Chapter 11

PROSPECTS FOR RADIOPROTECTION

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INTRODUCTION

One of the longest-sought and most elusive goals in radiobiology has been the development of a pharmacological agent that can mitigate the early damage produced in cells and tissues by ionizing radiation. The search for such an agent began in 1949 with the simultaneous demonstration by two different laboratories of survival in rodents exposed to a lethal dose of radiation and treated with the sulfur-containing compounds cysteine or glutathione.[1,2] Since that time, many diverse compounds have been shown to have protective activity (Table 11-1).

With new advances in immunology, biochemistry, radiobiology, and pharmacology, the achievement of that goal may be at hand. Over the longer term, newer concepts and techniques in molecular biology, arising from the so-called biotechnology revolution, are providing exciting approaches for developing specific and effective means to mitigate radiation injury.

The primary objective is to develop an agent or combination of agents that will substantially increase survival and enhance the postattack effectiveness of military personnel on a nuclear battlefield. This radioprotective agent differs from the medical interventions discussed in previous chapters in that it must be easily self-administered shortly before or after radiation exposure to reduce early molecular, cellular, and tissue damage. This chapter briefly reviews the relevant radiobiological concepts, presents the strategies and mechanisms for mitigating radiation injury, and discusses some of the more promising agents being investigated.

RADIATION INJURY

To understand the various strategies being used to mitigate ionizing radiation injury, it is first necessary to define ionizing radiation and to consider the events that occur in the development of ARS.

Ionizing Radiation

Ionizing radiation can be defined as any type of electromagnetic radiation (such as X or gamma rays) or particulate radiation (such as neutrons or alpha particles) that has sufficient energy to ionize atoms or molecules; that is, to eject electrons from their outer orbits.

In considering the effects of radiation on biological systems, it is important to distinguish the different types of ionizing radiation according to their LET. This term describes the amount of energy deposited by a particular type of radiation per unit of path length. Low-LET radiation (X and gamma rays) is sparsely ionizing because it causes few ionizations per micron of path length, whereas high-LET radiation (neutrons and alpha particles) is densely ionizing because it produces many ionizations per micron of path length. Generally, high-LET

radiation is much more efficient in producing biological damage than low-LET radiation.[3,4]

Biological Damage

Death from radiation injury is the result of a sequence of events that occurs over a period of less than a billionth of a second to several weeks (Figure 11-1).[28,29] The first step in this sequence is the transfer of radiation energy from the photon or particle to atoms and molecules in its path. This results in the production of the first discrete lesion in the sequence: a chemical alteration in macromolecules that are critical for biological function. Although the importance of membrane damage is still being evaluated, much of the evidence suggests that damage to DNA may be the most important factor in cell death.[28,30] This initial chemical injury can occur in one of two ways. If a critical biological molecule is in the radiation path, it becomes chemically altered by direct interaction with radiation energy. If that molecule is not in the radiation path, it can still become chemically altered indirectly, via reactions with free radicals and reactive oxygen species produced primarily from the radiolysis of water.

These free radicals and oxygen species are important in the overall scheme of radiation injury because their lifetime in solution is sufficiently long to allow them to diffuse and extend the damage beyond the primary path of radiation. In this way, the effects of ionizing radiation within the cell are greatly amplified. Most radiation injury from low-LET radiation is the result of this indirect damage, while that from high-LET radiation is from direct damage.[31] The net effect of direct and indirect damage is the disruption of molecular structure and function, leading to altered cell metabolism. When DNA is damaged, this is followed by altered cell division, cell death, depletion of stem-cell pools, organ system dysfunction, and, if the radiation dose is high enough, death of the organism.

There are several strategies for reducing radiation injury and mortality. Pharmacological agents can *protect* against indirect damage, *repair* damage once it occurs, or stimulate the *regeneration* of depleted cell populations (Figure 11-2). Spanning these strategies are new genetic approaches that are just beginning to be used in the development of advanced pharmacological agents. Combinations of agents that exploit the operative mechanisms in at least two of these strategies may substantially improve drug effectiveness.

PROTECTION AGAINST RADIATION INJURY

As indicated in Figure 11-2, almost nothing can be done pharmacologically to protect against the initial transfer of radiation energy to either water or critical biological molecules. The transfer occurs too rapidly (within 10^{-14} seconds after irradiation) and is a purely physical process.[28]

The failure of radioprotective agents to protect against direct damage to critical molecules indicates an inherent upper limit to the degree of protection that can be achieved pharmacologically. Because injury from high-LET radiation is due primarily to direct damage, and because the relative yields of water radiolytic products and reactive oxygen species decrease with increasing LET, protection against high-LET radiation injury is more difficult to achieve.[3] Protective agents would be most effective against a low-LET radiation hazard.

The earliest point at which a protective effect from pharmacological agents can be detected is around 10^{-12} seconds after irradiation.[29] At that time, the pharmacological agents can begin to repair chemical damage produced in the critical biological molecules and also react with the chemical intermediates that indirectly damage these molecules. Protection depends on the ability of chemical agents to reduce the intracellular concentration of free radicals and reactive oxygen species that are produced within the first millisecond after irradiation.

Mechanisms

The damage induced by the products of radiation interactions with water can be reduced either by inhibiting the formation of these reactive radical intermediates, or by eliminating them from the cellular environment. This can be accomplished by using agents that induce hypoxia or that scavenge toxic products.

Hypoxia. The formation of reactive oxygen species can be inhibited by the induction of hypoxia. The extent of radiation damage in a tissue is directly related to the degree of oxygenation of that tissue; agents capable of reducing oxygenation will mitigate the injury.[3,32] Many of these chemical agents are known to induce transient systemic or localized hypoxia.[3-5] Systemic hypoxia can be achieved in several ways: induction of hemodynamic cardiovascular alterations, interference with hemoglobin function, increased tissue oxygen utilization, and depressed respiratory-center function. At the cellular and molecular levels, localized hypoxia can be achieved by agents that take part in the chemical and biochemical reactions that use oxygen.

Induction of hypoxia is a widespread protective mechanism that accounts, at least in part, for the protective action of many different chemicals, drugs, and physiological mediators (Table 11-1). In spite of that, the usefulness of this mechanism must be considered with caution because of the potential effects of hypoxia on normal physiological function. This caution may apply more to agents that induce a systemic hypoxic state than to those that create localized hypoxia.

Scavenging. Free-radical scavenging and enzymatic detoxification refer to the ability of chemicals and endogenous enzymes to remove products of water radiolysis and highly reactive oxygen species before they can damage molecules of biological importance.[2,33] In essence, these are competitive reactions between protective agents and biological molecules. In aqueous solutions, protective

agents and enzymes react with free radicals and oxygen species to form relatively stable, nontoxic end products, thereby reducing the concentration of these reactive species and sparing the biological target. Many protectants are very efficient scavengers of water-derived free radicals.

Candidate Agents

Agents currently available as candidates for protection against indirect damage fall into three main groups: *aminothiols, naturally occurring antioxidants,* and *eicosanoids* (Tables 11-2 and 11-3). Research is also being conducted using various *genetic approaches.*

Aminothiols. The vast majority of agents that have been developed and tested in laboratory models for their ability to increase survival after irradiation are the aminothiols.[34] These compounds are chemical analogues of the sulfur-containing amino acid, cysteine. Like cysteine, they have a sulfhydryl group separated by two or three carbon atoms from a strongly basic nitrogen group (Figure 11-3).

As a group, the aminothiols are very effective protectants, and they must be present in the system during irradiation. Optimal protection in laboratory animals is generally obtained by intravenous injection 15-30 minutes before irradiation. The aminothiols function primarily through free-radical scavenging[5] and hydrogen transfer.[3,33] Hypoxia induction may also play a part in their functioning.[4,6]

One of the most significant events in the development of radioprotective agents was the 1969 synthesis of an aminothiol derivative known as WR-2721 (ethiofos).[35] This drug was developed through a program sponsored by the Walter Reed Army Institute of Research, and is the most thoroughly studied of over 4,000 compounds developed and tested to date. WR-2721 has been reported to reduce the effect of a radiation dose by a factor of 2.7 in mice given this drug intraperitoneally 30 minutes before exposure to gamma radiation.[36,37] This is the highest dose reduction factor (DRF) against mouse lethality at 30 days reliably reported for a single injection of a conventional radioprotectant. Increased 30-day survival is commonly interpreted as protection against death due to hemato-poietic-system failure.

WR-2721 also exerts differing protective effects for normal tissue and at least some types of solid tumors.[37] In addition, the drug significantly reduces the toxi-city of the tumor chemotherapeutic agents, cyclophosphamide and cisplatin,[38,39] without altering their chemotherapeutic effectiveness. For these reasons, WR-2721 is undergoing clinical trials as an adjunct to tumor radiation and chemotherapy. Two other potentially beneficial clinical side effects of this drug are that WR-2721 is a hypocalcemic agent and inhibits parathyroid hormone secretion.[40]

WR-2721 is still not available as a field-useable radioprotective agent because it induces nausea and vomiting.[41,42] Although no cumulative or irreversible toxicity has ever been observed in humans or experimental animals that received this drug (even at relatively high doses), the animals did show significant performance degradation after its parenteral administration.[43,44] Another problem that must be overcome is the drug's poor oral bioavailability, due primarily to first-pass metabolism by the intestinal mucosa during absorption.[45] In addition, the drug is hydrolyzed in the acidic environment of the stomach, a factor that is aggravated by its ability to slow gastric emptying.[46]

Research is in progress to overcome these bioavailability problems by using different formulations and by developing prodrugs that are not susceptible to first-pass metabolism. Researchers are also seeking to control or minimize the side effects by combining WR-2721 with antiemetics, or by using subtoxic amounts of the drug in combination with other agents that act synergistically or additively. A DRF of about 1.2 has been obtained with WR-2721 administered intraperitoneally to mice at a dose that produced no observable side effects or performance degradation.[44]

The side effects of WR-2721 and the pharmacological problems associated with its administration are serious obstacles that must be overcome before it can be fielded as a military radioprotective agent. However, a number of other compounds have been developed through the army's program that may more readily satisfy the requirements of a militarily useful agent (Table 11-4).

To compare these compounds for their potential military usefulness, it is necessary to consider a variety of characteristics in addition to the DRF. These include the route of administration, effective drug dose, and *therapeutic index*.[47] The therapeutic index, as used here, refers to the ratio between the toxic LD_{50} and the protective dose used to produce a specific DRF. Although it would also be advantageous to compare information on acute side effects produced by these agents at protective doses, the data are so limited that these factors cannot be included at this time.

The compound that stands out as the most promising candidate is the phosphorothioate WR-159243. Although its DRF is only 1.3, it is effective in mice when given orally, the protective dose is less than 50 mg/kg, and it has the highest safety margin or therapeutic index (7.5) of all the compounds listed. Other compounds with therapeutic indices greater than 2.5 that are being considered include *(a)* the sulfhydryl WR-76841, because of its oral effectiveness and relatively high therapeutic index (5.1); and *(b)* the thiosulfonates WR-1551, because of its oral effectiveness; WR-3302, because of its very low effective dose (5 mg/kg) and high therapeutic index (6.0); and WR-2926, because of its relatively low effective dose (50 mg/kg) and relatively high DRF (1.7). The thiosulfonate WR-1607 is particularly interesting because *(a)* it has a very low effective dose (5 mg/kg) in protecting against radiation-induced lethality, and *(b)*

it is one of the few compounds available that not only enhance survival but also ameliorate ETI.[48,49] This latter effect may be related to the drug's ability to minimize postirradiation hypotension.[48]

WR-2721 has a therapeutic index of only 1.4 at the dose required to produce a DRF of 2.7. However, the therapeutic index increases dramatically to 7.0 at a dose that produces minimal side effects (200 mg/kg). The DRF obtained at this dose is 1.2.

WR-3689 is identical in structure to WR-2721, except that WR-3689 possesses a terminal methyl group (Figure 11-3). When given intraperitoneally at a dose of 450 mg/kg, it provides a DRF of 1.7 with a therapeutic index of 2.5. When given orally at a similar dose (500 mg/kg), WR-3689 is still capable of providing significant protection (DRF: 1.2). With this regimen, the therapeutic index for WR-3689 increases to greater than 3.5 because the lethally toxic oral dose is much higher than that for the intraperitoneal dose.

Another potentially promising aminothiol compound is mercapto-propionyl-glycine (MPG). Under the trade name Thiola, this drug has been available in Japan since the 1970s. It has been used as a detoxifying agent for heavy-metal poisoning, among other clinical applications.[50] Evidence suggests that MPG is also radioprotective. In the most promising studies, it has provided a DRF of up to 1.4 when injected intraperitoneally at a dose (20 mg/kg) that is about 100-fold lower than its toxic dose (2,100 mg/ kg).[51,52]

Naturally Occurring Antioxidants. Naturally occurring compounds that function as antioxidants, such as certain vitamins, minerals, and enzymes, are also being evaluated. These are part of a natural biochemical defense system that has evolved to protect cells against free radicals and reactive oxygen species arising from normal metabolic processes. This defense can be divided into two components: compounds of low molecular weight that scavenge free radicals, and enzymes that detoxify reactive oxygen species (Figure 11-4).[53]

The low-molecular-weight compounds that function as free-radical scavengers in this defense system include vitamins A and E, which are lipophilic, and vitamin C, which is hydrophilic. The enzymatic arm of this system includes superoxide dismutase, which catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. The hydrogen peroxide produced by this reaction is removed from the system by two other enzymes: catalase and glutathione peroxidase. Selenium contributes to this scheme in that it is a cofactor for glutathione peroxidase.

Vitamin E has been shown to increase survival after irradiation.[22] Groups of mice were fed either a basal control diet or a diet supplemented with three times the normal daily mouse requirement of vitamin E (dl-alpha-tocopherol) for 1 week before an 8.5 Gy dose of cobalt-60 gamma radiation and for 30 days after

exposure. All of the control animals succumbed by day 30, whereas 60% of the vitamin E-fed animals survived. At 7.5 Gy, 10% of the controls survived, while 100% of the vitamin E-fed animals survived. DRFs for vitamin E have not been determined experimentally.

Vitamin A is also able to increase postirradiation survival when fed to mice as a dietary supplement.[23] In these experiments, mice were maintained on a basal control diet containing three times the daily mouse requirement of vitamin A for 1 week before irradiation from a cesium-137 source. Immediately following irradiation, they were maintained for the remainder of the experiment on *(a)* the basal diet, *(b)* a diet supplemented with about twenty-eight times the normal requirement of vitamin A, or *(c)* a diet supplemented with an amount of beta-carotene equivalent to about ten times the normal requirement of vitamin A. The vitamin A diet was able to produce DRFs of 1.12-1.25. The beta-carotene diet produced a DRF of 1.26. Significant mitigation of radiation lethality was also provided by vitamin A when diet supplementation was delayed for up to 2 days after irradiation, although delaying the supplementation for 6 days resulted in no increase in survival over the basal-diet-fed animals. Vitamin A fed to mice for 3 days before partial-body irradiation can substantially reduce the effects of localized (hind limb) X irradiation.[54]

In addition to its radioprotective ability, vitamin A or beta-carotene may also be able to promote recovery from burn injury by reversing postburn immunosuppression.[55] This point is significant because burns are expected to be the most common type of injury on the nuclear battlefield.

Selenium is protective when administered either orally or parenterally. When given orally as sodium selenite in drinking water (4 ppm)[21] or injected (1.6 mg/kg) 24 hours before exposure to 900 Gy of cobalt-60 radiation,[56] selenium was able to provide slight but significant increases in survival. The real potential for using selenium as a radioprotective agent lies in its ability to act synergistically with other agents. Selenium can also reduce the toxicity of the sulfur-containing radioprotective compound WR-2721.[56]

Vitamins A and E and selenium are being considered as potential radioprotectant candidates because they are normal dietary components, and considerable data on their toxicity, metabolism, and pharmacological action in the human are available. They are also effective when given orally.

The parenteral administration of superoxide dismutase has increased survival in mice exposed to ionizing radiation.[25] Intravenous injection of this enzyme to mice at a dose of 200 mg/kg given 1 hour before irradiation with X rays resulted in a DRF of 1.38. A single injection of only 35 mg/kg given 1 hour before irradiation with X rays was also able to increase survival (DRF: 1.12). The highest DRF reported for this enzyme is 1.56, achieved in mice given two intravenous

injections: one at a dose of 200 mg/kg given 1 hour before irradiation with X rays, and the other at a dose of 35 mg/kg given 1 hour after irradiation.[25]

Eicosanoids. The eicosanoids are a large group of potent inflammatory mediators derived from the 20-carbon fatty-acid precursor, arachidonic acid. The compounds in this family that are being examined for their ability to increase the survival of irradiated animals include 16,16-dimethyl prostaglandin E_2 ($DiPGE_2$, a synthetic analogue of the naturally occurring prostaglandin GE_2), leukotriene C_4 (LTC_4), and platelet-activating factor (PAF). In one study, $DiPGE_2$ was given subcutaneously to mice at a dose of 1.6 mg/kg and was able to elicit a DRF of 1.72, although severe diarrhea occurred at protective doses.[27] The optimal time for injection is 5-15 minutes before cobalt-60 gamma irradiation, but protection can still be achieved when the compound is given 1 hour before irradiation. LTC_4 has just recently been shown to be effective in increasing the survival of hematopoietic stem cells of mice exposed to cobalt-60 gamma radiation.[57] A DRF of 1.9, using 30-day lethality as the end point, has been achieved with LTC_4 in mice exposed to cobalt-60 gamma radiation.[58] Similarly, PAF is capable of producing fairly high DRFs (about 1.7).[59]

Genetic Approaches. Work is also under way, using molecular biology techniques, to define sequences in the DNA molecule that may be particularly sensitive to radiation. By precisely defining the mechanisms and sites of damage, it may be possible to develop protective agents that can be targeted to specific sensitive sites on the DNA molecule. Additionally, because enzymes are part of the body's natural defense against reactive chemical intermediates, it should be possible to identify the factors involved in regulating their synthesis and to define the encoding gene sequences. This may provide a means by which the synthesis can be activated to increase radioresistance.

REPAIR OF RADIATION INJURY

The aim of this strategy is to restore the chemical structure and normal function of damaged biological molecules so that the injury or death of critical cells is avoided.

As with protection, the effectiveness of the repair varies with the LET. High-LET radiation is densely ionizing and produces very intensive local chemical damage. As the density of damage increases, the ability of chemicals and enzymes to repair this damage becomes overwhelmed. Therefore, repair strategies are more effective for low-LET radiation. Repair can be achieved either chemically (by hydrogen transfer) or enzymatically.

Chemical Repair by Hydrogen Transfer

Radiation damage to a critical biological molecule results in the transformation of that molecule into an organic free radical. In this form, the molecule can then react with oxygen or other free radicals and become permanently altered chemically. However, if a suitable hydrogen donor is in the vicinity of the damaged molecule, it can compensate for the damage by donating or transferring a hydrogen atom.[3,33] Hydrogen atom transfer can be thought of as an instantaneous repair process, in which the original molecular structure is restored before the damaged critical molecule becomes permanently altered by further chemical reaction. Many of the agents that function as free-radical scavengers also have the ability to donate a hydrogen atom.

No radioprotective agents that function primarily or exclusively by chemical repair are available. However, the aminothiols, which act as free-radical scavengers, are all capable of hydrogen transfer and therefore can function in the repair strategy.[3]

Genetic Repair

Similar chemical alterations may also be induced by natural biological processes and disease states that generate free radicals. In the case of DNA, mammalian cells have evolved an elaborate and remarkably efficient system of enzymes that continually repair lesions in that critically important molecule. This system is complex, involving a number of different enzymes and a variety of regulatory molecules that control their synthesis and activity. One of the potentially useful features of this system is that it is inducible; that is, the synthesis of the repair enzymes and regulatory factors is activated when the need arises.

Strains of prokaryotic organisms exist that are capable of surviving very high doses of radiation. One that has received attention is *Deinococcus radiodurans*, which is an extremely radioresistant strain of bacteria.[60] Although study of these relatively simple prokaryotic systems may provide some insight into the genetic mechanisms involved in radiation sensitivity, their lack of complexity compared to mammalian cells is a limitation.

About 25 years ago, a radiosensitive mammalian cell line was isolated in tissue culture from a relatively radioresistant mouse lymphoma cell line.[61] With the advent of new biotechnology techniques, this extensively characterized strain is just beginning to be exploited to its full potential in uncovering genetic mechanisms in radiosensitivity.[62] Recent evidence indicates that the radiosensitivity of this cell line is due to a lower rate of DNA repair.[63] The genomes of the resistant parental line can be compared with the sensitive daughter line by DNA hybridization techniques, making it possible *(a)* to determine if genes are induced by radiation to activate the repair process, *(b)* to identify the genes that are so induced, and *(c)* to determine the proteins that are encoded.

It may be possible (as a long-term goal) to develop agents that will function exclusively in genetic repair. By increasing molecular repair capabilities, these agents may prevent the effects of cellular damage from overwhelming the organism.

REGENERATION AFTER RADIATION INJURY

The aim of this strategy is to increase survival by stimulating the function and regeneration of stem-cell populations that have decreased in number due to radiation-induced cell death. Conceptually, this strategy can be applied to any organ system (such as the hematopoietic system and the gastrointestinal system) that relies on stem-cell proliferation to provide mature differentiated cells for proper functioning. However, because hematopoietic stem cells are the most radiosensitive, only regeneration of the hematopoietic system is discussed here.

Regeneration is a feasible strategy for mitigating radiation injury at radiation doses below the threshold dose that would result in 100% death of hematopoietic stem cells. Figure 11-5 examines hematopoietic stem-cell survival as measured by the number of colony-forming units found in the spleens (E-CFU/spleen) of irradiated mice. Some of the mice were treated with the regenerating agent glucan. In the radiation control animals, which were not given glucan, the number of E-CFU/spleen decreased with increasing radiation dose. Similarly, the effectiveness of glucan in increasing the survival of these cells also decreased with increasing dose. This indicates that the effectiveness of these agents depends on the number of surviving stem cells. Above the threshold radiation dose that results in 100% stem-cell death (greater than 8.5 Gy in Figure 11-5), regeneration becomes ineffective.

The utility of this strategy depends on the threshold point, a factor that can be influenced greatly by partial shielding or by agents that operate in the protection or repair strategies. Because hematopoietic stem cells are among the most radiosensitive in the body, this threshold occurs at a fairly low radiation dose. For uniform whole-body radiation exposure, the threshold dose is approximately equal to the $LD_{100/30}$ radiation dose. However, in battlefield or accident situations, it is likely that the apparent threshold will be substantially higher. Because the inherent radiosensitivity of the hematopoietic stem cells would not change, other factors related to the nature of radiation exposure contribute to this apparent increase. In battlefield or accident situations, some element of shielding, either deliberate or coincidental, is likely to be present. This will provide inhomogeneous exposures, so that high levels of radiation reaching one part of the body may not reach others, thereby permitting increased stem-cell survival. In these situations, it may also be possible to increase stem-cell survival by minimizing the time spent in high-radiation fields and maintaining some distance from radiation sources. The contribution of these protective measures was evident in the

Chernobyl accident victims, in whom bone-marrow grafts apparently failed. These failures were due, at least in part, to host-versus-graft reactions initiated by surviving stem cells, even in patients who were exposed to doses of radiation much greater than those expected to completely deplete stem cells.

The effectiveness of minimal local shielding in protecting even small numbers of stem cells is demonstrated in experiments done with monkeys (Table 11-5).[64] Supportive therapy (fluid, platelets, and antibiotics) significantly increased the $LD_{50/30}$ of irradiated animals. In monkeys exposed to a lethal dose (8 Gy) of whole-body cobalt-60 radiation, supportive therapy extended survival for a few days but had no effect on 30-day survival rates because the radiation dose completely depleted the stem-cell population. However, when the tibias of these animals were shielded so that less than 1% of their bone-marrow stem cells survived, regeneration occurred and many of the animals survived.

Mechanisms

The regeneration of depleted stem-cell populations is brought about by agents that stimulate the proliferation and function of hematopoietic and immunopoietic stem cells. The precise biochemical mechanisms by which this stimulation occurs are complex and are as yet incompletely understood.

Exactly which cell type becomes stimulated depends on the type of agent involved (Figure 11-6). Nonspecific immunomodulators are exogenous agents that can bind to and stimulate a variety of different cell types, particularly macrophages. These agents are thought to induce the stimulated cells to release a variety of peptides (cytokines) that act specifically on immunopoietic and hematopoietic progenitor and stem cells to stimulate their growth and differentiation into mature, functional cells.[65]

Candidate Agents

Agents that mitigate radiation injury via regeneration can be grouped into two broad categories: *immunomodulators* and *cytokines*. Immunomodulators can be thought of as inducer molecules, and cytokines as effector molecules.

Figure 11-7 traces the development of early immunomodulators to cytokines. Original immunomodulators were generally crude whole-cell microbial preparations (such as *Bacillus Calmette-Guerin* [BCG] and *Corynebacterium parvum*) that were used because they could nonspecifically stimulate host immune responses. Later, the active components of these cells (such as endotoxin and zymosan) were identified and isolated from their cell walls. Further work led to the purification, identification, and synthesis of the specific portions of the cell fragments that were actually responsible for stimulating immune responses (such as lipid A from endotoxin and glucan from zymosan). Stimulation of cells by immunomodulators results in the release of cytokines, which act as specific

stimulators of host immune responses. Recent advances have seen the development of biologically defined molecules and recombinantly produced cytokines (such as interleukin-1 [IL-1] and granulocyte-macrophage colony-stimulating factor [rGM-CSF]), which are relatively nontoxic but allow specific manipulation of various components of the immune and hematological systems.

Immunomodulators. Immunomodulators are generally nonspecific immunostimulants that function as external stimuli for a broad range of cell types in the hematopoietic system. To reduce radiation injury, the most effective compounds appear to be those that act primarily on the macrophage. Glucan and trehalose dimycolate (TDM) are two immunomodulators that are currently being evaluated as potential mitigating agents for radiation injury.

Glucan is a beta-1,3-linked polysaccharide isolated from the cell wall of the yeast *Saccharomyces cerevisiae.* This agent is a potent immune modulator that is capable of enhancing a variety of immunopoietic and hematopoietic responses.[15,66]

The effect of glucan on preventing infection and enhancing the regeneration of bone-marrow cells after irradiation is shown in Figure 11-8. In this experiment, mice were injected with either saline or glucan 24 hours before exposure to 9 Gy of cobalt-60 gamma radiation.[15] The number of saline-treated mice showing evidence of infection increased substantially at 7-15 days following irradiation, and no detectable marrow regeneration was evident. However, glucan was able to reduce infection significantly and to produce a substantial increase in marrow regeneration. A temporal relationship was seen between the two effects: the ability of glucan to control infection occurred well before its demonstrable effect on marrow regeneration. This suggests a dual role for glucan in enhancing postirradiation survival: *(a)* this compound can stimulate the remaining mature, relatively radioresistant macrophages to control infection, and *(b)* it can also induce the stimulation of stem and progenitor cells to proliferate and repopulate the marrow.

These data correlate with the ability of this compound to increase the survival of irradiated animals. Administered intravenously to mice 24 hours before 9 Gy of cobalt-60 gamma radiation, glucan increased the 30-day survival from 0% in the saline-injected control mice to 63% in the glucan-treated mice (DRF: 1.2).[67] In addition, glucan is capable of slightly enhancing survival (DRF: 1.08) in mice when it is administered 1 hour after an exposure to 9 Gy of cobalt-60 gamma radiation.[68]

Trehalose dimycolate (TDM), also known as cord factor, is a glycolipid consisting of 6,6'-diesters of the sugar D-trehalose. It is isolated from the cell walls of *Mycobacteria, Nocardia*, and *Corynebacteria*, and is an active component of Freund's complete adjuvant. Like glucan, TDM is a potent immunostimulant that is capable of increasing host defense mechanisms against a variety of organisms and of increasing survival after irradiation.[16,17]

Cytokines. Cytokines are hormone-like peptides that function as molecular signals between cells. They are synthesized and released primarily by macrophages and lymphocytes that have been stimulated by inflammatory agents or immunomodulators. Included in this class of compounds are the interleukins-1 through -6, tumor necrosis factor (TNF), a variety of hematopoietic growth factors (such as granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erythropoietin), and the alpha-, beta-, and gamma-interferons. All of these act in a variety of ways to stimulate proliferation, differentiation, or function of cells in the hematopoietic system. Several have been examined for their ability to mitigate radiation injury, and of these, IL-1 and TNF have been found to be the most effective.

IL-1 is released by activated macrophages. It has a number of local immune effects, including the activation of resting T cells and the stimulation of cytokine synthesis and release.[69]

Although a significant increase in survival (43% for treated versus 0% for control mice) is seen when IL-1 is given 4 hours before irradiation, optimum survival (greater than 80% versus 0% in controls) is obtained when IL-1 is administered intraperitoneally 20 hours before exposure to 9.5 Gy of cobalt-60 gamma radiation.[70] Using that regimen, DRFs of up to 1.25 have been achieved with IL-1 in C57BL/6 mice. IL-1 is particularly attractive as a pharmacological means of mitigating radiation injury because only very small doses are required. The DRF of 1.25 was achieved with a dose of only 0.1μg of IL-1 per mouse (approximately 0.004 mg/kg). The effectiveness of IL-1 in mice is strain dependent. While all strains of mice examined showed different degrees of increased survival, optimum survival was obtained with the C57BL/6 strain.[71]

TNF is also released by activated macrophages. It was named for its ability to act as a direct cytotoxin for some tumor cells. Like IL-1, it has several local immune effects, including the stimulation of cytokine synthesis and release.[69] Unlike IL-1, however, its effectiveness does not depend on the mouse strain. In most strains, TNF is less effective than is IL-1, and in others it is more effective due to the variability in effectiveness of IL-1. TNF is optimally effective (with DRFs of 1.08-1.16) when given intraperitoneally 20 hours before irradiation at a dose of 5-10 g per mouse (approximately 0.2 mg/kg).[72]

Two other cytokines may be potentially useful agents: GM-CSF and interleukin-3 (IL-3). Several growth factors that are specific for different hematological cell populations have been discovered and can be produced by recombinant DNA methodologies. One of these, a specific human recombinant GM-CSF (rGM-CSF), accelerates marrow repair or engraftment and may contribute to increased nonspecific resistance. It functions by increasing the number of circulating granulocytes and platelets in normal animals and accelerating the recovery of these cells after irradiation. This factor was used in treating some victims of the radiation exposure accident in Brazil.

The effectiveness of this factor in ameliorating radiation-induced cytopenia can be seen from data obtained in the minimal-shielding experiment.[64] In that experiment, the survival of partially shielded monkeys that were given supportive therapy was enhanced. Unshielded animals rapidly became neutropenic and died within 15 days. In the shielded animals that survived beyond 30 days, peripheral granulocytes began to recover slowly between days 20 and 40. In contrast, shielded animals treated with a growth factor showed evidence of granulocyte recovery well before day 20, and granulocyte levels quickly reached supranormal levels. Therefore, it appears that this factor is a useful adjunct to radiation-injury therapy. However, its effectiveness as a regeneration agent in radioprotective regimens is much lower than that for IL-1 and TNF when it is given alone in the protocols described above for those cytokines.[70] In spite of that, evidence suggests that it may act synergistically when combined with other cytokines.[72]

IL-3 has not yet been evaluated for its ability to increase survival after irradiation. Unlike the described action of the cytokines (whose major target cells are primarily the more mature functional cells in the system), IL-3 is reported to act specifically in stimulating the growth of pluripotent stem cells.[69] Because hematopoietic stem cells are among the most radiosensitive in the body, this cytokine may be particularly effective as a regenerating agent.

COMBINATION AGENTS

Rationale

Agents that function in the three strategies (protection, repair, and regeneration) contribute in different ways to the mitigation of radiation injury. Each of the three strategies also has its limitations. Neither chemical nor enzymatic means of protection are able to minimize direct damage. In addition, it is almost impossible for any protective or repair agent either to completely eliminate all of the reactive intermediates formed or to repair all of the damaged molecules. Regardless of the efficiency of scavengers and repair agents and their concentration within the cell at the time of irradiation, some molecular damage and cell death will occur. The effectiveness of agents that function in the regeneration strategy is limited because the agents require a pool of surviving functional cells on which to work. That pool of highly radiosensitive hematopoietic stem cells becomes depleted after fairly low radiation doses.

It is reasonable to expect that optimal survival would be provided by an agent or combination of agents that would operate within two or more of these strategies. Such a formulation would maximize the effectiveness of each strategy and minimize its limitations. Protective and repair agents reduce the concentration of reactive species that are produced from the radiolysis of water and also repair the damage to critical target molecules. In so doing, the agents increase the surviving fraction of stem cells, progenitor cells, and mature cells of the hematopoietic

system after irradiation. By allowing stem cells to survive higher radiation doses, the net effect is to increase the threshold radiation dose that limits the effectiveness of regeneration agents. Regeneration agents further enhance the organism's survival by capitalizing on the advantages provided by protective and repair agents; that is, they maximize the proliferation and function of the extra cells provided.

It would be difficult to produce one drug that would be able to mitigate radiation injury by all three strategies. Two or more agents might be used either together or at intervals, but this is not desirable. The simplest dosing regimen (single dose) is the most desirable for military personnel under battlefield conditions. Therefore, the goal is a single treatment consisting of a combination of two or more agents that function in *(a)* either the protection or repair strategy, and *(b)* the regeneration strategy.

Examples of Combination Agents

The concept of using combinations of agents that function by different mechanisms to achieve protection was developed and studied in the 1950s and 1960s.[3] In many of the combinations examined, synergistic effects were seen. These results are particularly significant because increased protection with the combinations was often achieved using substantially lower doses of individual drugs than those required for protection when each agent was given alone. For example, one study examined various combinations of five different radioprotective agents: cysteine, beta-mercaptoethylamine (MEA), aminoethylisothiouronium (AET), glutathione, and serotonin.[73] AET, MEA, or serotonin used alone provided similar protection (DRF: 1.7), cysteine was less effective (DRF: 1.12), and glutathione was marginally protective (DRF: 1.05). The most effective regimen was a combination of all five agents, which produced a DRF of 2.8. In this combination, the doses were two-thirds that of the AET and one-half that of the MEA used when the drugs were given individually.

More recently, additive and synergistic effects were demonstrated with various combinations of aminothiols, antioxidant vitamins and minerals, immunomodulators, and cytokines. It is likely that a first-generation agent will be a combination of subtoxic doses of two or more of these agents. The effectiveness of several combinations is shown in Table 11-6.

Mitigation of Performance Decrement

Because a single, self-administrable agent is sought as a radioprotectant, it might also be necessary to include moderators of performance decrements in any regimen that is developed. While measures to enhance resistance to the lethal effects of radiation have been extensively studied, relatively little attention has been given to the application of pharmacological interventions to mitigate performance and behavioral deficiencies, even though these are of immediate

military concern. Although it is possible for radioprotective agents to prevent some performance decrements, drugs that increase survival generally have not enhanced performance. In fact, except for a few notable exceptions, they usually exacerbate radiation-induced performance decrements.[43,44] Groups of drugs are being developed that will, perhaps, stabilize performance by modulating cellular permeability, altering regional blood flow, and interrupting the release or action of various mediators. Drugs are being identified that can modulate postirradiation emesis, ETI, and other performance decrements.

Radioprotectants and Supportive Therapy

Radioprotectants will be most effective in personnel exposed to radiation doses within the ranges required to produce the hematopoietic subsyndrome (approximately 2.0-8.0 Gy) and mild gastrointestinal subsyndrome (approximately 8.0-10.0 Gy), and in whom no associated injuries are present. In the event of more severe radiation injury, or if radiation injury is combined with traumatic or burn injuries (a likely occurrence on the battlefield), then radioprotective measures alone will be insufficient, and additional supportive therapy will be required. Although the effectiveness of radioprotectants may be reduced in the face of more severe radiation injury or combined injury, their use at the time of irradiation will likely increase the effectiveness of supportive therapies provided days later.

Traumatic injury can reduce the ability of pharmacological agents to increase survival (Figure 11-9).[16] Mice that were given TDM dissolved in squalene within 2 hours after exposure to 10.25 Gy of cobalt-60 gamma radiation were protected against the infectious consequences of this exposure (70% survival versus 5% in vehicle-injected control animals). However, this protection was not seen in animals given a 1.0-by-1.5 inch skin wound and irradiated with only 8.5 Gy. In the irradiated and wounded mice, death began to occur about 1 week earlier than in the irradiated-only animals, and all mice died at the same rate regardless of treatment with TDM.

This difference in protective response between irradiated-only and combined-injury animals may be due to a more profound immunosuppression (Figure 11-10) and/or physiological perturbations. To avoid infection, the natural and artificial defenses must be in balance so that host resistance is sufficient to control the number of microorganisms. Therefore, as normal defenses are compromised (suppression), artificial interventions (enhancement) are required to maintain the resistance above the threshold for infection.

The potential synergy between therapeutic agents, such as antibiotics and substances that may be used as radioprotectants is indicated by recent data on the use of glucan and the antibiotic pefloxacin in the management of postirradiation mortality.[74] In this experiment, only 30% of mice given 7.9 Gy of whole-body cobalt-60 gamma radiation survived. Treatment with glucan alone at 1 hour after

irradiation, or incorporation of pefloxacin in the animal's drinking water for 24 days after exposure, had little or no effect on survival. However, if the two treatments were combined, survival was greater than 90%.

DEVELOPMENT OF A RADIOPROTECTIVE REGIMEN

A variety of factors must be considered in evaluating and developing candidate radioprotectant drugs for military use, and a compromise must be reached between the ideal and the achievable (Table 11-7).

Level of Protection

The problem of defining a suitable minimum level of effectiveness in promoting survival is still not fully resolved. For many years, the goal was that pharmacological agents should have a DRF of at least 2 against exposure to gamma radiation. This goal may have caused promising drugs with lower DRFs to be overlooked. Protection at a DRF greater than 2 is achievable in the laboratory, but the required doses produce side effects that are unacceptable in type and severity for military use.

Although it may not be possible to field a first-generation drug with a DRF of 2, it is likely that one can be developed with a DRF of less than 2. A reasonably achievable lower limit would be a DRF of about 1.4. Although this level of mitigation is generally considered to be low to moderate, it is far from trivial. First, because the animals used in research are immunologically naive, a laboratory-derived DRF of 1.4 is likely to underestimate the degree of protection actually realized in the field by normal, healthy persons. Second, as discussed previously, it is likely that many cases of accidental exposure will be accompanied by some degree of partial shielding. In light of evidence that even small amounts of partial shielding are beneficial, shielding should augment the effectiveness of pharmacological agents. Third, the difference between a dose of radiation that is lethal to 95% of a population (LD_{95}) and a dose lethal to 5% (LD_5) is commonly less than 1.0-2.0 Gy. Therefore, the use of an agent with a DRF of 1.4 for most species (including humans) can result in a reduction of the LD_{95} to a value near the LD_5. For persons exposed to doses greater than those resulting in LD_{95}, the use of an agent with a DRF of 1.4 may mean the difference between life and death, especially if even minimal postirradiation therapy is available. Over a large population, the net effect is a substantial increase in survival. It is possible that this level of protection might be achieved with minimal side effects.

Toxicity

Side effects are a major obstacle to the fielding of agents to mitigate radiation injury. Acute side effects (such as nausea, vomiting, and hypotension) are common, especially with the sulfur compounds. For a fieldable drug, any acute side effects will have to be reduced in severity so that military performance is not impaired. If that is not possible, these effects should be at least controllable by other conveniently applied therapies.

In addition, these agents must not significantly increase the user's vulnerability to chemical or biological agents or antidotes, exacerbate other battlefield injuries, negatively affect behavior, or interfere significantly with wound healing. The agent should have a wide safety margin (therapeutic index) to compensate for the "if one is good, then two must be better" philosophy.

Deliverability

A prime requirement for an agent that will be used by many people under battlefield conditions is that it be easily self-administered. The route of administration, drug dose, and simplicity of schedule are important. Oral administration is the most desirable route, but this may be difficult to achieve, at least for a first-generation agent. Transdermal administration (for example, via a dermal patch) is also acceptable, but is limited by the fact that only microgram or smaller quantities of the drug can be delivered. Most of the agents under study are effective in milligram to gram quantities. The major exceptions are the cytokines, which are effective in very small dosages, and may be administrable by the transdermal route. The transdermal route may have greater applicability for second- and third-generation agents developed via the genetic approaches described above. The next most acceptable route of administration is sublingual. The least acceptable practical method is intramuscular injection. Intravenous and subcutaneous injection and suppository administration are unacceptable routes for a self-administered field-deployed drug.

For oral, sublingual, or intramuscular administration, the drug dose must be small enough to be dispensed as a reasonably sized tablet or capsule, or in a manageable volume. If taken as a liquid, either orally or parenterally, the agent must also be soluble and stable in a vehicle that is appropriate for administration. Finally, for simplicity of use in the field, the agent should be designed as a single treatment, rather than a regimen of two or more different and sequentially spaced medications.

Other Factors

Ease of administration, simplicity of dose schedule, minimal side effects, and a wide safety margin are particularly important because it may be necessary to take a radioprotective drug repeatedly for several days. The agent should be

compatible with the other drugs and antidotes available to the soldier in the field, and it should have optimum effectiveness for an adequate duration (up to 6 hours following a single administration). Two hours of effectiveness should be considered the minimum. Finally, the agent should be formulated and packaged so that it has a shelf life of at least 5 years, to allow stockpiling. It should retain its potency under a wide variety of adverse conditions and, for an injectable, should not deteriorate in solution.

SUMMARY

Historically, the development of radioprotective agents has been dominated by the study of sulfhydryl compounds, particularly the aminothiols. These compounds function by a variety of mechanisms, almost all of which increase survival in the irradiated organism by minimizing the radiation-induced damage to critical biological molecules. The ability of aminothiols to provide high levels of protection has been demonstrated repeatedly. However, as a group, these compounds suffer from one major drawback: high levels of protection have been achieved only at doses that are accompanied by unacceptable side effects. Therefore, it has been necessary to look at less-toxic compounds in the search for a radioprotective agent.

Among the candidates being evaluated are naturally occurring dietary components (selenium and vitamins E and A) and drugs of low toxicity that are being used clinically (such as MPG). The drawback to these latter agents is that, generally, the protection achieved is relatively low.

The net effect of protective compounds, such as the aminothiols and dietary components, is an increase in the number of stem cells and progenitor cells that survive the initial radiation insult. To exploit this early benefit, agents that stimulate the proliferation and differentiation of those cells would help effect optimum repopulation of the organ systems that were depleted by radiation-induced cell death. The use of such regeneration agents (such as immunomodulators and cytokines) alone has been shown to enhance survival after irradiation, although the effect is relatively low. But when these agents are administered along with a protective agent, additive and synergistic effects are seen. Most important, these effects are often achieved using subtoxic doses of the individual agents.

Combining those agents that function in the protection or repair strategy with those that function in the regeneration strategy offers the advantages of *(a)* circumventing the side effects of aminothiols, *(b)* enhancing the effectiveness of relatively nontoxic agents that provide only mild protection when given alone, and *(c)* maximizing the therapeutic benefit provided by each agent.

The use of pharmacological agents to increase survival after irradiation will be most effective for personnel exposed to low or intermediate doses of radiation who have minimal associated traumatic or burn injuries. Indeed, in a mass-casualty situation, these agents may be the only type of medical intervention that is available. On the other hand, with smaller numbers of casualties, especially those with combined injuries, it is likely that additional supportive therapies will be available. When considered in this context, radioprotection should be thought of as part of the holistic management of radiation injury (Figure 11-11) Here, in the face of increasing injuries, various dose-reducing events occur to minimize the effect of the injury. The early application of radioprotector agents will minimize the need for subsequent interventions and will enhance the effectiveness of the interventions that are provided.

Many factors must be considered in defining the desired properties of a potentially fieldable first-generation agent. Since the development of WR-2721, emphasis has been placed on studying agents that produce DRFs greater than 2. This emphasis may actually have hampered efforts to field a suitable agent. Some agents with lower DRFs can provide significant protection and may be more appropriate for field use. Thus, the DRF used in evaluating a radioprotective drug need not be the maximum obtainable. Rather, the DRF should be that obtainable at doses resulting in minimal acute side effects and behavioral toxicity. The agent should also have a high therapeutic index, because it will most likely be self-administered. Whether or not the agent can be taken orally is an important consideration.

Based on the strategies and candidate agents now available, it should soon be possible to recommend several protective agents that sufficiently meet the requirements. After an agent has been recommended, it will be evaluated as a first-generation field-usable radioprotective drug (or drug combination) in humans. The agent should have a DRF greater than or equal to 1.4 and be effective when given as a single oral or intramuscular dose. The agent will probably be a combination of at least two of the candidate agents described above. Regardless of the number of candidates in this combination, it is likely that at least one will be a protective agent and one will be a regeneration agent.

Fielding a first-generation agent that satisfies most of the requirements discussed above is an achievable near-term goal that will satisfy, at least in part, a critical immediate need of the armed forces. Success will depend on making intelligent choices from the many available agents.

However, it is critical to note that fielding this first-generation agent is only an initial step. Much work needs to be done to develop an agent that is effective against high-LET radiation. This need will become increasingly urgent as the human presence in space expands. Second- and third-generation agents will be developed only through intense studies that are aimed at defining the mechanisms

of radiation injury on the molecular and cellular levels and determining how organisms can be stimulated to protect themselves against this injury.

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