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Animal Models for Medical Countermeasures to Radiation Exposure

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Abstract

Since September 11, 2001, there has been the recognition of a plausible threat from acts of terrorism, including radiological or nuclear attacks. A network of Centers for Medical Countermeasures against Radiation (CMCRs) has been established across the U.S.; one of the missions of this network is to identify and develop mitigating agents that can be used to treat the civilian population after a radiological event. The development of such agents requires comparison of data from many sources and accumulation of information consistent with the "Animal Rule" from the Food and Drug Administration (FDA). Given the necessity for a consensus on appropriate animal model use across the network to allow for comparative studies to be performed across institutions, and to identify pivotal studies and facilitate FDA approval, in early 2008, investigators from each of the CMCRs organized and met for an Animal Models Workshop. Working groups deliberated and discussed the wide range of animal models available for assessing agent efficacy in a number of relevant tissues and organs, including the immune and hematopoietic systems, gastrointestinal tract, lung, kidney and skin. Discussions covered the most appropriate species and strains available as well as other factors that may affect differential findings between groups and institutions. This report provides the workshop findings.

INTRODUCTION

As a result of the Project Bioshield Act of 2004, the U.S. government has initiated several programs, including one that established eight Centers for Medical Countermeasures against Radiation (CMCRs), under the purview of the National Institute of Allergy and Infectious Diseases (NIAID) with National Cancer Institute (NCI) involvement. Since a focus of these

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Centers is to develop agents for prophylaxis, mitigation and treatment of radiation injury, the testing of such countermeasures inevitably requires the use of animal model systems. The development and importance of animal models in radiation research has a historical context. In the 1950s, public concerns about the peaceful and military deployment of atomic power led to large-scale, mission-oriented research into the genetic consequences of radiation exposure. Most of this effort was performed in the U.S., Britain and Germany and was directed toward low-dose radiation experiments in inbred mice with the aim of evaluating the genetic risk of radiation exposure. While not fully successful in achieving their goal, these studies had a profound effect on biomedical science, laying down the foundation for research into human genetics, transplantation, cancer, immunity and other fields of scientific endeavor (1). A similar level of productivity from the research infrastructure that is currently being established would be expected to advance many fields of science, not just those in the radiation sciences.

Since their inception, the CMCRs have acknowledged the need for standardized animal model systems so that agents can be compared for efficacy in prophylaxis, mitigation and treatment of radiation injury within a framework that is consistent with Food and Drug Administration (FDA) requirements. In particular, the test systems must satisfy the FDA Animal Efficacy Rule, which details the evidence needed to demonstrate the effectiveness of new drugs in situations where human studies are not ethical or feasible (2). The Rule provides guidance for the experimental design of supportive and pivotal efficacy animal studies leading to the approval of drugs and/or biological agents and states that

1. there must be a reasonably well-understood pathophysiological mechanism for both the toxicity of the threat agent and its amelioration or prevention by the countermeasure under study;
2. the animal study end point must be clearly related to the desired benefit in humans, which is generally the enhancement of survival or prevention of major morbidity;
3. the data or information on the pharmacokinetics and pharmacodynamics of the product or other relevant data or information in animals and humans must be sufficiently well understood to allow for the selection of an effective dose in humans.

Past comparisons between agents with respect to efficacy have been hindered by the wide variations between different animal models with respect to species, strain, dose, dose rate, time, end point, level of supportive care, husbandry and protocol design. The CMCRs therefore jointly sponsored a workshop, “Animal Models for Medical Countermeasures,” held on January 18–19, 2008, in San Antonio, TX, with the aim of furthering the standardization and validation of animal models for radiation exposure; this workshop built upon a workshop held in 2003 (3).

The principal focus of the workshop was building a consensus with respect to the best animal models, assessment tools and end points for each of the organ systems considered to be most at risk after moderate radiation exposures. To streamline this process, the working group accepted a number of principles:

1. A consensus regarding radiation exposure conditions for testing was regarded as being essential. There was recognition that, as part of a nuclear incident, a body would be unlikely to receive a uniform dose and that there would be many other confounding variables, such as dose rate, concomitant exposure to burns or trauma, and infections resulting from immune suppression, as well as availability of timely supportive care. However, the efficacy of any medical intervention can be evaluated properly only in highly reproducible, well-controlled animal model

systems, and our goal was evaluating models in large part within the context of response to a uniform, relatively homogeneous whole- or partial-body exposure to moderate radiation doses at relatively high dose rate.

2. The organs at most risk were considered to be the immune system, the hematopoietic system, gastrointestinal tract, kidney, skin and lung. This breadth of scope resulted from acknowledging that moderate doses of total-body radiation compromise all organ systems to a greater or lesser extent and that little is known as to how the systemic effects of radiation exposure influence local organ function or how damage to one system affects others. Indeed, the dose hierarchy of failure for different tissues with time after radiation exposure dictates that, if early failure of one organ is prevented, another tissue could later fail (4). The well-established association between tissue turnover time and time to failure was also recognized.
3. Last, it was accepted that some approaches to the prophylaxis, mitigation and treatment of radiation injury might be organ-specific and that there is therefore a need to test agents in more than one model system, if for no other reason than that a compound that may mitigate damage to one organ system may compromise another.

Other General Discussion Points

There was overall agreement as to how best to evaluate the efficacy of an agent, either as a protector given before exposure or as a mitigator given after irradiation, based on sound radiobiological principles. For example, in situations where one type of target cell dominates the response, *in vivo* dose–response curves for a given normal tissue end point are known to be sigmoid and very steep, increasing rapidly with dose above a threshold; this is consistent with deletion of clonogenic cells within functional subunits. However, one consequence of steep dose–response curves is that what appears to be a dramatic increase in survival may in fact reflect only a small degree of sparing in terms of total dose. Thus animal survival after total-body irradiation (TBI) can increase from 37% to 87% with a change in dose of little more than 2 Gy (5). It was agreed, therefore, that the best way to measure effectiveness is in terms of the dose-modifying factor (DMF), i.e., the dose required for a given effect in the experimental group divided by the dose to give the same effect in the control group. As a point of reference, at a 50% survival level, one might expect an effective radioprotector or mitigator to have a DMF in excess of 1.15. A further consideration was that the steepness of the sigmoid curves might decrease with added drug treatment, which could indicate some additional source(s) of heterogeneity, such as might come from combined contributions to death from both bone marrow and intestine. Under such circumstances, a 50% survival rate alone may not be the best measure, and therefore effectiveness should be assessed using full dose–response curves.

Many other variables also were the subject of lively discussion over the 2 days of the workshop. Parameters such as age and sex of the subject, the quality of the radiation, the dose rate at which it is delivered, the homogeneity of dose and degree of shielding, microbial status, and supportive care were considered as important issues that need to be taken into account. While some of these issues are summarized in the general discussion, this report does not aim to be all-inclusive, but rather to focus more on its major goals: (1) to identify the animal models in which the radiation responses were best defined for each organ system and evaluate their utility, including dose/time/end-point relationships, genetic influences, the availability of animal strains and species, and the most relevant end point for meeting FDA requirements for human use, and (2) to establish the variables that might lead to discordant results between Centers and how best to control them by optimizing experimental designs so as to allow valid comparison between studies.

IMMUNE SYSTEM

The immune system responds rapidly and is inordinately sensitive to TBI, in large part because of the tendency of lymphocytes to undergo apoptosis. Indeed, the rate and extent of the decrease in peripheral blood lymphocyte number over the first few days after radiation exposure, along with clinical presentation and lymphocyte chromosomal aberrations, remain gold standards for establishing the medical management of exposed individuals. These also set the standards against which new alternative radiation biodosimeters must be judged. It is therefore clear that radiation-induced immune suppression and consequent infections are major factors in the acute radiation syndrome (ARS), making the use of antibiotics an essential part of patient management and the search for agents that boost immunity after radiation exposure a priority for the countermeasure programs.

Radiation-induced immune suppression is complex. It involves more than the loss of lymphocytes; other factors, such as altered cell trafficking, blocks in cell maturation, alterations in cellular differentiation and function, failure of a radiation-damaged bone marrow to replenish the immune system, and damage to immune cell “niches,” should also be considered as possible contributory mechanisms. The role played by the recovering immune system in the lymphodepleted host is even less clear, with some indication that this could have a negative impact on other organs, for example the lung (6). A longer-term concern is the impact of a chronically dysregulated immune system on life-shortening, as is seen in the A-bomb survivors.

Time–Dose Relationships and End Points

Decreasing peripheral lymphocyte counts over the first few days are reliable indicators of exposure of humans to radiation doses as low as 0.5 Gy; a 50% decrease within 24 h with a further decrease over 48 h indicates a lethal exposure. Granulocytes can transiently increase after <5 Gy (the abortive rise) before falling to a nadir that is variable in onset, taking 3–4 weeks to develop and possibly resulting in a prolonged neutropenia (7). Radioprotectors might affect the rate and extent of loss of lymphocytes over the first few days, but mitigators are more likely to influence primarily the recovery phase, especially if the agents are delivered a day or two after exposure. Thus, in animals that are receiving a known total-body radiation dose, the time to and extent of recovery of peripheral white cells can be a good measure of the extent of mitigation (8).

The factors that drive numerical and functional recovery of immune cells are still not well known, but cytokines used as mitigators clearly affect immune restoration. For example, the T-cell cytokine interleukin 7 (IL-7) may prevent radiation-induced immune defects in mice, in particular thymopoiesis (9). The use of immunodepletion combined with adoptive transfer of cells in preclinical and clinical models sheds light on some of the microenvironmental influences on recovery of the immune system after TBI. Under such circumstances, adoptively transferred T cells “rebound” in number and function depending on the extent of immunodepletion. Multiple mechanisms have been implicated, including radiation-induced depletion of regulatory T cells (Treg) and myeloid-derived suppressor cells that maintain homeostasis, increased production of cytokines, and immune cell stimulation by microbial products that translocate across the radiation-injured gut (10,11). “Rebound” is also likely to occur in residual T cells in an irradiated host. The possible stimulatory effects of microbial products offer several rationales for targeting Toll-like receptors (TLR) for radioprotection and mitigation, an approach that has been shown to have merit (12). The gut microbiota can play either beneficial or harmful roles during recovery after TBI, even in the absence of overt infection, and can influence the outcome in many different radiation scenarios.

The long-term effects of radiation on the immune system have been studied most notably in the A-bomb survivors. Reductions in mitogen-dependent proliferation and interleukin 2 (IL-2) production, decreases in helper T-cell (Th) populations, and increases in blood inflammatory cytokine levels have been observed (13), indicating that immune recovery may result in disturbed homeostasis with a chronic inflammatory component. In mice, alterations in the Th1/Th2 balance after TBI have been noted and ascribed to radioresistant natural killer (NK1.1) T cells (14), although further research is needed into how the immune system rebalances after exposure to different doses of radiation, and the roles of genetic and environmental influences. The involvement of the immune system in radiation-induced dysfunction in other tissues, such as lung, also requires further research.

While the simplest end point to assess radiation damage to the immune system is differential cell loss, it is clear from animal experiments that functional immune suppression is not due solely to cell death. For example, dendritic cell antigen-presenting function can be affected by radiation in both positive and negative ways and the maturation of these cells can be altered, biasing responses to some antigens (15,16). At this time, it is unclear what other immune subsets have their functions affected during and after recovery. Overall, the complexity of the immune system, with its many checks and balances, suggests that TBI is best thought of as an acute disturbance in the equilibrium between the different subpopulations and that, with time, there is a reprogramming process that has a strong likelihood of resulting in an imbalanced and disturbed functionality.

Variables

Lymphocyte subsets vary in their radiation sensitivity, so that the subset population numbers change differentially with time after exposure. An approximate rank order may be B cells > Treg > Th > cytotoxic T > memory T > NK cells, with differentiated cells such as macrophages, dendritic cells, granulocytes and mast cells showing less effect, at least in the short term. This ranking does not take into account the recovery times for the distinct cellular subsets, and the situation is further complicated by the fact that each immune cell subset itself contains a relatively radioresistant subpopulation (17,18) of varying size. The attributes that confer radioresistance on the subsets are not clear, but it is known that activation can dramatically decrease radiosensitivity, while age and sex also influence the extent of radiation-induced lymphocyte apoptosis (19,20). The differential sensitivity of lymphocyte subsets has functional consequences. B-cell-dependent, T-cell-independent antibody production is particularly sensitive to radiation (21), while immunological memory is relatively radioresistant. Thus vaccination, even just before exposure, may improve postirradiation resistance against a specific microorganism. In addition, $CD8^+$ T cells are frequently found to be more sensitive to radiation-induced apoptosis than $CD4^+$ T cells (22,23).

Species and Strains

Immune responses to a given antigen vary markedly between mouse strains; some strains seem biased toward making responses of a Th1-type, typified by cell-mediated responses that produce pro-inflammatory cytokines, while others prefer Th2-type responses that drive antibody secretion. Similar diversity exists between humans. The impact of this diversity in immune status on tissue radiation responses is not clear, but there is likely to be some overlap with the genes that influence tissue radiosensitivity (24). Genetic control over radiation-induced lymphocyte apoptosis has been suggested in humans (13,25), and it is likely to contribute to the considerable variation in the extent and rate of loss of cells after TBI (19). Thymocytes (26) and splenocytes (27) as well as crypt cells (28) of C57BL/6 mice are more sensitive to radiation-induced apoptosis than are those of the C3H/He strain. One should remember, however, that there is considerable variation in the sensitivity of

lymphocytes to apoptosis in different tissues (27) that depends on their location within the tissue and their activation status.

Other genetically determined differences between mouse strains have been documented that could affect tissue responses after irradiation. Some of these are due to spontaneous mutations, such as the TLR 4 mutation that makes C3H/HeJ mice resistant to endotoxin and to the radioprotective effects of this agent (29). In general, these strain differences suggest that radioprotectors and mitigators of the immune system should be tested in more than one mouse strain, preferably the C57BL/6 and C3H/HeN strains, for which the most data are available and that are known to demonstrate divergence in many tissue responses after irradiation.

Although the majority of studies on the effects of radiation on the immune system have been performed in rats and mice, other animal species have also been used. Canines and nonhuman primates are the most widely used subjects, although information comes more often from studies that are not primarily radiobiological, such as experiments on allogeneic bone marrow transplantation. The development of antibodies to markers of specific immune cell subpopulations has been slower for species other than mice, but the situation has improved considerably in recent years.

The beagle has been favored as a canine model for radiation studies on the hematopoietic and immune systems. Detailed investigations of the effects of ^{239}Pu inhalation found peripheral lymphopenia (30) and persistent T-cell dysfunction, although pulmonary immune responses to antigen seemed little affected (31), perhaps indicating the difficulty in extrapolating from systemic to local immune parameters. Long-term decreases in lymphocyte levels in dogs after external TBI also have been documented (32). In one interesting study, prenatal TBI (1.5 Gy from ^{60}Co) reduced primary humoral antibody responses and peripheral Th cells at 3 to 4 months of age (33). This was ascribed to radiation-induced defects in thymic development.

Rhesus macaques have been subjects of investigations of postirradiation hematopoiesis, with and without bone marrow transplantation. In the transplant setting, antibody and cytotoxic T-lymphocyte responses to foreign antigen challenge have been documented (34), although persistent immune dysfunction has also been found (35). In addition, sublethal TBI has been shown to suppress responses to simple antigens (36) and to vaccination with attenuated Venezuelan equine encephalomyelitis vaccine virus (37). The panel considered that more use could be made of existing nonhuman primate models of infectious disease, in particular through collaboration with scientists in the wider Project Bioshield research programs, to examine combined effects of radiation with potential infectious threats.

Summary

The primary immune system end point after TBI remains lymphocyte counts over the first few days of exposure and recovery over time. Functionality may be inferred in part from counts and phenotyping of peripheral cells, but it is unlikely to reflect the true immune status accurately. The most obvious acute consequence of immune suppression is bacterial sepsis, but subacute and long-term consequences of TBI are likely in the form of a persistent immune imbalance. This may result in viral reactivation or contribute to chronic inflammatory conditions that lead to life shortening. When testing radioprotectors or mitigators, more than one mouse strain should be used, and the C57BL/6J and C3H/HeN strains are the suggested choices because they appear to represent the spectrum of immune systems reasonably well. The canine model is popular for radiation inhalation studies, but the nonhuman primate would be the second model of choice for testing the effectiveness of radiation injury countermeasures on immune function.

HEMATOPOIETIC SYSTEM: ANIMAL MODELS FOR THE BONE MARROW

The hematopoietic or bone marrow syndrome is the most classically recognized ARS and appears at the lowest end of the dose range (1–6 Gy). Hematopoietic cells are highly sensitive to radiation damage and relatively low levels of exposure can result in bone marrow failure and potentially lethal hemorrhage or infections. The damaging effects of radiation on hematopoiesis have been well established (38–40), and animal models have been highly valuable in characterizing them (3,41–43). Indeed, it is because of the high sensitivity of the hematopoietic system to radiation relative to other organ systems that TBI has been developed and used successfully as a therapeutic conditioning regimen for patients with hematological malignancies undergoing hematopoietic cell transplantation (44).

However, despite the large body of research designed to assess the effects of radiation on hematopoiesis, specific therapies aimed at improving the acute survival of exposed individuals are lacking, and additional research is needed focusing on mitigating damage in the hematopoietic system (42,43). In particular, approaches that do not involve allogeneic hematopoietic cell transplantation are required for victims of radiation exposure who have not been exposed to marrow-ablative total-body radiation. Alternatively, in situations where transplantation truly is necessary, approaches that would improve the outcome when a major histocompatibility complex (MHC)-matched donor is not immediately available would be valuable.

Time–Dose Relationships and End Points

The hematopoietic syndrome after exposure to radiation is defined as the development of neutropenia, thrombocytopenia and anemia (38,39) and is due to the radiosensitivity of committed progenitor cells in these lineages (45,46). The time to appearance of each symptom is dependent on the various transit times from stem cell to mature functioning cell for each of the hematopoietic elements. With progressively increasing doses of radiation, the time to each of the deficits decreases slightly (38,39), and without recovery death will occur. The time to appearance of each of these symptoms, the extent of the induced deficit (nadir in the cell population count), and the time to recovery (return to baseline) of each of the cell lineages have been used as secondary end points to assess the extent of hematopoietic injury.

Because of the temporal predictability of the development of the above symptoms after TBI and the known corresponding dose requirements, the most widely used end point for acute radiation damage in the hematopoietic system is the LD_{50}. In humans, this is generally within the dose range of 2 to 8 Gy (~4 Gy in healthy adults with no medical intervention) (47), with the peak incidence of death occurring around 30 days after exposure, although deaths may continue up to 60 days postirradiation. The end point is therefore defined as the $LD_{50/60}$, i.e., the lethal dose that results in the death of 50% of the affected population within 60 days of irradiation. In mice, the temporal sequence and outcome of the bone marrow syndrome are significantly faster (death occurs within 30 days of irradiation), and the end point is defined as the $LD_{50/30}$ in mouse models.

Pluripotent hematopoietic stem cells in the bone marrow can survive doses of radiation that result in the hematopoietic syndrome, although with each incremental increase in dose, the numbers of surviving stem cells are reduced and the time to recovery of the multiple blood cell lineages is increasingly delayed. In principle, endogenous hematopoietic recovery could occur in humans after exposures to up to 9 or 10 Gy (single fraction); however, an extended period of pancytopenia begins as early as 4 to 7 days after significantly lower doses (13) and, to survive, a prolonged regimen of intensive supportive care, including broad-spectrum antibiotics and frequent transfusion of blood components, must be administered. Despite improvements in supportive care together with treatment using cytokines such as

granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), mortality from accidental radiation exposures of >5 Gy remains high (39,48). One explanation is the increased risk of overwhelming opportunistic infections, primarily bacterial pathogens translocated from the gastrointestinal tract.

To reduce the periods of profound neutropenia, thrombocytopenia and anemia, hematopoietic cell transplantation has been used in clinical settings as well as in a limited number of recent radiological accidents (44,49). The major complication of allogeneic hematopoietic cell transplantation is graft versus host disease (GVHD) due to alloreactive donor T lymphocytes causing potentially life-threatening normal tissue damage. The incidence and severity of GVHD is particularly increased if the donor and recipient are not human leukocyte antigen (HLA)-identical. Given the likely limitations in donor availability and compatibility after a terrorist event, animal models used to investigate mitigating agents in the hematopoietic system should include those that also would facilitate improvements in cell transplantation procedures.

Variables

Multiple animal models of the hematopoietic syndrome have been developed to study radiation countermeasures that could enhance survival after TBI (3,41–43). The working group suggested that to meet the requirements of the FDA Animal Efficacy Rule, optimal animal models should exhibit end points that closely resemble the known outcome of human exposure to radiation. Animals in all intra- and inter-institutional comparative studies should be irradiated during the same period of the day, either morning or afternoon, since known variations in the circadian rhythms of hematopoietic stem and progenitor cells can result in different outcomes. Finally, if possible, the bone marrow ablation and immunosuppression should be induced by a radiation delivery system that would mimic what might be expected to occur during a terrorist attack or other nuclear incident in terms of dose, dose rate and dose quality.

Appropriate statistical design of the studies is critical since the criteria for euthanasia must be defined and approved by each local Institutional Animal Care and Use Committee (IACUC), especially with respect to the use of large animal models, which necessarily involve U.S. Department of Agriculture regulated species. Following consistent criteria for euthanasia will lead to consistent measures of survival after irradiation. In addition, sample integrity must be maintained when euthanizing animals, and tissue samples should be obtained just prior to or immediately after euthanasia.

Species and Strains

Three species have been used in the majority of radiation studies on the hematopoietic syndrome; these are the mouse, canine and nonhuman primate (3,41–43). Several strains of mice have been used, the beagle is the predominant canine and the rhesus macaque (*Macaca mulatta*) is the major nonhuman primate. Cynomolgus macaques (*Macaca fascicularis*) have been used, but there is no published dose–response curve for lethality.

Most researchers have employed inbred mouse strains, although several have used hybrid strains, and there has been consideration of outbred strains. The most common mouse strains used are BALB/c, C3H/HeN, B6D2F1/J and C57BL/6. These strains show considerable variation in their response to TBI, as demonstrated by their range of $LD_{50/30}$ (6.5–9 Gy), with the BALB/c being most sensitive and the C57BL/6 most resistant. The BALB/c strain has been reported to have a double-strand DNA relative repair defect (polymorphism in DNA PKcs) that may account for its enhanced radiation sensitivity (50). Such strain variables have led to difficulties in directly comparing drug efficacy results in different

mouse strains, and the consensus small animal model should include at least two strains, such as the C57BL/6 and C3H/HeN strains.

As described above, the hematopoietic end point in the mouse is measured as survival at 30 days after TBI. However, whether 30 days is a sufficiently valid end point is still subject to debate, since prolonged neutropenia and thrombocytopenia may cause mortality at later times. In addition, "drift" in the $LD_{50/30}$ may occur within any laboratory. Therefore, the Workshop recommends that every laboratory establish the lethal dose–response relationship for each of its chosen mouse strains at least twice every year. It is also appropriate in this regard to design each study testing agent efficacy using radiation doses across the hematopoietic syndrome, e.g., $LD_{30/30}$, $LD_{50/30}$ and $LD_{70/30}$.

Two of the CMCR research groups provided decades of experience using the beagle and rhesus macaque as large animal models. These are well-characterized models for which the lethal dose–response relationships for the hematopoietic syndrome have been well established (51–53). Because of the increased life span and demand of hematopoiesis in a large animal (54) and because of the similarity in effect of supportive care in large animals and humans, it is possible to link the dose–effect relationships between the canine, nonhuman primate and human. Each animal can be followed sequentially, and the use of supportive care, comparable to that used in humans after myeloablative TBI conditioning for hematopoietic cell transplantation, is possible and recommended. These aspects underscore the usefulness of these well-characterized models for evaluating the response of the human hematopoietic system to radiation and its treatment.

Supportive care will be the "standard" of care for lethally irradiated personnel in the aftermath of a nuclear event. The database for large animals, both canine and nonhuman primate, is consistent and substantial in defining the efficacy of supportive care in enhancing the survival of lethally irradiated animals (42,55). Due to its efficacy, supportive care may well be the most effective radiation mitigator currently available. Supportive care in large animals includes (a) intravenous fluid/electrolyte support, (b) a stepwise algorithm for introduction of broad-spectrum antibiotic treatment in response to specific clinical signs and symptoms (56), and (c) transfusion support with irradiated blood products. Of note, large animal studies are not conducted in gnotobiotic settings. In contrast, supportive care in mice is more problematic and relies primarily on the use of acidified water (the use of defined gastrointestinal flora and barrier caging may also be considered as forms of supportive care). Since the FDA Animal Efficacy Rule requires a linkage between the animal models and the treatment protocol for humans, a number of investigators are currently assessing the utility of selected antibiotics and fluid administration in mouse lethality models of the hematopoietic and gastrointestinal syndromes to provide a more accurate baseline for assessing additional mitigating agents.

Summary

The most widely used and well-characterized end point for the assessment of acute hematopoietic radiation damage after TBI is the LD_{50}, measured over the appropriate period for the selected animal model (i.e., 30 days for the mouse model, 45–60 days for larger animal models). In addition, the time to onset, depth of the nadir in population counts, and rapidity of recovery time of each of the hematopoietic components, in particular the granulocytes and platelets, are considered useful secondary end points, offering an alternative to the use of "death as an end point" for the assessment of targeted mitigating agents. The mouse was deemed the most appropriate species for testing radioprotectors or mitigators in a small animal due to the breadth of the current literature, although more than one mouse strain should be used; the C57BL/6J and C3H/HeN strains are suggested. Both

the canine and the nonhuman primate were considered appropriate large animal models to test hematological countermeasures.

GASTROINTESTINAL TRACT

After injury to the bone marrow, the gastrointestinal (GI) tract ranks as the organ most sensitive to moderate-dose radiation exposure that can cause ARS (57). In fact, if part of the bone marrow is shielded, then acute radiation injury to the GI tract can become dose limiting for survival (58). As a consequence, radiation-induced injury of the GI tract has been a topic of radiobiological interest for decades.

The classical “target cell” model of intestinal radiation toxicity attributes radiation injury to death of clonogenic crypt epithelial stem cells (59,60). Other elements within the complex environment of the intestine also contribute to organ dysfunction: the enteric muscularis, immune system, microvasculature and nervous system, as well as the complex community of resident bacteria and fungi. A more pathobiologically based model of intestinal radiation injury emphasizes the subsequent functional consequences of cell loss, such as protein modifications and changes in redox status, as well as secondary effects due to inflammation and release of cytokines (61), while a more physiological approach emphasizes early vomiting and diarrhea as common gut-related symptoms of exposure that can greatly exacerbate fluid loss problems.

Although all cellular compartments may contribute to and modulate organ dysfunction, the key event in the pathophysiology of intestinal radiation toxicity is enterocyte depletion, with possible vascular damage contributing at higher radiation doses (62). Enterocyte depletion can lead to mucosal barrier breakdown, mucositis and secretory diarrhea. Indeed, death from pure GI radiation toxicity after total abdominal irradiation or TBI is the result of massive fluid and electrolyte loss (63), indicating a primary need for supportive care in humans. Even at radiation doses below the threshold for the full-blown GI syndrome, mucosal barrier breakdown allows bacteria to translocate into the circulation, which can cause sepsis and death in the setting of concomitant immune suppression (64). In the longer term, tissue remodeling subsequent to radiation damage alters the structure, motility and absorption of the gut, and fibrosis renders it more rigid and susceptible to adhesions, stenosis and perforation (65).

Time–Dose Relationships and End Points

Lethality as a result of intestinal failure is the primary end point for studying the GI syndrome, although the most rapid intestinal response in humans to TBI is vomiting, which can occur within minutes to hours after exposure, with the time to onset being dose-dependent; diarrhea may also occur within 48 h after higher-dose exposure. The classical histological end point in mice is the number of regenerating crypts measured in the intestine at defined times (small intestine: 3.5 days; colon: 5.5 days; stomach: 10 days). These clonogenic assays are highly quantifiable and repeatable; however, intestinal crypt cell survival does not necessarily correlate with animal survival due to the contribution to survival of possible concurrent damage to the immunohematopoietic system (58). Functional assays of GI injury include GI motility and permeability (66), bacterial translocation into the blood stream (67), and plasma levels of citrulline (68). A tertiary end point with currently undefined imaging techniques could be of value and would allow serial studies over the time course of an experiment.

Radiation-induced vomiting as an end point has been studied extensively in dogs by retrograde giant contraction (RGC), which is under the control of the vagus nerve (69). RGC precedes emetic episodes, but it may also occur without subsequent vomiting at lower doses

(69) and is therefore a more sensitive assay than vomiting frequency (70). The mechanisms underlying radiation-induced vomiting and delayed gastric emptying may be different because they do not directly correlate in dogs (71), nonhuman primates (72) or humans (73). Diarrhea is contributed to by giant migrating contractions (GMCs) that propagate rapidly in a caudad direction to propel the gut contents rapidly through the GI tract without allowing sufficient time for reabsorption (70). Abdominal irradiation has been shown to triple the frequency of colonic GMCs, with more than half originating in the small intestine (74). Both small intestinal and colonic GMCs will eventually revert to normal, although repetitive clusters of GMCs can occur for up to a week after exposure and are associated with discomfort and passage of diarrheal stools (sometimes bloody) with nearly every occurrence.

Variables

The current standard end point for studying the acute GI syndrome is animal lethality within 10 days after radiation exposure ($LD_{50/10}$). However, the timing of death appears to vary depending on the radiation dose, the species, strain and genetic background of the animal, the pathogen status of the animal colony, and the volume of the body that is irradiated (75). For example, when mice are exposed to total-body doses approaching those that result in severe enterocyte depletion (15 Gy), lethality after 10 days is principally due to the combined effects of GI toxicity and bone marrow failure (58,76). Intravenous delivery of bone marrow can rescue mice from the hematopoietic syndrome (58,76), as can shielding of 5% of bone marrow, such as one extremity (77); this latter technique may model a likely event in nuclear terrorism, where victims could have part of their bone marrow shielded. Regardless of the technique used for irradiation, when studying a potential radioprotector or mitigator of the GI syndrome, it is important to use radiation in the dose range that decreases crypt survival.

There are many additional potential contributory sources of heterogeneity in the assessment of GI toxicity. For example, the intestinal response to radiation can vary with the time of day (78); therefore, all cohorts of mice within each study should be irradiated at the same time of day. Experimental drift due to other variables may cause the radiation dose needed to induce the GI syndrome to change over time, even within the same laboratory. Thus appropriate controls (e.g., vehicle alone, using the same fluid injection volume) are required for every experiment, and investigators should not rely on historical control groups.

Experiments to study the GI syndrome should use a single fraction of relatively high-dose-rate radiation; fractionated or low-dose-rate radiation exposure will alter the dose–response curve. At least three doses of radiation were recommended: one dose at the $LD_{50/10}$ and the others above and below. However, because of the steep dose–response curves associated with the GI syndrome and the potential variability that may be introduced, it was recommended that rodents not receive supportive care such as antibiotics or saline or that it should be standardized carefully if given. Supportive care is required in larger animals such as dogs or nonhuman primates in accordance with IACUC recommendations.

Species and Strains

The choice of animal model of intestinal radiation toxicity will depend on the specific pathophysiological process being studied. For example, after abdominal irradiation, the prodromal syndrome with vomiting is best studied in the dog or ferret. Rodent models lack this prodromal syndrome, but they are outstanding models for studying enterocyte depletion, changes in absorption and secretion of the bowel, and bacterial translocation. Another major advantage offered by the mouse is the wide availability of strains with specific gene mutations for mechanistic studies. The relatively large size of the rat makes it a better model

for surgical studies in which part of the bowel is exteriorized or placed into the scrotum for segmental bowel irradiation (79). The large animal models used most widely to study intestinal radiation toxicity are the canine and nonhuman primate. Both primates and canines are capable of vomiting and diarrhea, and the associated specific contractions in the GI tract can be used to monitor response to drugs used to mitigate radiation sickness. Finally, both vomiting and diarrhea are important considerations in the development of mitigating agents since they exacerbate fluid losses and may limit the availability of oral mitigating agents for absorption.

For studies of countermeasures to protect against or mitigate the GI syndrome, the mouse is the preferred small animal model because

1. there is a wealth of quantitative information on enterocyte depletion and other effects of radiation exposure,
2. genetically inbred mouse strains limit experimental variability,
3. short gestation (3 weeks) and relatively large litter size (six or more) ensure ready availability of test subjects, and
4. a wide variety of mutant mouse strains exist for mechanistic studies.

No specific mouse strain is necessarily superior; however, as noted previously, there are documented differences between mouse strains with respect to their immune system (Th1 versus Th2), their response to cytokines, and the effect of various radiation response modifiers. The radiation dose that causes the GI syndrome varies considerably between different inbred mouse strains, indicating that there is a genetic component to its regulation (80). For this reason, it was recommended that more than one inbred strain be used to test agents that prevent and/or mitigate the GI syndrome. Radiation countermeasures that show promise in the mouse should then be tested in larger animal models such as the canine or nonhuman primate. Radiation GI toxicity in the dog is similar to that in humans, and efficacy in nonhuman primate models with full supportive care will likely predict efficacy in people similarly exposed to radiation.

Interestingly, colon specimens from human patients who have been treated with radiation have shown changes in overall innervation as well as in subtypes of neurons over time (81). This suggests that neuronal control may contribute to late intestinal malfunction and alterations in motility. The dominant model for mapping the enteric nervous system has been the guinea pig. Because of the wealth of information available on the neurotransmitters of the guinea pig intestine, this model may be used to further study the causes of contractile disorders identified in canine or primate models.

Summary

Candidate countermeasures to the GI syndrome should be identified by their ability to improve acute survival in mice ($LD_{50/10}$). TBI with or without bone marrow shielding or transfer provides suitable models for study providing the dose is high enough to cause crypt depletion associated with the GI syndrome. Appropriate follow-up studies therefore include crypt colony assays and functional assays. An agent should be considered for further study in larger animal models such as dogs or nonhuman primates when the countermeasure in mice

1. shows a radiation DMF of at least 1.15 at the $LD_{50/10}$,
2. shifts the dose–response curve to higher radiation doses by at least 1 Gy, or
3. increases the duration of survival at the $LD_{80/10}$ by at least 50%.

LUNG

The radiation sensitivity of the lung is evident from the fact that it is a dose-limiting organ in patients receiving TBI as part of a bone marrow transplantation regimen (82). This sensitivity was also evident after nuclear and radiological accidents, such as in Chernobyl, where a significant number of the early victims died from pneumonitis or as a direct result of their lung injuries (83). Because of this sensitivity, the lung has been an organ of interest in radiation research for decades; however, such work has frequently proven confounding. The lung is complex with regard to its functional anatomy; both its so-called subacute pneumonitis and its late fibrosis have long temporal progressions measured in months to years, and both responses involve an intimate but inadequately understood involvement of inflammatory and immune cells. In addition, the lung has a strong volume effect (84) with suggestions of regional radiosensitivity (85), it appears to have a “sympathetic” response when other organs are injured (86), and responses have a genetic component (87). Thus, despite a relatively broad historical database regarding the radiation response of this organ and well-defined morphological end points, the modeling of responses by this organ as part of a specific or targeted agent development strategy is highly problematic.

Time–Dose Relationships and End Points

Fortunately, many of the available animal models, including the mouse and the rat, have lung radiosensitivity equivalent to that of humans (88) and have similar timelines for the development of pneumonitis and fibrosis. Inflammatory pneumonitis develops at around 2–4 months after irradiation (89) whereas fibrosis is seen at 4–6 months (90), although there can be significant variations depending on the radiation dose and strain of the animal species used. A similar time course is seen in humans after a course of fractionated radiation; however, both the temporal course and dose response may vary considerably after a single high-dose-rate exposure. The temporal and morphological distinctions between the two lung response phases (pneumonitis versus fibrosis) should be considered when investigating the effectiveness of an agent to be used to mitigate the adverse effects of pulmonary irradiation. Fortunately, the majority of the recognized and distinct pathological changes after radiation injury described for humans in Table 1 (91) are seen in animals.

Because of the relative radiosensitivity of the lung, after a radiological or nuclear incident, those who have survived the ARS through delivery of appropriate supportive care are potential candidates for the development of pulmonary complications. Therefore, animal radiation models should follow the supportive care guidelines for bone marrow described above. However, since the TBI dose range that induces the hematopoietic syndrome in rodents is insufficient to cause pulmonary effects within a reasonable time, the working group proposed the use of a sublethal radiation dose immediately followed by an additional lung-only “top-up” dose. The sublethal doses would be 5 Gy for mice and 4 Gy for rats. An alternative that better controls the involvement of the hematopoietic system would be lethal TBI followed by lung-only top-up doses and by bone marrow reconstitution.

In addition to survival, proposed secondary end points were breathing rate analysis and serial imaging, both of which provide a noninterventional means of assessing lung function. Some groups have had success for decades using breathing rates to assess pneumonitis (92) but more limited success in monitoring fibrosis. Some groups also have successfully used CT (93), although this technique is not widely available and is less well standardized. Cytokine and growth factor expression are induced in the lung after irradiation (94,95) and may be considered as a tertiary end point, although to date no cytokines or growth factors have been shown to be directly causative in the development of pulmonary effects. Nonetheless, the up-regulation of such cytokines as tumor necrosis factor α, interleukins and transforming growth factor β and the accumulation of inflammatory cells at critical times

during progression to the late effects suggest that monitoring these factors may allow us to better understand the pulmonary response and offer potential targets for mitigation, although further work is needed to validate this approach.

Variables

As suggested above, the size of the dose that is required to induce pulmonary effects varies significantly with species and strain. For example, in murine models, investigators have consistently demonstrated that the C57BL/6 strain develops a robust fibrotic pulmonary response 6 months after a thoracic dose of approximately 15 Gy (96). In contrast, at a similar dose level, the C3H/HeN and other alveolitis-prone strains will have developed a lethal alveolitis (97) with significant mortality during the acute 8–16-week postirradiation period. Rats exposed to thoracic radiation in the dose range of 10–15 Gy also demonstrate considerable dose-related mortality (98); however, the development of the injury is relatively slow so that the end point is not reached for >6–12 months. Some investigators have observed a restoration of lung architecture at late times at the lower end of the dose range (~10–12 Gy) (99).

With respect to dose delivery, it was recommended that the radiation dose to the lung should be calculated at the midpoint in all models, although there will be inherent heterogeneities as part of such a calculation. These will be less evident in the small animal models but may need to be corrected for in large animals. The presence in the field of other organs, notably the heart, but also the liver, the thymus and the esophagus, may also affect the pulmonary response. Shaped fields will be necessary in the large animal model, but they are impractical in the small animal in the majority of research facilities, so extrapulmonary influences will need to be accounted for when assessing data. If the TBI + lung top-up model is used, care will need to be taken to deliver the lung dose as soon as is practically achievable after the TBI (or vice versa); otherwise calculations will need to be made to account for the split-dose effect. Delivery to the whole lung may be critical since survival is dependent on the volume irradiated to a lethal dose. To induce significant fibrotic damage in a more timely fashion, some investigators have used higher doses, of the order of 25–28 Gy, but have reduced mortality by administering the radiation to one lung only (100). However, the lung working group agreed that use of a hemithoracic model in the development of radiation countermeasures was inappropriate due to difficulties in interpreting results. Therefore, the group proposed the use of total thoracic doses in the range 10 to 15 Gy to the whole lungs of both mouse and rat models. The dose–response curves in nonhuman primates are less well known and will need to be derived on an institutional basis.

Species and Strain

Researchers investigating lung diseases in general have used a wide variety of animal models; however, a significant majority used either mice or rats. The mouse model has been a prototype for studies of cytokines and chemokines in response to radiation injury (96), whereas the rat has been more widely used in pulmonary function studies (99). Unfortunately, the pathology of the rodent lung differs considerably from that of the human with respect to its lobularity, the relative thickness of the septa and pleura (thin to absent in the rodent), and the blood supply to the pleura (101) (Table 2). Nonetheless, the similarities between the lung radiation dose responses and their timelines to that seen in humans led to both the mouse and the rat being proposed by the working group as preferred small animal models.

As was indicated above, there is a significant genetic component to the pulmonary radiation response (102,103), as is seen by a clear differential in the observed progression of subacute and late effects between certain murine strains (89,103). Mouse strains can be characterized

by their development of subacute pneumonitis/alveolitis as their primary lethal end point compared to those that display a minimal or mild pneumonitic response but a strong late fibrotic lesion (Table 3). The C3H/HeN and C57BL/6 strains, respectively, represent these subtypes, as well as being the two strains most reported on in the literature; therefore, these two strains were proposed as the leading candidates for murine screening assays. The authors are unaware of similar studies characterizing rat strains with respect to their pulmonary response to radiation injury; however, due to their utility in aging studies (104), the group suggested the use of the inbred Fischer 344 strains.

The three leading candidates for a large animal model, the pig, the dog and the nonhuman primate, all have their advantages and disadvantages for radiation lung studies. For example, pig lung has a similar physiology to that of the human, although the literature on its radiation responses after partial- (88) or whole-body irradiation is limited (105). In contrast, there is a wealth of research on the pulmonary response of the dog (106), of which a significant proportion was performed using inhaled radionuclides (107,108). However, as with rodents, the physiology of the dog differs from that of the human, with some investigators even grouping the dog with rodents with respect to its pulmonary anatomy and function (Table 2). Last, despite the similarities in the physiology of the nonhuman primate to the human, few investigators have used this model to study radiation effects in the lung. Nonetheless, given the above list of pros and cons and because of the breadth of data on the hematological response in the nonhuman primate, the lung working group proposed that the nonhuman primate be the large animal model of choice.

Summary

The proposed end points for the lung animal models should distinguish between the subacute versus late effects. They are

1. primary end point: survival,
2. secondary end points: changes in respiratory function and pathology, and
3. tertiary end points: alterations in expression of inflammatory cytokines or cells.

The recommended small animal model is the mouse or rat, although both of these species require strain-appropriate radiation doses to be delivered to the entire thorax. The well-characterized strain differences in the mouse pulmonary response prompts the recommendation that both the C57BL/6 and the C3H/HeN strains should be used since they represent the extremes of the range of observed human lung responses to radiation damage, with the proviso that mitigating agents may be found to be more effective in one strain than the other, depending on the targeted end point (pneumonitis or fibrosis). While both the dog and nonhuman primate satisfy the requirements for the large animal model, the nonhuman primate is the model of choice.

KIDNEY

Much of the concern regarding radiation accidents and radiological terrorism has focused on acute injuries to the hematopoietic system and GI tract. However, the kidney can also be damaged since it is one of the most radiosensitive late-responding organs in humans, with chronic renal injuries occurring after single doses as low as 4.5–6.0 Gy (109). For example, Fliedner *et al*. (110) reported that “very severe” renal injury (acute and chronic) occurred in 13 of 45 radiation accident victims who received doses high enough to cause severe hematological injury, and Maekawa (111) reported that two victims of the Tokai-mura criticality accident developed renal failure as part of their multiple organ system failure.

Time–Dose Relationships and End Points

In humans, renal radiation injury can appear in the first few months after renal exposure or it can manifest itself as late or chronic radiation nephropathy, which can occur years after the exposure and which may not necessarily follow an acute renal event (112). Interestingly, clinical radiation nephropathy has been relatively rare [e.g., in a review article published in 1972 (113), only 151 such cases had been identified], yet the effects of radiation on the kidney can be, and have been, life threatening (114).

Radiation nephropathy can be detected in animal models by most of the same noninvasive assays that are used to detect renal injury in humans: systolic blood pressure (115), blood urea nitrogen (BUN) (116), creatinine clearance (115), glomerular filtration rate (GFR) (12,117), and urine protein (115). In humans, GFR is the preferred noninvasive assay (118), but this is a difficult assay to perform routinely in animals. Fortunately, in radiation nephropathy, BUN and serum creatinine are tightly correlated (119), and data indicate that serum creatinine is a good marker of GFR (118). All of these noninvasive assays are measures of renal dysfunction in general; none are specific for radiation-induced renal failure. In both humans and animals, the gold standard for radiation nephropathy is histopathology (114,120). However, since BUN is tightly correlated with the degree of radiation-induced histopathological injury (121) and is strongly predictive of eventual renal failure (122), BUN serves as an excellent noninvasive surrogate marker.

Variables

The major sources of heterogeneity in assessment of radiation damage in the kidney that have been identified to date are animal age, radiation dose rate, and radiation volume. Data from both clinical (123) and animal (124) studies show that renal tolerance is affected by animal age, so age should be standardized across comparative studies. Renal tolerance is also dependent on dose rate (125), so dose rates should be monitored carefully and preferably kept above 0.5 Gy/min. Renal tolerance also is highly dependent on the volume irradiated (126), so care must be taken to keep the dose uniform across both kidneys.

Species and Strains

At doses below 10 Gy, bilateral renal irradiation (or TBI plus hematological rescue) can produce chronic renal injury in many species. For example, in the rat, radiation-induced renal failure can occur as early as 8 months after a single dose of 9.5 Gy, and renal dysfunction can be observed by 7 months after a single dose of 7.2 Gy (125). In the pig, renal dysfunction has been demonstrated at 3 months postirradiation after a single dose of 7.8 Gy (127). In dogs, detailed dose-response data are not available, although renal dysfunction has been shown at 6 months after a single dose of 10 Gy (128). Histopathological evidence of radiation nephropathy has been observed in Rhesus monkeys 6–8 years after a single total-body dose of 7.2–8.5 Gy (129). Mice appear to be the outliers, with single doses of 12 Gy and above being required to produce significant renal injury in less than about 9 months (130) and with 11–16 Gy being used in tubule regeneration assays at 60–68 weeks (131).

Because of the relative renal radioresistance of mice (130), the recommended small animal model is the rat. At this time, no specific recommendation can be made as to the most appropriate rat strain to be used since, with only a few exceptions [e.g., outbred CD(SD) rats (116)], almost all research on radiation nephropathy in rats in the U.S. (125) and in Europe (132) has been performed with a single strain (WAG/Rij). Although multiple mouse strains have been assessed in different laboratories, no systematic comparison of their renal radiosensitivity appears to have been performed.

For large animals, arguments can be made for pigs, dogs and nonhuman primates. Nonhuman primates may be the model most relevant to humans, but the follow-up period would need to be measured in years (129). Dogs have the advantage that there is extensive literature on canine renal physiology and on acute response to TBI (133). Porcine models have a number of advantages:

1. Pigs and humans are unusual among mammals in possessing a multipyrimidal, multipapillate kidney (134).
2. Pigs have been used extensively as models of urological conditions in humans (135).
3. Extensive literature exists on their response to renal irradiation.
4. The follow-up period for development of radiation may be shorter in pigs than in nonhuman primates or dogs (127,136).

Supportive care in the kidney animal model consists largely of reverse isolation to prevent infections during the period when animals are immune suppressed. Long-term reverse isolation may also be needed to prevent lung infections (125). Antibiotics as part of the supportive care should be used with caution, since many of them are nephrotoxic (e.g., antifungal agents and aminoglycoside antibiotics) and could therefore exacerbate radiation nephropathy (137).

One issue posed by the Animal Efficacy Rule (and one that may also affect other animal models) is that, for example, for a promising class of mitigating agents such as ACE inhibitors to be effective for mitigation and treatment in the rat radiation nephropathy model, they must be given for at least 3–6 weeks (138). Since the ACE inhibitors are available orally, they are given to the rats in drinking water, but humans generally take ACE inhibitors by mouth as tablets. If the FDA does not consider agents in drinking water to be equivalent to agents taken in tablet form, then animals would have to be given pills or gavaged daily for perhaps 6 weeks; this may be totally impractical in many species.

Summary

The preferred small animal model for radiation-induced renal injury is the rat; for large animals, arguments can be made for using pigs, dogs or nonhuman primates. Radiation can be delivered to the lower hemibody, given to the total body with some bone marrow shielded, or administered with a bone marrow transplant. Single doses of 8–12 Gy are sufficient to produce radiation nephropathy in these species, as long as adequate supportive care is provided to prevent acute injuries and chronic infections and the follow-up time is sufficiently long. However, it must be noted that the use of doses higher than 12 Gy to reduce follow-up time involves considerable risk since damage to other organ systems (e.g., GI tract) may interfere with the evaluation of renal injury. The gold standard end point is histopathology, but monitoring of blood urea nitrogen (BUN) provides a noninvasive surrogate assay.

SKIN

The scenarios in which skin injury is likely to be an important component of an accidental or terrorist-related radiation exposure are many (139). They include use of a conventional or improvised nuclear device (IND), which could result in mass casualties, a radiological dispersal device (RDD or “dirty bomb”), which will produce a smaller number of casualties (140), and experimental/industrial exposure accidents that tend to involve relatively few individuals. In many cases, outcomes may be complicated by additional injuries; for

example the detonation of a 10-kiloton device will likely inflict second-degree burns on the exposed skin of people at distances of up to 1.4 miles from ground zero (141).

The fact that the cutaneous syndrome often runs concurrently with or as a component of a multi-organ toxicity syndrome (142) has not been fully appreciated until recently. However, the previously described scenarios generally include a TBI component and, under such circumstances, cutaneous damage will significantly augment noncutaneous injuries, leading to lethality after otherwise easily tolerated radiation or physical injuries (142,143). Indeed, the extent or area of the skin exposure may result in a real-world clinical threshold—termed a “point-of-no-return” by Meineke—that, if exceeded, will impair the function of other organs (142,143). The mechanisms and the degree to which multiorgan toxicity is affected by skin injury have only been studied superficially in experimental models.

Time–Dose Relationships and End Points

Interventions for prevention or treatment of radiation toxicity to skin and subcutaneous soft tissues must address sequential pathogenic mechanisms. Erythema is one of the first manifestations of skin damage, followed by pigmentation, dry and moist desquamation, fibrosis and necrosis. A brief period of erythema can occur and disappear with a 1-mm skin surface dose of 0.3 Gy, usually within 24 h, and is caused by capillary congestion and probably histamine release (144). More prolonged erythema occurs at higher skin doses, with a time of onset and severity that depend on the dose and area of exposure (145). This inflammatory erythematous reaction may occur within a day and can be followed by depilation and desquamation at 1 to 2 weeks; this results in thinner and sparser hair that may have altered pigmentation. Changes in skin pigmentation may also occur, typically several weeks after the exposure, followed by a dry desquamation. The skin can become permanently depigmented after this “peel.” Cutaneous doses above 10 Gy can cause damage to dermal capillaries and sweat glands, which do not completely recover, leading to dry skin. Higher doses cause a dose- and time-dependent moist desquamation and blistering reaction. This peaks a week to a month after exposure and can be very slow to heal. Healing typically includes scarring and can progress to necrosis. The latter does not heal spontaneously and often requires a skin graft with vascular supply.

Late fibrosis can occur with minimal early reactions and occurs after single superficial doses as low as 11 Gy, is common with exposures over 20 Gy, and is dependent on area. Fibrosis is amplified when combined with a mechanical wound or burn and progresses over time, first replacing the dermis, followed by the epidermis and, in the most severe cases, infiltrating and then stiffening the underlying muscle. The result is a poorly healing tissue that is sensitive to trauma and immobilizes joints. The pace at which these fibrotic changes occur appears to be responsive to some vasoactive and anti-inflammatory compounds (146), perhaps in part by reduction of the ischemic inflammatory and reperfusion injuries.

The commonly used end points employed in the murine and porcine experimental models mimic those used in humans and include the acute cutaneous symptoms of erythema, dry and moist desquamation and ulceration, as well as the late symptoms of induration and fibrosis. Regarding the immediate and early toxicities, validated scoring scales recorded by researchers blinded to the treatment category along with quantitative measures of the healing time and wound area should be employed for animals (147,148), as with clinical scoring systems (149). For late toxicity, similar scales can be used and wound contracture (scar contracture), induration or, at the extreme of the dose range, loss of function (limb shortening, range of motion, strength) may be appropriate end points (146,150).

While the primary end points focus on specific skin effects, if an agent is intended to modulate the interaction between skin and systemic injuries, a secondary end point may be

survival. In this case, two experimental models might be appropriate. In one, the radiation dose selected would logically be sublethal. Skin exposures would then be escalated in dose or area to determine the impact of local injury on survival; such studies would follow an LD_{50} format. A complementary design would use one or more local injury exposure(s) together with escalating total-body doses. Using such models, potential agents would be assessed for their ability to shift the dose–response curves. A radiation protector or mitigator that produced a DMF of 1.15 or an increase in the LD_{50} of 1 Gy (whichever is less) would be considered efficacious. Finally, since evacuation of victims to off-site health resources can be critically important to their chances for survival, interventions that increase survival duration by 50% or at least 3 days also would be of significant benefit.

Other experimental end points may be vital to confirm the mechanism of action and to establish the best combinations of therapies. For example, agents that reduce certain types of toxicity might augment others, including late oncogenesis. Such end points have great value scientifically, but they are unlikely to be of primary value in the validation of agents for clinical utility or for approval by the FDA to treat the consequences of accidental or intentional radiation exposure.

Variables

Animal models should attempt to emulate the scenarios for the expected radiation exposure (41). For example, for the exposure scenario of a survivable nuclear bomb detonation without fallout, cutaneous damage will be dominated by skin injuries that are physical or thermal (141). Indeed, of those individuals who survived the Hiroshima detonation and received clinical treatment, 90% presented with burns, 83% with blast injuries, and only 37% with pure radiation injuries (141). Where fallout is involved, exposure to radioactive particles could produce single or multiple small superficial cutaneous doses in the range of 20 to 50 Gy, doses that are fully capable of inducing necrosis. In contrast, after a dirty bomb, lower doses are anticipated (e.g., ≤10 Gy, total or partial body) that could affect healing after any concurrent traumatic injury. This again suggests that two murine models may be needed: The first would use a broad range of radiation doses (20–45 Gy, depending on the mouse strain used) delivered to a localized volume as an initial screen for mitigator efficacy; the second would focus on combined injury to model the multiorgan syndrome and could include, for example, a low (survivable) total-body dose plus localized radiation or an independent trauma, such as burn or wound.

The radiation quality must also be considered. For example, β-particle or low-kV exposures may affect only the most superficial skin layers, while the higher-energy γ radiations (transuranics, ^{137}Cs) from a nuclear blast will have a greater depth of penetration. Based on their depth of penetration, therefore, the relevant X-ray energies that are needed for local skin injury in mice are in the range of 30 to 100 kV, whereas electrons are in the range of 1 to 2.5 MeV. Thermal neutrons might also be relevant for some studies; however, the complexity of neutron dose measurements and other logistical issues would make them difficult for initial testing.

A last and very important consideration for experimental design includes the size of the exposed skin area. While the number of surviving clonogens per unit area of skin may be the same, when combined with TBI, larger areas (e.g., 10–20% of skin area) will have a greater effect on lethality and therefore the nature of the intervention that is required for efficacy. Smaller skin areas (e.g., <10%) will be more relevant for interventions aimed at improving quality, rate or completeness of wound healing (151). Whether or not TBI is employed as part of a model design, the radiation doses to the cutaneous tissues that are isoeffective to the reactions seen in humans should be chosen, even though they may be twice as high in mice.

Species and Strains

The rodent is the most commonly studied model of radiation dermatitis. The wide variety of available mouse strains, including genetic variants, and the similarities in both the timing and the pathophysiological course of the reactions make results from this model relevant to human disease. Indeed, human and murine radiation-induced skin reactions are similar in nature and appearance (147). Although the skin pigmentation and dark hair color of many of the mouse strains makes the detection of erythema difficult, swelling and inflammation can be demonstrated easily beginning a few days after a 10-Gy exposure to approximately 1 to 3 cm^2 (e.g., a hind limb); as in humans, this response subsides a few weeks later. The various skin reactions in mice typically require approximately twice the dose in humans, in part due to their more cellular papilla and in part due to the necessarily small surface area irradiated in a rodent. Melanocytes clearly depopulate and, as in humans, the hair goes gray. Dry and moist desquamation are dependent on dose and area, as in humans, and fibrosis and necrosis occur as the dose is escalated (152). These various reactions have been studied in a variety of commonly used wild-type mouse strains, and much is known regarding specific differences (153); a number of scoring systems for cutaneous radiation responses have been described (147,148).

The pig is widely used as a large animal model. to study the skin effects of radiation exposure (154–156). The FDA often encourages experiments in the porcine model in support of the approval of an IND package for dermatological agents (157). Advantages over rodent models include the sparser hair and the structure of the cutaneous and subcutaneous tissues, which have a similar physiology to humans (156). However, the vast majority of data in this model have involved superficial or localized radiation volumes, with only a very limited number of studies incorporating TBI (158). This can be explained by the logistical problems involved in irradiating, and even handling, the standard adult pig, e.g., the Yorkshire White, which weighs approximately 55 kg at 6 months. Many investigators have therefore used juvenile animals, which unfortunately reduces the relevance of their findings to the adult human due to the growth-related changes and also limits the time available for follow-up because of the rapid growth in these animals. The use of miniature strains of pig does not necessarily alleviate this problem, since many of these pigs still achieve a relatively large size and are likely to have genetic abnormalities associated with their size differential. Despite these challenges, because of its physiological similarities to humans, the pig is a very logical second species for modeling the cutaneous syndrome as well as evaluating interventions for toxicity and efficacy.

Nonhuman primates have been used extensively for total- and partial-body irradiation experiments (159), but the cutaneous data are extremely limited. In addition, although much closer to the human with respect to the cutaneous and subcutaneous physiology, the hirsute nature of primates affects the relevance of these species.

Summary

There does not appear to be a clear, optimal species for study of radiation toxicity in skin and subcutaneous tissues, although the progressive effects of radiation have many gross, microscopic and mechanistic similarities in all species (19,160). However, in the opinion of the skin working group, mouse models were judged the most rational first species for use in a screening assay, with an emphasis on wild-type inbred or outbred strains. Since the cutaneous response of many mouse strains is known to vary with respect to dose tolerance, type of inflammation and severity of fibrosis, a screening paradigm should include both a more sensitive and a less sensitive strain that will encompass the expected range of human responses (153). The development of targeted agents that have species specificity suggests that, in some cases, the nonhuman primate may prove to be the only usable large animal

model. However, where pharmacokinetics allows, the pig should be considered as an appropriate second animal model.

OTHER FACTORS AFFECTING EXPERIMENTAL OUTCOME

Many variables affect the ARS that occurs after TBI. These include the species and strain of animal, age, sex, nutrition, microbial status and the level of supportive care. As mentioned above, these factors were discussed at various times during the workshop, and some will be considered further here.

Supportive Care

There is now considerable evidence in dogs and nonhuman primates that shows the contribution of supportive care to survival. In general, it was considered appropriate by the workshop participants to incorporate this into the experimental design for all larger species, but less so for mice, even though their water intake declines within days of TBI (161). The impact of supportive care is less well established in mice, and attempts at hydration may serve as an additional variable that is hard to control. In any event, it is critical that appropriate diluent controls using the same volumes are included when testing agents for efficacy. There are other aspects to supportive care that could be considered further; for example, dietary supplementation in mice has been shown to increase the LD_{50} for bone marrow death (162).

Microbial Status

It has been known for decades that the ARS in mice and humans depends on microbial status. Numerous, generally older, studies have shown that the total-body dose for lethality, whether for γ or X rays (163–165) or neutrons (166), was lower in conventionally housed mice than in germ-free (axenic) mice. It should be remembered that “germ-free” in these studies refers only to organisms that could be cultured using the techniques available at the time. Recently, it has been estimated that the gut may contain up to 500 different bacterial species, in addition to fungi and protozoans, most of which can be detected using molecular tools but not conventional tools (167).

The magnitude of the effect of microbial status has been calculated. For total-body X-ray doses less than 9 Gy, the difference in $LD_{50/30}$ values between conventional and axenic mice is between 50 and 2.50 Gy, depending largely on variations in the response of the former condition (162). It appears that the microbial flora contribute more to variations in the response to TBI than genetic variables such as strain and sex (females often being more radioresistant) (163). After higher radiation doses (>10 Gy X rays) that compromise the intestinal mucosa, conventionally housed mice survived for 50% less time than germ-free mice (163). This has been attributed to a faster turnover time for the intestinal epithelium under conventional conditions (164,165).

The greater lethality of radiation for conventionally housed mice is not due to increased cell death, as judged by bone marrow cellularity (167), and is generally considered to be the result of infection after immune suppression and mucosal compromise. Thus SPF mice that were free of *Enterobacter cloacae* lived significantly longer after TBI than SPF animals that harbored these organisms (168). Naturally, the interaction between antimicrobial therapy and radiation has been studied in some depth. It has been shown to be beneficial for rodents receiving sublethal irradiation (169), dogs (see earlier), and even humans who received lethal doses in the Tokai-mura accident (170). In keeping with this concept, probiotic bacteria have been shown to decrease the incidence of radiation therapy-induced diarrhea in humans (171).

The other side of this coin is the possibility that beneficial gut flora may maintain intestinal and immune function. This may explain why orally administered *S. enteritidis* spread more rapidly and systemically in germ-free than conventionally housed mice when their resistance was decreased by sublethal TBI (172). In one study, profound gut microflora depletion by antibiotics was associated with an unexpected *decreased* survival time after TBI (161). In another, quinolone antibiotics that were effective against exogenous infection in TBI hosts did not prevent endogenous infection (173). There is also growing evidence that certain antibiotics can protect cells against the lethal effects of irradiation *in vitro* and *in vivo* (169), indicating a direct effect on the host hematopoietic/immune system.

In summary, there is a compelling need to consider microbial status as a critical variable in the ARS. Standardization of the microbial status of rodents as SPF, as a minimum, is essential for the kind of studies described here. For species other than rodents, standard protocols for supportive care are required, including the administration of antibiotics. Further use of molecular phylotyping to define the microbial status is likely to be of great value, and further research is needed into the nature of the antibiotics and probiotics that should be administered as supportive care before and after a total-body exposure.

Dosimetry

Biological research is fraught with the systematic statistical uncertainties, variations in response, and other factors that yield a statistical uncertainty larger than that generally found in the physical sciences. The overall statistical uncertainty in radiobiology research can be simplistically given as

$$\sigma_{\text{experiment}} = \left[\left(\sigma_{\text{biology}} \right)^2 + \left(\sigma_{\text{dosimetry}} \right)^2 \right]^{1/2}.$$

Given the size of the error in the biological contribution, it is important that the physical errors are minimized. Furthermore, unless physical dose can be excluded as a variable in comparing studies, it is a waste to explore the source of the more complex biological variables that might contribute to differences in results between laboratories. The simplest way to standardize the dosimetry is to involve an active physics collaborator with substantial clinical experience to characterize the radiation fields to be used in the experiments and to calculate the doses the critical tissues receive. The recommendation to involve a clinical physicist has a hidden benefit in that this individual will have access to and knowledge in the use of state-of-the-art radiation measuring equipment that has current calibration factors traceable to the National Institute of Standards and Technology. In general, cable-connected detector systems have the lowest measurement uncertainty and the greatest accuracy. Devices such as thermoluminescence dosimeters, field-effect transistor detectors, and other readout separable systems can be used. The reading uncertainty tends to be larger, but they give greater positional accuracy because they can be implanted in a specific site in an animal and the local dose can be evaluated. Discussion with the physics collaborator should reach the best compromise between positional accuracy and dose accuracy.

Field Size

For simultaneous exposure of multiple animals, such as a tray of mice, the radiation field should have a uniform intensity. Frequently, this will involve the use of only the central half of the radiation field; as further assurance that all receive the same dose, the tray should be rotated. Treating individual animals with small field sizes has the concern that the field is defined as the distance between the 50% of maximum intensity on one edge to that point directly across the field, so that the maximum intensity is typically the center of the field.

This results in the intensity, and hence the dose, varying considerably over the irradiated volume.

Radiation Quality

There was considerable discussion at the workshop regarding security issues associated with isotope radiation sources and the possible move toward the use of orthovoltage X-ray systems for animal irradiations. Radiation biologists should be aware that 250 kVp X rays have a greater biological effect per cGy than do ^{60}Co γ rays or 4 to 18 MV X rays that must be taken into account. The variation in energy also has surface dose implications. For orthovoltage, the maximum dose is reached very close to the surface, whereas for ^{60}Co, it typically occurs at a depth of 5 mm, and for 6 MV X rays the depth is close to 16–17 mm. If there is any material between the source and the animal, such as the top of a cage or a container, this it will affect this calculation, and the effect will depend on how close the material is to the animal.

In summary, the variations between the experimental systems used in radiation biology are so great that it is impossible to generalize other than to say that unless physical dose is known accurately, it becomes difficult to make meaningful biological comparisons. Bad dosimetry leads to bad biology. The only firm recommendation that is applicable to all situations is to involve a qualified and experienced medical physicist in the design and the dosimetry of the experiments.

CONCLUSIONS

The CMCR Animal Models Workshop successfully identified areas where it was possible to introduce greater standardization in animal models and procedures that would allow more appropriate comparisons to be made between radioprotector and mitigator data from different Centers. The need for good dosimetry, consideration of the impact of the multiple elements of supportive care, microbial status, appropriate strains and species, and the nature of dose–response relationships were all discussed in depth within the framework of the best animal models for studying the effects of radiation exposure on the immune, hematopoietic, intestinal, lung, kidney and skin systems. Many recommendations were made that will be implemented within and hopefully outside the CMCRs. Radiation research has a glorious history of sound animal models. The field is now well placed to take advantage of these and move forward with the new concepts and ideas coming from within the radiation countermeasures programs.

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TABLE 1

Description of Radiation Pathology in Lung

8–16 weeks: *acute radiation pneumonitis*—Edema of alveolar septa; prominent hyperplasia of type II cells; moderate numbers of mononuclear cells and fibroblasts; increase in luminal macrophages
Months-years: *pulmonary fibrosis*—Early presence of hyaline membranes; myointimal proliferation with occasional foamy cells; progressive fibrosis and thickening of the septa; air spaces fill with connective tissue

Note. Modified from Fajardo *et al., Radiation Pathology*, 2001 (91).

TABLE 2

Mammalian Animal Models Grouped by Pulmonary Subtype

Mammalian lung subtype	Lobularity	Pleura/interlobular septa	Arterial supply to pleura
Type I (cow, sheep, pig)	Well developed	Thick collagenous structures	Bronchial artery
Type II (dog, cat, rodent)	Notably absent	Pleura thin; septa absent	Pulmonary artery
Type III (horse)	Imperfectly developed	Thick collagenous structures	Bronchial artery

Note. Modified from McLaughlin *et al*., 1961 (101).

TABLE 3

Summary of Strain-Dependent Differences in Mouse Lung (89,90)

	Acute phase (<28 weeks postirradiation)		Late phase (52 weeks postirradiation)
Strain	**Histological appearance**	**Pneumonitis (%)**	**Histological appearance**
C57BL/10J	Protein-rich edema containing fibrin		Extensive contracted fibrosis
C57BL/6J	Hyaline membranes		Extensive contracted fibrosis
C57L/J	fibrosis	12	Extensive contracted fibrosis
BALB/cJ	Protein-rich and protein-poor edema	28	Foci of contracted fibrosis
BALB/cCr//Alt	Hyaline membranes	29	Foci of contracted fibrosis
A/J	Small foci of fibrosis	26	Foci of contracted fibrosis
SWR/J		47	Foci of contracted fibrosis
C3HeB/FeJ	Protein-poor edema	50	No contracted fibrosis
C3H/HeJ	Wispy deposits in alveoli		No contracted fibrosis
CBA/J	No fibrosis	37	No contracted fibrosis