D.; Baltimore, D.; Bollum, F.; Gallo, R.; Korn, D. Nomenclature of eukaryotic DNA polymerases. *Science.* **190**:401–402; 1975.
46. Thelander, L.; Graeslund, A.; Thelander, M. Continual presence of oxygen and iron required for mammalian ribonucleotide reduction: Possible regulation mechanism. *Biochem. Biophys. Res. Commun.* **110**:859–865; 1983.

ABBREVIATIONS

GR—glutathione reductase
GSH—glutathione
BCyNU—N,N'1,3-bis(trans-4-hydroxycyclohexyl)-N'-nitrosourea
CCNU—1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
BCNU—1,3-bis(2-chloroethyl)-1-nitrosourea
GSSG—glutathione disulfide
α MEM—alpha-minimum essential media
FBS—fetal bovine serum
BSA—bovine serum albumin
SOD—superoxide dismutase
GPx—glutathione peroxidase
γ-GT—gamma glutamyl transferase

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RADIOPROTECTION BY POLYSACCHARIDES ALONE AND IN COMBINATION WITH AMINOTHIOLS

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ABSTRACT

We demonstrated that glucan, a beta-1,3 polysaccharide immunomodulator, enhances survival of mice when administered before radiation exposure. Glucan's prophylactic survival-enhancing effects are mediated by several mechanisms including (1) increasing macrophage-mediated resistance to potentially lethal postirradiation opportunistic infections, (2) increasing the D_0 of hematopoietic progenitor cells, and (3) accelerating hematopoietic reconstitution. In addition, even when administered shortly after some otherwise lethal doses of radiation, glucan increases survival. Glucan's therapeutic survival-enhancing effects are also mediated through its ability to enhance macrophage function and to accelerate hematopoietic reconstitution; glucan's therapeutic potential, however, is ultimately dependent on the survival of a critical number of hematopoietic stem cells capable of responding to glucan's stimulatory effects. Preirradiation administration of the traditional aminothiol radioprotectants WR-2721 and WR-3689 has been previously demonstrated to be an extremely effective means to increase hematopoietic stem cell survival. Therapeutic glucan treatment administered in combination with preirradiation WR-2721 or WR-3689 treatment synergistically increases both hematopoietic reconstitution and survival. Such combined modality treatments offer new promise in treating acute radiation injury.

INTRODUCTION

As manned spaceflight becomes more frequent and mission durations increase, the likelihood that astronauts may be acutely exposed to higher-than-anticipated radiation doses with little or no warning also increases. Solar flares, in particular, could acutely (within minutes or days) expose astronauts to radiation doses sufficient to produce a risk of lethality /1-3/. If such events are sufficiently predictable, it may be possible for astronauts on near-earth missions to return to earth and to avoid exposure; in circumstances involving deep-space missions, however, this maneuver will not be possible. In addition, truly unpredictable exposures may occur.

In light of these possible radiation exposures, and given the restraints of employing physical shielding in space, pharmaceutical agents capable of preventing the biological consequences of irradiation if administered before exposure, or pharmaceutical agents capable of enhancing recovery from irradiation even if administered after exposure, are desirable. Application of such pharmaceutical agents is limited by the biological consequences of radiation exposure.

Cell injury and/or death following irradiation generally results from damage to cellular DNA, cellular enzymatic systems, and cellular membrane systems /4, 5/. Such injury may either be caused directly by the radiation, or (more likely) caused indirectly by free radicals created primarily by the interaction of radiation with water in the cellular environment /4, 5/. If cellular injury is severe and is not adequately repaired, acute physiologic syndromes may occur, which ultimately may result in incapacitation or death. Hematopoietic dysfunction occurs after even low radiation exposures; after progressively higher radiation exposures, gastrointestinal, cardiovascular, and central nervous system dysfunction also occur /6/. Given the radiation events most likely to occur in space, hematopoietic dysfunction and its potentially lethal consequences are the most likely concerns for man in space.

The consequences of hematopoietic radiation injury are linked to blood cell development /7/. All of the mature elements of the blood are ultimately derived from bone marrow pluripotent hematopoietic stem cells (Figure 1). These cells have the unique capacity to both self-replicate and to give rise to highly proliferative progenitor cells committed to producing large numbers of mature cells of specific hematopoietic lineages. Many of the mature hematopoietic elements, such as the macrophages, granulocytes, platelets, and red cells, tend to be fairly radioresistant. These cells, however, have relatively short lifespans (hours for granulocytes, days for platelets,

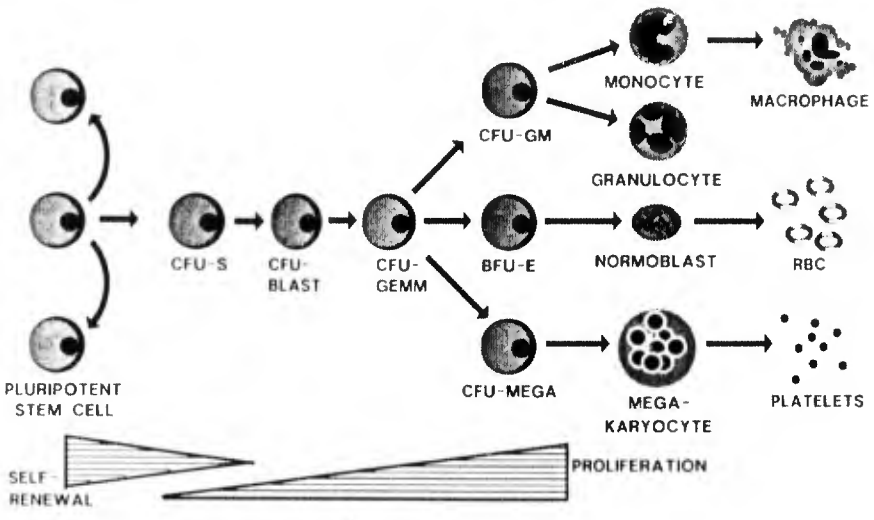

Fig. 1. Scheme for hematopoietic cell proliferation and differentiation.

weeks for macrophages, months for red cells), and new mature cells must be regenerated constantly. Unfortunately, the pluripotent hematopoietic stem cells from which the mature cells are ultimately derived, are some of the most radiosensitive cells in the body. Hence, following even low doses of radiation, these cells are destroyed and the potential to generate new hematopoietic elements is compromised. As mature blood cells present at the time of irradiation are lost through normal senescence, infection (due to lack of granulocytes, monocytes, and macrophages), hemorrhage (due to lack of platelets), and anemia (due to lack of red cells) ensue, and may result in death.

A major objective of our laboratory has been to find ways to enhance survival by enhancing hematopoietic reconstitution following radiation doses that injure the hematopoietic system. To accomplish this objective, we have extensively evaluated the use of immunomodulators for both preventing and treating such injury. Immunomodulators have traditionally been defined as immune-augmenting or immune-restoring agents; however, it is now recognized that many immunomodulators also function as potent hematopoietic regulators /8/. Immunomodulators may be of either biologic (bacteria, yeast, fungi) or synthetic origin, and it is generally accepted that these agents mediate their effects indirectly by inducing endogenous cellular regulators (i.e., cytokines) that control immunologic and hematopoietic functions /8/.

This paper focuses on the biologic immunomodulator glucan, a beta-1,3 polysaccharide isolated from the inner cell wall of the yeast *Saccharomyces cerevisiae*, and describes its protective and therapeutic applications for treating hematopoietic radiation injury both when used alone, and when used in combination with traditional aminothiol radioprotectants.

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 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.

Glucans

Glucans used in these studies were endotoxin-free particulate (glucan-P) or soluble (glucan-F) preparations obtained from Tulane University School of Medicine (New Orleans, LA). The preparation of these glucans has been described by DiLuzio *et al.* /9/. Mice were intravenously (i.v.) administered glucan-P or glucan-F at doses of

75 mg/kg or 250 mg/kg, respectively. Preliminary experiments demonstrated that at these doses the two glucan preparations produced approximately equal biological effects. Initially only particulate glucan was available for experimentation. Subsequently, methods to solubilize particulate glucan were developed. Because soluble glucan is more clinically applicable than particulate glucan (which, when administered i.v., induces side effects such as microemboli, hepatosplenomegaly, and granuloma formation; /10/), radioprotection studies originally performed with particulate glucan were subsequently repeated with soluble glucan. In subsequent therapeutic studies, only the more clinically relevant soluble glucan was used.

WR-2721 and WR-3689

Stock WR-2721 and WR-3689 were obtained from the Walter Reed Army Institute of Research (Washington, DC) and kept frozen at -20°C until use. Immediately before each use, these agents were dissolved in pyrogen-free saline. Exposure of the materials to light was minimized. WR-2721 was administered intraperitoneally (i.p.; 200 mg/kg) 30 minutes before irradiation; WR-3689 was administered by oral gavage (p.o.; 500 mg/kg) 60 minutes before irradiation.

Indomethacin

Indomethacin (Sigma, St. Louis, MO) was administered subcutaneously (s.c.; 5 mg/kg) 24 hours before glucan administration.

Irradiation

Mice were placed in ventilated Plexiglas containers and exposed to bilateral total-body gamma rays at a dose rate of 0.4 Gy/minute. The AFRRI ^{60}Co source was used for all irradiations. Dosimetry was determined by ionization chambers as previously described /11/ with calibration factors traceable to the National Institute of Standards and Technology.

Survival Assays

Irradiated mice were returned to the animal facility and treated routinely. Survival was monitored daily for 30 days; on day 31 surviving mice were euthanized by cervical dislocation. The percentage of mice surviving at 30 days postexposure was used to construct survival curves.

Cell Suspensions

Each cell suspension represented a pool of tissues from three mice. Cells were flushed from femurs with 3 ml of McCoy's 5A medium containing 10% heat-inactivated fetal bovine serum (HIFBS; Hyclone, Logan, UT). Spleens were pressed through stainless-steel mesh screen, and the cells were washed from the screen with 6 ml of medium. The total number of nucleated cells in each suspension was determined by counting cells on a Coulter counter.

Peritoneal Exudate Cell Collection and Macrophage Isolation

Mice were anesthetized with halothane (Halocarbon Laboratories, Hackensack, NJ) and injected i.p. with 5 ml of calcium- and magnesium-free Hanks' Balanced Salt Solution containing 2 units/ml of preservative-free heparin (Abbott Laboratories, North Chicago IL). Following gentle massage, the peritoneal lavage fluid was withdrawn through a 20-guage needle inserted into the cavity, and the cell content of the fluid was determined by counting cells on a Coulter counter. For macrophage cell isolation, 1 ml of peritoneal cells was cultured in 35-mm petri dishes (Corning, Corning, NY) in Dulbecco's Modified Essential Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% HIFBS at a concentration of 2.5×10^6 cells per dish. Macrophages were allowed to attach for 2 hours at 37°C in 5% CO_2. Plates were then washed three times with DMEM to remove nonadherent cells, and the adherent macrophages were used as described for individual assays.

Peripheral Blood Cell Counts and Serum Collection

Blood was obtained from halothane-anesthetized mice by cardiac puncture using a heparinized syringe attached to a 20-guage needle. White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts were performed using a Coulter counter. In some instances, blood was collected in nonheparinized syringes, transferred to centrifuge tubes, and allowed to clot. Blood samples were then centrifuged at 400 x g for 10 minutes and the serum was collected and stored at -70°C until use.

Exogenous Spleen Colony-Forming Unit Assay

Exogenous spleen colony-forming units (CFU-s) were evaluated by the method of Till and McCulloch /12/. Recipient mice were exposed to 9 Gy of total-body radiation to oblate endogenous hematopoietic stem cells. Three to five hours later, 5×10^4 bone marrow or 5×10^5 spleen cells from normal or glucan-treated mice were injected i.v. into the irradiated recipients. Twelve days after transplantation, the recipients were euthanized by cervical dislocation and their spleens were removed. The spleens were fixed in Bouin's solution, and the number of grossly visible spleen colonies was counted.

Endogenous Spleen Colony-Forming Unit Assay

Endogenous spleen colony-forming units (E-CFU) were also evaluated using a method of Till and McCulloch /13/. Mice from various treatment groups were exposed to total-body radiation to partially oblate endogenous hematopoietic stem cells. Twelve days later, mice were euthanized by cervical dislocation and the number of spleen colonies was determined as described above.

Granulocyte-Macrophage Colony-Forming Cell Assay

Hematopoietic progenitor cells committed to granulocyte-macrophage differentiation (GM-CFC) were assayed by a modification /14/ of the semi-solid agar technique originally described by Bradley and Metcalf /15/ and Pluznik and Sachs /16/. The upper agar-medium mixture for cell suspensions consisted of equal volumes of 0.66% agar and double-strength supplemented CMRL 1066 medium (Gibco, Grand Island, NY). The medium was supplemented with final concentrations of 10% HIFBS, 5% trypticase soy broth, 5% heat-inactivated horse serum, antibiotics, and L-serine. The agar mixture for the lower feeder layer consisted of equal volumes of 1% agar and supplemented medium. Endotoxin serum (5% v/v) was added to each 1-ml feeder layer as a source of colony-stimulating factor (CSF). Colonies (>50 cells) were counted after 10 days of incubation in a 37°C humidified environment containing 5% CO_2.

Granulocyte-Macrophage Colony-Stimulating Factor Assay

Serum levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) in normal and in glucan-treated mice were determined by incorporating 50 µl of test serum instead of endotoxin serum into the lower agar feeder layers in the GM-CFC assay. Normal mouse bone marrow cells were then plated in the upper agar layer. Cultures were incubated and GM-CFC colonies were scored as described above.

Hydroxyurea Suicide Assay

The percentage of GM-CFC in S-phase of the cell cycle was determined by hydroxyurea (HU) suicide. HU was administered to mice i.p. in the dose of 900 mg/kg 3 hours before tissues were harvested for GM-CFC assays.

Bacteriological Assays

To evaluate the occurrence of opportunistic infections in irradiated mice, livers of animals were monitored for bacterial translocation. Mice were euthanized by cervical dislocation and the livers were aseptically removed, homogenated, and streaked onto two BHI agar plates (Difco, Detroit, MI). One plate was incubated for 48 hours at 37°C and 5% CO_2 to isolate aerobic bacteria. The other plate was incubated for 96 hours in an anaerobic jar to isolate anaerobic bacteria. Microorganisms were identified by standard criteria /17, 18/.

Reticuloendothelial System Carbon Clearance Assay

The rate of removal of colloidal carbon from the circulation was used to measure the phagocytic capacity of various macrophage populations comprising the reticuloendothelial system /19/. Individual mice were injected i.v. with 0.25 ml of diluted colloidal carbon (160 mg/kg; C11/143a, Gunther Wagner, Hanover, West Germany), and its removal from the circulation was measured. At 1, 2, 4, 6, 8, and 10 minutes after carbon injection, ~0.2 ml of blood was removed from halothane-anesthetized mice by cardiac puncture, and 50 µl was immediately dispersed into 4 ml of 0.1% sodium carbonate solution. At the end of the blood collections, the absorbance of each sample was spectrophotometrically measured against a 0.1% sodium carbonate blank at 650 nm. Time (in minutes) was semilogarithmically plotted against absorbance. A regression line was calculated, and the time required for the absorbance to be halved (T1/2) was determined.

Macrophage 5'Nucleotidase Assay

Macrophage 5'nucleotidase (5'N) activity, which has been shown to decrease as macrophage activation increases /20/, was used to assess peritoneal macrophage activation in irradiated mice. Washed dishes of adherent macrophages were lysed with 200 µl of 0.05% Triton X-100 (Sigma Chemical, St. Louis, MO) in distilled water, and the cell lysate was used to measure 5'N activity. Specifically, 5'N was assayed with 0.15 mM ^{3}H-adenosine monophosphate (AMP) as substrate in 50 mM Tris buffer (pH 9.0) containing 12 mM magnesium chloride /21/. The specific activity is expressed as nmol AMP hydrolyzed per minute per mg cell protein at 37°C. The protein content of the macrophage cell lysates was determined by the Bio-Rad procedure (Bio-Rad Labs, Rockville Center, NY) with bovine gamma globulin as a standard.

Statistics

Results of replicate experiments were pooled and are represented as 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 generalized Savage (Mantel-Cox) procedure. In some instances, probit survival curves were plotted and analyzed according to Finney /22/ to determine $LD_{50/30}$ values (radiation dose lethal for 50% of mice within 30 days postexposure). $LD_{50/30}$ values were used to calculate dose reduction factors (DRF's /23/).

RESULTS AND DISCUSSION

Administration of Glucan Preirradiation

Survival effects. Studies performed in the late 1970's in normal mice demonstrated glucan's ability to function as a potent hematopoietic stimulant capable of dramatically enhancing hematopoietic proliferation at the multipotent stem cell level, as well as, at the level of committed granulocyte-macrophage, pure macrophage, and erythroid progenitor cells /24-26/. Because primitive immunomodulators (e.g., endotoxin /27/) capable of enhancing hematopoiesis had previously been demonstrated to enhance survival when administered to mice before hematopoietically damaging doses of radiation, we suspected that glucan may function similarly. Studies evaluating this possibility demonstrated glucan's "radioprotective" effect (Figure 2). Optimal effects were observed when glucan was administered 20 hours before exposure. When administered at this optimal time, glucan-P and glucan-F, respectively, produced DRF's of 1.21 and 1.17 (Figure 3); however, 250 mg/kg of glucan-F was required to produce an effect approximately equal to 75 mg/kg of glucan-P. Although similar to the primitive immunomodulator endotoxin in its radioprotective capacity /27/, glucan is much less toxic than endotoxin, and hence more clinically applicable as an immunomodulator "radioprotectant". In fact, glucan is ultimately metabolized to glucose within macrophages and used as a food source.

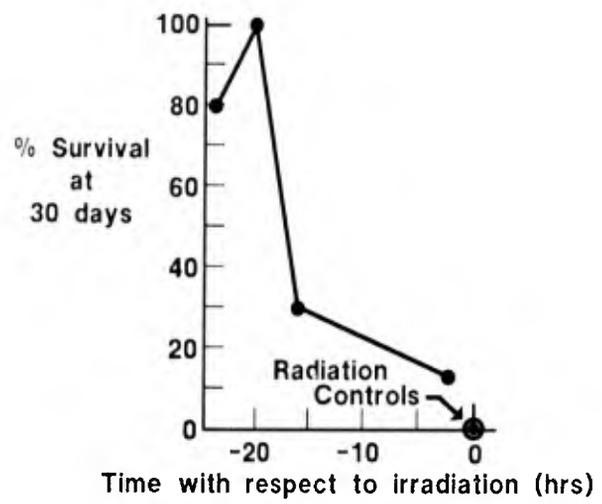

Fig. 2. Effects of glucan-P administration time on survival of irradiated mice. C3H/HeN mice were intravenously injected with glucan-P (75 mg/kg) at time intervals indicated before being exposed to 8.5 Gy ^{60}Co. Composite data from 20 mice at each time point.

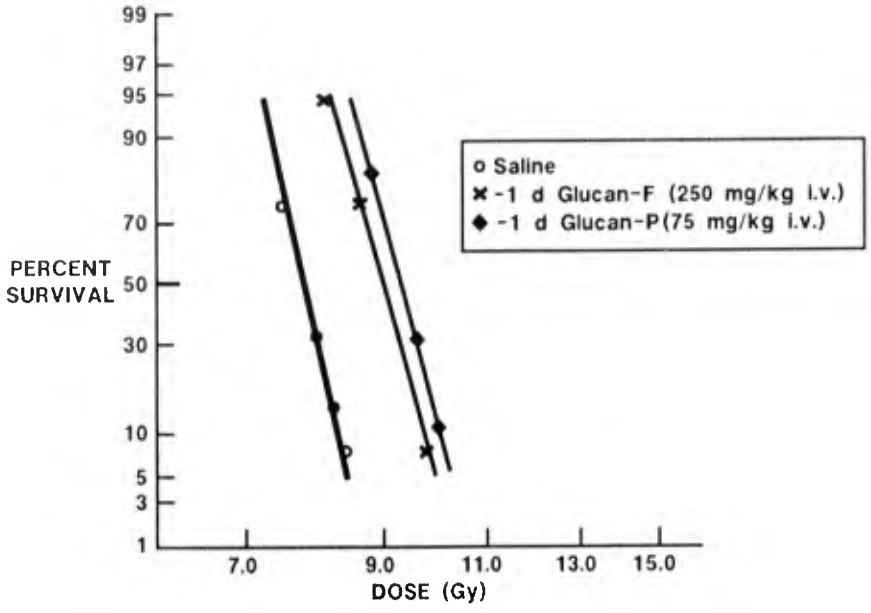

Fig. 3. Effects of glucan-P and glucan-F on survival of irradiated mice. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to indicated doses of ^{60}Co. Composite data from at least 50 mice at each radiation dose for each treatment group.

Mechanisms of action. Multiple mechanisms appear to contribute to the survival enhancement observed in mice treated with glucan before hematopoietically damaging radiation doses. It has been demonstrated that traditional aminothiol radioprotectants typically enhance survival by protecting cells from radiation-induced lethality /28/. To evaluate whether glucan may function in a similar manner, mice were administered glucan 20 hours before increasing doses of radiation (0.25-2.50 Gy), and 24 hours following irradiation the survival of GM-CFC in the bone marrow and spleen (both of which are hematopoietic organs in the mouse) was evaluated. Based on D_0 values (i.e., the radiation dose required to reduce GM-CFC survival to 37%), both glucan-P and glucan-F protected bone marrow and splenic GM-CFC from radiation-induced lethality (Figure 4). Consistently, glucan-P treatment resulted in greater progenitor cell survival than glucan-F treatment. These results confirmed previous studies demonstrating the ability of polysaccharides administered within 20 minutes before irradiation to alter the D_0 of hematopoietic progenitor cells /29/.

Because cells in late S-phase of the cell cycle have been demonstrated to be more radioresistant than cells in other phases of the cell cycle /30/, HU suicide studies were performed to determine if glucan's ability to alter GM-CFC D_0 values could be correlated to the cell cycle status of these progenitor cells at the time of irradiation. Twenty hours following administration, both glucan-P and glucan-F clearly increased the percentage of GM-CFC in S-phase of the cell cycle (Figure 5). Although the HU suicide assay does not specifically kill cells only in late S-phase, these HU studies confirmed glucan's ability to shift GM-CFC into S-phase of the cell cycle at a time that coincides with maximum glucan-induced radioprotection, i.e., 20 hours following glucan administration.

Based on the ability of preirradiation glucan treatment to both increase progenitor cell survival and to stimulate progenitor cell cycling, we suspected that accelerated hematopoietic regeneration would also be detected in glucan-treated, irradiated mice. Time course experiments evaluating the recovery of bone marrow and splenic GM-CFC progenitors in mice administered glucan 20 hours before otherwise lethal irradiation confirmed this hypothesis (Figure 6).

It has long been recognized that the major cause of death following radiation-induced myelosuppression is infection arising primarily from translocated endogenous gram-negative gastrointestinal bacteria /31, 32/. Agents capable of enhancing survival in the radiation dose range inducing the hematopoietic syndrome have typically been associated with accelerated hematopoietic regeneration. Because of this, it has generally been assumed that an accelerated ability to regenerate new hematopoietic elements, especially ones such as granulocytes which are important in controlling bacterial infections, allows the host to better resist opportunistic infections and, hence, enhances survival. Studies evaluating the effect of glucan on bacterial translocation in irradiated mice demonstrated that both glucan-P and glucan-F decrease bacterial translocation (Figure 7). Interestingly, however, reduced bacterial translocation was observed as early as days 7 and 10 postirradiation, at which time signs of

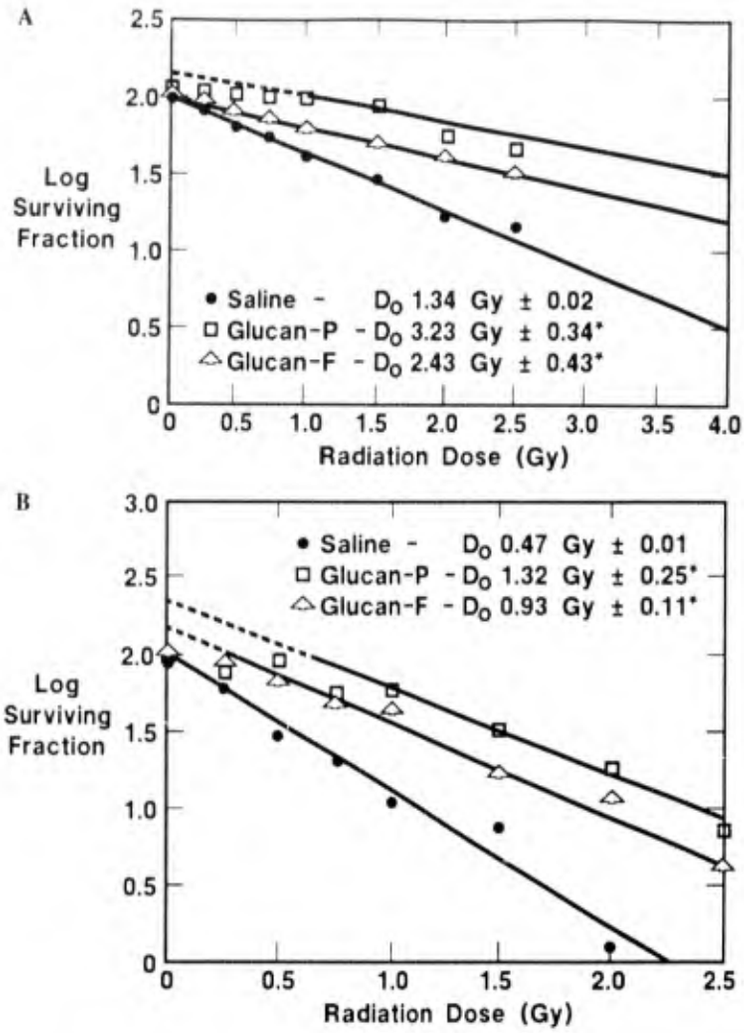

Fig. 4. Effects of glucan-P and glucan-F on GM-CFC D_0 values. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to the indicated doses of ^{60}Co. Twenty-four hours later, bone marrow (A) and spleen (B) were assayed for GM-CFC. * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from three experiments.

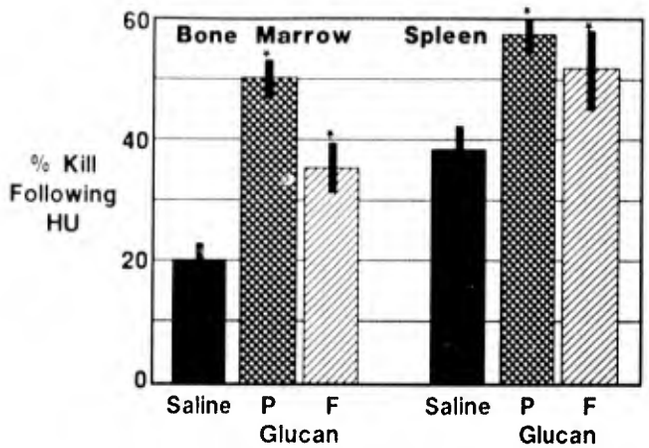

Fig. 5. Effect of *in vivo* hydroxyurea treatment on GM-CFC. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg). Seventeen hours later, mice were intraperitoneally injected with hydroxyurea (900 mg/kg) and 3 hours later (i.e., 20 hours after glucan administration) bone marrow and spleen were assayed for GM-CFC. * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from three experiments.

hematopoietic regeneration had not yet been detected (Figure 6 versus Figure 7). Hence, regeneration of new hematopoietic elements could not account for the early decrease in bacterial translocation observed in glucan-treated mice.

Alternative possibilities were examined to explain the phenomenon of reduced bacterial translocation in the absence of hematopoietic regeneration. Studies performed in the 1960's and 1970's demonstrated that glucan is a potent macrophage activator /33, 34/. It is also well known that macrophages, especially activated macrophages, can play a critical role in controlling infection /35/, as well as, be a potent source for the production and release of several endogenous cytokines capable of regulating hematopoietic and immunologic proliferation and function /36/. In addition, macrophages are extremely radioresistant /37, 38/ and survive several weeks following radiation exposure /38/. Hence, the possibility that activated macrophages contribute to survival enhancement in glucan-treated, irradiated mice was evaluated. Because we hypothesized that bacterial translocation should be reduced if the ability of macrophages comprising the reticuloendothelial system (RES) exhibit an accelerated clearance capacity, the carbon clearance technique was used to evaluate RES clearance capacity in mice treated with glucan 20 hours before irradiation. Clearance time was accelerated in glucan-F-treated mice at all postirradiation time points evaluated, while in glucan-P-treated mice, clearance was actually prolonged at early times, then accelerated

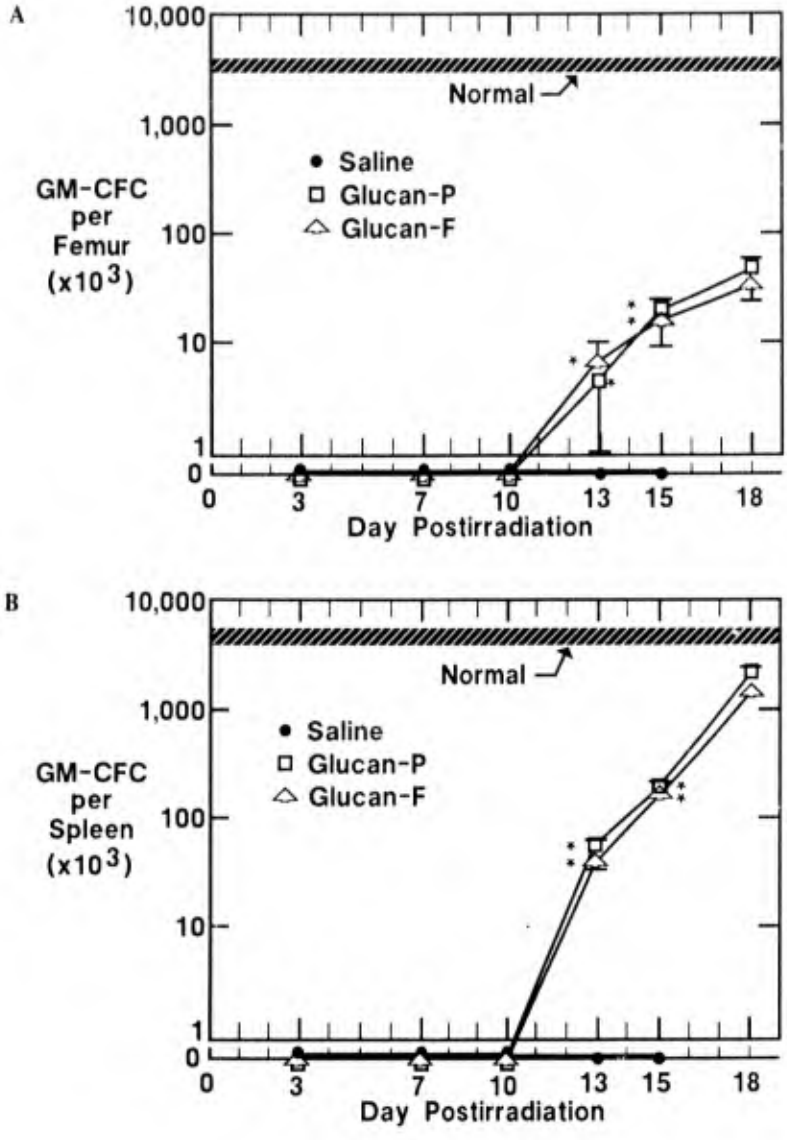

Fig. 6. Effects of preirradiation glucan treatment on GM-CFC progenitor cell recovery. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to 8.5 Gy ^{60}Co. At the times indicated, bone marrow (A) and spleen (B) were assayed for GM-CFC. * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from two experiments. Not enough saline mice survived to assay on day 18, precluding statistical comparisons at this time point.

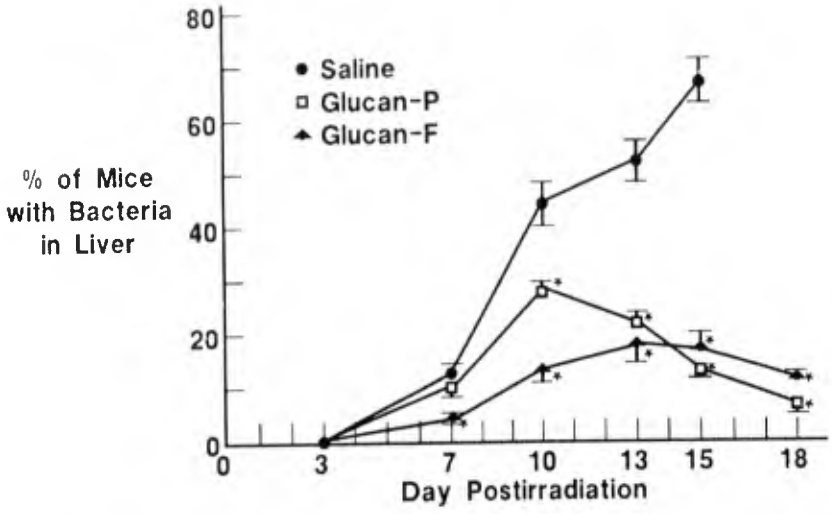

Fig. 7. Effect of preirradiation glucan treatment on hepatic bacterial translocation. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to 8.5 Gy ^{60}Co. On the days indicated, mice from each treatment group were euthanized, and livers were aseptically removed, homoginated, and assayed to determine presence of bacteria. Organisms detected were *Proteus mirabilis, Staphylococcus aureus,* and *Escherichia coli.* * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from 10 mice at each assay point. Not enough saline mice survived to assay on day 18, precluding statistical comparisons at this time point.

at later times postirradiation (Figure 8). The early prolongation of clearance capacity observed only in glucan-P-treated mice may be related to the particulate nature of glucan-P. Macrophages from glucan-P-treated mice typically appeared to be gorged with glucan particles for approximately 10-14 days following glucan administration /Patchen, unpublished observation/. Such gorged macrophages may simply not be capable of phagocytizing additional material until glucan particles are metabolized. When the 5'N assay was used to biochemically assess the activation state of macrophages obtained from glucan-P- and glucan-F-treated mice, both glucans induced a dramatic state of activation that was evident early postexposure and persisted for at least 18 days (Figure 9).

The composite results described above lead us to postulate that, in addition to protecting hematopoietic progenitor cells from radiation injury (as suggested by the D_0 results), glucan also enhances survival through multiple

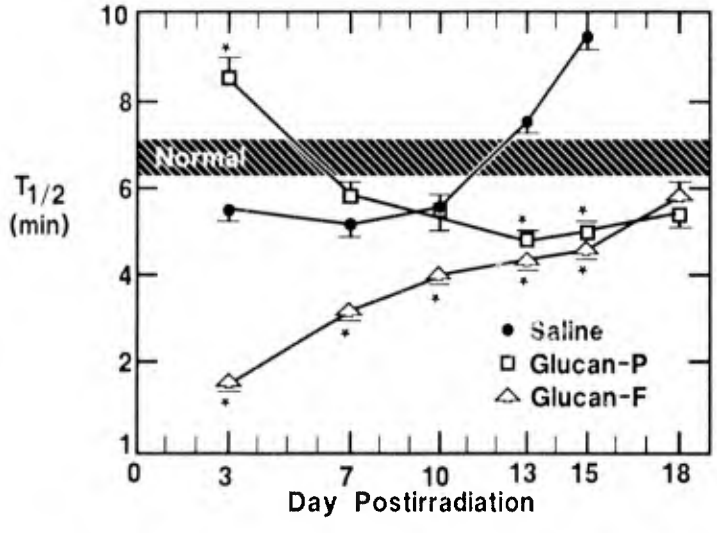

Fig. 8. Effect of preirradiation glucan treatment on carbon clearance capacity. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to 8.5 Gy ^{60}Co. On the days indicated, clearance capacity of intravenously injected carbon was measured. * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from 10 mice at each assay point. Not enough saline mice survived to assay on day 18, precluding statistical comparisons at this time point.

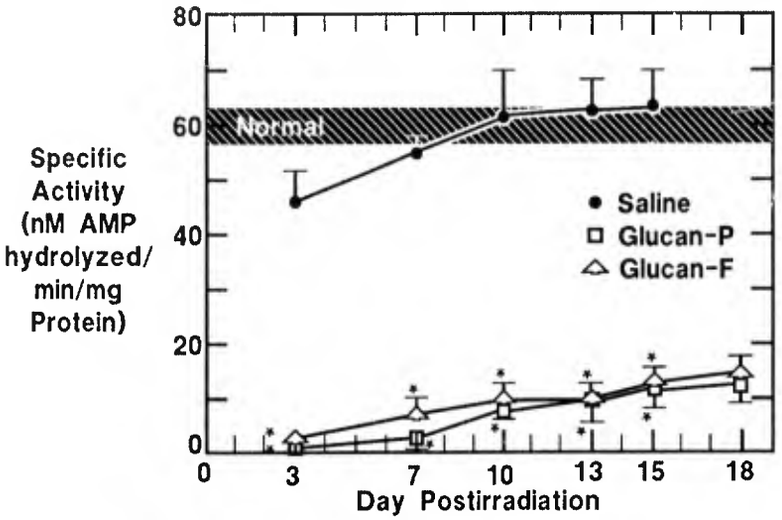

Fig. 9. Effect of preirradiation glucan treatment on 5'nucleotidase activity. C3H/HeN mice were intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg) 20 hours before being exposed to 8.5 Gy ^{60}Co. On the days indicated, peritoneal macrophages were isolated from mice and assayed for 5'nucleotidase activity. * $p < 0.05$, with respect to saline values. Mean ± standard error of values obtained from three experiments. Not enough saline mice survived to assay on day 18, precluding statistical comparisons at this time point.

macrophage-mediated events (Figure 10). In essence, glucan administration appears to activate macrophages, which, being radioresistant cells, survive the radiation exposure. Once activated, these macrophages play a role in both nonspecifically defending the host against opportunistic infections and in producing and releasing several cytokines capable of stimulating the proliferation of protected hematopoietic progenitor cells. The latter part of this hypothesis is supported by the fact that, following glucan administration in normal mice, large amounts of GM-CSF, a cytokine required for the proliferation of GM-CFC progenitors, has been previously demonstrated to be released into the serum /39/ and to be produced primarily in tissues containing large numbers of macrophages /40/. In addition, both glucan-P and glucan-F have been demonstrated to induce macrophage production and release of interleukin-1 (IL-1 /41/). This cytokine, in the presence of other hematopoietic growth factors, has been demonstrated to synergistically enhance hematopoietic proliferation /42/. The importance of IL-1 induction in mediating the survival enhancing effects of glucan has been further substantiated by the observation that one specific mouse stain, the C3H/HeJ strain, does not produce IL-1 in response to glucan-F, and is not radioprotected by glucan-F. However, IL-1 itself, or substances capable of inducing IL-1 production in this mouse strain are radioprotective /41, 43/.

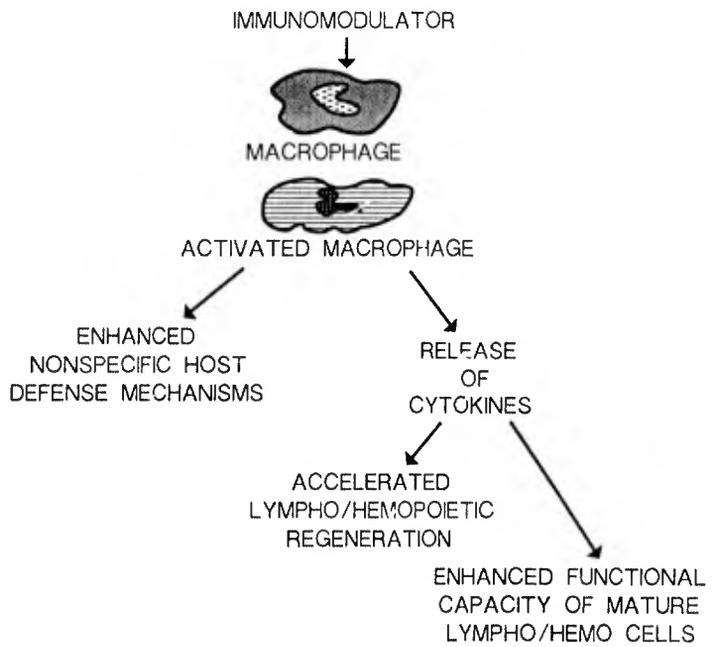

Fig. 10. Scheme of glucan's macrophage-mediated survival enhancing mechanisms.

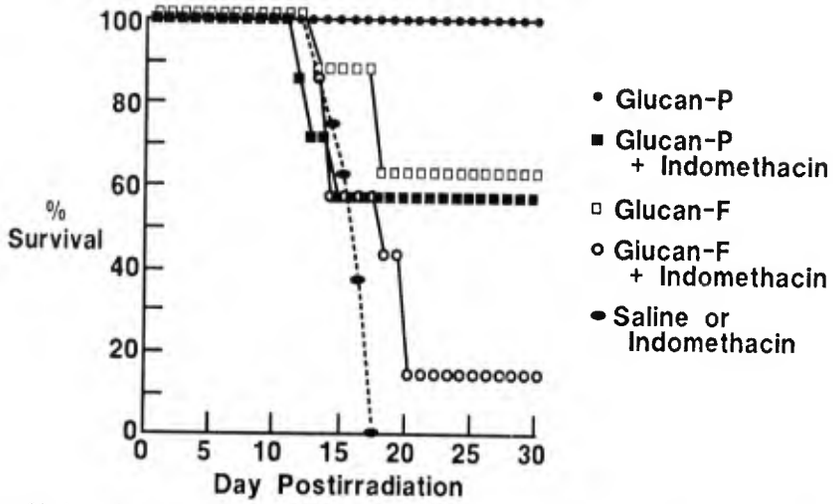

Fig. 11. Effect of indomethacin on glucan-induced radioprotection. C3H/HeN mice were subcutaneously injected with indomethacin (5 mg/kg) 24 hours before being intravenously injected with saline, glucan-P (75 mg/kg), or glucan-F (250 mg/kg). Twenty hours following saline or glucan injections, mice were exposed to 8.5 Gy ^{60}Co. Composite data from at least 30 mice for each treatment group.

In addition to prophylactic glucan administration initiating macrophage-mediated events that postirradiation contribute to survival enhancement, the possibility also existed that macrophage-derived products produced during the 20-hour period between glucan administration and irradiation may contribute to survival enhancement. Both glucan-P and glucan-F have been demonstrated to induce large quantities of macrophage-derived prostaglandins /44/, and administration of the prostaglandin-E_2 (PGE_2) to mice shortly before irradiation has been demonstrated to be radioprotective /45/. To evaluate the role of PGE_2 induction in glucan-mediated radioprotection, mice were pretreated with indomethacin (an inhibitor of PGE_2 synthesis) before glucan administration and survival was monitored. In these studies, the radioprotective effects of both glucan-P and glucan-F were reduced, suggesting that glucan-induced PGE_2 may also play a role in the "protective" aspect of glucan-mediated survival enhancement (Figure 11).

Hence, it appears that the preirradiation survival enhancing effects of both glucan-P and glucan-F are mediated by similar mechanisms, which include prevention of hematopoietic stem cell death, increased host defenses against opportunistic infections, and the induction of hematopoietic growth factor production, which additionally contributes to accelerated hematopoietic regeneration.

Administration of Glucan Postirradiation

Survival effects. Based on the fact that a large part of the survival-enhancing effects observed with prophylactically administered glucan appeared to be mediated through activated macrophages, we suspected that even postirradiation it may be possible to stimulate these radioresistant cells and, hence, to enhance survival. Experiments evaluating this possibility demonstrated the therapeutic ability of glucan to enhance survival (Figure 12).

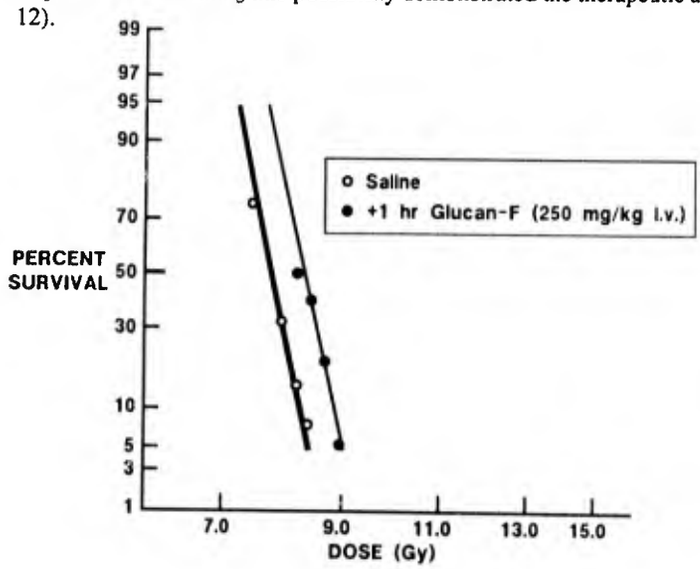

Fig. 12. Effect of postirradiation glucan administration on survival of irradiated mice. C3H/HeN mice were exposed to indicated doses of ^{60}Co and intravenously injected with glucan-F (250 mg/kg) 1 hour postexposure. Composite data from at least 50 mice at each radiation dose for each treatment group.

TABLE 1 Effects of Postirradiation Glucan-F Treatment on Survival, GM-CFC Content, CSF Activity, Macrophage 5'Nucleotidase Activity, and Carbon Clearance Capacity in C3H/HeN Mice Exposed to 8.0 Gy ^{60}Co

	Time Postirradiation														
	Saline							Glucan-F							
Assay	3h	6h	1d	3d	7d	10d	14d	3h	6h	1d	3d	7d	10d	14d	Normal
Survival (%)	100	100	100	100	100	70	10	100	100	100	100	100	100	90	-
GM-CFC/ Femur	-	-	-	0	0	0	0	-	-	-	2.0 ± 0.1	2.9 ± 0.2	5.5 ± 0.4	12.8 ± 0.4	3650 ± 275
CSF Activity (GM-CFC/ 10^5BMC)	1 ± 1	0	0	-	-	-	-	39 ± 2	12 ± 3	2 ± 1	-	-	-	-	0
5'Nucleotidase (specific activity)	-	-	-	52.8 ± 2.6	62.2 ± 3.4	71.4 ± 3.6	73.8 ± 3.7	-	-	-	2.6 ± 0.1	5.0 ± 0.4	5.4 ± 0.3	6.5 ± 0.3	60.0 ± 2.9
Carbon Clearance T 1/2 (min)	-	-	-	5.1 ± 0.2	5.8 ± 0.3	8.0 ± 0.2	7.7 ± 0.4	-	-	-	2.1 ± 0.1	3.8 ± 0.2	3.8 ± 0.1	4.8 ± 0.2	6.7 ± 0.4

-, not tested.

Mechanisms of action. The survival-enhancing effect of therapeutically administered glucan (DRF 1.08; Figure 12) was less than that of prophylactically administered glucan (DRF 1.17; Figure 2). This difference suggested that although macrophage-mediated mechanisms may still contribute to survival enhancement when glucan is therapeutically administered, additional truly "protective" mechanisms that contribute to survival enhancement when glucan is prophylactically administered are absent. Studies performed in irradiated mice treated with glucan 1 hour after exposure confirmed that macrophage activation, enhanced RES clearance capacity, increased production of hematopoietic growth factors, and acceleration of hematopoietic regeneration do occur in these mice (Table 1).

Administration of Aminothiols Preirradiation and Glucan Postirradiation

The survival-enhancing effects of therapeutically administered glucan appeared to be limited by the survival of a critical number of hematopoietic stem cells capable of responding to glucan's stimulatory effects. For example, if instead of 8.0 Gy (as illustrated in Table 1), mice were exposed to 8.5 Gy, then administered glucan 1 hour postexposure, macrophage activation, increased carbon clearance capacity, and elevated GM-CSF production were still detected, however, hematopoietic regeneration did not occur and most of the mice died /Patchen, unpublished observations/.

Aminothiols have been demonstrated to be some of the most effective radioprotectants evaluated to date /46, 47/. Operationally, these agents are limited by the fact that the most radioprotective doses also induce the most serious side effects, one of which is extreme performance decrement /48/. In space, maintaining crew performance could be critical not only to the mission but also to subsequent crew survival; the use of a performance-degrading

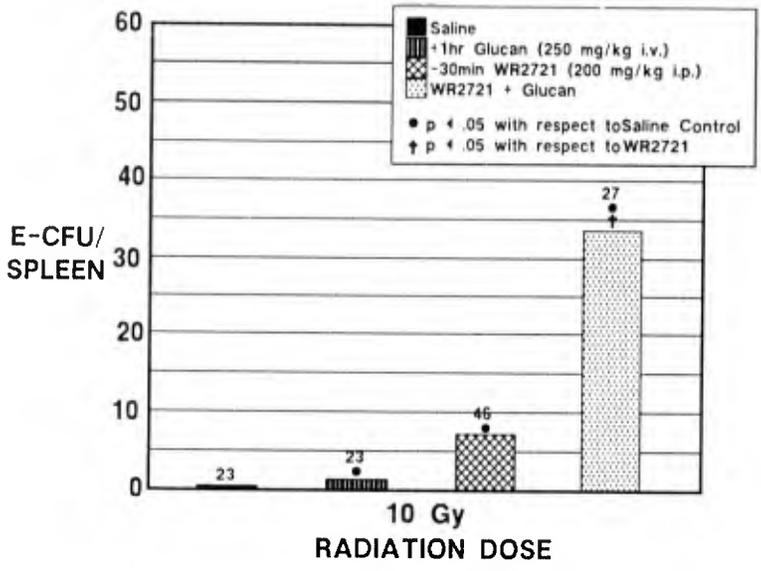

Fig. 13. Effect of preirradiation WR-2721 and therapeutic glucan treatment on endogenous spleen colony formation in irradiated mice. C3H/HeN mice were intraperitoneally injected with WR-2721 (200 mg/kg) 30 minutes before being exposed to 10 Gy ^{60}Co; glucan-F (250 mg/kg) was intravenously injected 1 hour after irradiation. * $p < 0.05$, with respect to saline values. + $p < 0.05$, with respect to WR-2721 values. Mean of values obtained from number of mice indicated above each bar.

radioprotective agent would simply not be desirable. However, we hypothesized that it may be possible to use low (nontoxic and non-performance degrading) doses of aminothiols to protect a small fraction of hematopoietic stem cells, which postirradiation could be stimulated to regenerate using therapeutic glucan. In addition to stimulating hematopoietic regeneration from aminothiol-protected stem cells, therapeutic administration of glucan should also enhance survival through its previously discussed macrophage-mediated mechanisms. This possibility was evaluated in experiments in which mice were administered the aminothiol WR-2721 (200 mg/kg, i.p.) 30 minutes before irradiation and glucan-F (250 mg/kg, i.v.) 1 hour after irradiation. Figure 13 clearly illustrates the synergistic effect of this combined treatment on hematopoietic regeneration. Similar synergistic effects were subsequently demonstrated when survival enhancement was evaluated (Figure 14). Additionally, when an orally administerable aminothiol, WR-3689, was substituted for WR-2721, similar synergistic enhancement of survival was observed (Figure 15).

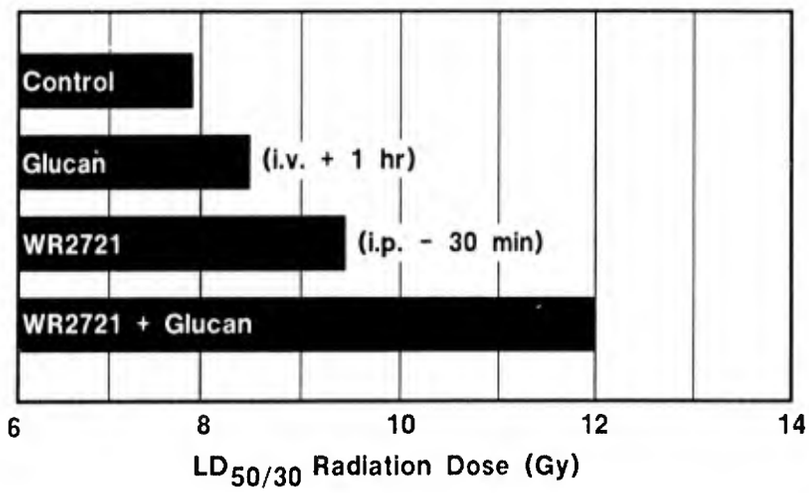

Fig. 14. Survival-enhancing effect of therapeutic glucan treatment combined with low-dose WR-2721 protection. C3H/HeN mice were intraperitoneally injected with WR-2721 (200 mg/kg) 30 minutes before being exposed to ^{60}Co doses ranging from 7 Gy to 15 Gy; glucan-F (250 mg/kg) was intravenously injected 1 hour after irradiation. $LD_{50/30}$ values calculated from composite data from at least 200 mice for each treatment group.

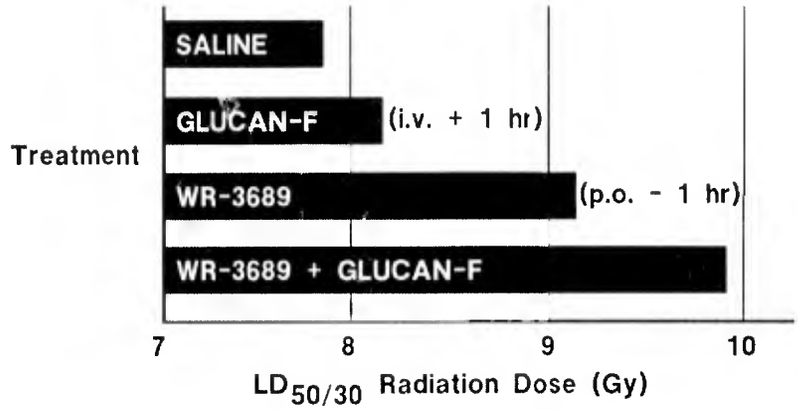

Fig. 15. Survival-enhancing effect of therapeutic glucan treatment combined with low-dose WR-3689 protection. C3H/HeN mice were orally gavaged with WR-3689 (500 mg/kg) 1 hour before being exposed to ^{60}Co doses ranging from 7 Gy to 15 Gy; glucan-F (250 mg/kg) was intravenously injected 1 hour after irradiation. $LD_{50/30}$ values calculated from composite data from at least 200 mice for each treatment group.

CONCLUSIONS

Studies presented in this paper demonstrated that both prophylactic and therapeutic administration of the immunomodulator glucan enhances survival. Glucan is but one of a variety of new generation immunomodulators, which, when administered before irradiation, can enhance survival /49/. Unlike many other immunomodulators, however, glucan offers the advantage of being nontoxic and is ultimately metabolized to glucose. Compared with the best radioprotectants known to date, the aminothiols, prophylactic use of glucan (or any other immunomodulator) offers no advantage in terms of increased DRF's. Even low nontoxic doses of aminothiols can produce DRF's greater than those produced with optimal doses of immunomodulators. However, because the optimal timing of aminothiol administration (< 60 minutes before exposure) differs from the optimal timing of immunomodulator administration (20-24 hours before exposure), one agent may be more appropriate than the other depending on specific radiation exposure scenarios. In addition, combinations of glucan and WR-2721 (each administered at their optimal times before irradiation) have been demonstrated to result in additive protective effects /50/

In contrast to the aminothiol radioprotectants, glucan (and several other immunomodulators /49/) enhances survival even when administered after radiation exposure. Such a capability allows unpredictable radiation injury to be therapeutically treated. The effectiveness of such therapy, however, appears to be ultimately limited by the survival of some critical number of hematopoietic stem cells that, when stimulated by the immunomodulator can repopulate the host. In the case of predictable radiation exposures, therapeutic immunomodulators such as glucan may be used in combination with low (nontoxic and non-performance degrading) doses of aminothiols to synergistically increase hematopoietic regeneration and survival. Using aminothiols in such treatments provides an additional advantage in that aminothiols also can reduce long-term carcinogenic effects of radiation exposure /51/.

Recently, recombinant hematopoietic growth factors, such as GM-CSF and granulocyte colony-stimulating factor (G-CSF), have also been shown to enhance survival when administered after life-threatening radiation exposures /52, 53/. G-CSF has also been demonstrated to enhance the effectiveness of WR-2721 /Patchen, manuscript in preparation/. These agents, however, must be administered once or twice a day for 10-24 days following exposure to obtain effects similar to those seen with a single immunomodulator injection 1 hour after exposure. If medical personnel are limited, as may be the case in space, the advantages of one therapy over the other are obvious.

All studies described in this paper were performed using ^{60}Co gamma radiation. The space environment presents multiple radiation threats, including high-speed protons and electrons, cosmic rays, and energetic particles /54/. Whether the novel survival-enhancing measures described in this paper will also mitigate the consequences of these additional radiation threats remains to be evaluated.

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REFERENCES

1. G.R. Heckman and W.J. Wagner, this issue.

2. G. Reitz and R. Facius, this issue.

3. N.S. Nachtwey, T. Yang and G.D. Badhwar, this issue.

4. L. Giambarresi and A. Jacobs, in: *Military Radiobiology*, eds. J.J. Conklin and R.I. Walker, Academic, New York 1987, p. 265.

5. L. Giambarresi and R. Walker, in: *Medical Consequences of Nuclear Warfare*, eds. R.I. Walker and T.J. Cerveny, TMM Publications, Falls Church, VA 1989, p. 245.

6. T.J. Cerveny, T.J. MacVittie and R.W. Young, in: *Medical Consequences of Nuclear Warfare*, eds. R.I. Walker and T.J. Cerveny, TMM Publications, Falls Church, VA 1989, p. 15.

7. R. Mon[illegible]y, in: *Military Radiobiology*, eds. J.J. Conklin and R.I. Walker, Academic, New York 1987, p. 111.

8. J.W. Hadden, F. Spreafico, Y. Yamamura, K. Austen, P. Dukor and K. Masek, *Advances in Immunopharmacology*, Pergamon, New York, 1989.

9. N.R. DiLuzio, D.L. Williams, R.B. McNamee, B.F. Edwards and A. Kilahama, *Int. J. Cancer* 24, 773 (1979).

10. N.R. DiLuzio, *Trends Pharmacol. Sci.* 4, 344 (1983).

11. J. Schulz, P.R. Almond, J.R. Curningham, J.G. Holt, R. Loevinger, N. Suntharalingam, K.A. Wright, R. Nath and D. Lempert, *Med. Phys.* 10, 741 (1983).

12. J.E. Till and E.A. McCulloch, *Radiat. Res.* 14, 213 (1961).

13. J.E. Till and E.A. McCulloch, *Radiat. Res.* 18, 96 (1963).

14. T.J. MacVittie, *J. Reticuloendothel. Soc.* 26, 479 (1979).

15. T.R. Bradley and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.* 448, 287 (1966).

16. D.H. Pluznik and L. Sachs, *J. Cell. Physiol.* 66, 219 (1965).

17. E.H. Lennette, A. Balows and W. Hausler, *Manual of Clinical Microbiology*, American Society of Microbiology, Washington, 1980.

18. V.L. Sutter, D.M. Citron and S.M. Finegold, *Wadsworth Anaerobic Bacteriology Manual*, Mosby, St. Louis, 1980.

19. H.B. Herscowitz, H.T. Holden, J.A. Bellanti and A. Ghaffar, *Manual of Macrophage Methodology*, Marcel Decker, New York, 1981.

20. P.S. Morahan, P.J. Edelson and K. Gass, *J. Immunol.* 125, 1312 (1980).

21. P.J. Edelson and Z.A. Cohn, *J. Exp. Med.* 144, 1581 (1976).

22. D.J. Finney, *Probit Analysis*, Cambridge, London, 1971.

23. D.J. Finney, *Statistical Methods in Biological Assays*, MacMillan, New York, 1978.

24. C. Burgaleta and D.W. Golde, *Cancer Res* 37, 1739 (1979).

25. M.L. Patchen and E. Lotzova, *Exp. Hematol.* 8, 409 (1980).

26. M.L. Patchen and T.J. MacVittie, *Immunopharmacology* 5, 303 (1983).

27. E.J. Ainsworth, *Pharmacol. Ther.* 39, 223 (1988).

28. D.E. Davidson, M.M. Grenan and T.R. Sweeney, in: *Radiosensitizers: Their Use In The Clinical Management Of Cancer*, ed. L.W. Brady, Masson, New York 1980, p. 309.

29. J.R. Maisin, S. Topalova, A. Kondi-Tamba and G. Mattelin, *Pharmacol. Ther.* 39, 255 (1988).

30. W.K. Sinclair, *Radiat. Res.* 33, 620 (1968).

31. B. Benacerraf, *Bacteriol. Rev.* 24, 35 (1960).

32. C.W. Hammond, M. Tompkins and C.P. Miller, *J. Exp. Med.* 99, 405 (1954).

33. S.J. Riggi and N.R. DiLuzio, *Am. J. Physiol.* 200, 297 (1961).

34. W.R. Woolcs and N.R. DiLuzio, *Science* 142, 1078 (1963).

35. F.M. Griffin, *Adv. Host Defense Mech.* 1, 31 (1982).

36. D.W. Golde and J.C. Gasson, *Sci. Am.* July (1988).

37. J.R. Schmidtke and F.J. Dixon, *J. Immunol.* 108, 1624 (1972).

38. E.S. Buescher and J.I. Gallin, *Blood* 63, 1402 (1984).

39. M.L. Patchen and T.J. MacVittie, *J. Immunopharmacol.* 8, 407 (1986).

40. M.L. Patchen and E. Lotzova, *Biomedicine* 34, 71 (1981).

41. E.K. Gallin, M.L. Patchen, and S. Green, *Int. J. Immunopharmacol.* (submitted).

42. M.A. Moore, *Immunol. Res.* 8, 165 (1989).

43. E.K. Gallin, S.W. Green, M.L. Patchen and G.N. Schwartz, *Armed Forces Radiobiology Research Institute Annual Review, Abstracts* C15 (1988).

44. G.J. Bowers, M.L. Patchen, T.J. MacVittie, E.F. Hirsch and M.P. Fink, *Surg. Forum.* 36, 66 (1985).

45. T.L. Walden, M.L. Patchen and S.L. Snyder, *Radiat. Res.* 109, 440 (1987).

46. J.M. Yuhas, *Radiat. Res.* 44, 621 (1970).

47. L. Giambarresi and A. Jacobs, Radioprotectants, in: *Military Radiobiology*, eds. J.J. Conklin and R.I. Walker, Academic, New York 1987, p. 265.

48. M.R. Landauer, H.D. Davis, J.A. Dominitz and J.F. Weiss, *Pharmacol. Biochem. Behav.* 27, 573 (1987).

49. M.L. Patchen, M.A. Chirigos and I. Brook, *Comment. Toxicol.* 2, 217 (1988).

50. M.L. Patchen, T.J. MacVittie and J.F. Weiss, *Int. J. Radiat. Oncol. Biol. Phys.* 18 (1990).

51. C.K. Hill, B. Nagy, C. Peraino and D.J. Grdina, *Carcinogenesis* 7, 665 (1986).

52. M.L. Patchen, T.J. MacVittie, B.D. Solberg and L.M. Souza, *Int. J. Cell Cloning* 8, 107 (1990).

53. T.J. MacVittie, R.L. Monroy, M.L. Patchen and L.M. Souza, *Int. J. Radiat. Biol.* 57, 723 (1990).

54. W.H. Hess, *The Radiation Belt and Magnetosphere*, Blaisdell, 1968.

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● *Session F: Thiol Modulation and Protection*

POSTIRRADIATION TREATMENT WITH GRANULOCYTE COLONY-STIMULATING FACTOR AND PREIRRADIATION WR-2721 ADMINISTRATION SYNERGIZE TO ENHANCE HEMOPOIETIC RECONSTITUTION AND INCREASE SURVIVAL

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These studies tested whether WR-2721 could be used to protect hemopoietic stem cells, which after irradiation could be stimulated by granulocyte colony-stimulating factor (G-CSF) to proliferate and reconstitute the hemopoietic system. Female C3H/HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before ^{60}Co irradiation and G-CSF (2.5 μg/mouse/day, s.c.) from days 1–16 after irradiation. In survival studies, saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments resulted in LD50/30 values of 7.85 Gy, 8.30 Gy, 11.30 Gy, and 12.85 Gy, respectively. At these LD50/30 values, the dose reduction factor (DRF) of 1.64 obtained in combination-treated mice was more than additive between the DRF's of G-CSF-treated mice (1.06) and WR-2721-treated mice (1.44). Bone marrow and splenic multipotent hemopoietic stem cell (CFU-s) and granulocyte-macrophage progenitor cell (GM-CFC) recoveries were also accelerated most in mice treated with WR-2721 + G-CSF. In addition, mice treated with WR-2721 + G-CSF exhibited the most accelerated peripheral blood white cell, platelet, and red cell recoveries. These studies (a) demonstrate that therapeutically administered G-CSF accelerates hemopoietic reconstitution from WR-2721-protected stem and progenitor cells, increasing the survival-enhancing effects of WR-2721 and (b) suggest that classic radioprotectants and recombinant hemopoietic growth factors can be used in combination to reduce risks associated with myelosuppression induced by radiation or radiomimetic drugs.

Radioprotection, WR-2721, Hemopoiesis, Granulocyte colony-stimulating factor.

INTRODUCTION

The aminothiol WR-2721 [s-2(3-aminopropylamino)-ethyl phosphorothioic acid; ethiofos; gammaphos] is one of the most effective radioprotectants yet discovered (4, 7, 10). Free radical scavenging, hydrogen atom donation, and induction of hypoxia have been suggested as mechanisms through which WR-2721 protects cells from radiation-induced lethality (1, 42, 45). As a survival-enhancing agent in experimental animals, WR-2721 is most effective when relatively high doses are administered 30 min to 3 hr before radiation exposure (7). However, WR-2721 doses that yield the largest radiation dose reduction factors (DRF's) are also the most toxic (17, 45). In clinical trials with cancer patients, WR-2721 has been shown to reduce a variety of side effects associated with radiotherapy and/or chemotherapy, such as hemopoietic toxicity, lung toxicity, gastrointestinal toxicity, ototoxicity, and peripheral neuropathy (12, 14, 20, 41). However, toxic side effects including hypotension, emesis, somnolence, and hypocalcemia have also been noted in clinical trials following high doses of WR-2721 (12, 41).

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 (28, 31). 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 (28). The enhanced survival observed with WR-2721 + glucan was attributed to sequential effects of cell protection me-

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diated by WR-2721 and increased hemopoietic proliferation mediated by glucan. Glucan is a potent inducer of several hemopoietic cytokines (26, 27, 29, 36); therefore, we hypothesized that specific hemopoietic cytokines may be used in place of glucan to stimulate hemopoiesis and to increase the survival-enhancing effects of WR-2721.

One such hemopoietic cytokine is granulocyte colony-stimulating factor (G-CSF; 48). G-CSF administration increases granulocyte regeneration in drug-induced (37, 44) and radiation-induced (18, 30) myelosuppressed animals. Recently, clinical studies have confirmed the usefulness of G-CSF in preventing and/or treating myelosuppression (2, 3, 9, 24, 33).

The studies described in this paper demonstrate the ability of G-CSF to accelerate hemopoietic regeneration from stem and progenitor cells protected by a low dose of WR-2721, and the effects of such hemopoietic stimulation on survival enhancement.

METHODS AND MATERIALS

Mice

C3H/HeN female mice (~20 g) were used.* 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.

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 i.p. 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 μg 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 (34) with calibration factors traceable to the National Institute of Standards and Technology.

Survival assays

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. Each treatment group within each experiment consisted of 10 mice. Experiments were repeated three times. The percentage of mice surviving each radiation dose at 30 days postexposure was used to construct survival curves. LD50/30 values (i.e., radiation dose lethal for 50% of mice within 30 days postexposure) were extrapolated from survival curves and used to calculate dose reduction factors (DRF's; LD50/30 value for treated mice divided by LD50/30 value for radiation control mice).

Hemopoietic assays

To evaluate multipotent hemopoietic progenitor cell recovery, spleen colony-forming units (CFU-s) were assayed by the method of Till and McCulloch (40). Recipient mice were exposed to 9.25 Gy of total-body irradiation to eradicate endogenous hemopoietic stem cells. Three to five hours later, $1–5 \times 10^4$ bone marrow or $1–5 \times 10^5$ 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, fixed in Bouin's solution, and the number of grossly visible spleen colonies was counted. Hemopoietic progenitor cells committed to granulocyte and/or macrophage development were assayed using a previously described (28) 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. The cell suspensions used for CFU-s and GM-CFC assays represented tissues from three normal, irradiated, G-CSF-treated, WR-2721-treated, or combination-treated mice at each time point and were prepared as previously described (28). To perform peripheral blood cell counts, blood was obtained from halothane-anesthetized mice by cardiac puncture using a heparinized syringe attached to a 20-gauge needle.

* Charles River Laboratories, Raleigh, NC.

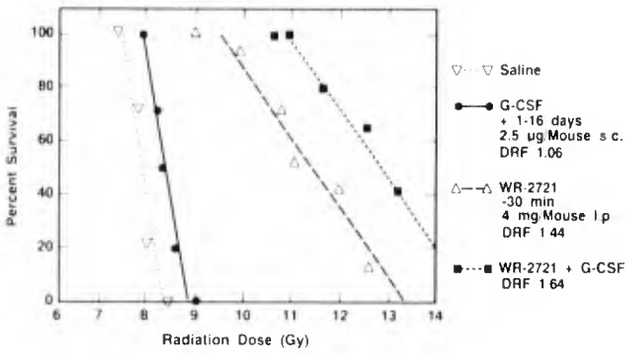


Fig. 1. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on survival of irradiated mice. C3H/HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before ^{60}Co irradiation and G-CSF (2.5 µg/mouse/day, s.c.) on days 1–16 after irradiation. Each data point represents results obtained from 30 mice.

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 (8). Differences were considered significant when P was less than 0.05.

RESULTS

Effects of G-CSF, WR-2721, and WR-2721 + G-CSF on survival.

Figure 1 illustrates the ability of G-CSF, WR-2721, and WR-2721 + G-CSF to enhance survival in irradiated mice. The LD50/30 for saline-treated mice was 7.85 Gy ± 0.06 Gy. Consistent with our previous experiences (28, 31), preirradiation administration of 4 mg of WR-2721 significantly increased survival and altered the slope of the survival curve; the LD50/30 for WR-2721-treated mice was 11.30 Gy ± 0.15 Gy. Compared to saline, WR-2721 administration resulted in a DRF of 1.44. Postirradiation treatment with G-CSF alone also significantly enhanced survival, but less than WR-2721 administration. G-CSF treatment alone yielded an LD50/30 of 8.30 Gy ± 0.08 Gy, which resulted in a DRF of 1.06. Administration of both WR-2721 and G-CSF 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.

Effects of G-CSF, WR-2721, and WR-2721 + G-CSF on hemopoiesis

To evaluate hemopoietic responses induced by G-CSF, WR-2721, and WR-2721 + G-CSF, recoveries of bone marrow and splenic cellularity (Table 1), CFU-s (Fig. 2), and GM-CFC (Fig. 3) were assayed at 10, 17, and 24 days following 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 long enough 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. For example, by day 24 postexposure bone marrow CFU-s (Fig. 2A) and bone marrow GM-CFC (Fig. 3A) numbers in WR-2721-treated mice, respectively, had recovered to only 2% of normal and 7% of normal, compared to 14% of normal bone marrow CFU-s numbers and 15% of normal bone marrow GM-CFC numbers observed in WR-2721 + G-CSF-treated mice. Splenic CFU-s and GM-CFC recoveries were more dramatic than those observed in the bone marrow (Figs. 2B and 3B). By day 24 postexposure, WR-2721 + G-CSF-treated mice exhib-

Table 1. Effect of G-CSF, WR-2721, and WR-2721 + G-CSF on cellularity, following a 10.75 Gy radiation exposure*

	Treatment			
	Saline	G-CSF	WR-2721	WR-2721 + G-CSF
Cells per femur ($\times 10^6$)				
Day 10	0.37 ± 0.02	0.52 ± 0.09	1.46 ± 0.15	2.32 ± 0.25‡
17	†	†	2.14 ± 0.38	2.91 ± 0.16
24	†	†	2.23 ± 0.27	5.23 ± 0.25‡
Cells per spleen ($\times 10^6$)				
Day 10	12.0 ± 0.2	13.0 ± 0.5	14.1 ± 0.6	15.2 ± 0.8
17	†	†	69.9 ± 1.9	223.3 ± 23.2‡
24	†	†	194.6 ± 16.3	153.4 ± 26.1

*Mean ± standard error of values obtained from three experiments.
†No mice survived to be assayed at this time point.
‡$p < 0.05$, with respect to WR-2721 values.

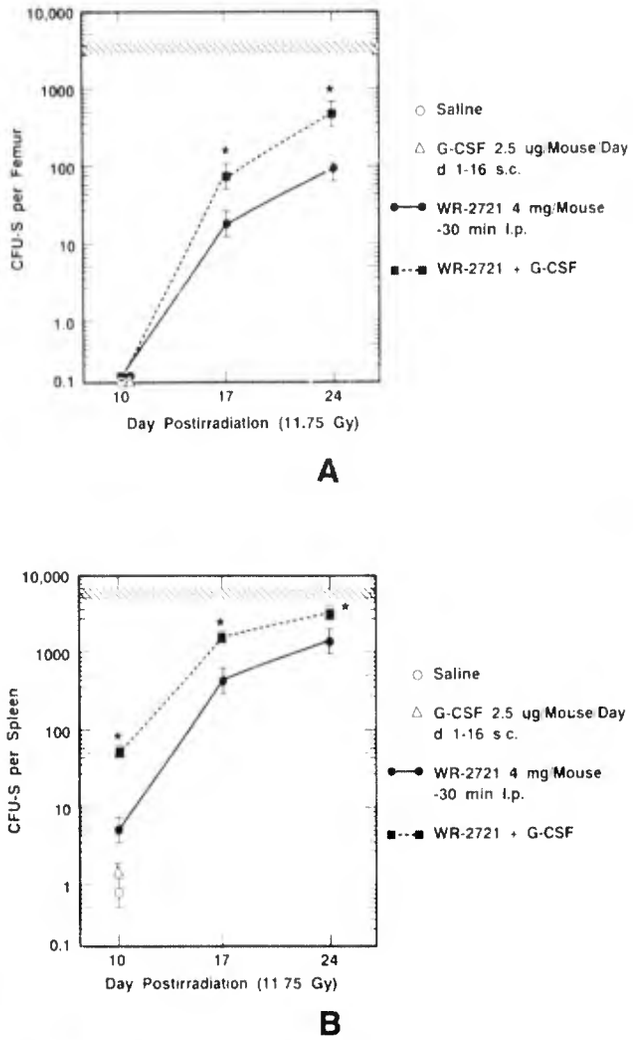


Fig. 2. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on bone marrow (A) and splenic (B) CFU-s recovery in mice exposed to 10.75 Gy. C3H/HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before ^{60}Co irradiation and G-CSF (2.5 μg/mouse/day, s.c.) on days 1–16 after irradiation. Data represented as the mean ± standard error of values obtained from three replicate experiments. $*p < 0.05$, with respect to WR-2721 values.

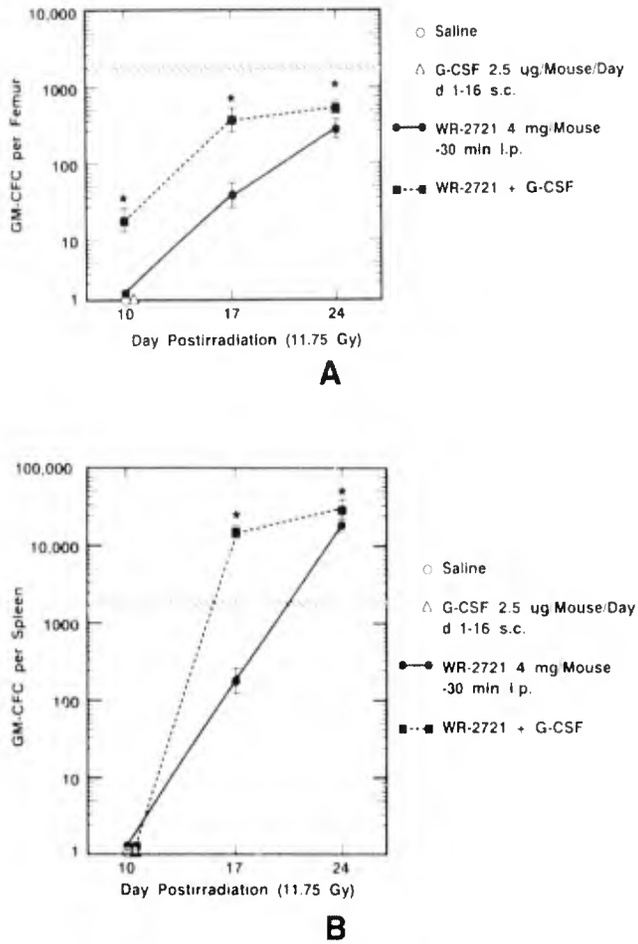
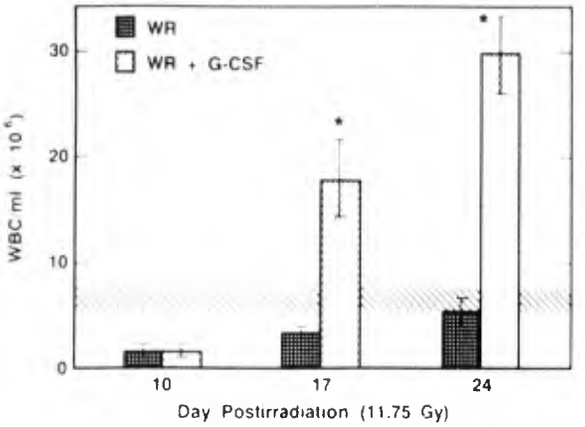


Fig. 3. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on bone marrow (A) and splenic (B) GM-CFC recovery in mice exposed to 10.75 Gy. C3H/HeN mice were administered WR-2721 (4 mg/mouse, i.p.) 30 min before ^{60}Co irradiation and G-CSF (2.5 μg/mouse/day, s.c.) on days 1–16 after irradiation. Data represented as the mean ± standard error of values obtained from three replicate experiments. $*p < 0.05$, with respect to WR-2721 values.

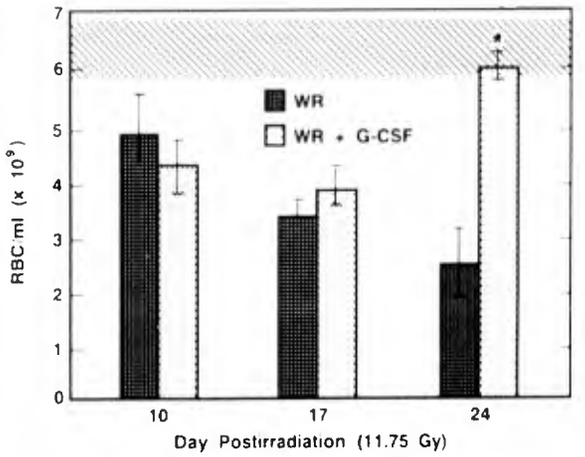
ited 61% of normal CFU-s numbers, compared to only 36% in WR-2721-treated mice. Even more dramatic than splenic CFU-s recovery was splenic GM-CFC recovery. Already at day 17 postirradiation, splenic GM-CFC numbers in WR-2721 + G-CSF-treated mice had surpassed normal levels, reaching 791% of normal. This compared to only a 15% of normal recovery at the same time in WR-2721-treated mice. 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 (Fig. 4). WBC's in combination-treated mice surpassed normal levels by day 17 postirradiation, reaching 293% of normal, compared to only 37% of normal in WR-2721-treated mice. RBC recovery in WR-2721 + G-CSF-treated mice occurred later, reaching normal levels at day 24 postexposure; at this time RBC numbers in WR-2721-treated mice were still only 37% of normal. Although PLT recovery was also accelerated in combination-treated mice, at 24 days postirradiation recovery to only 46% of normal was observed. However, this compared to a PLT recovery of only 16% of normal in WR-2721-treated mice.

DISCUSSION

The effectiveness of chemotherapy and radiation for the treatment of cancer is limited by adverse effects of these treatments on critical normal tissues within the body. Hemopoietic tissues, in particular, are extremely sensitive to chemotherapy and radiation, hence, hemopoietic suppression and associated risks of infection and hemorrhage are common problems associated with therapies used in the treatment of cancer. Means to avoid drug- or radiation-induced damage to normal tissues, or means to



A



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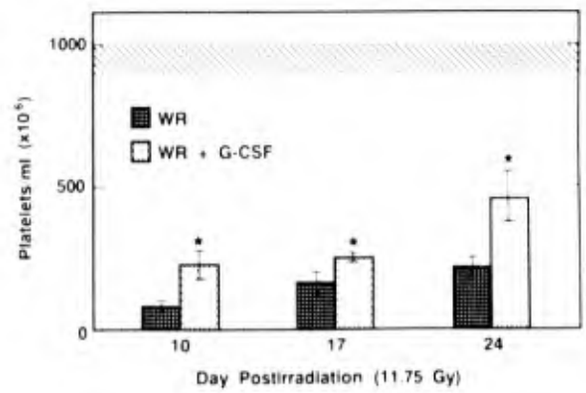

C

Fig. 4. Effect of saline, G-CSF, WR-2721, and WR-2721 + G-CSF treatments on peripheral blood WBC (A), RBC (B), and PLT (C) recovery 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 μg/mouse/day, s.c.) on days 1–16 after irradiation. Data represented as the mean ± standard error of values obtained from three replicate experiments. *$p < 0.05$, with respect to WR-2721 values.

enhance the regeneration of surviving normal tissues following cancer treatment could be extremely beneficial.

Regarding the first possibility — protecting normal tissues from adverse effects of chemotherapy or radiation — WR-2721 has been shown to hold promise. Interestingly, this agent was not developed with this purpose in mind. WR-2721 was initially synthesized by the U.S. Army Antiradiation Drug Development Program in its search for agents to protect military personnel from radiation exposures associated with nuclear warfare (7). Of ~4,400 agents evaluated in the Program, WR-2721 proved to be the most radioprotective. It was later discovered that, in addition to protecting against radiation, WR-2721 could also protect against adverse effects of chemotherapeutic agents (13). Furthermore, WR-2721 selectively protected normal but not neoplastic tissues (43, 46, 47) and even reduced mutagenic and carcinogenic effects associated with exposure of normal tissues to radiation and drugs (15, 22, 25). Hence, WR-2721 attracted significant clinical attention as an agent potentially capable of reducing the toxicity of conventional doses of chemotherapy and radiation without loss of antitumor activity as well as allowing for the safe administration of higher than usual clinical doses of chemotherapy or radiation to increase tumor cytotoxicity (20). In humans, Phase I, II, and III clinical trials have confirmed beneficial effects of WR-2721 when administered before chemotherapy or radiation (14, 20, 41). Significant protection against hemopoietic toxicity repeatedly has been observed, with WR-2721-treated patients exhibiting reduced incidents of neutropenia, thrombocytopenia, and infectious complications otherwise associated with severe bone marrow suppression (6, 13). The data presented in our studies confirm the ability of WR-2721 to protect hemopoietic stem and progenitor cells in irradiated hosts.

As eluded to in the opening paragraph of this discussion, a second approach may also exist to mitigating adverse effects of chemotherapy and radiation; rather than "protecting" cells from radiation- or drug-induced lethality, it may be possible to accelerate the regeneration of normal cells that survive the therapy. However, for this approach to be successful, tissues of interest must contain target cell populations with significant amplification potential that can be specifically induced to proliferate and differentiate into mature functional cells. Fortunately, the hemopoietic system (one of the most sensitive to drug- and radiation-induced damage) contains such target cells — the hemopoietic stem cells and progenitor cells — which proliferate and differentiate in response to specific hemopoietic growth factors (32, 33, 35). Over the past 20 years multiple glycoprotein hemopoietic factors have been identified that regulate hemopoietic proliferation and differentiation (32, 35). G-CSF, one specific hemopoietic growth factor, can significantly increase total granulocyte numbers in normal animals (5, 21) and in drug-induced myelosuppressed animals (23, 37, 39, 44), enhance resistance to infection in neutropenic cyclophosphamide-treated animals (19), and increase hemopoiesis and survival after cyclophosphamide and radiation (16, 18, 30, 38). Clinical trials have also clearly demonstrated granulocytopoietic effects of G-CSF in humans (2, 3, 9, 11, 24, 33).

The data presented in our studies reconfirm the ability of both WR-2721 and G-CSF to individually enhance survival in irradiated mice (7, 28, 30, 31). Furthermore, our studies clearly illustrate the benefit of using these two agents in combination. The enhanced survival observed in combination-treated mice (Fig. 1) was no doubt due to accelerated hemopoietic regeneration. G-CSF was able to stimulate the production of mature functional blood cells (Fig. 4) by amplification and differentiation of stem and progenitor cell populations protected by WR-2721 treatment (Figs. 2, 3).

Although WR-2721 provides a variety of beneficial effects against drug and radiation damage, a variety of undesirable and potentially detrimental side effects have also been observed following WR-2721 administration (12, 17, 41, 45). These include locomotor decrements, emesis, hypotension, hypocalcemia, and somnolence. These side effects are dose-related; with currently recommended clinical WR-2721 doses these effects have been somewhat reduced and are clinically manageable. However, it should be kept in mind that applications other than the treatment of cancer patients undergoing chemotherapy and radiation may exist for radioprotectants. For example, other possible applications may include protection of individuals involved in the clean-up of radioactive accidents, decontamination of fall-out areas, and space travel. In these circumstances, continued mental and physical performance would be required of individuals administered radioprotectants, thus, agents inducing toxic or performance-degrading side effects would be undesirable. With this in mind, the WR-2721 dose chosen for use in our studies was purposefully lower than doses that induce optimal protective effects in mice (7). Hence, using only a suboptimal WR-2721 dose we have demonstrated significant hemopoietic and survival enhancing effects when G-CSF therapy is combined with WR-2721 treatment.

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. These studies clearly establish a role for the postirradiation use of hemopoietic growth factors in combination with classical radioprotectants.

REFERENCES

1. Alper, T. Cellular radiobiology. London: Cambridge University Press; 1979.
2. Bonilla, M. A.; Gillio, A. P.; Ruggerio, M.; Kernan, N. A.; Brochstein, J. A.; Abboud, M.; Fumagalli, L.; Vincent, M.; Gabrilove, J. L.; Welte, K.; Souza, L. M.; O'Reilly, R. J. Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N. Engl. J. Med. 320:1574–1580; 1989.
3. Bronchurd, M. H.; Scarffe, J. H.; Thatcher, N.; Crowther, D.; Souza, L. M.; Alton, N. K.; Testa, N. G.; Dexter, T. M. Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br. J. Cancer 56:809–813; 1987.
4. Brown, D. Q.; Graham, W. J.; MacKenzie, L. J.; Pittock, J. W.; Shaw, L. M. Can WR-2721 be improved upon? Pharmacol. Ther. 39:157–168; 1988.
5. Cohen, A. M.; Zsebo, K. M.; Inoue, H.; Boone, T. C.; Chazin, V. R.; Tsai, L.; Ritch, T.; Souza, L. M. In vivo stimulation of granulopoiesis by recombinant human granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 84: 2484–2488; 1987.
6. Constine, L. S.; Zagars, G.; Rubin, P.; Kligerman, M. M. Protection by WR-2721 of human bone marrow function following irradiation. Int. J. Radiat. Oncol. Biol. Phys. 12: 1505–1508; 1986.
7. Davidson, D. E.; Grenan, M. M.; Sweeney, T. R. Biological characteristics of some improved radioprotectors. In: Brady, L. W., ed. Radiation sensitizers: their use in the clinical management of cancer. New York, NY: Masson Publishing; 1980: 309–320.
8. Finney, D. J. Statistical methods in biological assays. New York, NY: MacMillan Press; 1978.
9. Gabrilove, J. L.; Jakubowski, A.; Scher, H.; Sternberg, C.; Wong, G.; Grons, J.; Yagoda, A.; Fain, K.; Moore, M. A. S.; Clarkson, B.; Dettgen, H. F.; Alton, K. Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional cell carcinoma of the urothelium. N. Engl. J. Med. 318: 1414– 1422; 1988.
10. Giambarresi, L.; Jacobs, A. Radioprotectants. In: Conklin, J. J., Walker, R. I., eds. Military radiobiology. New York, NY: Academic Press; 1987:264–301.
11. Glaspy, J. A.; Golde, D. W. Clinical trials of myeloid growth factors. Exp. Hematol. 18:1137–1141; 1990.
12. Glover, D.; Fox, K. R.; Weiler, C.; Kligerman, M. M.; Turrisi, A.; Glick, J. H. Clinical trials of WR-2721 prior to alkylating agent chemotherapy and radiotherapy. Pharmacol. Ther. 39:3–7; 1988.
13. Glover, D.; Glick, J. H.; Weiler, C.; Hurowitz, S.; Kligerman, M. M. WR-2721 protects against the hematologic toxicity of cyclophosphamide: a controlled phase II trial. J. Clin. Oncol. 4:584–588; 1986.
14. Glover, D.; Grabelsky, S.; Fox, K.; Weiler, C.; Cannon, L.; Glick, J. Clinical trials of WR-2721 and cis-platinum. Int. J. Radiat. Oncol. Biol. Phys. 16:1201–1204; 1989.
15. Grdina, D. J.; Sigdestad, C. P. Radiation protectors: the unexpected benefits. Drug Metabol. Rev. 20:1342; 1989.
16. Kobayashi, Y.; Okabe, T.; Urabe, A.; Suzuki, N.; Takaku, F. Human granulocyte colony-stimulating factor produced by Escherichia coli shortens the period of granulocytopenia induced by irradiation in mice. Jpn. J. Cancer Res. 78:763–766; 1987.
17. Landauer, M. R.; Davis, H. D.; Dominitz, J. A.; Weiss, J. F. Dose and time relationships of the radioprotector WR-2721 on locomotor activity in mice. Pharmacol. Biochem. Behav. 27:573–576; 1987.
18. MacVittie, T. J.; Monroy, R. L.; Patchen, M. L.; Souza, L. M. Therapeutic use of recombinant human G-CSF (rhG-CSF) in a canine model of sublethal and lethal whole-body irradiation. Int. J. Radiat. Biol. 57:723–736; 1990.
19. Matsumoto, M.; Matsubara, S.; Matsuno, T.; Tamura, M.; Hattori, K.; Nomura, H.; Ono, M.; Yokota, T. Protective effect of human granulocyte colony-stimulating factor on microbial infection in neutropenic mice. Infect. Immun. 55:2715–2720; 1987.
20. McCulloch, W.; Scheffler, B.; Schein, P. WR-2721 (ETHYOL): Reduction in toxicity of anticancer therapy without loss of efficacy. In: Howell, S. B., ed. Proceedings of the Sixth International Symposium on Platinum and Other

Metal Coordination Compounds in Cancer Chemotherapy. New York, NY: Plenum Publishing; (In Press).
21. Metcalf, D. The molecular control of normal and leukemic granulocytes and macrophages. Proc. R. Soc. Lond. (Biol.) 230:389–423; 1987.
22. Milas, L.; Hunter, N.; Stephens, C.; Peters, L. J. Inhibition of radiation carcinogenesis in mice by s-2-(3-aminopropylamino)-ethyl phosphorothioic acid. Cancer Res. 44:5567–5569; 1984.
23. Moore, M. A. S.; Warren, D. J.; Synergy of interleukin-1 and granulocyte colony-stimulating factor: in vivo stimulation of stem-cell recovery and hemopoietic regeneration following 5-fluorouracil treatment of mice. Proc. Natl. Acad. Sci. USA. 84:7134–7138; 1987.
24. Morstyn, G.; Campbell, L.; Souza, L. M.; Alton, N. K.; Keech, J.; Green, M.; Sheridan, W.; Metcalf, D.; Fox, R. Effect of granulocyte colony-stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet i:667–671; 1988.
25. Nagy, B.; Dale, P. J.; Grdina, D. J. Protection against cis diaminodichloroplatinum cytotoxicity and mutagenicity in V79 cells by 2-[(aminopropyl)-amino]ethanethiol. Cancer Res. 46.1132–1135; 1986.
26. Patchen, M. L.; Lotzova, E. The role of macrophages and T-lymphocytes in glucan-mediated alterations of murine hemopoiesis. Biomed. 34:71–77; 1981.
27. Patchen, M. L.; MacVittie, T. J. Hemopoietic effects of intravenous soluble glucan administration. J. Immunopharmacol. 8:407–425; 1986.
28. Patchen, M. L.; MacVittie, T. J.; Jackson, W. E. Postirradiation glucan administration enhances the radioprotective effects of WR-2721. Radiat. Res. 117:59–69; 1989.
29. Patchen, M. L.; MacVittie, T. J.; Solberg, B. D.; D'Alesandro, M. M.; Brook, I. Radioprotection by polysaccharides alone and in combination with aminothiols. Adv. Space Res. (In press).
30. Patchen, M. L.; MacVittie, T. J.; Solberg, B. D.; Souza, L. M. Therapeutic administration of recombinant human granulocyte colony-stimulating factor accelerates hemopoietic regeneration and enhances survival in a murine model of radiation-induced myelosuppression. Int. J. Cell. Cloning 8:107–122; 1990.
31. Patchen, M. L.; MacVittie, T. J.; Weiss, J. F. Combined modality radioprotection: the use of glucan and selenium with WR-2721. Int. J. Radiat. Oncol. Biol. Phys. 18:1069–1075; 1990.
32. Robinson, B. E.; Quesenberry, P. J. Review: Hematopoietic growth factors: overview and clinical applications, Part I. Am. J. Med. Sci. 300:163–170; 1990.
33. Robinson, B. E.; Quesenberry, P. S. Hemopoietic growth factors: overview and clinical applications, Part II. Am. J. Med. Sci. 300:237–244; 1990.
34. Schulz, J.; Almond, P. R.; Cunningham, J. R.; Holt, J. G.; Loevinger, R.; Suntharalingam, N.; Wright, K. A.; Nath, R.; Lempert, D. A protocol for the determination of absorbed dose for high energy photon and electron beams. Med. Phys. 10:741–771; 1983.
35. Sieff, C. A. Hematopoietic growth factors. J. Clin. Invest. 79:1549–1557; 1987.
36. Sherwood, E. R.; Williams, D. L.; McNamee, R.; Jones, E.; Browder, W.; DiLuzio, N. Enhancement of interleukin-1 and interleukin-2 production by soluble glucan. Int. J. Immunopharmacol. 9:261–267; 1987.
37. Shimamura, M.; Kobayashi, Y.; Yuo, A.; Urabe, A.; Okabe, T.; Komatsu, Y.; Itoh, S.; Takaku, F. Effect of human recombinant granulocyte colony-stimulating factor on hematopoietic injury in mice induced by 5-fluorouracil. Blood 69: 353–355; 1987.
38. Talmadge, J. E.; Tribble, H.; Pennington, R.; Bowersox, O.; Schneider, M. A.; Castelli, P.; Black, P. L.; Abe, F. Protective, restorative, and therapeutic properties of recombinant colony-stimulating factors. Blood 73:2093–2103; 1989.
39. Tamura, M.; Hattori, K.; Nomura, H.; Oheda, M.; Kubota, N.; Imaseki, I.; Ono, M.; Ueyama, Y.; Nagata, S.; Shirafuji, N.; Asand, S. Induction of neutrophilic granulocytosis in mice by administration of purified human native granulocyte colony-stimulating factor (G-CSF). Biochem. Biophys. Res. Commun. 142:454–460; 1987.
40. Till, J. E.; McCulloch, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14:213–222; 1961.
41. Turrisi, A. T.; Kligerman, M. M.; Glover, D. J.; Glick, J. H.; Norfleet, L.; Gramkowski, M. Experience with Phase I trials of WR-2721 preceeding radiation therapy. In: Nygaard, O. F., Simic, M. G., eds. Radioprotectors and anticarcinogens. New York, NY: Academic Press; 1983:681–694.
42. Ward, J. F. Chemical aspects of DNA radioprotection. In: Nygaard, O. F., Simic, M. G., eds. Radioprotection and anticarcinogens. New York, NY: Academic Press; 1983:73–85.
43. Wasserman, J. H.; Phillips, T. L.; Ross, G.; Kane, L. J. Differential protection against cytotoxic chemotherapeutic effects on bone marrow CFU's by WR-2721. Cancer Clin. Trials. 4:3–6; 1981.
44. Welte, K.; Bonilla, M. A.; Gillio, A. P.; Boone, T. C.; Potter, G. K.; Gabrilove, J. L.; Moore, M. A. S.; O'Reilly, R. J.; Souza, L. M. Recombinant human granulocyte colony-stimulating factor: Effects on hematopoiesis in normal and cyclophosphamide-treated primates. J. Exp. Med. 165: 941–948; 1987.
45. Yuhas, J. M. Biological factors affecting the radioprotective efficiency of s-2[3-aminopropylamino]ethyl phosphorothioic acid (WR-2721): LD50/30 doses. Radiat. Res. 44:621–628; 1970.
46. Yuhas, J. M. Active versus passive absorption kinetics as the basis for selective protection of normal tissues by s-2-[3-aminopropylamino)ethyl phosphorothioic acid. Cancer Res. 40: 1519–1524; 1980.
47. Yuhas, J. M.; Spellman, J. M.; Culo, F. The role of WR-2721 in radiotherapy and/or chemotherapy. In: Brady, L. W., ed. Radiation sensitizers: their use in the clinical management of cancer. New York, NY: Masson Publishing; 1980: 303–308.
48. Zsebo, K. M.; Cohen, A. M.; Murdock, D. C.; Boone, T. C.; Inoue, H.; Chazin, V. R.; Hines, D.; Souza, L. M. Recombinant human granulocyte colony-stimulating factor: molecular and biological characterization. Immunobiol. 172:175–184; 1986.

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Electrophysiological consequences of exposure of hippocampal slices to dihydroxyfumarate, a generator of superoxide radicals

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In an effort to understand the damaging actions of free radicals to neuronal electrophysiology, the superoxide generator, dihydroxyfumarate (DHF), was evaluated in slices of guinea pig hippocampus. Using field potential recording techniques, population spikes and population synaptic potentials were recorded in field CA1. Slices were exposed to 3 mM DHF either alone or in the presence of a protectant. DHF did not alter the ability of the afferent volley to generate a synaptic potential, but it did impair the ability of the synaptic potential to elicit a population spike. In addition, DHF induced lipid peroxidation as measured by the thiobarbituric acid assay. Superoxide dismutase (SOD) provided no protection. Instead, SOD treatment promoted DHF damage to synaptic potentials. Catalase alone mitigated the actions of DHF, but only in SOD plus catalase was the DHF-induced electrophysiological deficit and lipid peroxidation completely antagonized. The iron chelator, Desferal, did not protect but promoted synaptic damage. Desferal may be ineffective because of the nitroxide radical formed upon its reaction with DHF. The hydroxyl radical scavenger, dimethylsulfoxide, prevented lipid peroxidation and reduced the DHF-induced deficit but did not completely prevent the impairment of spike generation. These data suggest that DHF exerts its actions through generation of hydrogen peroxide which would further react with tissue iron to produce hydroxyl radicals.

INTRODUCTION

Evidence is increasing for the role of free radicals in the etiology of a number of pathological conditions in the brain. The strongest evidence for free radical involvement is with ischemia/reperfusion injury. Spin trap experiments have demonstrated the presence of a free radical adduct following an ischemic insult[4,18,39]. Intrinsic antioxidants such as ascorbic acid[10,13] and glutathione[6,12] are expended. In addition, exogenously applied free radical scavengers[10,11,24] or the antioxidant enzyme superoxide dismutase[19,20] can provide protection. The source of the free radicals is uncertain, but could arise from activation of microglia[5], conversion of endothelial cell xanthine dehydrogenase to xanthine oxidase[34] and/or a respiratory burst with reperfusion resulting in incomplete reduction of oxygen[21].

In an effort to understand the pathologies that could result from exposure to free radicals, our laboratory has been investigating the electrophysiological consequences of exogenously applied free radical generators in hippocampal brain slices. Hydroxyl radicals can be generated through the Fenton reaction by peroxide interaction with iron or other transition metals within the tissue[17]. Treatment of hippocampal brain slices with peroxide results in a decrease in synaptic efficacy, an impaired ability to generate spikes orthodromically[28] and an increase in lipid peroxidation[31]. Both inhibitory and excitatory synaptic potentials are reduced by peroxide exposure[29]. Ionizing radiation causes tissue damage predominantly through generation of free radicals. A variety of free radical species are likely to be generated. Tolliver and Pellmar[38] found that gamma radiation, like peroxide, impaired spike generation and reduced synaptic efficacy. In contrast, X-radiation caused an increase in synaptic efficacy rather than a decrease, as well as an impairment of spike generation[32].

The present study compares the actions of superoxide radicals with the Fenton-generated hydroxyl free radicals and the radiation-generated mixture. Dihydroxyfumarate (DHF), which autoxidizes to generate superoxide[1,16,26], was used as the free radical source. As with all other free radical sources tested, spike generation was impaired by DHF. In contrast, synaptic efficacy was unaffected. The presence of superoxide dismutase (SOD) promoted synaptic damage and provided no protection of spike generation. The actions of DHF were fully blocked only by a combination of SOD and catalase. The data suggest that peroxide production is critical for DHF effects.

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MATERIALS AND METHODS

Slices of hippocampus were prepared from male guinea pigs as previously described[28]. Tissue was incubated in artificial cerebrospinal fluid (ACSF) for at least 1 h at room temperature before use in any experiments. The composition of the ACSF was (in mM): NaCl, 124; KCl, 3.0; $CaCl_2$, 2.4; $MgSO_4$, 1.3; KH_2PO_4, 1.24; glucose, 10; and $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2. ACSF was made daily; all drugs were added immediately before use. Protectants were used at concentrations previously reported to be effective against 3 mM DHF damage in cardiocytes[3] or against 1.8 mM peroxide damage in hippocampal slices[31]. DHF, deferoxamine mesylate (Desferal, desferrioxamine), catalase, SOD, dimethylsulfoxide (DMSO) and most other reagents were obtained from Sigma Chemical Company, St. Louis, MO. Malonaldehyde bis(dimethyl acetal) (MDA) was purchased from Aldrich Chemical Company, Milwaukee, WI.

Electrophysiology

All electrophysiology experiments were performed in a submerged slice chamber (Zbicz design[41]). ACSF was continuously perfused at 30 ± 1°C at a rate of 1–2 ml/min. A bipolar stimulating electrode was positioned in the stratum radiatum of the hippocampus to stimulate afferents to field CA1. A glass recording electrode containing 2 M NaCl was positioned in the stratum (s.) pyramidale to record the somatic response of the neurons to stimulation, the population spike. The population spike is the summation of the extracellularly recorded action potentials of cells in the proximity of the recording electrode. A second glass recording electrode was positioned in the s. radiatum to monitor the afferent volley, a measure of the presynaptic fibers firing, and the synaptic potential (population postsynaptic potential, population PSP). The population PSP was quantitated by the maximal negative slope of the potential, since the amplitude was frequently obscured by the reflection of the population spike. To calculate the maximal slope, PSP slope was calculated every 70 μs beginning after the peak of the afferent volley. The population spike amplitude was calculated from the difference between the maximal negativity and the average of the early and late positive peaks. The potentials were recorded with high gain AC amplifiers and digitized, stored and analyzed on a PDP 11-23 computer.

The tissue was always initially monitored in AC F to ensure that the recordings were stable before any treatment was initiated. The actions of DHF or fumarate were tested by a 30-min application of the agent, followed by a 30-min wash period. To test protective agents, the tissue was exposed to the protectant alone for 30 min, followed by a 30-min exposure to the combination of DHF and protectant. The actions of DHF in the presence of protectant were always compared to the protectant alone. This protocol also allowed us to evaluate the direct effects of each protectant. SOD, catalase and desferal had no effect on hippocampal electrophysiology. In the present study, in contrast to a previous report from our laboratory[31], 50 mM DMSO caused a slight, stable increase in the recorded potentials. The reason for the discrepancy is uncertain but should not influence the present results.

Throughout the experiment, the afferent pathway was stimulated with constant current (0.1–1 mA; 300 μs, 0.2 Hz) at a stimulus intensity that evoked a population spike approximately 30% of maximal. At the end of each treatment, an input/output curve was obtained by varying the stimulus intensity between 0 and 1 mA. In early experiments this was done manually and 4 responses were averaged at each stimulus intensity to obtain a representative curve. In later experiments, the curves were computer generated and only single responses were obtained. Two curves were generated consecutively to ensure that the responses were representative.

Input/output (I/O) curves were analyzed as previously described[32,38]. Briefly, responses from each experiment were normalized to a standard maximal amplitude (population spike, 5 mV; population PSP, 0.5 mV/ms; volley, 2 mV). Data from all experiments for each treatment were averaged and standard errors calculated. Best fit sigmoid curves[38] were determined by a computer graphics program (RS/1, BBN Software Products Corporation). To determine if two curves were significantly different from one another, the residual sum of squares for the curves fit to the data points of each condition were compared with the residual sum of squares for the curve defined by all the data points under both conditions. Significance was accepted at $P < 0.05$. This statistic would indicate if treatment with DHF (with or without protectants) caused a change in the electrophysiological response. The percent change in the curves was quantified by the average of the change in the area under the best fit curve for each experiment (modified from Balestrino et al.[2]). The various treatments were compared using these values and significance was tested by analysis of variance (ANOVA one way; significance accepted at $P < 0.10$).

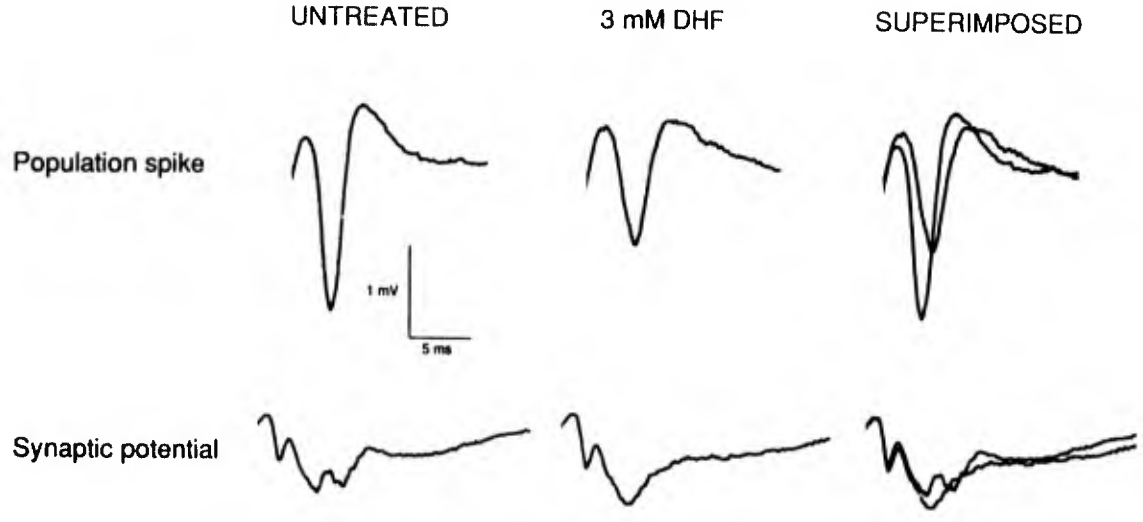

Fig. 1. Electrophysiological responses to stimulation of afferents in s. radiatum recorded simultaneously in the s. pyramidale (population spike) and s. radiatum (synaptic potential) of field CA1 of hippocampal slices of the guinea pig. Each trace is the average of 4 responses. DHF (3 mM) decreased the amplitude of the population spike without affecting the afferent volley or the initial slope of the synaptic potential. Traces from the untreated and 3 mM DHF treated conditions are superimposed on the right. The amplitude of the synaptic potential is increased by 3 mM DHF because of the decreased reflection of the population spike in s. radiatum.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test using the methods modified from Kovachich and Mishra[22] as described previously[31]. Hippocampal slices from each animal were divided into 3 treatment groups: untreated control, protectant alone, and protectant plus DHF. Three slices were used in each group to provide sufficient tissue for the assay. Each treatment was repeated in 8 animals. Untreated control tissue and tissue treated with protectant alone were incubated in appropriate solutions at 30 ± 1°C for 1 h. Tissue treated with protectant plus DHF was exposed to only the protectant for the first 30 min followed by exposure to DHF in the continued presence of protectant for the final 30 min. After treatment, the tissue was blotted dry and weighed to obtain a wet weight. Tissue was homogenized in 1 ml of 20% trichloroacetic acid with 0.5 mM EDTA. Following addition of 2 ml 67% thiobarbituric acid in 20 mM NaOH, the solution was boiled for 10 min and then centrifuged for 10 min at 2400 rpm. Absorbance was measured at 530 nm.

RESULTS

Actions of DHF

Previous studies in cardiac myocytes[3,23] used DHF at a concentration of 3 mM to evoke free radical effects. The same concentration of DHF was tested for its actions on hippocampal excitability (Figs. 1 and 2A). DHF was found to decrease the amplitude of the population spike without altering the afferent volley or the initial slope of the synaptic response (Fig. 1). Because the reflection of the population spike in the stratum radiatum is reduced, the waveform recorded through this electrode is altered and the amplitude of the population PSP is actually larger. This observation points out the necessity of measuring the initial slope rather than the amplitude of the PSP. Fig. 2A illustrates the average I/O curves obtained before and during exposure to 3 mM DHF in 7

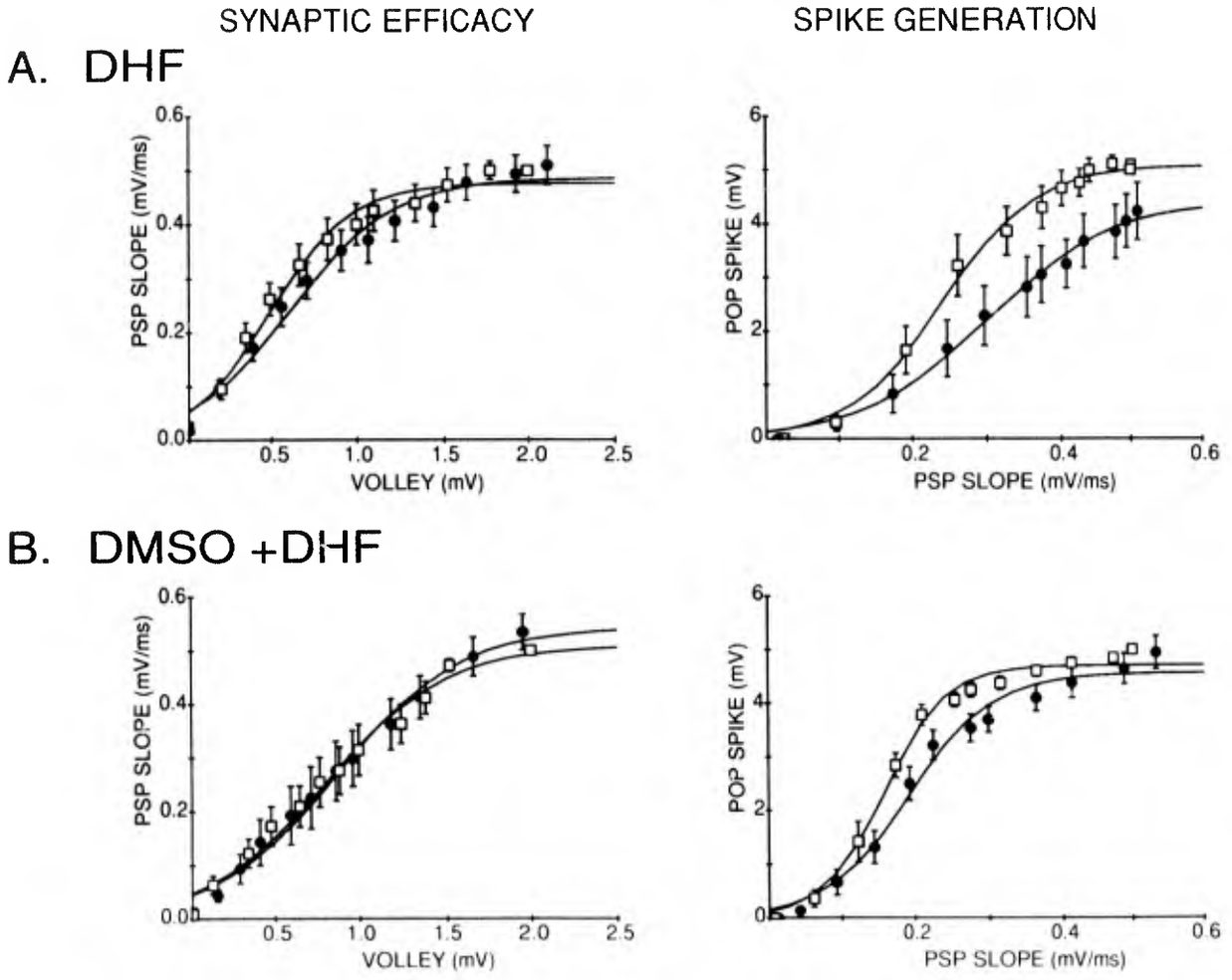

Fig. 2. Plot of afferent volley vs PSP slope reflects the ability of the presynaptic fibers to evoke a synaptic potential (synaptic efficacy). Plot of PSP slope vs population spike reflects the ability of the synaptic potential to generate spikes in the postsynaptic neurons (spike generation). A: effects of 3 mM dihydroxyfumarate. DHF had no significant effect on synaptic efficacy but caused a significant decrease in spike generation. Open squares show data from untreated controls. Filled circles represent data obtained in the presence of 3 mM DHF. Bars represent standard errors. Standard errors on symbols without bars are smaller than the symbol size. B: effect of 50 mM DMSO on actions of 3 mM DHF. Control curves (open squares) show data obtained in the presence of DMSO alone. Filled circles show data in DMSO plus 3 mM DHF. There is no significant change in synaptic efficacy with addition of DHF. The change in spike generation with addition of DHF in the presence of DMSO is still significant but is less than the change in the absence of DMSO shown in A above.

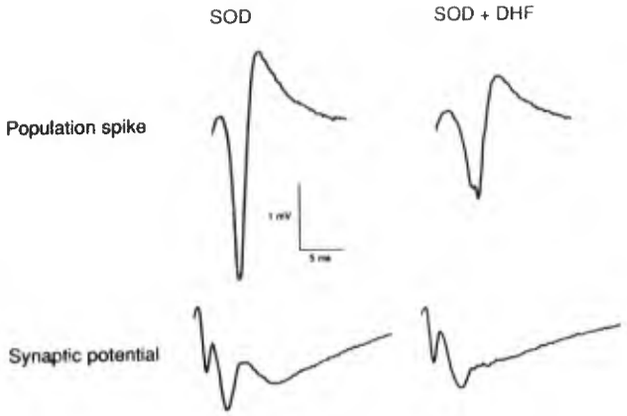

Fig. 3. Electrophysiological traces recorded from a slice treated with SOD. Each trace is the average of 4 consecutive responses. Population spikes and synaptic potentials were recorded simultaneously from s. pyramidale and s. radiatum respectively. Addition of 3 mM DHF in the presence of SOD caused a decrease in the maximal negative slope of the synaptic potential and a decrease in the amplitude of the population spike.

TABLE I

Malonaldehyde levels in hippocampus following DHF treatment

Each value (nmol MDA/mg w.wt.) is the mean ± S.E.M. of 8 experiments. Slices from each animal were divided into 3 groups. (1) untreated, incubated for 1 h in ACSF; (2) drug alone, treated with the protectant alone for 1 h; (3) drug + DHF, treated with the protectant alone for 30 min followed by treatment with protectant plus 3 mM DHF for 30 min.

Protectant	*Untreated*	*Drug alone*	*Drug + DHF*
None	122.4 ± 9.0	–	168.5 ± 8.9*
Desferal	125.8 ± 20.3	121.9 ± 13.8	125.1 ± 17.0
DMSO	131.6 ± 11.7	141.9 ± 10.5	130.4 ± 16.7
SOD/catalase	142.9 ± 12.0	152.9 ± 9.9	156.2 ± 20.8

* Significantly different from untreated control by two-tailed paired *t*-test ($P < 0.05$).

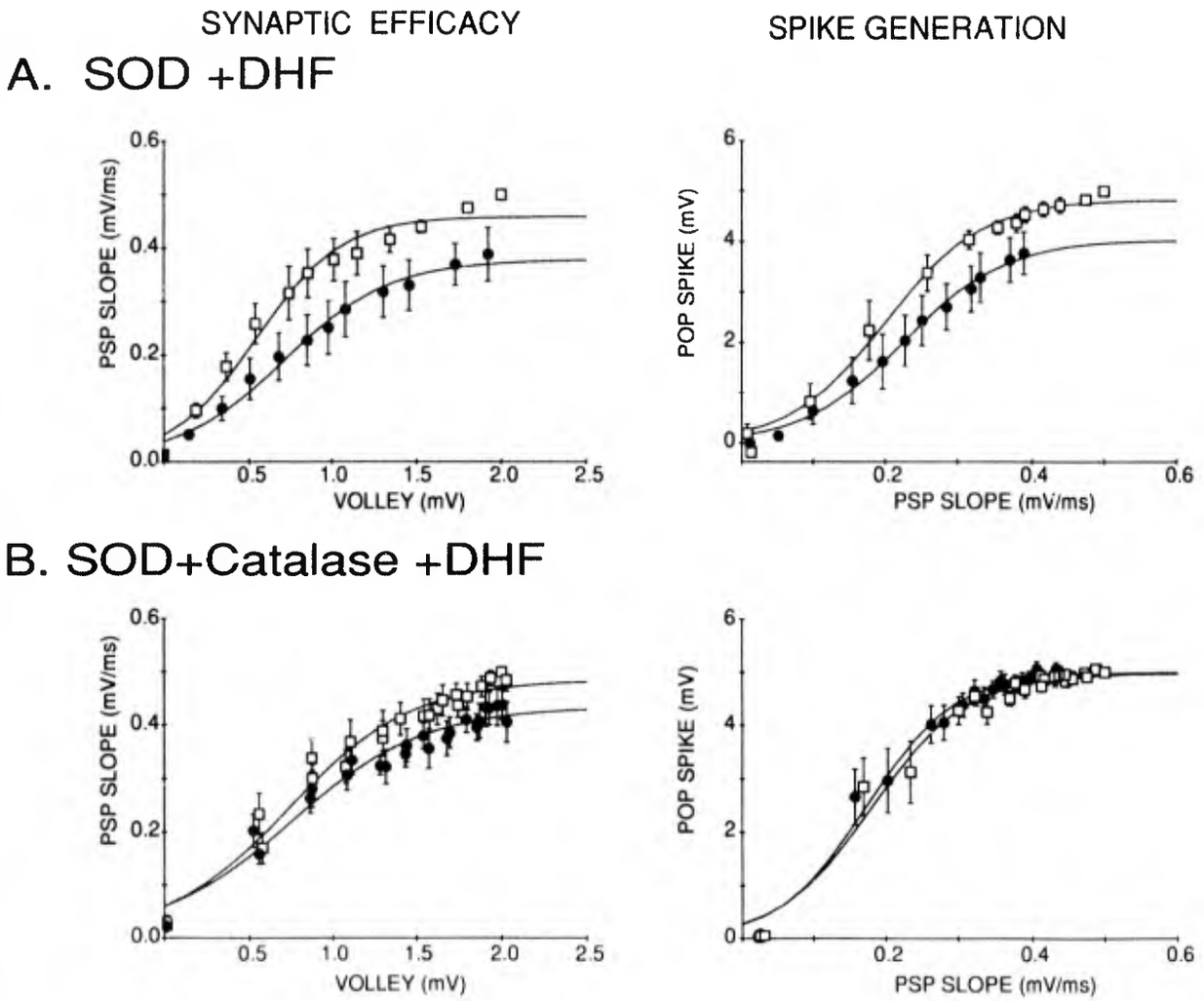

Fig. 4. Plots reflecting synaptic efficacy (volley vs PSP slope) on left and spike generation (PSP slope vs population spike) on right. A: in the presence of SOD, 3 mM DHF caused a significant decrease in both synaptic efficacy and spike generation. Open squares represent data obtained in slices exposed to SOD; filled circles represent data obtained in the same slices following addition of 3 mM DHF. B: when slices were treated with both SOD and catalase, addition of 3 mM DHF did not produce any significant damage. Control curves (open squares) were obtained from slices with both SOD and catalase present. DHF curves (filled circles) were obtained in the same slices with DHF, SOD and catalase present.

slices. DHF significantly decreased (62.3 ± 8.6% of untreated control, $P < 0.05$) the ability of the synaptic potential to evoke a population spike (impaired spike generation) (see also Fig. 6). In contrast, DHF produced no significant change (94.7 ± 8.4% of untreated control, $P > 0.05$) in the ability of the afferent volley to elicit a synaptic response (synaptic efficacy) (see Fig. 7). The actions of DHF were readily reversible with washout of the free radical generating compound, recovering to 90 ± 7% of untreated control within 30 min. Control experiments were performed using the structurally similar, but non-radical producing, fumaric acid. At a concentration of 3 mM, fumaric acid was without any significant electrophysiological effects ($n = 7$).

A lower concentration of DHF (1 mM) caused smaller changes in the electrophysiological responses ($n = 5$). Spike generation was decreased to 88.3 ± 9.5% of control ($P < 0.05$) while synaptic efficacy was not impaired. With 6 mM DHF ($n = 4$), both spike generation (39.4 ± 3.6%) and synaptic efficacy (61.3 ± 6.2%) were decreased. These effects resembled those previously reported for peroxide[28] and for gamma radiation[38]. However, 6 mM fumaric acid also decreased synaptic efficacy, although there was no concurrent effect on spike generation ($n = 3$). Consequently, a free radical mechanism for the reduction in synaptic transmission by 6 mM DHF was questionable. All subsequent experiments limited the DHF concentration to 3 mM.

Antioxidant enzymes

DHF is known to produce superoxide free radicals[1,16,26]. If this is the effective oxygen radical, one would predict that SOD should protect against the damage produced by DHF. Pretreatment of hippocampal tissue with 121 U/ml SOD caused no direct change in the electrophysiological responses. Subsequent treatment with 3 mM DHF in combination with SOD produced more severe electrophysiological damage than with DHF alone ($n = 6$). In the presence of SOD, the synaptic response, as well as the population spike, was reduced (Fig. 3). Synaptic efficacy was decreased to 70.7 ± 8.0% of SOD control while spike generation was still impaired (72.1 ± 6.0% of SOD control) (Fig. 4A).

Superoxide can spontaneously dismutate in an aqueous environment to form hydrogen peroxide. Peroxide can interact with tissue iron and create the very damaging hydroxyl radical. The enzyme catalase catalyses the breakdown of peroxide to water and oxygen. If peroxide is contributing to DHF actions, catalase would be expected to provide protection. While catalase (260 U/ml) had no direct effect on hippocampal excitability, it did mitigate the actions of DHF ($n = 10$). With catalase present, DHF only reduced spike generation to 93.7 ± 5.8% of catalase control (Fig. 6), although this was still

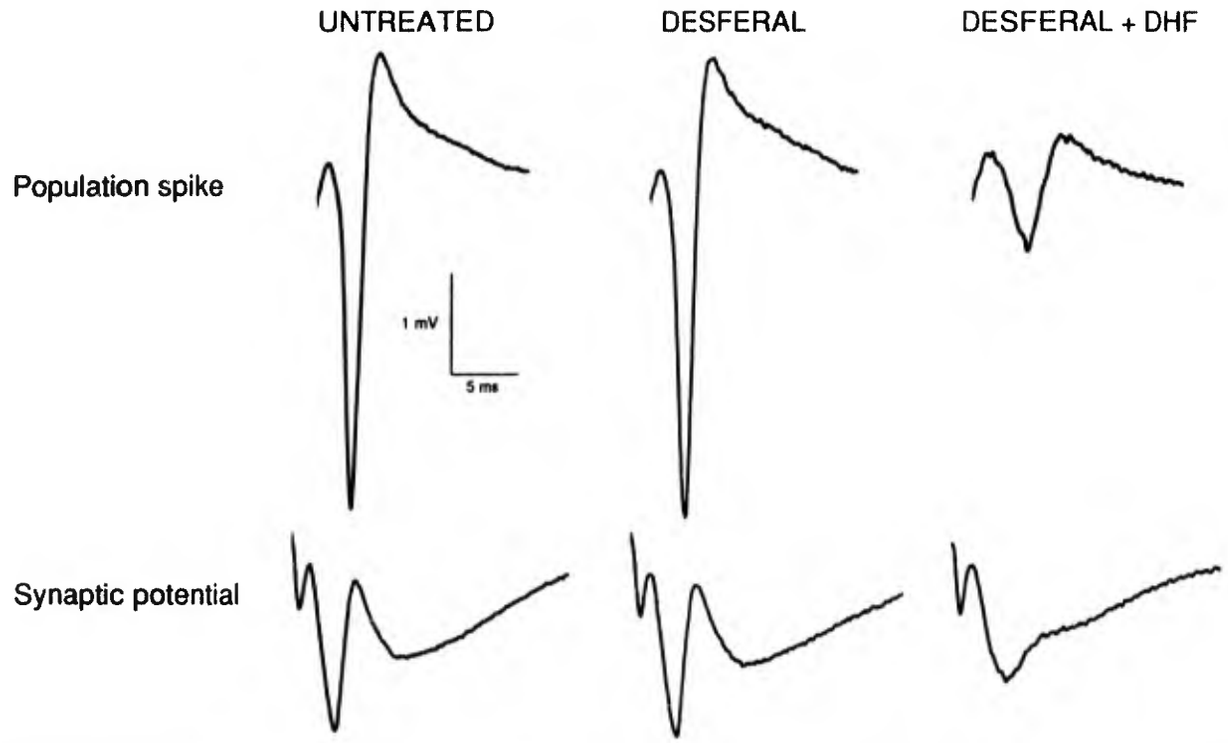

Fig. 5. Electrophysiological traces recorded from a slice treated with Desferal. Population spike and synaptic potentials were recorded simultaneously. Traces are the average of 4 consecutive responses. Desferal (1 mM) did not produce any change in the responses compared to the untreated controls. Following addition of 3 mM DHF in the continued presence of Desferal, the population spike was greatly reduced. In addition, the maximal negative slope of the synaptic potential was reduced.

a statistically significant deficit. Unlike SOD, catalase did not promote the reduction of synaptic efficacy (93.1 ± 3.7% of catalase control) (Fig. 7).

Application of both SOD and catalase together should eliminate both peroxide and syperoxide radicals. In the absence of DHF, they did not alter hippocampal electrophysiology. SOD plus catalase (n = 6) appeared to produce nearly complete protection from DHF damage (Fig. 4B). The changes in synaptic efficacy (90.1 ± 3.9% of SOD + catalase control) (Fig. 7) and spike generation (101.2 ± 4.6% of SOD + catalase control) (Fig. 6) were not significant. DHF damage to spike generation in tissue treated with SOD plus catalase was significantly less than that in untreated tissue (ANOVA $P < 0.10$) (Fig. 6). These results would suggest that much of the damage from DHF results from the formation of peroxide.

Iron chelation

Hydroxyl radicals have been previously shown to mediate the actions of peroxide in producing electrophysiological damage in the hippocampal slice preparation[31]. Pretreatment of the tissue with 1 mM Desferal provided protection from the neurophysiological deficits elicited by peroxide, indicating that the Fenton reaction was necessary for peroxide damage[31]. To evaluate the contribution of iron to the deficit seen with DHF, 1 mM Desferal was added to the perfusate. In the presence of Desferal, the damage evoked by DHF was not blocked (Fig. 5). On the contrary, the synaptic potential and the population spike were both significantly reduced (n = 6). As with SOD treatment, Desferal promoted a DHF-induced decrease in synaptic efficacy (63.8 ± 12.7% of Desferal control) (Fig. 7) and provided no protection for spike generation (54.5 ± 16.6% of Desferal control) (Fig. 6).

Free radical scavenger

Since the hydroxyl radical is a likely candidate for the mediator of DHF action, a scavenger of this radical may be expected to afford some protection from the electrophysiological deficits. DMSO (50 mM) has been previously found to protect against peroxide damage in the hippocampal slice[31]. The same concentration of DMSO

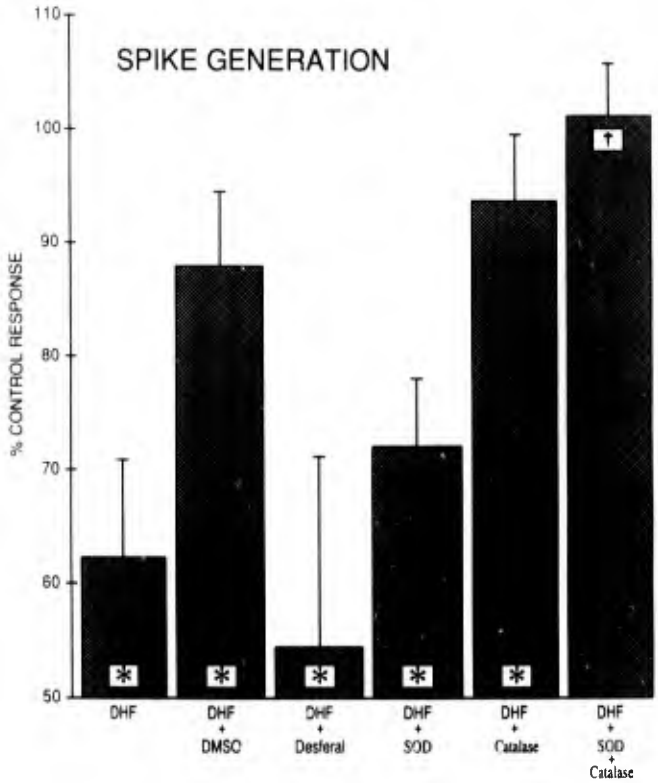

Fig. 6. Effect of treatments on the impairment of spike generation produced by 3 mM DHF. Values were calculated from changes in the I/O curves reflecting spike generation (population PSP vs population spike). Damage was calculated as a percent change in the area under the curves obtained in DHF plus protectant compared to protectant alone. * represents a statistically significant difference ($P < 0.05$) between input/output relationship in DHF-treated tissue compared to DHF-untreated tissue under each condition. † represents a statistically significant difference ($P < 0.10$, ANOVA, one way) between the DHF-induced damage in protectant-treated tissue compared to DHF-induced damage in protectant-untreated tissue. DHF produced a statistically significant (*) decrease in the ability of slices to generate spikes as seen in the I/O curves. Neither Desferal nor SOD mitigated the actions of DHF; spike generation was significantly impaired (*). While DMSO and catalase each reduced the deficit produced by DHF, a significant impairment was still observed (*). Only in the presence of SOD and catalase together was the action of DHF completely prevented. No significant impairment of spike generation resulted in the presence of SOD + catalase. Treatment with SOD + catalase provided significant protection from the DHF reduction of spike generation (†).

limited the DHF impairment of spike generation (Fig. 2B), although a significant decrease was still evident (87.9 ± 6.5% of DMSO control; $n = 8$) (Fig. 6). DMSO did not significantly affect the interaction of DHF with synaptic efficacy (102.5 ± 6.0% of DMSO control) (Fig. 7).

Lipid peroxidation

Malonaldehyde (MDA) was measured by the thiobarbituric acid test. In untreated hippocampal tissue, levels of MDA were 122.4 ± 9.0 nmol MDA/mg w.wt., in agreement with levels previously reported for similarly handled tissue[31]. Treatment with 3 mM DHF for 30 min produced a significant increase in this level to 168.5 ± 8.9 nmol MDA/mg wet weight. The actions of a 1-h exposure to Desferal, DMSO and SOD plus catalase were evaluated both alone and in the presence of 3 mM DHF for the final 30 min. None of the 3 treatments had a significant effect of their own compared to paired untreated controls. All of the 3 treatments did prevent the DHF-induced increase in MDA (Table I).

DISCUSSION

Free radical system

DHF provides an effective source of superoxide radicals which can be further converted to other active oxygen species. DHF slowly autoxidizes to generate superoxide and dihydroxyfumarate radicals[1,16,27]. Electron spin resonance spectral analysis has demonstrated the appearance of the oxygen radical in the absence of any catalysts[27]. Superoxide can spontaneously dismutate to hydrogen peroxide although superoxide dismutase accelerates this reaction[14,15]. Peroxide itself is considered to be minimally damaging to biological tissue[17,31] but it can react with tissue iron through the Fenton reaction to generate hydroxyl radicals which are extremely reactive oxidants. Catalase catalyses the conversion of peroxide to water and thereby prevents the formation of hydroxyl radicals. Oxygen-free radicals are known to promote damage to biological substrates. Free radicals can oxidize cellular proteins[7,8], induce peroxidation of membrane lipids[17,36] as well as damage the DNA molecule[25,40].

Determination of active radical species

In the present study we found that DHF produced specific electrophysiological damage to the spike generating system in slices of hippocampus from the guinea pig. Synaptic efficacy was not significantly affected. Pretreatment of the hippocampal slice with SOD provided no protection from DHF damage. Rather, an additional

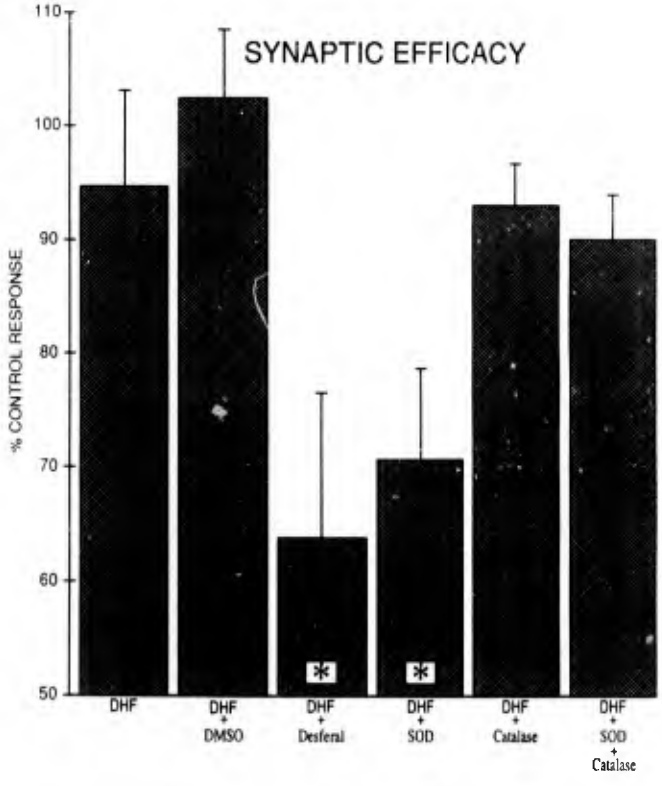

Fig. 7. Effect of treatments on synaptic efficacy in the presence of 3 mM DHF. Values were calculated from changes in the I/O curves reflecting synaptic efficacy (afferent volley vs population PSP). Damage was calculated as a percent change in the area under the curves obtained in DHF plus protectant compared to protectant alone. * represents a statistically significant difference ($P < 0.05$) between input/output relationship in DHF-treated tissue compared to DHF-untreated tissue under each condition. DHF itself caused no significant effect on synaptic efficacy. In addition, there was no change in synaptic efficacy with DHF in the presence of DMSO, Catalase or SOD + catalase. In the presence of Desferal or SOD, DHF produced a significant decrease in synaptic potentials (*).

effect of DHF to decrease the synaptic potentials was induced in the presence of the enzyme. In contrast, catalase was an effective protectant, although its protection was not complete. The combination of SOD and catalase provided nearly complete protection; no significant damage from DHF occurred in their presence. These results suggest that peroxide, not superoxide, is the oxygen species critical to electrophysiological effects of DHF in the hippocampal slice preparation. The aggravating effects of SOD can be explained by the increased enzymatic generation of peroxide. Since the combination of SOD and catalase was more effective than the catalase alone, it is possible that superoxide radicals are contributing to the electrophysiological effects as well. These results contrast somehwat with those of Barrington et al.[3]. Working with isolated cardiac myocytes, they observed that *either* SOD or catalase alone could delay the DHF-induced changes to cardiac action potentials and prevent the loss of cell excitability. A role for superoxide in addition to other radicals was hypothesized.

Previous studies in the hippocampus used hydrogen peroxide as a free radical generating system. Peroxide impaired the generation of spikes and reduced synaptic potentials[28,29]. The hydroxyl radical, and not peroxide itself was suggested as the active oxygen species[31]. Iron was essential for the electrophysiological damage; the iron chelator, Desferal, was effective in protecting against peroxide actions[31]. In addition, the hydroxyl radical scavenger DMSO was found to be an effective protectant in this system[31]. The actions of DHF were similarly mitigated by DMSO in the present study. Desferal, on the other hand, was not at all protective of the electrophysiological damage. In fact, although the iron chelator had no direct electrophysiological effects, it significantly enhanced the DHF damage. As with SOD, a decrease in the synaptic response appeared with Desferal pretreatment.

The results with Desferal do not rule out the possible contribution of hydroxyl radicals. With electron spin resonance techniques, Desferal has been shown to react with superoxide to generate a nitroxide free radical[9,26]. The nitroxide radical has a half life of approximately 10 min at room temperature but is sufficiently reactive to impair enzyme activity[9]. In the presence of DHF, Desferal could be reacting with the superoxide to generate a nitroxide radical. If this new radical is damaging to the hippocampal electrophysiology, instead of preventing damage through iron chelation, Desferal could accentuate it. Since Desferal was able to protect against DHF-induced lipid peroxidation, the nitroxide radical may be producing electrophysiological deficits in spike generation through an alternate mechanism.

Cellular mechanisms of action

Although the actions of SOD and catalase strongly suggest that peroxide is the active oxygen species involved with DHF exposure in the hippocampal slice preparation, the electrophysiological effects were not identical to those produced by exogenously applied peroxide. Peroxide decreases both spike generation and synaptic efficacy[28]. No concentration of peroxide has been observed to produce only one of the two actions (unpublished observations). DHF did not produce a significant decrease in synaptic efficacy except at higher concentrations where fumarate toxicity is a complicating factor. In the presence of SOD, however, DHF was able to promote synaptic damage. DHF is not likely to penetrate the cell membrane[1]. Consequently, the superoxide must be generated in the bathing solution. The permeability of superoxide is also limited. The negatively charged radical is thought to have access to cells through anion channels. Yet in neurons, anion conductance is very low[35,37] and superoxide may not traverse the neuronal cell membrane. This implies that any superoxide contribution would be at the cell membrane. Extracellular generation of peroxide from superoxide is supported by the observation that exogenously applied catalase with SOD is effective in preventing the DHF-induced damage. Peroxide, on the other hand, is freely permeable through the plasma membrane. Yet the actions observed in the present study are limited to spike generating mechanisms. Perhaps the mode of generation of peroxide limits its accessibility to all cellular domains. We previously hypothesized that lipid peroxidation was the underlying mechanism responsible for deficits in spike generation[30,33,38]. This suggests an action at the cell membrane, requiring little diffusion of the free radical species from the extracellular space. In contrast, a peroxide-induced decrease in synaptic efficacy has been previously hypothesized to result from oxidation of cellular proteins[30,38] and this would likely require access to intracellular sites. This mechanism would suggest that only when the rate of formation of peroxide from superoxide is enhanced by exogenously added SOD would the distribution of peroxide change to allow synaptic damage.

Conclusions

The data suggest that DHF generates a free radical species that produces electrophysiological damage in the hippocampal slice preparation. Based on the protective effects of catalase but not SOD, peroxide rather than superoxide, is critical to the mechanism of damage. Because peroxide itself produces little effect[31], hydroxyl radicals probably mediate most of the free radical damage in this preparation.

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REFERENCES

1 Autor, A.P., McLennan, G. and Fox, A.W., Oxygen free radicals generated by dihydroxyfumarate and ionizing radiation. Cytotoxic effect on isolated pulmonary macrophages. In R.S. Bhatnager (Ed.), *Molecular Basis of Environmental Toxicity*, Ann Arbor Service Publishing Inc., Ann Arbor, MI, 1980, pp. 51–67.

2 Balestrino, M., Aitken, P.G. and Somjen, G.G., The effects of moderate changes of extracellular K^+ and Ca^{2+} on synaptic and neural function in the CA1 region of the hippocampal slice, *Brain Research*, 377 (1986) 229–239.

3 Barrington, P.L., Meier Jr., C.F. and Weglicki, W.B., Abnormal electrical activity induced by free radical generating systems in isolated cardiocytes, *J. Mol. Cell. Cardiol.*, 20 (1988) 1163–1178.

4 Cao, W., Carney, J.M., Duchon, A., Floyd, R.A. and Chevion, M., Oxygen free radical involvement in ischemia and reperfusion injury to brain, *Neurosci. Lett.*, 88 (1988) 233–238.

5 Colton, C.A. and Gilbert, D.L., Production of superoxide anions by a CNS macrophage, the microglia, *FEBS Lett.*, 223 (1987) 284–288.

6 Cooper, A.J.L., Pulsinelli, W.A. and Duffy, T.E., Glutathione and ascorbate during ischemia and post-ischemic reperfusion in rat brain, *J. Neurochem.*, 35 (1980) 1242–1245.

7 Davies, K.J.A., Protein damage and degradation by oxygen radicals. I. General aspects, *J. Biol. Chem.*, 262 (1987) 9895–9901.

8 Davies, K.J.A. and Goldberg, A.L., Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes, *J. Biol. Chem.*, 262 (1987) 8220–8226.

9 Davies, M.J., Donkor, R., Dunster, C.A., Gee, C.A., Jonas, S. and Willson, R.L., Desferrioxamine (Desferal) and superoxide free radicals, *Biochem. J.*, 246 (1987) 725–729.

10 Demopoulos, H.B., Flamm, E.S., Pietronigro, D.D. and Seligman, M.L., Free radical pathology and the microcirculation in the major central nervous system disorders, *Acta Physiol. Scand.*, Suppl., 492 (1980) 91–119.

11 Demopoulos, H.B., Flamm, E.S., Seligman, M.L. and Pietronigro, D.D., Oxygen free radicals in central nervous system ischemia and trauma. In A.P. Autor (Ed.), *Pathology of Oxygen*, Academic, New York, 1982, pp. 127–155.

12 Domanska-Janik, K. and Wideman, J., Regulation of thiols in the brain. 2. Effect of hypoxia on the activities of cytoplasmic NADPH-producing enzymes in different parts of the rat brain, *Resuscitation*, 3 (1974) 37–41.

13 Flamm, E.S., Demopoulos, H.B., Seligman, M.L., Posner, R.G. and Ransohoff, J., Free radicals in cerebral ischemia, *Stroke*, 9 (1978) 445–447.

14 Fridovich, I., The biology of the oxygen radicals. The superoxide radical is an agent of oxygen toxicity; superoxide dismutases provide an important defense, *Science*, 201 (1978) 875–880.

15 Fridovich, I., Superoxide radical: an endogenous toxicant, *Annu. Rev. Pharmacol. Toxicol.*, 23 (1983) 239–257.

16 Halliwell, B., Generation of hydrogen peroxide, superoxide and hydroxyl radicals during the oxidation of dihydroxyfumaric acid by peroxidase, *Biochem., J.*, 163 (1977) 441–448.

17 Halliwell, B. and Gutteridge, J.M.C., Oxygen radicals and the nervous system, *Trends Neurosci.*, 8 (1985) 22–26.

18 Imaizumi, S., Tominaga, T., Uenohara, H., Yoshimoto, T., Suzuki, J. and Fujita, Y., Initiation and propagation of lipid peroxidation in cerebral infarction models, *Neurological Res.*, 8 (1986) 214–220.

19 Imaizumi, S., Woolworth, V., Fishman, R.A. and Chan, P.H., Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats, *Stroke*, 21 (1990) 1312–1317.

20 Kitagawa, K., Matsumoto, M., Oda, T., Ninobe, M., Hata, R., Handa, N., Fukunaga, R., Isaka, Y., Kimura, K., Maeda, H., Mikoshiba, K. and Kamada, T., Free radical generation during brief period of cerebral ischemia may trigger delayed neuronal death, *Neuroscience*, 35 (1990) 551–558.

21 Kogure, K., Arai, H., Abe, K. and Nakano, M., Free radical damage in the brain following ischemia, *Progr. Brain Res.*, 63 (1985) 237–259.

22 Kovachich, G.B. and Mishra, O.P., Lipid peroxidation in rat brain cortical slices as measured by the thiobarbituric acid test, *J. Neurochem.*, 35 (1980) 1449–1452.

23 Kramer, J.H., Mak, I.T. and Weglicki, W.B., Differential sensitivity of canine cardiac sarcolemmal and microsomal enzymes to inhibition by free radical-induced lipid peroxidation, *Circ. Res.*, 55 (1984) 120–124.

24 Martin, D., Beer, M. and Betz, A.L., Dimethylthiourea reduces ischemic brain edema without affecting cerebral blood flow, *J. Cerebral Blood Flow Metabol.*, 10 (1990) 352–357.

25 Mello Filho, A.C., Hoffman, M.E. and Meneghini, R., Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron, *Biochem. J.*, 218 (1984) 273–275.

26 Morehouse, K.M., Flitter, W.D. and Mason, R.P., The enzymatic oxidation of Desferal to a nitroxide free radical, *FEBS Lett.*, 222 (1987) 246–250.

27 Nilsson, R., Pick, F.M. and Bray, R.C., EPR studies on reduction of oxygen to superoxide by some biochemical systems, *Biochim. Biophys. Acta*, 192 (1969) 145–148.

28 Pellmar, T.C., Electrophysiological correlates of peroxide damage in guinea pig hippocampus in vitro, *Brain Research*, 364 (1986) 377–381.

29 Pellmar, T.C., Peroxide alters neuronal excitability in the CA1 region of guinea-pig hippocampus in vitro, *Neuroscience*, 23 (1987) 447–456.

30 Pellmar, T.C. and Neel, K.L., Oxidative damage in the guinea pig hippocampal slice, *Free Rad. Biol. Med.*, 6 (1989) 467–472.

31 Pellmar, T.C., Neel, K.L. and Lee, K.H., Free radicals mediate peroxidative damage in guinea pig hippocampus in vitro, *J. Neurosci. Res.*, 24 (1989) 437–444.

32 Pellmar, T.C., Schauer, D.A. and Zeman, G.H., Time- and dose-dependent changes in neuronal activity produced by X radiation in brain slices, *Radiation Res.*, 122 (1990) 209–214.

33 Pellmar, T.C., Tolliver, J.M. and Neel, K.L., Radiation-induced impairment of neuronal excitability, *Comments Toxicol.*, 2 (1988) 253–263.

34 Rosen, G.M. and Freeman, B.A., Detection of superoxide generated by endothelial cells, *Proc. Natl. Acad. Sci. U.S.A.*, 81 (1984) 7269–7273.

35 Scholfield, C.N., Electrical properties of neurones in the olfactory cortex slice in vitro, *J. Physiol.*, 275 (1978) 535–546.

36 Tappel, A.L., Lipid peroxidation damage to cell components, *Fed. Proc.*, 32 (1973) 1870–1874.

37 Thompson, S.M., Deisz, R.A. and Prince, D.A., Relative contributions of passive equilibrium and active transport to the distribution of chloride in mammalian cortical neurons, *J. Neurophysiol.*, 60 (1988) 105–124.

38 Tolliver, J.M. and Pellmar, T.C., Ionizing radiation alters neuronal excitability in hippocampal slices of the guinea pig, *Radiation Res.*, 112 (1987) 555–563.

39 Tominaga, T., Imaizumi, S., Yoshimoto, T., Suzuki, J. and Fujita, Y., Application of spin-trapping study to rat ischemic brain homogenate incubated with NADPH and Fe-EDTA, *Brain Research*, 402 (1987) 370–372.
40 Ward, J., Blakely, W. and Joner, E., Mammalian cells are not killed by DNA strand breaks caused by hydroxyl radicals from hydrogen peroxide, *Radiation Res.*, 103 (1985) 383–392.
41 Zbicz, K.L. and Weight, F.F., Transient voltage and calcium-dependent outward currents in hippocampal CA3 pyramidal neurons, *J. Neurophysiol.*, 53 (1985) 1038–1058.

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Investigation of the existence and biological role of L-arginine/nitric oxide pathway in human platelets by spin-trapping/EPR studies

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The aim of the present study was to apply spin trapping/EPR spectroscopy to investigate the existence and biological role of the L-arginine/nitric oxide pathway in human platelet aggregation. Three different spin traps were used: two nitroso, 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) and 2-methyl-2-nitrosopropane (MNP), and a nitrone, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). The effect of spin-trap concentration on the collagen-induced human platelet aggregation was compared to the anti-aggregatory effect caused by L-arginine. The results show that the nitroso spin traps (DBNBS and MNP) are more effective than L-arginine in preventing platelet aggregation. DMPO has virtually no effect on the collagen-induced aggregation except at a high concentration (300 mM). Furthermore, activation of platelets with a low concentration of collagen (17 μg/ml) and in the presence of DBNBS or MNP yields several EPR-detectable spin adducts. Some of the observed spin adducts do not correspond to those originating from the interaction of a free radical, nitric oxide (NO˙) gas, with the spin traps [Arroyo, C. M. & Kohno, M. (1991) *Free Radical Res. Commun. 14*, 145–155]. Only one adduct of DBNBS, with a relative intensity of 0.1, observed in the washed-platelet experiment and in the presence of superoxide dismutase, is similar to the EPR spectrum obtained following a reaction of pure NO˙ gas with DBNBS. This suggests that the EPR spectrum of the DBNBS adduct consisting of a triplet may originate from the production of NO˙ by these cells. Additional DBNBS and MNP spin adducts were generated during platelet activation in the presence of Ca^{2+} and of a cytosol-depleted L-arginine preparation from washed platelets to which L-arginine was subsequently added. The formation of these DBNBS and MNP spin adducts were inhibited by N^{ω}-methyl-L-arginine (MeArg, 100 μM), suggesting that these originated from a product of NO synthase. Furthermore, the formation of DBNBS and MNP spin adducts in platelet suspensions was enhanced by the presence of superoxide dismutase; however, their formation was prevented by the endothelial-derived relaxing factor (EDRF) inhibitors methylene blue and hemoglobin. The results from the MeArg and EDRF inhibitor experiments support the existence of the L-arginine/NO pathway in platelets. In addition, the prevention of spin-adduct formation by EDRF inhibitors, suggests that the mechanisms of EDRF formation and the L-arginine/NO pathway in endothelial cells and platelets are similar. The potent platelet anti-aggregatory effect shown by DBNBS and MNP, in addition to the results obtained from experiments involving MeArg and EDRF inhibitors, raise doubts with respect to the true identity of EDRF which has previously been suggested to be identical to NO˙ originating from L-arginine. The results suggest that EDRF may be a nitroxylated compound, a hydroxyguanidinium cation radical, with strong nucleophilic properties and with pharmacological properties similar to NO˙. It is possible, following an electron reduction, that such a hydroxyguanidinium cation radical could yield NO˙.

Platelets possess a soluble guanylate cyclase that induces, when activated, an increase in cyclic GMP. Recent evidence suggests that activation of guanylate cyclase occurs in the presence of nitric oxide (NO˙), a product originating from the oxidation of the terminal guanidino group of L-arginine (Palmer et al., 1987). Studies dealing with vascular endothelium (Moncada et al., 1989) have suggested that NO˙ synthesis from L-arginine occurs through the action of a soluble enzyme, NO synthase. The mechanism, referred to as the L-arginine/NO pathway, is now known to exist in various cellular systems (Moncada et al., 1989; Palmer et al., 1988; Marletta et al., 1988; Garthwaite et al., 1988; Amber et al., 1988). Furthermore, it has been proposed (Moncada et al., 1989) that the L-arginine/NO pathway is responsible for the stimulation of soluble guanylate cyclase. Although in human

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Abbreviations. DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonate; MNP, 2-methyl-2-nitrosopropane; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; MeArg, N^{ω}-methyl-L-arginine; SOD, superoxide dismutase; EDRF, endothelial-derived relaxing factor; IC_{50}, half-maximal inhibitory concentration; Nbs, thionitrobenzoic acid.

Enzyme. Superoxide dismutase (EC 1.15.1.1).

platelets aggregation is induced by collagen and inhibited by L-arginine (Radomski et al., 1990), collagen and L-arginine induce an increase in cyclic GMP levels suggesting the existence of the L-arginine/NO pathway in these cells. Support for this pathway in platelets originates from cytosol experiments. In these experiments, an increase in cyclic GMP was observed, not only in the presence of L-arginine, but also in the presence of sodium nitroprusside, a compound known to induce the release of NO· (Feelisch and Noack, 1987).

Although the exact function of NO· generated in platelets is unclear, its production by macrophages, identified via measurement of NO_2^-/NO_3^- (Hibbs et al., 1988), has been associated with a cytotoxic/cytostatic effect. NO· production in cultured aortic endothelial cells has been related to the endothelium-derived relaxing factor (EDRF). The similarity in chemical and pharmacological actions of EDRF and NO· (vascular smooth muscle relaxation), have led Palmer et al. (1987) and Ignarro et al. (1987a, b) to suggest that the EDRF released from intact vascular components is NO·. However, neither the chemical or pharmacological assays used can discriminate between NO· and labile nitroso compounds that rapidly liberate NO·. Therefore, it is not yet clear whether EDRF is NO· or a labile nitroso precursor, or a mixture of both. For this reason, the spin-trapping technique, although having its limitations when applied to this type of study (Janzen, 1971, 1980; Thornalley, 1986; Arroyo et al., 1988), should play an important role in determining the structure of EDRF and its related mechanism.

Since NO· is a free radical, in principle, the spin trapping of NO· or NO-related intermediates could appear to be a simple and straightforward. However, it must be noted that these experiments, and specifically in the case of nitroso spin traps, deal with the addition of a NO· radical to an NO functional group on the spin trap. This raises some questions regarding the nature of the potential spin adducts and the location of the unpaired nitroxide electron spin density in the spin adduct. Using EPR and spin trapping, Arroyo et al. (Arroyo et al., 1990) have characterized the receptor-mediated formation of a precursor of NO· derived from L-arginine in cultured mouse neuroblastoma cells (clone N1E-115). Furthermore, this intermediate activates guanylate cyclase in these cells. In the experiments involving neuroblastoma cells, the inhibition by N^{ω}-methyl-L-arginine (MeArg) of a labile nitroso precursor of nitric oxide trapped by 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) and cyclic GMP formation was used as evidence for the existence of the L-arginine/NO pathway.

Since L-arginine is known to inhibit platelet aggregation by a mechanism not clear at this time (Caren and Corbo, 1973; Houston et al., 1983), using the techniques of EPR and spin trapping the present study investigates the existence and biological role of the L-arginine/NO pathway in human platelets.

MATERIALS AND METHODS

Isolation of platelets

Blood (60 ml) from healthy volunteers who had not taken any drugs for at least 14 days was collected by venopuncture into a plastic flask containing sodium citrate (1:9 by vol.). Platelet-rich plasma was prepared by centrifugation ($800 \times g$, 8 min). To prepare washed platelets ($1.5-3.5 \times 10^5/\mu l$), prostacyclin (300 ng/ml) was added to platelet-rich plasma and the mixture centrifuged for 18 min as described by Radomsky and Moncada (1983). The supernatant was removed and the platelet pellet was washed once in 20 ml calcium-free oxygenated buffer A (0.32 M sucrose, 10 mM Hepes and 1 mM DL-dithiothreitol, pH 7.4) containing 300 ng/ml PGI_2. The cells were then resuspended in buffer (5 ml) without prostacyclin. Indomethacin (10 μM) and calcium (1 mM) were added to the final platelet suspension. Aggregation was induced by collagen (5–20 μg/ml) and was monitored for 10 min in an Aggretec TE-500 dual-channel aggregometer, Tokyo Erma Optical Works, Ltd, Japan, by the method of Born and Cross (1963).

Preparation of platelet cytosol

Washed platelets were centrifuged ($150000 \times g$, 30 min) and resuspended in buffer A (5 ml). Cell membranes were then disrupted by sonicating twice for 5 s with a Branson B-12 sonicator (Danbury, CT, USA). The supernatant was passed through a 3-ml column of AG^R 50W-X8 cation-exchange resin (Bio-Rad Laboratories, Richmond, CA, USA) to remove endogenous arginine. The existence of the L-arginine/NO pathway in platelet cytosol was determined by the formation of DBNBS or 2-methyl-2-nitrosopropane (MNP) adducts in the presence of L-arginine and calcium.

EPR detection of DBNBS, MNP and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) adducts

DBNBS was obtained from Sigma Chemical Company (St. Louis, MO, USA) and was prepared in the absence of light by dissolving DBNBS (33 mg/ml) in double-deionized and double-distilled H_2O. MNP (Aldrich, Milwaukee, WI, USA) was also prepared in the absence of light by dissolving it (22 mg/10 ml) in double-deionized double-distilled water and stirring (approximately 2 h) in a water bath at 45 °C in the absence of light. DMPO was a gift from Shonan Analytic Center (Tokyo, Japan) and was purified according to the method of Buettner and Oberley (1978) by repeatedly treating the DMPO solution with activated charcoal until all the free-radical impurities were eliminated (verified by EPR). The concentration of DMPO was determined spectrophotometrically ($\lambda = 236$ nm, $\varepsilon = 7220\ M^{-1} \cdot cm^{-1}$; Samuni et al., 1989). The experiments were carried out in darkness to prevent any photolytic degradation of DBNBS, MNP or DMPO. Aqueous solutions of DBNBS (40 mM), MNP (13 mM) or DMPO (300 mM) were added to platelet suspensions in buffer A and incubated (2 min, 37 °C) in the presence of Ca^{2+} (1–3 μM). The activation of platelets was initiated with the addition of collagen (17 μg/ml, final concentration) and monitored by EPR no later than 1 min after the reaction started. In the case of MNP, the EPR signal obtained immediately (first 4 min) was broad with poor resolution, possibly due to the low concentration of MNP in the aqueous phase. Therefore, in order to determine the nature of MNP adducts, the samples were allowed to sit in the dark for about 1 h permitting spin-adduct accumulation and better resolution of their EPR signals. The time scale of the formation and decay of the spin adducts of DBNBS range from 30 s to a half-life of 27.5 min, respectively. The EPR spectra were recorded at room temperature on a JEOL-JES-FE2XG X-band spectrometer (JEOL Ltd, Tokyo, Japan) in a 100-kHz magnetic field. Unless otherwise stated, the instrumental conditions were as follows: field intensity, 335.5 mT; modulation width, 0.1 mT; instrument gain, 6.3×10^3; response, 1–3 s; sweep time, 4 min/360 mm or 8 min/360 mm; microwave power, 8 mW. The EPR spectra were obtained using a quartz flat cell

(JEOL Co. Ltd, Tokyo, Japan; 10 × 45 × 0.4 mm). The spectra were analyzed by comparison with computer-generated EPR spectra.

Reagents

Collagen was from Hormon-Chemie, Munich, FRG; L-arginine, DL-dithiothreitol and bovine hemoglobin were from Sigma Chemical Company (St. Louis, MO, USA). MeArg was from Calbiochem, San Diego, CA, USA. Methylene blue trihydrate, D-arginine, potassium ferricyanide, glutathione and indomethacin were from Wako Pure Chemical Industries, Ltd, Japan. Recombinant human superoxide dismutase and prostacyclin were gifts from Nihon Kayaku, Tokyo and Ono Pharmeutical Co., Tokyo, Japan, respectively. NADPH was obtained from Boehringer Mannheim, FRG. All reagents were dissolved and diluted in double-deionized double-distilled water and were incubated with washed platelets or platelet-rich plasma for 2 min prior to the addition of the aggregating agent (collagen).

RESULTS

Collagen (8–40 μg/ml) induces a concentration-dependent platelet aggregation in platelet-rich plasma and in washed platelets resuspended in buffer A. To determine the optimal collagen concentration which induces maximum response, several platelet-rich plasma suspensions were incubated with different concentrations of collagen. The results obtained showed that collagen concentrations of 8.5, 17 and 40 μg/ml respectively induced 65%, 90–100% and 70% platelet aggregation. These aggregation measurements were measured in the presence of indomethacin (10 μM). Therefore, a collagen concentration of 17 μg/ml was used in all experiments involving the activation of platelets.

It is of interest to determine what effect the spin traps have on the collagen-induced platelet aggregation and how this effect compares to the anti-aggregatory effect caused by L-arginine. Fig. 1 shows the results obtained after addition of collagen to several platelet suspensions each containing a different concentration of L-arginine or different concentrations of one of the spin traps (DBNBS, MNP, DMPO). The half-maximal inhibitory concentration (IC_{50}) values for L-arginine, D-arginine, DBNBS, MNP and DMPO concentrations, at which the platelet aggregation is inhibited by 50%, are summarized in Table 1. Compared to L-arginine, D-arginine showed virtually no difference in its capacity to inhibit platelet aggregation; however, the results indicate that L-arginine is approximately 4.5 times less effective than DBNBS and approximately 15 times less effective than MNP in inhibiting platelet aggregation. The results also show that the nitrone DMPO is far less effective in its capacity to inhibit platelet aggregation than L-arginine and the nitroso spin traps (DBNBS and MNP).

To determine whether free-radical intermediates are generated during the activation of platelets, initial experiments were performed in which platelets in platelet-rich plasma were activated in the presence of DBNBS. This spin trap has previously been shown to be effective in trapping intermediates generated in neuroblastoma cells activated with carbamylcholine (Arroyo et al., 1990). Collagen (17 μg/ml) activation of platelets ($1-2\times10^8$ cells/ml) in th presence of Ca^{2+} (1 μM) and DBNBS (40 mM) yields the EPR spectra shown in Fig. 2. Control experiments carried out under similar conditions, but

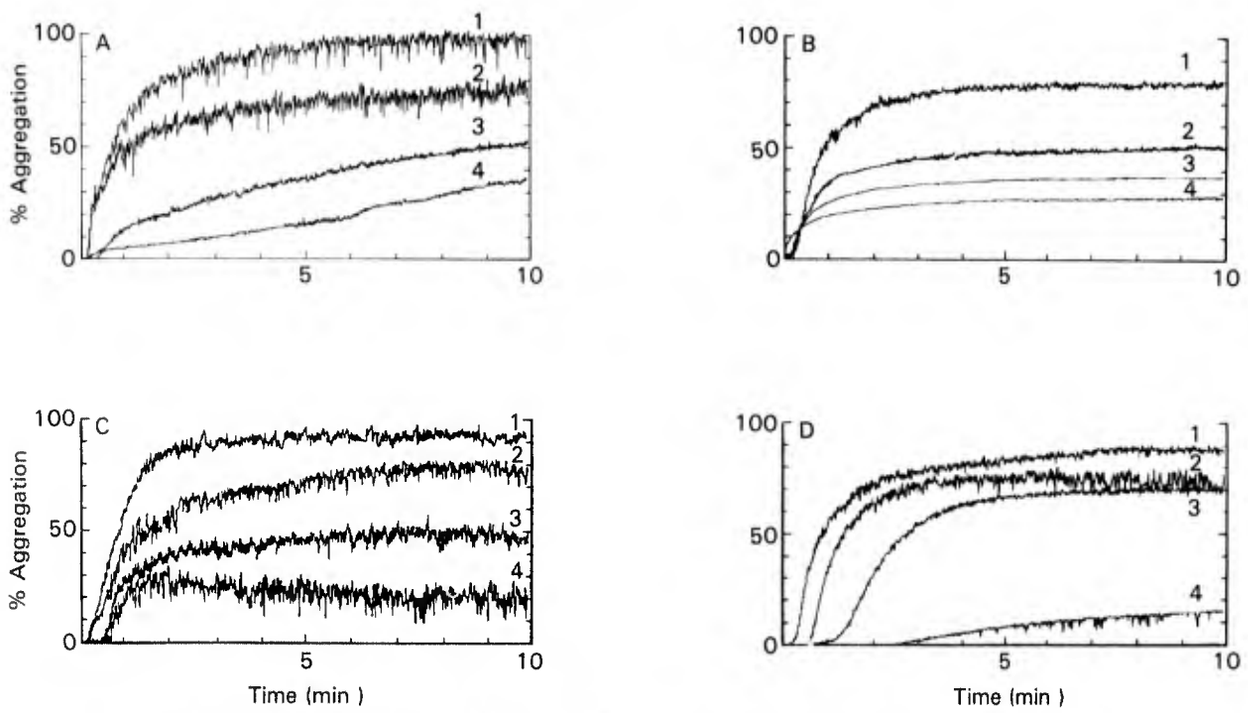


Fig. 1. *Effect of L-arginine and spin traps (DBNBS, MNP and DMPO) on collagen-induced platelet aggregation.* The first experiment in each panel (1) correspond to the collagen-induced platelet aggregation (controls) in the absence of L-arginine or spin traps. (A) L-Arginine effect. (2–4) L-Arginine concentrations of 20, 40 and 80 mM, respectively. (B) DBNBS effect. (2–4) DBNBS concentrations of 9, 17 and 28 mM, respectively. (C) MNP effect. (2–4) MNP concentrations of 1.3, 2.6 and 4.3 mM, respectively. (D) DMPO effects (2–4) DMPO concentrations of 130, 240 and 330 mM, respectively

Table 1. *Inhibition of platelet aggregation by arginine and spin traps*

Compound	IC_{50}
	mM
L-Arginine	40
D-Arginine	35
DBNBS	9
MNP	2.6
DMPO	260

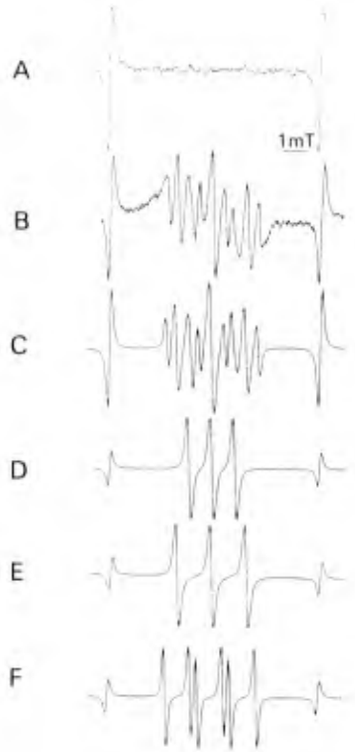


Fig. 2. *Spin-adduct EPR spectra obtained following collagen activation of platelets in platelet-rich plasma in the presence of the spin trap DBNBS.* (A) Control, corresponding to the EPR spectrum obtained following incubation of DBNBS with platelets in the absence of collagen. A similar EPR spectrum is also obtained when DBNBS is incubated with collagen alone. (B) EPR spectrum obtained following the collagen activation of platelets in the presence of DBNBS. (C) Overall computer simulation (addition of computer-generated spectra D–F) that matches the experimental spectrum in B. (D–F) Computer-generated EPR spectra which match the various spin adducts superimposed in the experimental EPR spectrum B

in the absence of platelets or collagen, or after incubating DBNBS with collagen (5 min), yielded no EPR signals (Fig. 2A). The large signals flanking the EPR spectra correspond to an internal instrumental Mn^{2+} standard. The EPR spectrum in Fig. 2B was obtained when platelet-rich plasma was incubated (37 °C, 2 min) with DBNBS and Ca^{2+} followed by incubation with collagen (1 min). This EPR spectrum consists of three superimposed spin-adduct EPR spectra; two consisting of broad triplets and the third to a triplet of doublets. Fig. 2C shows the overall computer stimulation that matches the experimental spectrum in Fig. 2B and was obtained by adding the computer-generated EPR spectra (Fig. 2D–F) corresponding to the three superimposed experimental spin-adduct EPR spectra in Fig. 2B. The broad triplets (Fig. 2D and E), which indicate that the unpaired

Table 2. *Spectral parameters used to obtain the simulated spectra of the observed DBNBS and MNP spin adducts*

Spin adduct in Fig.	Nuclear spin	HFCC	Line width	Intensity
		mT		
2D	1.0	0.93	0.20	0.25
2E	1.0	1.39	0.20	0.50
2F	1.0	1.33	0.16	0.90
	0.5	1.02	–	–
3B	1.0	0.89	0.32	0.53
	1.0	1.40	0.21	0.36
	0.5	0.78	–	–
3D	1.0	1.53	0.10	0.16
	1.0	1.57	0.18	0.18
	1.0	0.22	–	–
	0.5	0.21	–	–
5B	1.0	1.33	0.20	0.90
	0.5	1.02	–	–
	1.0	0.93	0.2	0.5

HFCC, hyperfine coupling constant

nitroxide electron in each of these spin adducts interacts with a single nitrogen nucleus, are best matched by computer simulations using for one (Fig. 2D) a nitrogen hyperfine coupling constant, $a_N = 0.93$ mT, with a relative signal intensity of 0.25, and for the other (Fig. 2E), $a_N = 1.39$ mT, with a relative signal intensity of 0.5 (Table 2). The third spin-adduct EPR spectrum (Fig. 2B), consisting of a triplet of doublets, indicates that the unpaired nitroxide electron is interacting the nucleus of a β hydrogen. This EPR spectrum is best matched by a computer simulation (Fig. 2F) using hyperfine coupling constants as reported in Table 2.

Collagen activation, in the presence of DBNBS (40 mM) and Ca^{2+} (1 μM), of washed platelets resuspended in buffer A yields the EPR spectrum shown in Fig. 3A. This EPR spectrum consists of two superimposed DBNBS spin-adduct EPR spectra. One corresponds to a triplet of doublets similar to the triplet of doublets observed in Fig. 2B and F and can be computer-simulated using hyperfine coupling constants, as indicated in Table 2. Although the triplet of doublets in Fig. 3A does not fit computer simulation identical to the triplet of doublets in Fig. 2B, the similarity between both these triplets of doublets suggests that they originate from the same intermediate. The difference in the computer simulations could be attributed to the superimposition of various spin-adduct EPR spectra and the line widths corresponding to these adducts. It is also possible that changes in the environment (e.g. ionic strength) could lead to differences in the spin adduct EPR spectra corresponding to identical intermediates. The second spin adduct EPR spectrum consists of a broad triplet which is simulated using EPR parameters reported in Table 2. This EPR spectrum is similar to the triplet described in Fig. 2B and D, suggesting that it originates from the same intermediate. The addition of the computer-generated EPR spectra for both DBNBS spin adducts yields the overall computer simulation (Fig. 3B) for the experimental EPR spectrum in Fig. 3A. The spin adduct with an EPR spectrum consisting of a triplet ($a_N = 1.39$ mT), described in Fig. 2B and E, is not observed in the experiments involving activated washed

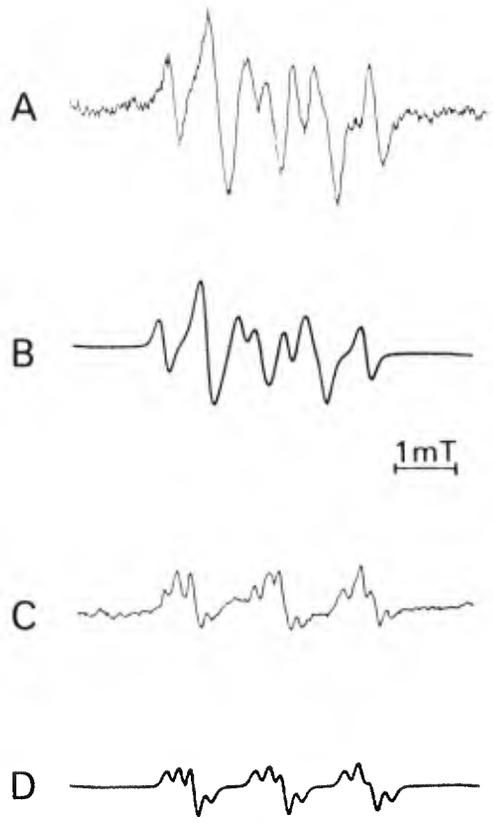


Fig. 3. *Spin-adduct EPR spectra obtained following collagen activation of washed platelets in the presence of DBNBS (A) or MNP (instrument gain, 10^4; C).* (B, D) Overall computer simulations corresponding to the experimental EPR spectra in A and C, respectively. These computer-generated EPR spectra were obtained by addition of the simulations for the superimposed (see text) spin adducts in A and C

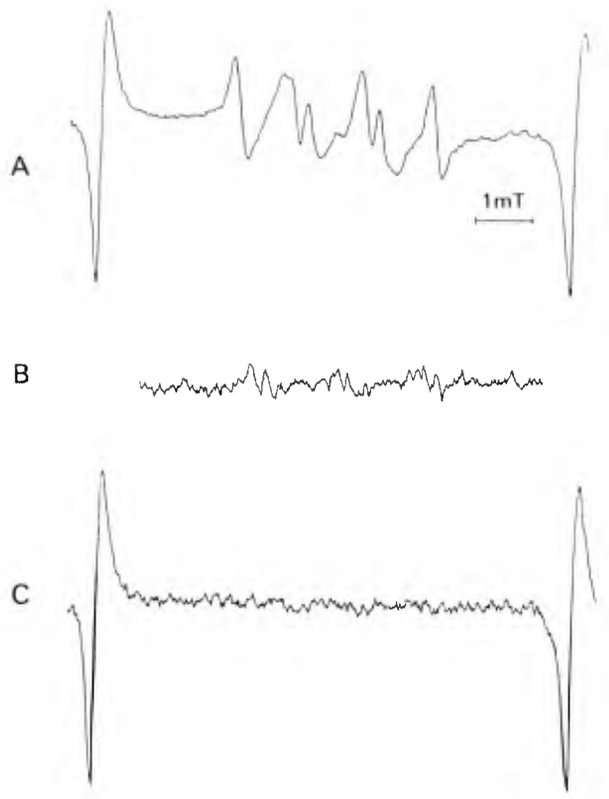


Fig. 4. *Spin-adduct EPR spectra obtained during activation of platelet cytosol following addition of* L-*arginine (A, B) or MeArg (C) to arginine-depleted platelet cytosol.* NADPH and Ca^{2+} were added to the arginine-depleted platelet cytosol prior to the addition of L-arginine or MeArg. DBNBS was used in A and C. MNP was used in B

platelets, suggesting, therefore, that this spin adduct originates from a reactive component in the plasma and not from the activated platelets. In an attempt to gain further information as to the nature of the DBNBS spin adducts, the experiments were performed in the presence of other spin traps (MNP and DMPO). When the experiment described in Fig. 3A is carried out in the presence of MNP (13 mM) instead of DBNBS, the EPR spectrum shown in Fig. 3C is obtained. This EPR spectrum consists of two superimposed MNP spin-adduct EPR spectra. One consists of a broad triplet which is matched by computer simulation using a nitrogen hyperfine coupling constant, $a_N = 1.53$ mT, as shown in Table 2. The second MNP spin-adduct EPR spectrum consists of a triplet of quartets. This indicates that the unpaired nitroxide electron is interacting similarly with the nuclei of a secondary nitrogen and hydrogen (Table 2). The addition of the computer-generated EPR spectra for the MNP spin adducts yields the overall computer simulation (Fig. 3D) that best matches the experimental EPR spectrum in Fig. 3C. When DMPO (300 mM) was used as the spin trap in platelet suspensions (controls) and in collagen-activated platelet suspensions, no EPR signals were detected.

In order to support the existence of the L-arginine/NO pathway in platelets, and to determine whether the spin adducts observed in the experiments described in Fig. 3 originate from this pathway, L-arginine (100 μM) was added to a mixture containing arginine-depleted platelet cytosol, NADPH (1 mM), Ca^{2+} (1 μM) and spin trap (40 mM DBNBS or 13 mM MNP). The EPR spectra shown in Fig. 4A and B were obtained when the experiments were performed in the presence of DBNBS and MNP, respectively. In control experiments, no spin adducts were observed in the absence of L-arginine or NADPH. Although the EPR spectrum of Fig. 4B is weak, it appears to consist mainly of a triplet of quartets which can be matched by a computer simulation using spectral parameters identical to those for the triplet of quartets in Fig. 3C and D. The formation of the spin adducts in Fig. 4A and B are dependent on the presence of Ca^{2+} and are only formed in the presence of L-arginine, and not when this amino acid is substituted by D-arginine. The cation requirement (Ca^{2+}) and the enantiomeric specificity (L-arginine vs D-arginine) suggests an enzymatic process leading to the formation of an intermediate originating from L-arginine from which the spin adducts are formed. The question remains whether such an enzymatic process is that involving the L-arginine/NO pathway. To address this question, the experiments were carried out in the presence MeArg instead of L-arginine. MeArg, an L-arginine analog, is known to inhibit the enzymatic process which leads to the formation of NO˙ from L-arginine. Fig. 4C shows the result obtained from this experiment and indicates that when MeArg (100 μM) is used instead of L-arginine, the formation of the spin adducts is completely inhibited. This result strongly suggests that the enzymatic process which leads to the formation of the spin adducts observed in Fig. 4A and B is the one involving the L-arginine/NO pathway. In addition, since the spin-adduct EPR spectra obtained in Fig. 4A and B are matched by computer simulations similar to those used for the DBNBS and MNP spin adducts in Fig. 3 (Table 2), suggests that the spin adducts generated in the experiments involving washed platelets (Fig. 3) also originate from the L-arginine/NO pathway.

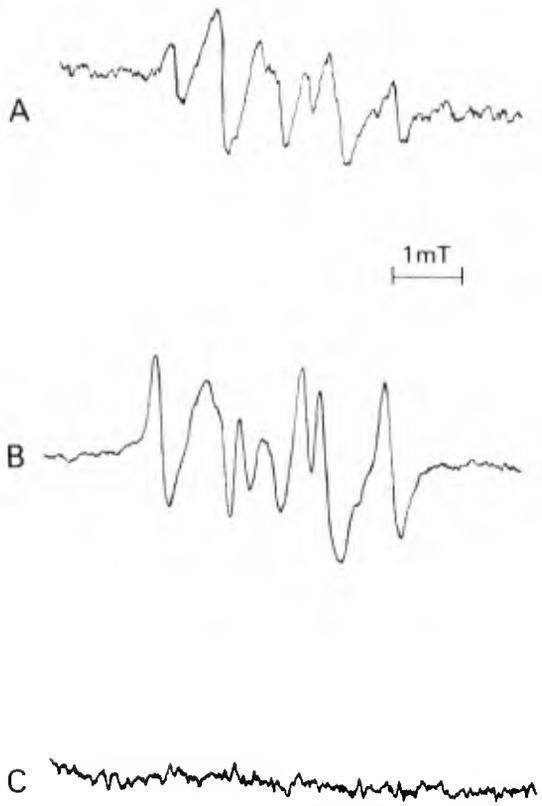


Fig. 5. *DBNBS spin-adduct EPR spectra obtained during collagen activation of washed platelets in the absence (A) and presence (B) of SOD, and in the presence of methylene blue (C).* Instrument gain in B, 4×10^3

Additional evidence, suggesting that the spin adducts produced in the experiments with activated platelets originate from an enzymatic process involving the L-arginine/NO pathway, is given in experiments in which platelets are activated in suspensions containing the spin trap and either superoxide dismutase (SOD) or other well-known inhibitors of the enzymatic synthesis of NO from L-arginine (methylene blue or hemoglobin). It is known that superoxide radical anion ($^{\cdot}O_2^-$) is a major mode of inactivation of the products generated in the L-arginine/NO pathway (Grylewski et al., 1986), and that its presence induces platelet aggregation (Salvemini et al., 1989), an effect opposite to the effect produced by L-arginine. Since it is not uncommon that production of $^{\cdot}O_2^-$ occurs in activated cells, it could be expected that the presence of SOD in platelet suspensions would lead to the potentiation of the observed spin-adduct EPR spectra (Figs. 3 and 4). On the other hand, the presence of methylene blue or hemoglobin in the activated platelet suspensions should prevent the formation of the spin adducts. Fig. 5 shows the results obtained when identical washed platelet suspensions are activated in the presence and absence of SOD or methylene blue (or hemoglobin). Fig. 5A shows the DBNBS spin adduct EPR spectra obtained in a fashion identical to that in the experiments described in Fig. 3. However, when the experiment is performed in the presence of SOD (100 U/ml), the EPR signal in Fig. 5A is potentiated by a factor of three (Fig. 5B). Furthermore, Fig. 5C shows that formation of the spin adduct observed in Fig. 5A is completely inhibited when methylene blue (50 μM) is present during the activation of washed platelets. The same result as that shown in Fig. 5C is obtained when hemoglobin (100 μM) is used instead of methylene blue. It must be noted that similar to the case shown in Fig. 2B, the triplet of doublets in Fig. 5B is best matched by a computer simulation using hyperfine coupling constants, $a_N = 1.33$ mT and $a_H = 1.02$ mT, with line width and relative intensity of 0.2 mT and 0.9, respectively. The same is observed for the spin-adduct EPR spectrum corresponding to a triplet in Fig. 5B. The computer simulation that best matches this triplet is obtained using a nitrogen hyperfine coupling constant of $a_N = 0.93$ mT and line width and relative intensity of 0.2 mT and 0.1, respectively. These spectral parameters are also similar to those observed for the triplet in Fig. 2B and D. The similarity of the spin-adduct EPR spectra in Figs 2B and 5B further support the hypothesis that the spin adducts in Fig. 3A (or Fig. 5A) and the spin adducts in Fig. 2B (or Fig. 5B) originate from the same intermediates.

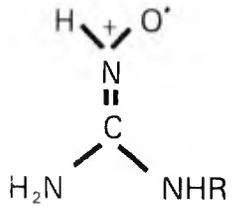
Since the EPR spectra in Fig. 4A and B, respectively, correspond to spin adducts identical to those described by the EPR spectra in Fig. 3A and C, indicating that the spin adducts originating from the experiments involving arginine-depleted platelet cytosol (Fig. 4), and which are not formed in the presence of known L-arginine/NO pathway inhibitors, are the same spin adducts produced following activation of the washed platelets (Figs 3 and 5), which are potentiated by SOD and inhibited by methylene blue or hemoglobin. Therefore, the results shown in Figs 3–5 strongly suggest that the spin adducts generated by the activated platelets are produced from intermediates originating from the L-arginine/NO pathway.

DISCUSSION

The spin-trapping results presented in this work show that upon collagen activation of platelets in the presence of nitroso spin traps, two DBNBS and two MNP spin adducts were formed (Fig. 3). The third DBNBS spin adduct, consisting of a triplet (Fig. 2), $a_N = 1.39$ mT, was observed only in the experiments using platelet-rich plasma. Since this spin adduct was produced only in experiments involving platelet-rich plasma its formation was attributed to an active component in the plasma. A similar DBNBS spin adduct, consisting of a triplet ($a_N = 1.36$ mT), was obtained when glutathione was reacted with $^{\cdot}OH$, generated in a Fenton system (Fe^{2+}/H_2O_2) or by ultraviolet irradiation of H_2O_2, to form oxidized glutathione which is trapped by DBNBS. It is therefore possible that the DBNBS spin adduct (triplet, $a_N = 1.39$ mT), observed only in the platelet-rich plasma experiments, was produced following the reaction of DBNBS with an activated sulfur center. The formation of two DBNBS and two MNP spin adducts in activated washed platelets, in addition to the threefold enhancement of the DBNBS spin adducts in the presence of SOD and the prevention of their formation in the presence of well-known L-arginine/NO pathway or EDRF inhibitors (MeArg, methylene blue, hemoglobin), strongly suggests that these spin adducts originate from the L-arginine/NO pathway in platelets.

Since DBNBS and MNP are similar in that they are nitroso spin traps, and since the DBNBS and MNP spin-adduct EPR spectra (Fig. 3), consisting of triplets with hyperfine coupling constants $a_N = 0.89$ mT (or $a_N = 0.93$ mT, Fig. 5B) and $a_N = 1.53$ mT, respectively, exhibit a similar EPR pattern (triplet) and were generated in analogous experiments using washed platelets, these DBNBS and MNP adducts may originate from the reaction of the spin traps with the same intermediate from the L-arginine/NO pathway. Furthermore, the DBNBS spin-adduct EPR spectrum consisting of a triplet ($a_N = 0.93$ mT and line width 0.2 mT; Fig. 5B), obtained in the washed-platelet experiments in the presence of SOD, is similar to

the EPR spectrum ($a_N = 0.959$ mT) obtained following the reaction of pure NO· gas with DBNBS (Arroyo and Kohno, 1991). This suggests that the EPR spectrum of the DBNBS adduct consisting of a triplet may originate from the production of NO· by these cells. With regard to the additional predominant spin-adduct EPR spectra (Fig. 3) consisting of a triplet of doublets (DBNBS) and a triplet of quartets (MNP), the triplet of doublets (DBNBS) indicates that the unpaired nitroxide electron is interacting with the nucleus of a β hydrogen and the triplet of quartets (MNP) indicates that this unpaired electron is interacting with the nuclei of a secondary nitrogen and hydrogen (not observed in experiments with DBNBS). This suggests that the unpaired electron of the spin-trap NO functional group experienced two different electron-spin-density distributions, leading us to postulate that the intermediate trapped is possibly a hydroxyguanidinium cation radical originating from the N-terminal guanidinium portion of L-arginine. The hydrogen coupling and the additional nitrogen coupling observed in the experiments with MNP, supports that this spin trap reacts with a labile nitrogen-containing intermediate, possibly a hydroxyguanidine derivative from L-arginine formed prior to the production of NO·. Theoretical self-consistent field molecular-orbital calculations indicate that the hydroxyguanidinium ion can dissociate in alkaline or neutral medium by the loss of the hydroxyl proton (Sapse et al., 1981). Therefore, this strongly nucleophilic agent can attack a positive receptor in NO synthase, acting in this way as an anti-aggregatory agent.



Hydroxyguanidinium cation radical

Cellular production of NO· from the N-terminal guanidinium group of L-arginine has been documented in several reports (Collier and Vallance, 1989). Furthermore, since NO· and EDRF have similar pharmacological properties, it is generally thought that NO· is EDRF. However, it is conceivable that EDRF may be some other nitrogen-oxide-containing intermediate formed prior to NO· in the L-arginine/NO pathway, and that NO·, although having similar properties to EDRF, is a product of EDRF degradation.

Although it has been established that NO· not only inhibits platelet aggregation, but also inhibits platelet adhesion to vascular surfaces (Radomski et al., 1987), it has also been established that the cellular production of NO· originates from the N-terminal guanidino group of L-arginine. Therefore, it is not surprising that the collagen-induced aggregation of platelets is prevented in the presence of L-arginine. However, the large anti-aggregatory effect exhibited by the nitroso spin traps (in contrast to the nitrone DMPO) suggests that this anti-aggregatory effect is not limited to NO·, and that other forms of nitric-oxide-containing compounds may also play a role in this mechanism. In neuroblastoma cells, where the L-arginine/NO pathway has also been established, DBNBS was shown to have an inhibitory effect on guanylate cyclase (Arroyo et al., 1990). Similar to this effect in neuroblastoma cells, it is possible that the effect of DBNBS on platelet aggregation is mediated via an inhibition of guanylate cyclase, indicating that this nitroso trap reacts with the radical intermediate species that stimulates guanylate cyclase via a heme-dependent mechanism (data not shown). Since the anti-aggregatory effect of the nitroso spin traps is substantially larger than the effect of L-arginine may suggest that other intermediates, containing nitroso functional groups formed before or after the production of NO·, may play a predominant role in the biochemical and pharmacological effects (EDRF) originating from the L-arginine/NO pathway. It is known that NO· is unstable in aqueous solutions, especially in the presence of oxygen. This again raises the question whether in cellular systems, where oxygen is always present, NO· is actually EDRF exerting its effects prior to its reacting and disappearance.

Another alternative is the formation of an *S*-nitrosothiol. It is known that the enzymatic activity in the L-arginine/NO pathway require the presence of glutathione or other thiol-containing compounds (e.g. dithiothreitol). The pharmacology of *S*-nitrosothiols is essentially the same as that exhibited by NO·. Furthermore, activation of guanylate cyclase by certain nitroso compounds, nitrite, nitrite esters and nitrate esters, has been attributed to the generation of labile *S*-nitrosothiols (Ignarro, 1990). *S*-Nitrosothiols activate cytosolic guanylate cyclase (Ignarro et al., 1980a, b, c) by a heme-dependent mechanism (Mellion et al., 1983), elevate vascular (Ignarro et al., 1981; Ignarro and Kadowitz, 1985) and platelet (Mellion et al., 1983) cyclic GMP levels, relax artery and vein walls (Ignarro et al., 1981; Ignarro and Kadowitz, 1985), inhibit platelet aggregation (Mellion et al., 1981) and produce a potent vasodilatory response *in vivo* (Ignarro et al., 1981).

The question still remains whether the DBNBS (triplet of doublets) and MNP (triplet of quartets) spin adducts observed during the activation of platelets were produced before or after generation of NO·. Recently, Clancy et al. (1990) reported the reaction of NO· with thionitrobenzoic acid (Nbs) and found that in the presence of oxygen NO· is stabilized by Nbs in aqueous solutions. Furthermore, this report also showed that the *S*-nitrosothiol derivative of Nbs was more effective than NO· in inhibiting ADP-induced platelet aggregation. In order to determine whether the spin adducts observed in this work originated from a product of a *S*-nitrosothiol radical intermediate, dissolved NO· was added to a solution of Nbs in the presence of the nitroso spin traps. The experiment was also carried out by adding the spin traps to the reaction mixture after addition of dissolved NO·. Analogous reactions were carried out using oxidized and reduced forms of glutathione. The results showed no spin-adduct formation in any of these reactions. These results would appear to suggest that the spin adducts observed were formed at some intermediate step in the L-arginine/NO pathway prior to the formation of NO·. However, these results do not exclude the possibility that nitroso spin traps exert a large inhibitory effect on platelet aggregation, forming a labile nitroxide intermediate which rapidly reacts with a thiol-containing compound consequently exerting the anti-aggregatory effect. Neither do the results exclude the possibility that the L-arginine anti-aggregatory effect may be caused by another intermediate, possibly a hydroxyguanidinium cation radical formed as a product of auto-oxidation of L-arginine. The spin-trapping and aggregation experiments presented in this work raise questions regarding the true identity of EDRF and whether EDRF is actually NO· or other reactive nitric-oxide-containing compounds.

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REFERENCES

Amber, I. J., Hibbs, J. B. Jr., Taintor, R. R. & Vavrin, Z. (1988) *J. Leukocyte Biol. 43*, 187–192.

Arroyo, C. M., Dickens, B. F., Kramer, J. H., Leiboff, R. H., Mergner, G. W., Mak, I. T. & Weglicki, W. B. (1988) *Life Sci. 49*, 905–910.

Arroyo, C. M., Forray, G., El-FaKahany, E. E. & Rosen, G. M. (1990) *Biochem. Biophys. Res. Commun. 170*, 1177–1183.

Arroyo, C. M. & Kohno, M. (1991) *Free Radical Res. Commun. 14*, 145–155.

Born, G. V. R. & Cross, M. J. (1963) *J. Physiol. (Lond.) 168*, 178–195.

Buettner, G. R. & Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun. 83*, 69–74.

Caren, R. & Corbo, L. (1973) *Proc. Soc. Exp. Biol. Med. 143*, 1067–1071.

Clancy, R. M., Miyazaka, Y. & Cannon, P. J. (1990) *Ann. Biochem. 191*, 138–143.

Collier, J. & Vallance, P. (1989) *Trends Pharmacol. Sci. 10*, 427–431.

Feelisch, M. & Noack, E. A. (1987) *Eur. J. Pharmacol. 139*, 19–30.

Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature 336*, 385–388.

Gryglewski, R. J., Palmer, R. M. J. & Moncada, S. (1986) *Nature 320*, 454–456.

Hibbs, J. B., Taintor, R. R., Vavrin, Z. & Rachlin, E. M. (1988) *Biochem. Biophys. Res. Commun. 157*, 87–94.

Houston, D. S., Gerrard, J. M., McCrea, J., Glover, S. & Butler, A. M. (1983) *Biochim. Biophys. Acta 734*, 267–273.

Ignarro, L. J., Edwards, J. C., Gruetter, D. Y., Barry, B. K. & Gruetter, C. A. (1980a) *FEBS Lett. 110*, 275–278.

Ignarro, L. J., Barry, B. K., Gruetter, D. Y., Edwards, J. C. & Ohlstein, E. O. (1980b) *Biochem. Biophys. Res. Commun. 94*, 93–100.

Ignarro, L. J. & Gruetter, C. A. (1980c) *Biochem. Biophys. Acta 631*, 221–231.

Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H. & Hyman, A. L. (1981) *J. Pharmacol. Exp. Ther. 218*, 739–749.

Ignarro, L. J. & Kadowitz, P. J. (1985) *Annu. Rev. Pharmacol. Toxicol. 25*, 171–191.

Ignarro, L. J., Buga, G. M. N., Wood, K. S., Byrns, R. E. & Chaudhurt, G. (1987a) *Proc. Natl Acad. Sci USA 84*, 9265–9269.

Ignarro, L. J., Byrns, R. E., Buga, G. M. & Woods, K. S. (1987b) *Circ. Res. 61*, 866–879.

Ignarro, L. J. (1990) *Annu. Rev. Pharmacol. Toxicol. 30*, 535–560.

Janzen, E. G. (1971) *Acc. Chem. Res. 4*, 31–40.

Janzen, E. G. (1980) in *Free radicals in biology* (Pryor, W. A., ed.) vol. 4, pp. 115–154, Academic Press, New York.

Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry 27*, 8706–8711.

Mellion, B. T., Ignarro, L. J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L. & Kadowitz, P. J. (1981) *Blood 57*, 946–955.

Mellion, B. T., Ignaro, L. J., Meyers, C. B., Ohlstein, E. H. & Ballot, B. A. (1983) *Mol. Pharmacol. 23*, 653–664.

Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1989) *Biochem. Pharmacol. 38*, 1709–1715.

Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature 327*, 524–526.

Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature 333*, 664–666.

Radomski, M. W. & Moncada, S. (1983) *Thromb. Res. 30*, 383–389.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1987) *Lancet I*, 1057–1058.

Radomski, M. W., Palmer, R. M. J. & Moncada, S. (1990) *Proc. Natl Acad. Sci USA 87*, 5193–5197.

Salvemini, S., de Nucci, G., Sneddon, J. M. & Vance, J. R. (1989) *Br. J. Pharmacol. 97*, 1145–1150.

Samuni, A., Krishna, C. M., Riesz, P., Finkelstein, E. & Russo, A. (1989) *Free Radical Biol. & Med. 6*, 141–148.

Sapse, A. M., Herzig, L. & Synder, G. (1981) *Cancer Res. 41*, 1824–1828.

Thornalley, P. J. (1986) *Life Chem. Reports 4*, 57–112.

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RADIOPROTECTION BY METALS: SELENIUM

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ABSTRACT

The need exists for compounds that will protect individuals from high-dose acute radiation exposure in space and for agents that might be less protective but less toxic and longer acting. Metals and metal derivatives provide a small degree of radioprotection (dose reduction factor $\leq$ 1.2 for animal survival after whole-body irradiation). Emphasis is placed here on the radioprotective potential of selenium (Se). Both the inorganic salt, sodium selenite, and the organic Se compound, selenomethionine, enhance the survival of irradiated mice (^{60}Co, 0.2 Gy/min) when injected IP either before (-24 hr and -1 hr) or shortly after (+15 min) radiation exposure. When administered at equitoxic doses (one-fourth LD_{10}; selenomethionine = 4.0 mg/kg Se, sodium selenite = 0.8 mg/kg Se), both drugs enhanced the 30-day survival of mice irradiated at 9 Gy. Survival after 10-Gy exposure was significantly increased only after selenomethionine treatment. An advantage of selenomethionine is lower lethal and behavioral toxicity (locomotor activity depression) compared to sodium selenite, when they are administered at equivalent doses of Se. Sodium selenite administered in combination with WR-2721, S-2-(3-aminopropylamino)ethylphosphorothioic acid, enhances the radioprotective effect and reduces the lethal toxicity, but not the behavioral toxicity, of WR-2721. Other studies on radioprotection and protection against chemical carcinogens by different forms of Se are reviewed. As additional animal data and results from human chemoprevention trials become available, consideration also can be given to prolonged administration of Se compounds for protection against long-term radiation effects in space.

INTRODUCTION

The search for effective radioprotectors is determined by the desired application. Historically, development has focused on protection against acute high-dose radiation injury and for protection of normal tissues of cancer patients who are undergoing therapy. The search has been dominated by the study of sulfhydryl compounds, particularly the phosphorothioates /1/. Good protection against lethality in experimental animals has been achieved with WR-2721 and its derivatives, but high levels of protection are accompanied by side effects, including behavioral toxicity, that may be unacceptable in some situations /2, 3/. With respect to development of radioprotectors for uses where behavioral toxicity would be an unacceptable side effect, performance decrement must be evaluated. In a series of studies, Landauer and coworkers demonstrated that the most effective doses of protectors have the greatest behavioral side effects, as measured by alterations in locomotor behavior /4, 5/.

The problem of behavioral toxicity of single protectors or radioprotective regimens would be more acute in space, where performance is an important factor. On the other hand, the presence of trained personnel and adequate preparation should also allow for parenteral administration of the more potent protectors, e.g., phosphorothioates, in the event of impending high-dose exposures that might occur, such as with a solar flare. Developing radioprotectors for use in space involves considerations that may not be as relevant for other uses. For example, the use of some doses of WR-2721 (and related compounds) during spaceflight may be complicated by the effect of this compound on calcium metabolism /6/, causing exacerbation of the hypocalcemia induced by weightlessness.

Protectors that are less toxic and longer acting are also needed, although they may not be as effective as phosphorothioates. Among the less active protectors are metals and metal complexes. Metals are among the many endogenous factors that influence cellular radiosensitivity, causing either a pro-oxidant or antioxidant effect /7/. Iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), and Se are important in enzyme and protein functions, which are often involved in the removal of toxic free radicals or their products, including those arising from

radiation-induced free radicals /8/. These include: superoxide dismutases (Zn, Cu, and Mn), catalase (Fe), ceruloplasmin (Cu), metallothionein (Zn), glutathione peroxidases and related proteins (Se). In this paper we concentrate on the radioprotective properties of Se.

METHODOLOGY

Male CD2F1, (BALB/c x DBA/2)F1, mice, approximately 25 g, obtained from Charles River Laboratories were quarantined and acclimated for at least 2 wks before experimentation. During quarantine, mice were examined for pathological or serological indications of disease and *Pseudomonas* infection (representative sampling). Mice were housed eight/MicroIsolator cage on hardwood chip contact bedding, maintained in conventional animal holding rooms in an AAALAC-accredited facility. Rooms were maintained at 21°C ± 1°C with 50% ± 10% relative humidity and 12 room changes of 100%-conditioned fresh air per hr. Mice were maintained on a 12-hr light-dark cycle, and provided acidified water (pH 2.5) *ad libitum*. Research was conducted according to the principles enunciated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council.

Sodium selenite and seleno-D,L-methionine (Sigma Chemical Co., St. Louis, MO) and WR-2721 (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) were injected intraperitoneally (IP) in neutralized saline (pH 7-8) in a volume that was 1% of the mouse body weight. Drug doses used for radioprotection studies were administered at fractions of the LD_{10} dose, which were approximately 800 mg/kg for WR-2721, 16 mg/kg for selenomethionine, and 3.2 mg/kg for sodium selenite. Doses of Se compounds are expressed as mg Se/kg body weight. When the radioprotective effects of sodium selenite and selenomethionine were compared, mice in Plexiglas boxes were irradiated unilaterally with ^{60}Co (Theratron 80, 0.2 Gy/min). When combinations of sodium selenite and WR-2721 were studied, mice in Plexiglas boxes were irradiated bilaterally with ^{60}Co (AFRRI ^{60}Co source, 1 Gy/min). Behavioral toxicity (performance decrement) of radioprotectors was assayed by automated quantitation of spontaneous locomotor activity /5, 9/. Statistical analyses of survival data were done by the Mantel-Cox procedure and behavioral toxicity by Dunnett's test /10/.

RESULTS

Radioprotection by Sodium Selenite and Selenomethionine

Determination of the lethal toxicity of sodium selenite and selenomethionine indicated that a larger amount of Se could be administered to mice in the organic form than in the inorganic form; the LD_{10} for selenomethionine was 16.0 mg/kg body weight and for sodium selenite was 3.2 mg/kg body weight. Radioprotection studies and behavioral toxicity experiments were done using fractions of the LD_{10} dose. Comparison of the behavioral toxicity of selenite and selenomethionine injected at equivalent doses of Se (1.6 mg/kg) is shown in Figure 1. No significant behavioral toxicity was observed with selenomethionine at this dose. Significant decrements in locomotor activity were observed up to 1 hr after sodium selenite treatment, with a nadir at 0.5 hr. When

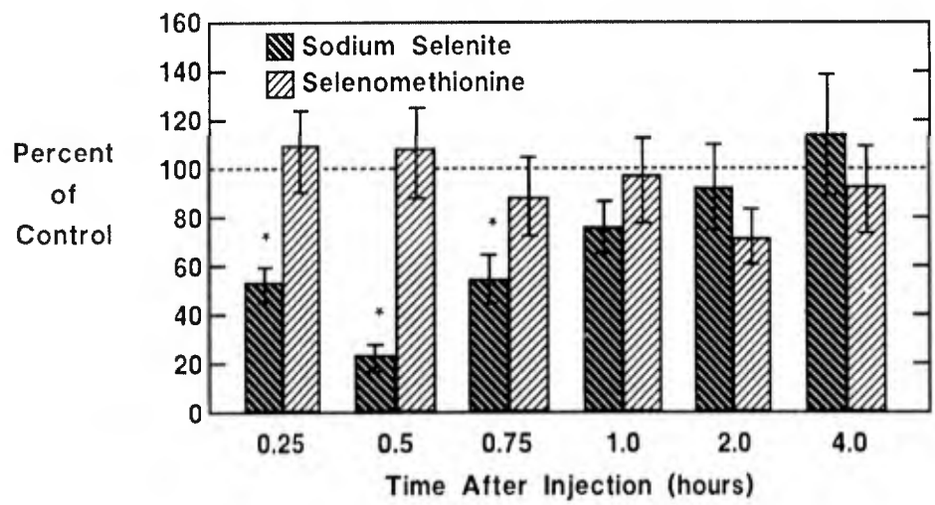

Fig. 1. Comparison of behavioral toxicity (locomotor activity) of sodium selenite and selenomethionine at equivalent doses of Se (1.6 mg/kg). The dashed line at 100% represents the mean locomotor activity of the saline-treated control group. N=11/group. * $p<0.05$, significantly different from controls.

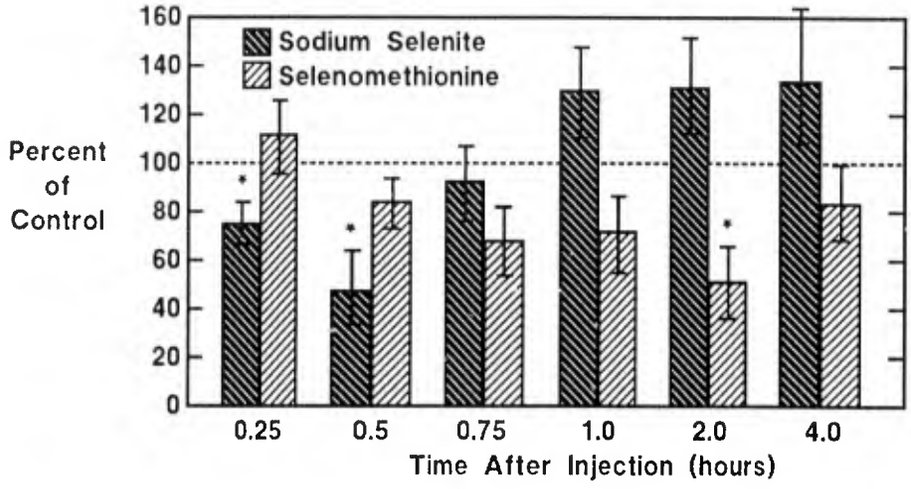

Fig. 2. Comparison of behavioral toxicity (locomotor activity) of sodium selenite (0.8 mg/kg Se) and selenomethionine (4.0 mg/kg Se) at equitoxic doses (one-fourth LD_{10}). The dashed line at 100% represents the mean locomotor activity of the saline-treated control group. N=11/group. * $p<0.05$, significantly different from controls.

behavioral toxicity was compared at equitoxic doses, based on the LD_{10}, locomotor activity was significantly depressed 2 hr after administration of selenomethionine (4.0 mg/kg or one-fourth LD_{10}) (Figure 2). Sodium selenite administration at 0.8 mg/kg or one-fourth LD_{10} significantly depressed locomotor activity at 0.25 hr and 0.5 hr, with a return to normal at 0.75 hr.

Radioprotection was observed when either sodium selenite (0.8 and 1.6 mg/kg) or selenomethionine (0.8, 1.6, and 4.0 mg/kg Se) were injected before or shortly after irradiation. In general, time of administration did not significantly influence percentage of survivors. At 9 Gy, approximately 80% of mice survived when they were treated with either 4.0 mg/kg selenomethionine or 0.8 mg/kg sodium selenite, whether the drug was administered at -24 hr, -1 hr, or +15 min with respect to irradiation. Figure 3 shows the survival patterns of mice irradiated at 9 Gy with a low dose rate (0.2 Gy/min) and treated 15 min after irradiation.

At 10 Gy (the highest radiation dose at which protection could be observed), there was still a statistically significant increase in survival of irradiated mice when selenomethionine was injected at one-fourth LD_{10} (4.0 mg/kg Se), whereas survival after sodium selenite treatment was inconsistent. Table 1 details the radioprotective effects of selenomethionine at 9-Gy and 10-Gy exposures.

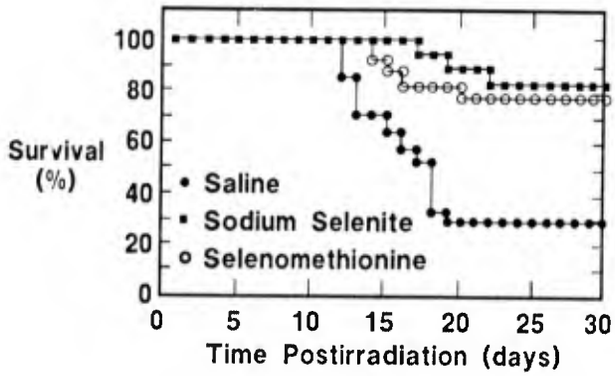

Fig. 3. Survival of irradiated mice (9 Gy, 0.2 Gy/min ^{60}Co) after administration (IP, +15 min) of sodium selenite (0.8 mg/kg Se) or selenomethionine (4.0 mg/kg Se) at equitoxic doses (one-fourth LD_{10}). N=18-28/group. Treated groups were significantly different from controls, $p<0.001$.

TABLE 1 Effect of Time of Administration and Radiation Dose (^{60}Co, 0.2 Gy/min) on Radioprotection by Selenomethionine (4 mg/kg Se)[1]

	30-Day Survivors	
Time	9 Gy	10 Gy
Saline controls	23%	0%
-24 hr	93%***	36%***
-1 hr	71%**	21%*
+15 min	79%***	35%**

[1]N = 14-28/group.
*p<0.05, **p<0.01, ***p<0.001, significantly different from saline-treated control group.

Radioprotection by Combinations of Sodium Selenite and WR-2721

The effect of Se as sodium selenite on the acute toxicity and radioprotective effect of WR-2721 was determined. Injection of 1.6 mg/kg sodium selenite 24 hr before WR-2721 (800-1200 mg/kg, IP) decreased the lethal toxicity of WR-2721 significantly /11/. The radioprotective effect of WR-2721 was enhanced when selenite was injected 24 hr before WR-2721 (200-400 mg/kg IP 0.5 hr before irradiation) at 1 Gy/min (Table 2). The DRF for 30-day survival after 400 mg/kg WR-2721 was 2.6 with sodium selenite, compared to 2.2 without selenite pretreatment. One-hundred percent survival could be obtained at 20 Gy and 22 Gy when a dose of 600 mg/kg WR-2721 was given after pretreatment with sodium selenite /11/. Sodium selenite injection alone (1.6 mg/kg·Se) 24 hr before ^{60}Co irradiation increased survival (DRF = 1.1).

In subsequent studies, we used lower doses of sodium selenite and WR-2721. Mice were pretreated with sodium selenite, or selenite and WR-2721 were administered simultaneously IP. Table 3 summarizes data obtained in various studies /3, 11, and unpublished/.

TABLE 2 Effect of Pretreatment With Sodium Selenite on Radioprotective Efficacy (^{60}Co, 1 Gy/min) of WR-2721[1]

	30-Day Survivors					
Radiation dose (Gy)	Control (saline)	Selenite only	WR-2721 (200 mg)	Selenite + WR-2721 (200 mg)	WR-2721 (400 mg)	Selenite + WR-2721 (400 mg)
8.5	50%**	100%**	-	-	-	-
9.0	0%***	100%***	-	-	-	-
10.0	0%	0%	-	-	-	-
14.0	-	-	69%*	100%*	-	-
16.0	-	-	0%***	88%***	100%	100%
18.0	-	-	-	-	75%*	96%*
20.0	-	-	-	-	17%***	83%***
22.0	-	-	-	-	6%*	38%*
24.0	-	-	-	-	0%*	31%*

-, not tested.
[1]Sodium selenite (1.6 mg/kg Se) injected IP 24 hr before WR-2721 treatment IP. N=16-24/group.
*p<0.05, **p<0.01, ***p<0.001, significant difference between groups with and without selenite. Data from /11/.

TABLE 3 Effects of Sodium Selenite Administered in Combination with WR-2721

Sodium Selenite Dose (mg/kg Se)	Time of Administration	Endpoint and Effect of Sodium Selenite on WR-2721
0.8, 1.6	-24 to -3 hr	Decreased lethal toxicity
0.4, 0.8, 1.6	-24 to -3 hr	Increased radioprotection with 0.8 and 1.6; inconclusive with 0.4
0.8	Simultaneously with WR-2721	Increased radioprotection
0.8	Simultaneously with WR-2721	Decreased lethal toxicity
0.8	Simultaneously with WR-2721	Increased behavioral toxicity in nonirradiated mice
0.4	-24 hr	No difference in behavioral toxicity in irradiated or nonirradiated mice

Radioprotection by WR-2721 increased when the agents were administered simultaneously IP, as well as when mice were pretreated with sodium selenite (0.8 mg/kg Se). Although the lethal toxicity of WR-2721 decreased when sodium selenite (0.8 mg/kg Se) was administered simultaneously, the behavioral toxicity did not.

DISCUSSION

A number of investigators have studied radioprotection by Se compounds /12, 13, 14/. Not all studies published on survival of irradiated rodents showed protection. We estimated, based on published studies and our studies, that protection did not exceed a DRF of 1.2 with any Se compound. Our studies were performed at a relatively low dose rate (0.2 Gy/min), and we do not know whether similar results would be obtained at higher dose rates.

The present study provides the first clear indication that the survival of irradiated experimental animals is increased significantly when Se compounds are administered either before or after irradiation. Although Se compounds, such as selenomethionine, may act as free radical scavengers /15/, this is probably not the only mechanism of radioprotection, because protection is obtained when the agents are injected 24 hr before, shortly before, and after irradiation. This window of radioprotection is similar to that observed with immunomodulators, such as endotoxin and interleukin-1, and differs from radioprotection by most sulfhydryl compounds, such as the phosphorothioates /3/. That certain antioxidants or free radical scavengers are also active when administered postirradiation provides evidence that protection involves modulation of later reactions, e.g., interaction of radiation-induced radicals of biomolecules with reactive oxygen species evolved during normal cellular processes /7, 16/.

Immunological mechanisms cannot be discounted as contributing to the overall radioprotective effect, because Se enhances functions of immunocompetent cells /17/. Sodium selenite injection at -20 hr increased hematopoietic stem cells (endogenous spleen colony-forming unit assay) and contributed to accelerated hematopoietic regeneration when used together with WR-2721 and glucan /18/. It is unlikely that compounds that can be administered after radiation will have a greater effect than those that must be administered before radiation that intercept or immediately repair damage or enhance repair mechanisms. However, from a practical point of view it is important to develop postirradiation therapeutic agents to enhance biochemical, physiological, and immunological responses. The availability of compounds, especially "natural agents" to protect against acute and long-term effects of low doses of radiation when administered before or after exposure would be especially beneficial for space travel.

According to Foye /12/, Se compounds have been generally ineffective *in vivo*, although *in vitro* studies suggest that Se derivatives often are more effective radioprotectors than their sulfur analogs /15/. Radioprotection in rodents has been demonstrated with both inorganic and organic Se compounds. Effective inorganic compounds studied were colloidal Se /19/ and sodium selenite /20/; it is unclear whether sodium selenate is active /20/. Organic compounds having a positive effect in either mice or rats include 2-aminoselenoazoline /21/, selenourea, selenocystine, selenoxanthene, selenoxanthone, and selenochrome /19/.

There are conflicting reports on the protective effects of selenomethionine with respect to survival. The present study clearly shows protection when mice are treated parenterally with selenomethionine, and a previous study showed a small degree of protection in rats /19/. Studies in which selenomethionine was provided in the diet as selenous yeast or selenomethionine indicate no protection against acute /22, 23/ or chronic /24/ radiation exposure, although another group reported protection of rats with selenous yeast /25/. Our studies suggested a small degree of protection when sodium selenite was given in drinking water (4 ppm) to mice fed a semisynthetic diet. After 2 weeks of treatment before irradiation and during the 30-day observation period postirradiation, survival increased by 12 percent after exposure to 9-Gy ^{60}Co radiation, which increased further (25%) when vitamin E was added to the diet /26/. The results further indicated that the increased Se intake, and especially increased Se/vitamin E intake, helped maintain serum and liver levels of glutathione peroxidase that were depressed 1-4 days after 9-Gy radiation exposure /26/. This study supported the concept of synergistic effects of vitamin E and Se in protection from oxidative damage /27/. However, a double deficiency of vitamin E and Se, did not affect the survival of irradiated mice /28/, although a preliminary study of this group indicated an effect /29/. Cekan and coworkers demonstrated a decreased incidence of radiation-induced malformations when diets of female mice were supplemented with selenomethionine /30/ and when sodium selenite was injected during pregnancy /31/. Generally, it is easier to observe radioprotection when Se derivatives are injected rather than administered in the diet. Most of the radioprotection observed with Se compounds results from "pharmacological" doses, although in some models, radioprotection by doses in the "nutritional range" may also be possible /30/.

There appears to be only *in vitro* evidence that Se might protect against radiation-induced carcinogenesis /32/, although many animal studies have clearly demonstrated that inorganic and organic Se protect against chemical carcinogenesis /33-36/. Because of this evidence, L-selenomethionine and sodium selenate have been proposed for human clinical trials on chemoprevention /33/. The majority of animal studies have been done using either sodium selenite or selenomethionine /35, 36/. Other organic compounds with some activity in mice include p-methoxybenzeneselenol, benzylselenocyanate, selenobetaine, and selenobetaine methyl ester /36/. Ebselen, a mimic of glutathione peroxidase in which the Se moiety is not available for metabolism, was inactive /36/. As proposed for Se chemopreventive agents /36/, the most effective Se-containing radioprotective compounds may have to be metabolized. Figure 4 shows the various routes of metabolism of active Se compounds, as suggested by Ganther, Ip, and others /34-36/.

The differences in metabolism of sodium selenite and selenomethionine may account for differences in radioprotection and toxicity. The delayed behavioral toxicity of selenomethionine compared to sodium selenite (Figure 2) may indicate a slower metabolism of the former compound, with toxicity resulting from a metabolite. Other studies have indicated that selenomethionine lacks other toxic effects (e.g., hypothermia) observed with sodium selenite /37/. Sodium selenite reacts readily with glutathione to yield selenodiglutathione, and hydrogen selenide forms as a key intermediate after several steps. Hydrogen selenide is also a key intermediate in the metabolism of selenomethionine but not of all Se compounds. Some of the intermediates formed may have radioprotective effects by themselves. Both selenite and selenomethionine can serve as precursors of the amino

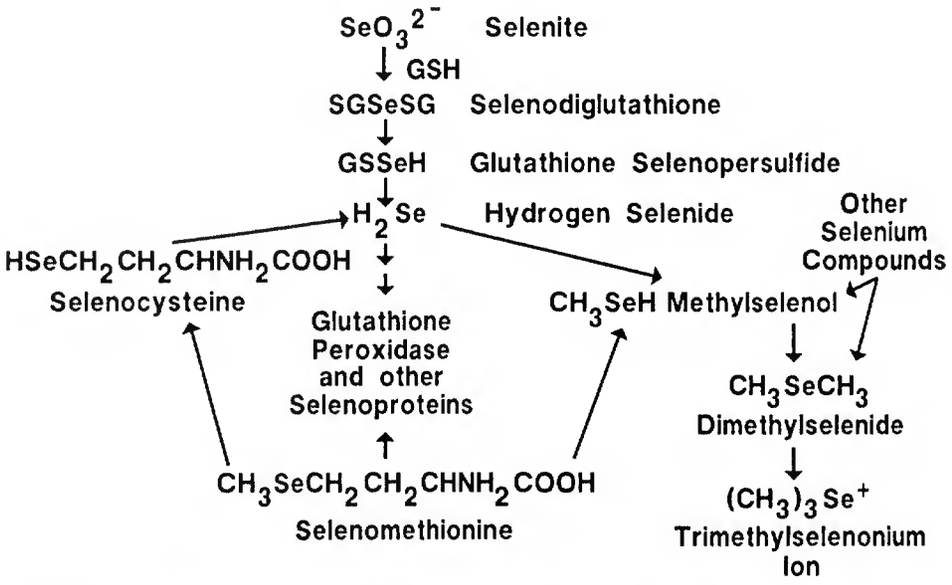

Fig. 4. Proposed pathways for the metabolism of sodium selenite, selenomethionine, and other protective Se compounds.

acid selenocysteine, which is incorporated into glutathione peroxidase, and of other antioxidant selenoproteins. Glutathione peroxidase and various selenoproteins may provide the antioxidant (radioprotective) activity. However, it is possible that selenomethionine itself provides protection because it exhibits strong glutathione peroxidase activity /38 and unpublished/.

The relative importance of glutathione peroxidase activity in contributing to the radioprotective effects of Se compounds requires further study. Glutathione peroxidase(s) catalyzes the reduction of various organic hydroperoxides and hydrogen peroxide to lipid alcohols or water, respectively, with glutathione as the hydrogen donor. Glutathione peroxidase activity in tissues can be increased in humans by dietary supplements of various Se-containing compounds, but the activity remains constant when the Se level in whole blood reaches a certain level /34/. Studies on the effects of Se may be obscured by the fact that there are multiple forms of glutathione peroxidase that may be regulated differently by the level of available Se. Other selenoproteins that are not well characterized also may be important physiological antioxidants. The increase in radioprotection and decrease in lethal toxicity observed on combined treatment with selenite and WR-2721 may be due partially to the increase in glutathione peroxidase in bone marrow and other tissues after pretreatment with sodium selenite /8/. It is also possible that other endogenous protective organometallic compounds form when sodium selenite is injected (Figure 4) and contribute to the radioprotective effect of WR-2721. Results of studies on radioprotection by combinations of selenomethionine and phosphorothioates are not yet available. The interaction of Se, the free thiol of WR-2721, and oxygen may also affect cell radiosensitivity /39/.

Although earlier work suggested that glutathione peroxidase was an important factor in cell radiosensitivity /40/, recent *in vitro* studies, in contrast to animal studies, have demonstrated that adding Se to cultured cells with subsequent induction of glutathione peroxidase did not result in radioprotection of the cells /41, 42/. In a similar way, other *in vitro* studies have shown that large increases in glutathione /43/ or superoxide dismutase /44/ do not always result in radioprotection. It is becoming clear that a balance of the antioxidant enzymes (e.g., glutathione peroxidase, superoxide dismutase, catalase) is more important than changes in any one enzyme /6, 44/. Despite incomplete information on the optimum levels of glutathione peroxidase and other endogenous antioxidant systems in humans, we propose that it is important to maintain optimum cellular levels, especially when an individual would be susceptible to oxidative damage and physiological protectors may not be functioning at full capacity, including spaceflight. Future studies on chemoprotection by Se compounds in humans, including determination of maximum safe doses, will provide important information on the potential of these compounds for use in spaceflight to protect against the acute and long-term effects of radiation exposure.

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REFERENCES

1. D.E. Davidson, M.M. Grenan and T.R. Sweeney, Biological characteristics of some improved radioprotectors, in: *Radiation Sensitizers, Their Use in the Clinical Management of Cancer*, ed. L.W. Brady, Masson, New York 1980, p. 309.

2. V. Bogo, Behavioral radioprotection, *Pharmacol. Ther.* 39, 73 (1988).

3. J.F. Weiss, K.S. Kumar, T.L. Walden, R. Neta, M.R. Landauer and E.P. Clark, Advances in radioprotection through the use of combined agent regimens, *Int. J. Radiat. Biol.* 57, 709 (1990).

4. M.R. Landauer, T.L. Walden and H.D. Davis, Behavioral effects of radioprotective agents in mice: Combination of WR-2721 and 16, 16 dimethyl prostaglandin E_2, in: *Frontiers in Radiation Biology*, ed. E. Riklis, VCH Publishers, New York 1990, p. 199.

5. M.R. Landauer, H.D. Davis, K.S. Kumar and J.F. Weiss, Behavioral toxicity of selected radioprotectors, this issue.

6. D. Glover, L. Riley, K. Carmichael, B. Spar, J. Glick, Z. Agus, E. Slatopolsky, M. Attie and S. Goldfarb, Hypocalcemia and inhibition of parathyroid hormone secretion following administration of WR-2721 (a radioprotective and chemoprotective agent), *N. Engl. J. Med.* 309, 1137 (1983).

7. J.F. Weiss and K.S. Kumar, Antioxidant mechanisms in radiation injury and radioprotection, in: *Cellular Antioxidant Defense Mechanisms*, Vol. II, ed. C.K. Chow, CRC Press, Boca Raton 1988, p. 163.

8. K.S. Kumar, Y.N. Vaishnav and J.F. Weiss, Radioprotection by antioxidant enzymes and enzyme mimetics, *Pharmacol. Ther.* 39, 301 (1988).

9. M.R. Landauer, H.D. Davis and T.L. Walden, Behavioral and physiological effects of leukotriene C_4, *Prostaglandins Leukot. Essent. Fatty Acids* 39, 247 (1990).

10. D.J. Finney, *Statistical Methods in Biological Assays*, MacMillan, New York 1978.

11. J.F. Weiss, R.L. Hoover and K.S. Kumar, Selenium pretreatment enhances the radioprotective effect and reduces the lethal toxicity of WR-2721, *Free Radic. Res. Commun.* 3, 33 (1987).

12. W.O. Foye, Radioprotective drugs, in: *Burger's Medicinal Chemistry*, ed. M.E. Wolff, John Wiley & Sons, New York 1981, p. 11.

13. D.L. Klayman and E.S. Copeland, Radioprotective agents, in: *Kirk-Othmer: Encyclopedia of Chemical Technology*, Vol. 19, John Wiley & Sons, New York 1982, p. 801.

14. R.J. Shamberger, *Biochemistry of Selenium*, Plenum Press, New York 1983, p. 294.

15. F. Shimazu and A. L. Tappel, Selenoamino acids as radiation protectors *in vitro*, *Radiat. Res.* 23, 210 (1964).

16. J.F. Weiss, Y.N. Vaishnav, E. Radha and K.S. Kumar, Cellular antioxidant mechanisms and radioprotection, in: *Recent Trends in Radiobiological Research*, ed. P. Uma Devi, Scientific Publishers, Jodhpur 1990, p. 99.

17. L. Kiremidjian-Schumacher and G. Stotzky, Selenium and immune responses. A review, *Environ. Res.* 42, 277 (1987).

18. M.L. Patchen, T.J. MacVittie and J.F. Weiss, Combined radioprotection: The use of glucan and selenium with WR-2721, *Int. J. Radiat. Oncol. Biol. Phys.* 18, 1069 (1990).

19. A. Breccia, R. Badiello, A. Trenta and M. Mattii, On the chemical radioprotection by organic selenium compounds *in vivo*, *Radiat. Res.* 38, 483 (1969).

20. Z.M. Hollo and S. Zlatarov, The prevention of X-ray death by selenium salts given after irradiation, *Naturwissenschaften* 47, 328 (1960).

21. I. Kozak, L. Kronrad and Z. Dienstbier, Distribution and behavior of isoselenium salts in organism. III. Radioprotective effect of selenium derivatives, *Strahlenther. Onkol.* 150, 539 (1975).

22. H.D. Hurt, W.H. Allaway and E.E. Cary, The effects of dietary selenium on the survival of rats exposed to whole body irradiation, *Fedn. Proc.* 299, 499 (1970) Abstr.

23. A.M. Connor, K.P. McConnell and C.P. Sigdestad, Absence of radioprotection in mice fed enriched seleno-methionine yeast, in: *Abstracts of Papers for the 32nd Annual Meeting of the Radiation Research Society*, Orlando 1984, p. 106.

24. H.D. Hurt, E.E. Cary, W.H. Allaway and W.J. Visek, Effect of dietary selenium on the survival of rats exposed to chronic whole body irradiation, *J. Nutr.* 101, 363 (1971).

25. I. Djujic, M. Demajo, M. Mandic and M. Spasic, Influence of pretreatment with selenous yeast on radiation sensitivity, in: *Abstracts of 3rd International Conference on Anticarcinogenesis and Radiation Protection*, Dubrovnik 1989, p.112.

26. A.J. Jacobs, W.A. Rankin, V. Srinivasan and J.F. Weiss, Effects of vitamin E and selenium on glutathione peroxidase activity and survival of irradiated mice, in: *Proceedings of the 7th International Congress of Radiation Research*, ed. J.J. Broerse, G.W. Barendsen, H.B. Kal and A.J. van der Kogel, Martinus Nijhoff, Amsterdam 1983, D5-15.

27. C.K. Chow, Interrelationships of cellular antioxidant defense systems, in: *Cellular Antioxidant Defense Systems*, vol. II, ed. C.K. Chow, CRC Press, Boca Raton 1988, p. 217.

28. G. Batist, A. Reynaud, A.G. Katki, E.L. Travis, M.C. Shoemaker, R. F. Greene and C.E. Myers, Enzymatic defense against radiation damage in mice. Effect of selenium and vitamin E depletion, *Biochem. Pharmacol.* 35, 601 (1986).

29. C.E. Myers, A. Katki and E. Travis, Effect of tocopherol and selenium on defenses against reactive oxygen species and their effect on radiation sensitivity, *Ann. N.Y. Acad. Sci.* 393, 419 (1982).

30. E. Cekan, P. Slanina, K. Bergman and B. Tribukait, Effects of dietary supplementation with selenomethionine on the teratogenic effect of ionizing radiation in mice, *Acta Radiol.* 24, 459 (1985).

31. E. Cekan, B. Tribukait and H. Vokal-Borek, Protective effect of selenium against radiation-induced malformations in mice, *Acta Radiol.* 24, 267 (1985).

32. C. Borek, A. Ong, H. Mason, L. Donahue and J.E. Biaglow, Selenium and vitamin E inhibit radiogenic and chemically induced transformation *in vitro* via different mechanisms, *Proc. Natl. Acad. Sci. USA* 83, 1490 (1986).

33. C.W. Boone, G.J. Kelloff and W.E. Malone, Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: A review, *Cancer Res.* 50, 2 (1990).

34. Proceedings of the Workshop, Strategies Needed to Develop Selenium Compounds as Cancer Preventive Agents, *J. Am. Coll. Toxicol.* Vol. 5, 1986.

35. C. Ip, Selenium inhibition of chemical carcinogenesis, *Fedn. Proc.* 44, 2573 (1985).

36. C. Ip and H.E. Ganther, Activity of methylated forms of selenium in cancer prevention, *Cancer Res.* 50, 1206 (1990).

37. C. Watanabe, T. Suzuki, T. Ohba and Y. Dejima, Transient hypothermia and hyperphagia induced by selenium and tellurium compounds in mice, *Toxicol. Lett.* 50, 319 (1990).

38. K.A. Caldwell and A.L. Tappel, Glutathione peroxidase activity of selenocystine and selenomethionine, *Biochemistry* 3, 1643 (1964).

39. K.S. Kumar and J.F. Weiss, Effect of metals on oxygen consumption by radioprotective thiols *in vitro*, in: *Frontiers in Radiation Biology*, ed. E. Riklis, VCH Publishers, New York 1990, p. 209.

40. S.L. Marklund, N.G. Westman, G. Roos and J. Carlsson, Radiation resistance and the CuZn superoxide dismutase, Mn superoxide dismutase, catalase, and glutathione peroxidase activities of seven human cell lines, *Radiat. Res.* 100, 115 (1984).

41. B.E. Sandstrom, K. Grankvist and S.L. Marklund, Selenite-induced increase in glutathione peroxidase activity protects human cells from hydrogen peroxide-induced DNA damage, but not from damage inflicted by ionizing radiation, *Int. J. Radiat. Biol.* 56, 837 (1989).

42. B.E. Sandstrom, J. Carlsson and S. Marklund, Selenite-induced variation in glutathione peroxidase activity of three mammalian cell lines: No effect on radiation-induced cell killing or DNA strand breakage, *Radiat. Res.* 117, 318 (1989).

43. A. Russo and J.B. Mitchell, Radiation response of Chinese hamster cells after elevation of intracellular glutathione levels, *Int. J. Radiat. Oncol. Biol. Phys.* 10, 1243 (1984).

44. M. D. Scott, S. R. Meshnick and J. W. Eaton, Superoxide dismutase amplifies organismal sensitivity to ionizing radiation, *J. Biol. Chem.* 264, 2498 (1989).

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ATTN: LAWRENCE BERKELEY LABORATORY

UNIVERSITY OF CINCINNATI
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