

Chapter XIV: Countermeasures Development Based on the Concept of an Evolution of “Radiation Disease

The Evolution of “Radiation Diseases”

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Introduction

Physics provides us with a relatively simple explanation for the powerful cytotoxic nature of ionizing radiation. Low LET radiations result in about 10^5 ionization events per cell per Gy, with 1% in the nucleus. This means that exposure to whole body doses of ionizing radiation of sufficient magnitude to cause clinical symptoms will have affected every cell in the body, and many will die. Such clarity is, however, not so evident in the biology of the radiation tissue injury that ensues, despite over 100 years of research. Complex processes drive the evolution of radiation diseases, starting seconds after exposure and in some cases lasting a lifetime. Any clinical symptoms that appear are just the tip of the iceberg of underlying molecular and cellular processes (Figure 1). Multiple events may be observed that depend on many variables, including dose, dose rate, quality of radiation, time, the extent of damage, genetics, the microbiome and numerous other confounders. This complex ever-changing molecular and cellular landscape presents a huge challenge for the mechanistic interpretation of the action of radiation and, in particular, the development of countermeasures whose efficacy can only be properly evaluated using identical, well-defined and validated biological endpoints. This chapter will examine the interdependence or independence of the classical mortality endpoints and associated intercurrent processes that shape the evolution of the multiple facets of radiation “diseases”.

Preclinical studies of the classical radiation syndromes generally have lethality as their endpoint. This seeming simplicity belies an inherent complexity, which is why the term “syndrome”, i.e. a set of tissue-related symptoms, is used. This complexity can be minimized in part by studying mortality in model systems where a tight radiation dose-time window can be identified, typically associated with tissue-specific symptoms. Acute radiation syndromes (ARS) have well-defined, discrete time-dose relationships for mortality and, as a result, ARS tend to be the target of most countermeasures development, although late lethality syndromes and potentially lethal or non-lethal manifestations of radiation damage are equally valid targets that loom large for ARS survivors. These later facets of radiation disease tend to present as chronic diseases and may appear to be more stochastic than deterministic in nature, which makes their quantitation even more difficult. One problem with their study is that, while the signs and symptoms for ARS are tangible and reproducible, this is not always the case for later facets of radiation diseases, which result from an organism having to integrate complex internal and external signals over an extended time period, hence the diverse and often obscure pathoetiologies. For example, the life-shortening effects of radiation exposure are well known but their causes are not.

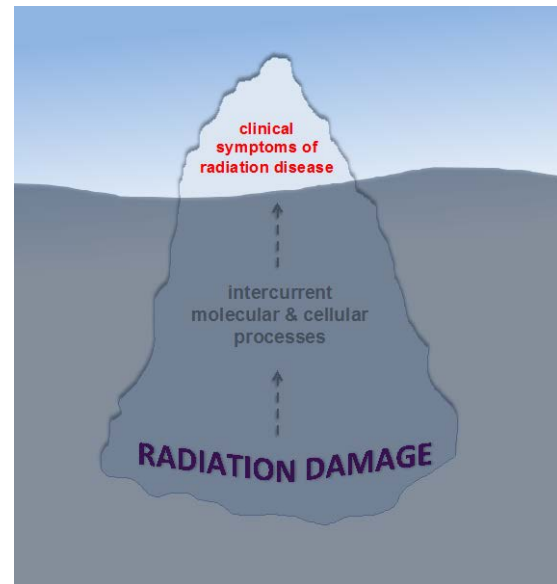


Figure 1: Radiation disease is just the tip of an iceberg

The classic ARS are dealt with in more detail elsewhere so, in this chapter, we will discuss only those features that constrain and distinguish them for the purposes of comparison with less classical diseases that can evolve and how these impact countermeasures development. It should be noted that our knowledge of radiation diseases comes largely from animal models. While the relevance of ARS models to the human condition is generally accepted, as is the consistency of their occurrence across species, less is known about chronic radiation diseases.

Radiation Syndromes – are they linked?

Radiation syndromes are classically defined as a set of symptoms that are associated with a potentially fatal failure of a specific tissue system within a characteristic dose-time framework. In animal models, the probability of single dose radiation lethality increases rapidly from 0% to 100% over a narrow dose range and can be modeled by a probit S-shaped cumulative curve. Steep dose-survival curves are an attractive research tool as a small change in dose can translate into a large increase in survival, making biologically and statistically significant differences more achievable. From a purely scientific point of view the steepness of the curve gives some indication as to the homogeneity of the system. If a countermeasure were to change this steepness, it would indicate additional factors at play. Another way of looking at this is to say that if the control dose-survival curves are less than steep, heterogeneity is likely already present with the possibility of more than one endpoint.

Distinct ARS have been recognized since the 1950s (1) and follow the general dose and

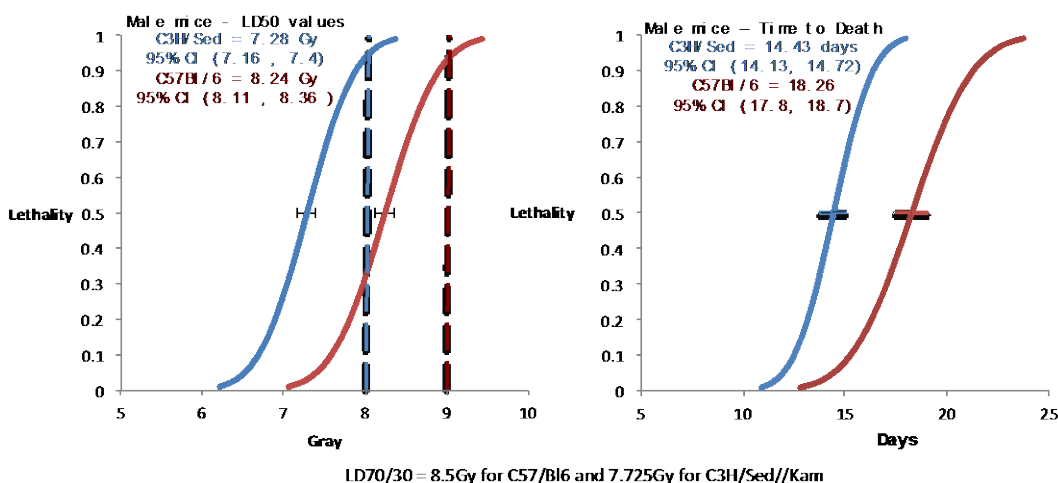


Figure 2: a) Probit mortality plots for H-ARS with dose for 2 strains of mice after WBI. b) Mortality with time plotted by probit for LD70/30 doses in the same 2 strains.

time framework that is shown for hematopoietic ARS (H-ARS) assessed by probit plots in Figure 2 for 2 mouse strains exposed to whole body irradiation (WBI). Classic radiobiological target cell theory considers the dose-response curve (Figure 2a) to result from the random nature of radiation cell kill and the probability of elimination of the last surviving clonogen required to maintain tissue function. In contrast, the median survival time (MST) is classically believed to reflect the turnover time of the tissue, which like the dose response, displays genetic variation (Figure 2b). The source of this genetic variation in radiation response is generally obscure, even though it has been recognized

since the days when Little first developed inbred strains at the Jackson Laboratories - an institution founded for "research in cancer and the effects of radiation," and when Russell, who moved from the Jackson Labs, performed his "megamouse" radiation genetic experiments at Oakridge. Possible sources of variation include a propensity to undergo different forms of radiation cell death, or the size of the "target" cell pool, or differences in the type or strength of the inflammatory responses that are generated; in any event, such differences greatly impact the development of a countermeasure, which has to be inherently effective across many genetic backgrounds.

Death is, of course, in and of itself a process, and ascribing it to a single cause is often as much a philosophical as scientific question. For instance, classic H-ARS is often labeled "bone marrow syndrome" since lethality has most often been ascribed to severe neutropenia and thrombocytopenia due to loss of hematopoietic progenitor cells, and, indeed, transplantation with myeloerythroid-restricted progenitor cells can protect WBI mice from H-ARS (2), which may provide enough time for surviving multipotent stem cells to repopulate the system. At the same time, other causes of death, such as infection as a result of immune suppression and/or gut barrier insufficiency, cannot be excluded as causes of H-ARS lethality.

One way to minimize errors in interpretation of ARS data is to closely examine the relationship between single radiation dose exposures and median survival time (MST). Such an exercise is illustrated in cartoon form for H-ARS and for gastro-intestinal ARS

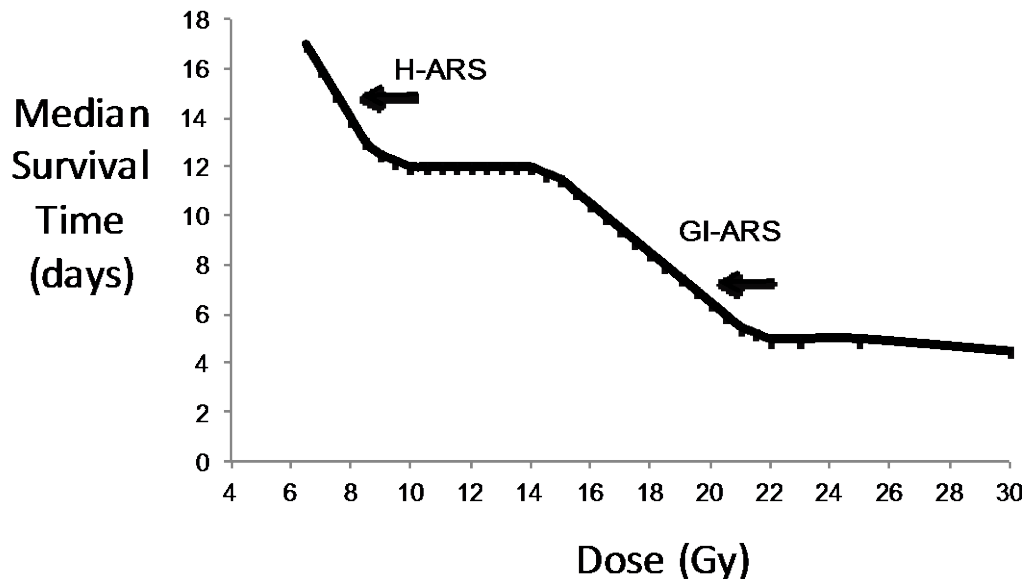


Figure 3: Dose vs MST relationships for H-ARS and GI-ARS showing the dose-dependency of lethal syndromes with intervening dose independent steps.

(GI-ARS) in Figure 3. It makes the point that the MST is independent of radiation dose except during the time when lethality is rapidly increasing, i.e. when dose and MST are inversely related (3, 4). The implication is that these lethal ARSs behave as discrete entities. Similar MST-dose relationships are generally found for all ARS, and across species (2). It is true for the earliest lethal radiation syndrome, which has been labeled

cerebrovascular (CVS)/central nervous system (CNS-ARS) and occurs within 1-2 days of very high WBI doses (2). It is also true for certain well-defined late syndromes, such as the lethality from radiation pneumonitis that occurs in C3H mice between 12 and 20 weeks after single thoracic radiation (LTI) doses of 13-15 Gy (5).

The reason for the shape of this curve is not clear, but the plateau phase can be equated to the turnover time of the involved tissue, while one hypothesis for the inverse relationship between MST and dose is a decrease in the number of ante-mortem divisions traversed by stem/progenitor cells with increasing dose, although there are other possible explanations, such as an “avalanche” effect due to recruitment of cells with damaged DNA into division leading to their demise (6) (see 3). The shift in MST with dose in the lethal ranges is not large, perhaps 1.5 days/Gy (3), but is conceptually important because a narrow dose-time window can be determined within which to expect specific symptomology, helping to define the endpoint. From a countermeasures perspective, defining a narrow MST-dose window makes it likely that the countermeasure is counteracting a specific system failure. On the other hand, a wide MST-dose window would suggest heterogeneous endpoints and, therefore, multiple targets.

Time-Dose Lethalities Outside Classic Syndromes

Unfortunately, the MST-dose paradigm described above rarely encompasses all the mortality data for a given model, especially in the lower dose range. Reality is more complex. Assessing the mortality (or other hazard) distribution over a dose spectrum

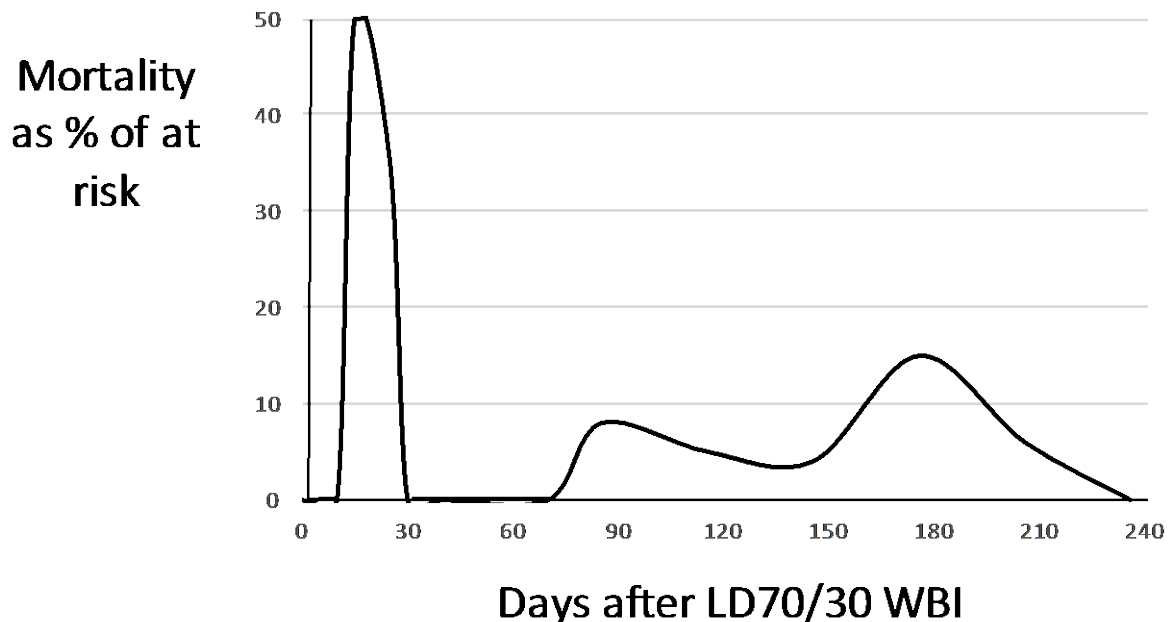


Figure 4: Mortality with time after LD70/30 dose of WBI delivered to C3H mice.

and throughout the complete lifespan of the animals requires a considerable amount of data and a long-term study that is rarely performed or achieved. It is clear, though, that once an animal passes the dose-time window for a given lethal syndrome, other potential hazards loom ahead. Figure 4 shows a cartoon representation of the incidence

of mortality in a large number of C3H mice given a LD70/30 radiation dose. Mortality increases rapidly after day 10, due to H-ARS, decreases to zero on day 30 to 70, only to increase again with 2 waves of non-hematopoietic mortality, peaking at 3 and 6 months. Indeed, none of the 30% of mice that survived H-ARS lived a normal life span. The administration of bone marrow protects against H-ARS lethality but Peters and Travis et al. showed that Balb/c mice receiving LD50/30 WBI doses with bone marrow rescue still developed increased late mortalities similar to those in figure 2 (7) (<http://www.iaea.org/inis/collection/NCLCollectionStore/Public/20/029/20029409.pdf?>). Patients receiving high-intensity cancer treatments with hematopoietic cell transplantation also develop early potentially fatal radiation disease. Two year survivors were found to be 8.4 times more likely to be frail than their siblings and had a cumulative incidence of subsequent all-cause mortality of 39.3% at 10 years compared to 14.7% in those without frailty (8). Individuals receiving such treatments were found to have a high incidence of cardiovascular disease with co-morbidities and mortalities (9), similar to that seen in Japanese A-bomb (10, 11), and the mice shown in figure 2 (McBride, unpublished). Peters and Travis et al. (7) also observed that late radiation mortality was more sensitive to low dose-rates (around 1-5 cGy/min) than H-ARS or GI-ARS, with dose-rate mattering little for H-ARS, moderately for GI-ARS, and most for late effects, is important for individuals unfortunate enough to be exposed to low dose-rate radiations and for the design of experiments to test countermeasures.

Waves in the hazard function for late mortalities after total thorax only irradiation (TLI) could also be extricated from data pooled from multiple experiments in C3H mice receiving various TLI doses (6). The first peak occurred after 13 and 15 Gy between 90-

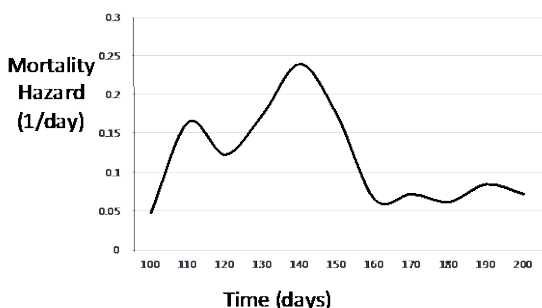


Figure 5: Cartoon showing variation in hazard for mortality over time in C3H mice given LTI doses of 12.25-15 Gy with periodically occurring peaks every 33 days – reconstructed from (6). The total mortality was 45%.

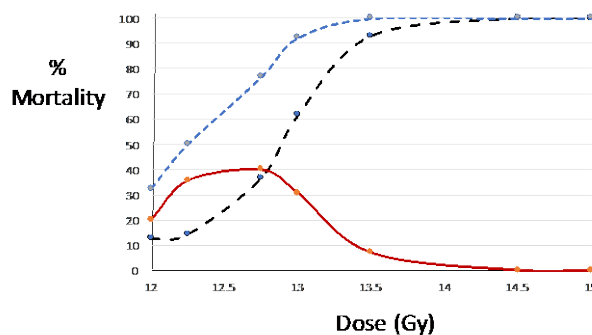


Figure 6: Percent mortality versus LTI dose after 150-200 days (solid, red line), 90-140 days (large, black dash), and the combined data (small, blue dash) – reconstructed from (6).

120 days, with a second peak around 140 days and additional peaks out to 200 days (Figure 5). These waves in the hazard function per day had a calculated average periodicity of 33 days (Figure 5; 6). The first two peaks were associated with pneumonitic pathology, but this was not true for most of the late mortalities (12), which occurred primarily at lower doses of 12-13 Gy and independent of the incidence of prior deaths (Figure 6). This heterogeneity in the thoracic radiation response is illustrated by the observations that the radioprotector, WR2721, differentially minimizes very late

effects (13), whereas T cell depletion decreases pneumonitis but not later effects (14). Down et al. (15) has suggested that late changes in lung function and associated lethality can result from pleural effusions, which may be consistent with cardiovascular disease, while others have considered fibrosis as the cause (8). Censoring data at <140 days gives a steeper mortality curve (Figure 6) and focuses the endpoint on lung pneumonitis, which is an important consideration when designing mitigator experiments. In general, late complications tend to have a broad dose-MST response curve, which is consistent with fibrosis being a major component and the fact that it is possible to live with a considerable amount of organ fibrosis as long as sufficient functional tissue is left. This highlights the point that assessors of radiation countermeasures need to carefully consider lethality endpoints. The time-dose relationships can help in this regard, but they can be misleading and may be less useful for late effects such as fibrosis.

Conclusions on MST-Dose Relationships

In spite of their non-mechanistic nature, well-considered MST-dose relationships have proven convenient when identifying lethal radiation syndromes for both medical and research purposes. A critic might say that they merely provide a conceptual framework to indicate the organ system that might be failing, and that the use of syndromic “labels” may well have delayed the development of appropriate radiation countermeasures by failing to identify other targets. This is a fair comment. For example, it should be self-evident that death within 3-10 days of WBI is not necessarily due to GI insufficiency and it would be wrong to assume so simply on the basis of an observed MST. Mortality is a limited endpoint that cannot automatically be ascribed to a specific cause. Another major limitation is that clinical data on radiation lethality are not readily available, and therefore preclinical findings cannot easily be extrapolated to the human condition. This was why the FDA established the Animal Rule (<http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm>) that requires testing in more than one species and some indication as to mechanism of action for drugs that cannot be tested in humans, as is the case for radiation mitigators. Having said that, consideration of dose-time relationships and associated endpoints is important and failure to take them into consideration may be one reason why countermeasure findings are difficult to reproduce between laboratories and to reproduce in different genetic models (16).

The bottom line is that the development of radiation countermeasures requires clear endpoint definition; well-defined MST relationships for mortality help in this regard, but all radiation exposures cause multiple effects with an inherent degree of randomness. Complications arise 1) from the waves of lethality that occur over time, and 2) from the impact of systemic factors on local tissue radiation responses; the latter affects the choice of the radiation model (WBI or partial body) for countermeasure development. Some of these issues will be discussed after consideration of intercurrent molecular and cellular processes and the possible influences of systemic factors that – in their entirety – form the bulk of the iceberg underlying the observed radiation syndromes.

Intercurrent Radiation Diseases

The concept that radiation generates intercurrent events, that influence processes within a tissue and at a distance, is not new. When Mole in 1953 used the term

“abscopal” to describe events “at a distance from the irradiated volume but within the same organism,” he was in fact considering how the effect of WBI on one normal tissue influences the response of another and may cause symptoms “by interfering with ...cellular interdependence, with organization” in tissues (17). He noted that, earlier, Lea had proposed the reverse i.e. that a normal environment might affect the behavior of irradiated cells (18).

Here, we are discussing intercurrent pathological processes that are generated in irradiated tissues and that act both locally and/or systemically on other irradiated or non-exposed tissues. Responses can be rapidly initiated soon after radiation exposure that trigger cascadic processes that evolve progressively over time, and often appear to be associated with more readily identified clinical symptoms in dose and time.

Radiation-induced Molecular Signals

Ionizing radiation generates DNA damage and oxidative stress in cells, which rapidly activates molecular signaling pathways in response, subsequently receiving further input from sensors of cell damage and death. Canonical pathophysiological cascades are set in motion that, initially, are pro-inflammatory in nature. Their immediate role is to harness the immune defenses. Later, feedback control circuits are triggered to control the resulting inflammation and to correct the associated pathology with the aim of restoring homeostasis. Multiple cell systems are affected, both within and outside the irradiated field. Both WBI and local irradiation send signals that mobilize and activate bone marrow cells that enter irradiated and non-irradiated sites by interacting with a systemically activated vasculature. In addition, organ systems are thrown into disarray as a result of the loss of radiosensitive subpopulations of cells, for which they attempt to compensate to maintain tissue functionality. Depending on dose and volume and site of exposure, some physiological systems may be permanently lost. These “forever dead” systems are not necessarily critical with respect to short- or even long-term function after irradiation, but can have indirect effects on systems trying to regain homeostasis and maintain the imbalance and the progression of late clinically important outcomes. Tissues that have a small number of functional subunits or tissue rescuing units (19) will tend to be more radiosensitive. For example, at the lower end of the dose range, complete hair loss due to follicular damage, skin pallor and hair greying due to loss of melanocytes, or sterility due to loss of germ cells is likely. Even if such losses do not affect overall survival, they may have a significant effect on quality of life, for example, by creating hormonal or endocrine imbalances. Certainly, these effects strongly echo Mole’s thoughts mentioned earlier on how WBI might interfere with cellular interdependence and organization (17).

The downstream radiation response period involves multiple molecular and cellular effector mechanisms. Pro-inflammatory cytokine cascades are rapidly activated after radiation exposure, as are cascades of inflammation-related plasma proteins belonging to the complement, kinin, coagulation, and fibrinolysis systems, many of which are acute phase protein reactants (20). The major cytokines expressed include tumor necrosis factor-alpha (TNF- α), interleukin 1beta (IL-1b), basic fibroblast growth factor (bFGF), interferon (IFN), and vascular endothelial growth factor (VEGF, otherwise known as vascular permeability factor) (20). Another element in this coordinated response is the upregulation of cell adhesion molecules that are critical for extravasation

of immune cells and damage recognition. Damage-associated molecular pattern molecules (DAMPs) that are released by damaged and dying cells signal through [pattern recognition receptors](#) (PRRs), such as the transmembrane Toll-like (TLR) or cytosolic nucleotide binding (NOD) receptor family members, to initiate further cytokine cascades and other inflammation-related products (21-23). However, many DAMPS, such as the prototypical DAMP, high-mobility group box 1 protein (HMGB1), are biologically active and, following irradiation, can maintain and amplify inflammation (21), ironically leading to further tissue injury.

Intrinsic attempts to control inflammation-associated damage come in the form of anti-oxidant and anti-inflammatory innate immune cells and molecules. These include T cell and macrophage subsets, such as Tregs, myeloid-derived suppressor cells, M2 macrophages and cytokines, like IL-10 and TGF- β . One result of immune cell infiltration, is a change in the profile of the cytokines that are produced changes over time. Another time-related mechanism seen following irradiation is stress-induced premature senescence (SIPS), with the emergence of a “senescence-associated secretory phenotype” (SASP) with characteristically high levels of IL-6, C-reactive protein, etc. The SASP will influence both the local microenvironment through bystander action and also events at distant sites (24-26). How these potential mechanisms interdigitate to result in what looks like waves in lesion evolution is unclear, but alternating cycles of immune activation and suppression seem a likely component.

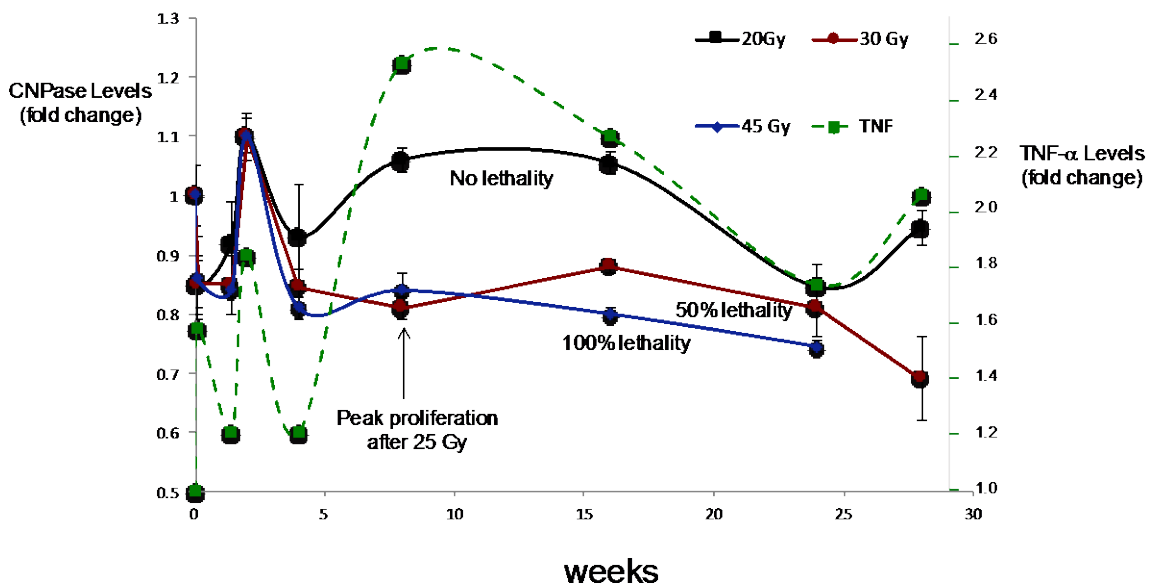


Figure 7: Fluctuation of TNF- α mRNA levels (green, dashed) days, weeks, and months after 25 Gy whole brain irradiation in C3H mice and their correlation with oligodendrocyte levels after 20 (black), 30 (red), and 45 Gy (blue) measured by CNPase expression.

It is easy to assume that radiation responses evolve progressively with time when, in fact, they appear as waves, with repeating cycles of inflammation, bouts of oxidative stress, further cell loss and/or cell proliferation, and associated alterations in tissue functionality that may or may not be lethal. The oscillations can be ascribed to feedback mechanisms that are induced to control the damage process, although they themselves

may have been damaged by radiation. Clinical symptoms, morbidity, and even mortality is not seen until the extent of tissue damage and the loss of function become too great. A brief snapshot of a body of work on how these processes can evolve (27-30) is shown in Figure 7. It illustrates the periodicity of events in the evolution of radiation-induced brain demyelination in mice using a single classic pro-inflammatory cytokine (TNF- α) and one cell type (oligodendrocytes) for simplicity to demonstrate this balletic evolution of response, although many other cytokines and cell populations are involved and could be used. TNF- α is seen to be induced in the brain within minutes of radiation exposure, but then fluctuates wildly over subsequent months. Oligodendrocyte levels also fluctuate, but result in dose-related demyelination only at >3 months. It is important to note that oligodendrocyte dose-responsiveness is seen only from one month onwards. The dose-responsiveness of these changes over time raise serious questions as to whether early radiation-induced changes can predict late outcomes. It also highlights the point that, in all radiation effects that are associated with cell depletion, the margin between life and death, or symptoms and no symptoms, is likely due to a small number of surviving cells that make recovery possible despite extensive cell loss.

A critical question that should be raised is the relationship among these processes, one to the other. For instance, is TNF- α and other molecular components of the cascade causing damage or are they just a surrogate biomarker of radiation damage? Are the “waves” necessarily related to one another or could they be generated independently? Since TNFR2 knock out mice develop radiation-induced brain damage earlier and after lower radiation doses, one suspects a direct involvement (31). The TNFR2 receptor is known in many situations to counter the effects of the pro-inflammatory TNFR1 death receptor, arguing that these processes are intrinsic to the manifestation of radiation damage. However, it should be noted that similar neurological symptoms can be associated with diverse pathologies. Indeed, TNF- α is highly pleiotropic, having major vascular effects, including increasing vascular permeability. It also can be directly cytotoxic to oligodendrocytes and contributes to astrogliosis. Furthermore, TNFR2 knock out mice develop seizures relatively early after brain irradiation (around 3-4 months), whereas the most extreme forms of demyelination are seen at 6 months. The radiation-induced seizures are in keeping with findings in other (non-radiation) seizure models, where TNFR1 signaling appears to contribute to neuropathology while TNFR2 suppresses this pathway and is neuroprotective (32, 33). In fact, the radiation-induced seizures in TNFR2 knockout animals seem subtly different from the radiation endpoints generally seen in wild type mice, which is reflected in their different time-dose relationship. This highlights the point that, if any genetic model is used to explore the mechanism of action of mitigators, care is needed in data interpretation as the mechanistic endpoint may have altered.

Similar intercurrent pathological processes seem to follow radiation exposures in all other tissues, which leads to the question as to how much they contribute to the many radiation disease states. At this point, many questions remain as to how these waves of responses evolve with time, how they relate to outcome, and how they can be influenced by mitigators. It seems though that, through these avenues, the body struggles to control pathological responses. In an evolutionary sense, these avenues exist to initiate healing of damaged tissues, such as following pathogen invasion or

physical insult, with the ultimate aim being homeostasis restoration. Where radiation may differ from other challenges is by causing DNA damage that can remain latent in some cell types, but may eventually be expressed when the cells proliferate, either as part of normal tissue turnover or as a natural response to tissue damage. Under such circumstances, cells with latent DNA damage may complete one or a few divisions before dying a mitotic death. Given the role of the innate immune system in these responses, as well as in the maintenance of homeostasis, it is relevant to look at the role of bone marrow-derived myeloid cells in the evolution of radiation disease.

The Bone Marrow Axis and Systemic Factors in Radiation Diseases

The natural involvement of the immune system in tissue damage responses complicates any mechanistic interpretation that is based solely on elimination of critical target cells. A few examples will illustrate this point:

GI-ARS is often reported as occurring over a wide range of radiation doses, but broad dose ranges do not jibe well with MST-dose relationships for ARS, suggesting underlying complexity or heterogeneity in the data sets. At least some of this heterogeneity can be explained by the finding that lethality occurring in C3H mice before 10 days after WBI doses of 12 to 17 Gy can be mitigated through transplantation of bone marrow cells (10, 11), whereas this approach is ineffective after higher WBI doses (of up to 22.5 Gy). When delivered to the abdomen alone, doses of 12-17 Gy cause no lethality, yet higher doses still do. In microcolony assays, epithelial crypt stem/progenitor cells show the same dose response, irrespective of whether WBI or abdominal irradiation is given and whether or not bone marrow was injected. Our interpretation of these data is that “GI-ARS” (defined by time to death) in the high dose range is due to failure of epithelial stem/progenitor cells, while this is not true at lower doses, where the term “GI-ARS” has been used, but the cause of death is in fact obscure. We appreciate that others have interpreted the above findings to indicate a contributory role for marrow-derived cells in preventing “gut death” in the lower dose range, but this does not solve the heterogeneity issue. The recent observation that CD11b+ myeloid cells in the intestinal stem cell niches can operate through the WNT pathway to mitigate the radiation-induced loss of crypt progenitors and stromal cells after 18 Gy abdominal irradiation (34), goes some way to tease out the mechanisms, but questions remain as to the cause of death in the lower dose range.

Bone marrow-derived cells may, of course, play a different role if lethality is due to sepsis. Radiation-associated sepsis can be caused by bacterial translocation across a damaged mucosal epithelial layer, perhaps acting in combination with radiation-induced immune suppression (35). The role of microbes, especially after WBI, is an old story, but it still needs to be carefully evaluated in each model to avoid introducing confusion. The fact that radiation alters the composition of the microbiome (36, 37) and creates a microenvironment that favors opportunistic pathogens (38) may be important for both ARS and late effects. In this regard, studies showing that barrier protection, in conjunction with appropriate medical regimens, shifts the effective dose ranges of both H-ARS and GI-ARS may eventually reveal the endpoints that should be assessed in the development of certain countermeasures (39). For example, intestinal endothelial cell apoptosis has been postulated as a cause of GI-ARS (40), especially in the low WBI dose range. Additional factors, such as villus shortening, with subsequent difficulties in

absorption and peristalsis leading to diarrhea, may also contribute to lethality, although in our opinion these are poor endpoints due to their lack of specificity: mitigating diarrhea should not be a primary aim of countermeasure development.

The evidence for involvement of myeloid cells in the low dose range of “GI-ARS” is strong since, as suggested earlier, attribution of “GI-ARS” may have confused countermeasure development; GI-specific injury in the high dose range, as measured by crypt damage, is more convincing with respect to mechanism. It is also likely present in CNS-ARS lethal syndrome after WBI, which is generally associated with clear evidence of rapid vessel and capillary damage in association with neutrophil infiltrates (1). This syndrome is considered by many to be consequent to vascular leakage and edema with increased intracranial pressure rather than direct brain tissue injury (hence CNS-ARS). However, if whole brain irradiation is delivered, as opposed to whole body, significantly larger doses are needed for lethality, so it is likely that the etiology of the disease is altered, even if the time frame and symptoms are similar. Bone marrow-derived cells have also been implicated in the healing of localized radiation damage in skin, lung, and kidney. For example, sub-lethal WBI doses are sufficient to cause a deficit in murine skin wound healing that can only be attained with substantially higher doses of local irradiation, in the order of 13 Gy or more (41). Another case in point is radiation lung disease, which is clearly associated with infiltration of bone marrow-derived cells (42-44). The composition of this infiltrate varies over time, is dependent on the endpoint (whether pneumonitis and/or fibrosis, etc), and at least in murine models, is generally tailored to the intrinsically variable cytokine environments characteristic of different strains (42-44). In addition, T lymphocytes seem critical for radiation pneumonitis, but are not involved in late mortality (14, 45).

The reality is that diverse systemic elements are likely involved in all radiation pathologies. Bone marrow-derived cells are just one contributor to the complex evolution of radiation disease. The question that arises is the extent to which they mitigate or exacerbate the outcome in the absence of other interventions, and whether or not they contain useful countermeasure targets. Such would be the case where systemic inflammation is clearly part of the disease and multiple organs show pathology, as in very late effects with lung fibrosis or cardiovascular or kidney disease. On the other hand, where tissues fail apparently due to the loss of a critical population of target cells (parenchyma) within a tight dose-time window, the role of bone marrow-derived cells is less clear. In either case, unless care is taken, mitigators may be merely addressing the symptoms rather than the problem (46).

Inflammation in Radiation Diseases

One could argue that “inflammation” is a rather poor word to describe the immensely complex processes triggered by a wide range of potential pathological challenges, including radiation. “Inflammation” is not one process, but is multifaceted. Classically, the difference between acute and chronic inflammation is the lymphocyte involvement in the latter, but this simple distinction is totally inadequate to describe the many inflammatory conditions that can be found. This subject has been reviewed (20), and will not be covered in detail here, but it is worth making a few points.

Ultimately, “inflammation” can only be defined properly by its individual cells and products. For example, interleukin-6 (IL-6) is frequently described as pro-inflammatory or as an acute phase protein. However, IL-6 at pathological levels enhances TGF- β production and late fibrosis, i.e. it can drive an archetypical anti-inflammatory response (47). This illustrates the importance of understanding that the immune system is a balance of opposing forces that can be present at the same time, even if one predominates in a disease situation and that the role of an individual cytokine can vary depending on the circumstances. Also, baseline conditions can vary depending on the genetics and the environment and may determine outcome. For example, C3H mice that succumb to pneumonitis following thoracic irradiation have failed to switch off the TNF-related response, unlike C57BL/6 mice that go on to develop fibrosis (43). As with the data in figure 7, early radiation responses in these two mouse strains often show little significant difference, but, over time, the pro- versus anti-inflammatory responses can differentially predominate, leading to alternative outcomes.

One important aspect of inflammation that is not often taken into account in radiation disease are the thrombo-inflammatory cascades, and we will consider these before discussing dysregulated, chronic inflammatory states and their contribution to late radiation damage.

Thrombo-Inflammatory Events and Vascular Damage in Radiation Diseases

Thrombo-inflammatory events are tightly linked to vasculature damage and may act as a nidus for subsequent progressive post-radiation manifestations (48, 49). A drastic and somewhat paradoxical example of the power of this thrombo-inflammatory axis is that, for decades, radiation was used in the clinic as a hemostatic tool against cancer-related bleeding (50). The association between vascular radiation injury and inflammation was described more than a century ago by the pioneers of radiation research, including the Curies and Becquerel (51, 52). Thereafter, there were many contentious discussions as to whether normal tissue damage was vascular or parenchymal in nature. Under any circumstances, vascular damage is an inevitable and integral part of any tissue response to radiation that can take many and varied forms and at varying times after exposure. It is a rarely considered target for countermeasure development, but, at the very least, its participation in acute and chronic inflammation confounds the interpretation of radiation effects.

Understanding vascular damage is complicated by the fact that the endothelial vessel lining exhibits significant phenotypic heterogeneity, not only across different tissues and organs, but also between different segments of vascular loops within the same organ, and even between neighboring endothelial cells of the same organ and blood vessel type (53). The associated supportive structures, such as the smooth muscle cells, basement membranes and matrix, also vary extensively and will influence the magnitude and type of the vascular response, which may be why the venous microvasculature expresses radiation damage more readily than arterioles. It follows that radiation responses are highly context dependent. Vascular responses can be initiated by only a few gray (Gy) and may have minimal effect on tissue function (54), while microvascular failure at high doses is generally considered the cause of CNS-ARS lethality. In addition to dose, the volume irradiated will be important. For example, after WBI, the systemic, cumulative impact of vascular damage will be considerably more

clinically relevant than after partial body irradiation. The impact on tissue function will also vary with tissue, time, and many other factors and will involve multiple components of the thrombo-inflammatory cascade. Nonetheless, it is clear that the vasculature has to be considered as a major force in the evolution of radiation diseases and a vital target for countermeasure development.

The acute microvascular cascade evolves over minutes to days after irradiation, reflecting the classic events that occur during acute inflammation (55). For convenience, a summary of acute and late vasculature-related events is presented in Table 1 (not all of which will be discussed here). Within a day of radiation exposure, endothelial cells may be lost through apoptosis caused by direct radiation damage or mediated by members of the TNFR family of death receptors (56). As has been mentioned, some investigators consider GI-ARS to be a direct consequence of endothelial cell apoptosis involving the sphingomyelin pathway (57), although this has been strongly disputed. What is not disputed is the fact that moderate radiation doses inflame the microvasculature, activating endothelial cells to express pro-inflammatory cytokines and/or receptors and cascades involving plasma proteins and cell adhesion molecules, such as selectins and ICAM-1 (58-61). These events proceed in distinct localized patterns in the vasculature of the irradiated tissue (62). Activated endothelial cells tether neutrophils and other myeloid cells, which, in turn bind activated platelets (63) that produce a burst of radical oxygen species (ROS). Myeloid pro-inflammatory cytokines, in a positive forward loop associated with increased oxidative stress, generate further inflammation, amplifying the response. This oxidative burst by myeloid cells also may be required for the transmigration process across the endothelium, which itself can lead to damage influencing other vascular functions after irradiation, including in the kidney (64). Indeed, the many mitigators that have demonstrated anti-oxidant properties may function by inhibiting this oxidative burst and abrogating its downstream consequences (65). The short-term functional effects of radiation on the vessels are vasodilation, increased blood flow to the site, increased vascular permeability and migration of inflammatory cells into the tissue parenchyma, along with plasma exudation (66, 67).

One consequence of the radiation-induced activation of death receptor pathways and thrombo-inflammation is loss of microvasculature. This can be assessed histologically by measuring the mean vascular density in irradiated and non-irradiated tissue; for example, after staining using anti-CD31 antibodies. CT imaging can be used (68). Furthermore, vessels may collapse or develop thickened basal membranes. Since the initial vascular damage is localized, over time, they also may establish an inflammatory non-resolving nidus within a tissue that may serve as a focus for lesion progression. Such a nidus may lead to the localized expression of late damage that is frequently seen in irradiated tissues and explain the random nature of disease expression between tissues and individuals.

ACUTE EVENTS

- Endothelial cell apoptosis through radiation or Fas death receptor-induced pathways

- Plasma cascade complement, kinin, coagulation, fibrinolysis systems engaged
- Pro-inflammatory cytokine production, such as TNF- α , IL-1, IL-6, VEGF, bFGF, and DAMPS, such as HMGB1.
- Endothelial cell hypertrophy and spreading
- Increased cell adhesion molecule expression such as selectins, ICAM-1
- Vascular contraction and dilation of arterioles leading to increased blood flow and redness
- Neutrophil margination, rolling and adhesion to activated endothelium
- Platelet adhesion
- Migration of neutrophils, monocytes, and lymphocytes, normally in that order, into extracellular spaces
- Increased vascular capillary permeability
- Plasma exudation, edema
- ROS production
- Collateral damage to parenchymal cells
- Activation of blood extrinsic coagulant and pro-thrombotic systems
- Clotting, vascular stasis, hypoxia, ischemia
- Vascular pruning, failure of neo-angiogenesis, possible switch to vasculogenesis

LATE EVENTS

Chronic inflammation

Mononuclear cell infiltrates with prominent mixed lymphocytes

Growth arrest, senescence, and production of senescent-associated secretory phenotype (SASP) by fibroblasts and smooth muscle cells

Chronic cytokine production, such as IL-6, IL-8, TGF- β , and ROS production

Vascular rupture, capillary dilatation

Thickening of vascular walls

Decrease in vasculature, hypoxia, ischemia

Telangiectasia, thrombosis, stenosis, fibrosis, necrosis

Table 1: Acute and late events in inflammation

In addition to the consequences of direct cell loss, radiation blocks the angiogenesis which normally would be initiated to restore normal blood flow and hemostasis. This complex process involves migration, proliferation, sprouting, and differentiation of endothelial cells interacting with the subendothelial space lining the vessel lumen or the vascular adventitia (69) and it is not clear which step is most sensitive to the prior

radiation injury. Compensatory vasculogenesis is a possible alternative tissue survival pathway to angiogenesis that most likely involves infiltration of bone marrow-derived endothelial progenitor or stem cells, although this also may be compromised following WBI (versus local irradiation). However, vasculogenesis tends to be a less effective tissue support system and may not be able to replace larger volumes of lost microvasculature (70). One measure of this defect is seen in the tumor bed effect that slows the growth of tumors in pre-irradiated normal tissues (71). In any event, vascular loss is a common result of radiation damage (68, 72) that may precipitate a hypoxic and ischemic microenvironment and progress into late damage expression, such as fibrosis and necrosis (Table 1), and generate a M2 suppressive macrophage lineage (42, 73). Ironically, hypoxia increases reactive oxygen species (ROS) levels by the electron transport chain and changes the metabolic status of the tissue through HIF-1 expression towards glycolysis and Nrf2 antioxidant products (e.g. NADPH and glutathione) (73). This metabolic adaptation can drive stem cell reprogramming through TGF- β , but an alternative outcome is collagen production and fibrosis (74). The contribution of microvascular loss to damage is likely to vary with dose and the affected tissue, but in the case of radiation-induced cardiovascular disease, fibrosis is a likely direct cause of cardiomyelopathy (68). Again, it is worth noting that the dose required to cause cardiac damage after WBI is generally less than that required to the heart alone (75-77).

Secondary to the primary vascular effects, serum lipid values have been shown to change after WBI (68), likely as part of a metabolic switch associated with inflammation. As Baker et al. have described in their rat studies, "From 20 days after TBI, a progressive increase in total serum cholesterol was seen. Low-density lipoprotein cholesterol progressively increased to a peak value of 82 ± 8 mg/dl at 80 days compared with 13 ± 3 mg/dl in unirradiated rats. There was also a transient increase in triglyceride levels 40 days after TBI, which then declined to values present in unirradiated rats by 100 days". Similar changes in the metabolic profile were found in A-bomb survivors (78). Interestingly, changes in lipid profiles are also found in inflammatory breast cancer patients where they impact outcome after radiation therapy, as well as modulating radiosensitivity and tumor initiating cells (79). Lipid metabolism may therefore be an important target for countermeasure development. The general link between lipid metabolism and inflammation is only now becoming recognized through studies on obesity and diabetes, but, as such, this is likely to be a major part of an integrated radiation-induced disease profile. In support of this are the studies mentioned earlier on cardiovascular disease and the findings that cardiac disease and diabetes have been recorded as late effects of WBI in non-human primate models (80, 81).

Some Vascular-Related Inflammatory Events and their Relevance to Countermeasures

A brief examination of the vascular-related processes mentioned in the previous section reveals a number of potential targets for countermeasure development. Each will have different dose and time parameters. Unlike mortality endpoints, these processes are often hard to reliably quantify.

Radiation-induced vascular permeability can be determined by assessing leakage of various tracers. The more popular tracers include fluorescein isothiocyanate (FITC)-dextrans, which comes in various molecular sizes (82), albumin, Evans blue dye,

(^{99m}Tc)-diethylenetriamine pentaacetic acid (83, 84), immunoglobulin G, or peroxidase (85). These tracers have different levels of sensitivity and are generally limited in that they require high doses of radiation to detect leakage. In addition, they give only semi-quantitative assessments of localized changes in permeability. There are no other well-established ways to measure systemic leakage after WBI except using proteomic changes in serum/plasma, whose complexity makes normal shotgun mass spectrometry approaches difficult. Dynamic changes in plasma volume after irradiation, especially after WBI, have to be taken into consideration and necessitate careful standardization (86-88). The use of sites that have natural body fluid filters with extravasation limits may be superior for this purpose, although the assays are less established. For example, in the eye, non-invasive flare photometry has been adapted for clinical assessment of proteomic changes in patients receiving WBI for bone marrow transplantation (89). Another model site is the kidney where vascular radiation damage in the glomerulus may result in proteinuria, measurable in the urine (90-92). The use of metabolomics also seems to offer advantages over more conventional assays (93). Nonetheless, despite the current uncertainties in providing real-time and meaningful determinations of the extent of radiation injury, the biological contributions that acute microvascular events make to the radiation-induced symptoms and disease stress the importance of using even basic vascular supportive care aimed at maintaining fluid and electrolyte balance.

Inflammation is an obvious target for mitigator intervention in vascular and tissue events. Cytokines are readily assessed in tissues and plasma at the RNA or protein level, and there are many multiplex, generally antibody-based (ELISA), commercial kits available for this purpose with varying degrees of sophistication. Unfortunately, assay standardization is often left solely to the manufacturer and results will vary with the chosen system. In general, responses are observed with doses of more than a few gray, though rarely are they linear with respect to dose and they vary markedly with time. Indeed, the measurement of cytokines contains many pitfalls. For one, levels of most pro-inflammatory cytokines are normally very low in serum and they are heavily influenced by half-lives and rates of secretion. Another huge cause for concern are platelets that tend to get easily activated during sample handling and, as a result, release significant amounts of cytokines, distorting results enormously. Analyses of cell and tissue mRNA or protein levels by immunohistochemistry can be more informative, but many cytokines have cell-bound forms and knowledge of their juxtacrine effects in mediating cell-cell interactions is sparse. In such cases, genetic approaches may be best, but as noted earlier, radiation endpoints may be affected by genetic manipulations. In vitro assessment using isolated cell cultures is fraught with artefacts, being very dependent on the culture conditions. In isolation, cytokine expression levels do not form a good endpoint for mitigator studies, but genetic models and inhibitory approaches can certainly inform on the general role of cytokine-mediated pathways in radiation responses and their mitigation. Arguably, one of the more radiation-sensitive endpoints in the integrated response appears to be up-regulation of ICAM-1, which is seen with doses as low as 2 Gy. The relevance of this response is illustrated by the findings that radiation-induced vascular permeability and immune cell infiltration into tissues can be mitigated by administration of anti-ICAM-1 antibody (82, 94), although we know of no reports on this modifying ARS. In keeping with a role for vasculature damage in ARS, endothelial progenitor cell transplantation has been shown to mediate hematopoietic

recovery and mitigate against ARS (95).

The activation and transmigration of the myeloid cell system following irradiation is also in line with the neutrophilia that is observed within hours, even after bone marrow-ablating doses of WBI and has been seen in all species studied to date. Following radiation injury, immature promyelocytic and neutrophilic myelocytic cells are mobilized from the bone marrow, and myelopoiesis is initiated (3), shifting the myeloid:lymphoid balance to favor the former. Interestingly, neutrophils and platelets have long been considered as critical controllers of the hematopoietic ARS (1). These, and subsequent, observations have raised a number of possibilities with respect to countermeasures, in particular, suggesting that these populations might be valuable cellular targets for mitigation. Furthermore, given the role of myeloid cells discussed earlier, they may protect against syndromes other than H-ARS. Finally, monitoring the various subpopulations may act as a simple marker of countermeasure efficacy. Consistent with these criteria, recent work has shown that mobilized myeloid cells are an essential element in the radiomitigation activity of 4-nitrophenyl sulfonyl piperazines, compounds that have demonstrated efficacy in several models of radiation lethality (McBride, in prep).

Encouragingly, over the past few decades, a plethora of agents have been used with varying degrees of success in the prevention or treatment of acute, transient and even chronic radiation injury, often with some degree of anti-inflammatory action (65). However, there is still a dire need to mitigate the less obvious acute or delayed radiation effects in order to achieve long-term satisfactory treatment. Efforts are needed to develop treatments for, or countermeasures against, the radiation “disease” that take into account the complex range of both the immediate and subsequent biological (and psychological) conditions that are seen following localized or whole body exposure in order to provide a complete therapeutic strategy.

Short-term survivors of Radiation Exposures and Later Disease

In this review, we have used dose-time relationships as a framework for the evolution of radiation diseases. Clearly, surviving WBI-related ARS, whether by luck or successful clinical intervention, does not necessarily result in long-term health, as late diseases can emerge with serious morbidities that decrease life-span (96-98). Waves of mortality emerge, as do waves of associated intercurrent molecular events that are most obviously observed as inflammatory responses. Examples of a direct proven causal linkage between inflammation and any mortality phase are few and far between, but there is ample evidence for myeloid cells being activated and mobilized within hours of WBI that seem to have a protective role in ARS and some other radiation diseases, with most the clear involvement being in H-ARS, where they are critical targets. In other ARS, and some other radiation mortalities, they seem to modify responses by other target cell populations. In contrast, for late morbidities and mortalities, systemic inflammation, and in particular cardiovascular disease, seems to provide both the conveyor belt and the underlying etiology for the radiation diseases. In essence, individuals lose the control mechanisms that hold their inflammatory responses in check, with a resultant pathology.

Our findings in mice surviving LD70/30 doses of WBI (Figure 4) are that they exhibit late morbidities including cardiomyelopathy, and inflammatory lesions in the lung, liver and kidney over the entire post-injury time period with several peaks in mortality that shorten their life-span (Figure 4). One interpretation is that they are undergoing radiation-induced premature aging, which is not a new concept (99, 100), but we prefer the term multi-organ disease syndrome (MODS), which is a general rubric for these complex disease endpoints in humans, that also encompasses increased frailty which is also seen in radiation-induced late disease (103). The good news is that, in our hands, 50% of the mice receiving a regimen of novel mitigators after LD70/30 doses of WBI were alive 1 year, compared with 0% of controls, and although late mortality was still present after ARS mitigation, it was much decreased in incidence.

Although this form of life shortening has often focused on carcinogenesis as a cause, non-cancer-related chronic conditions that tend to occur very late are being increasingly recognized as being of equal, if not greater, importance. Similar morbidities and mortality are also reported in mice receiving WBI plus bone marrow rescue (7) or LTI (7), in WBI non-human primates (80, 81), in A-bomb survivors (101, 102), and in accidents, such as at Tokai-mura (103, 104). In the clinic, recent advances in cancer therapy have resulted in encouraging increases in survivorship, but this has come at a cost. Increased late effects and life shortening are perhaps most obvious in bone marrow transplant patients (8, 9), but the phenomenon likely results from many types of cancer therapy. The similarities with frailty in the transplant population is of interest. Frailty in this population has been ascribed to chronic inflammation, cardiovascular disease, metabolic syndrome, low oxygen utilization, decreased physiologic reserve, and diminished resistance to stressors (9). Quality of life is often impacted in the form of weight loss, easy exhaustion, muscular weakness, decreased walking speed, etc.. In all these clinical situations, late debilitating diseases emerge that are of uncertain etiopathogenesis and incidence, and have few treatment options. Obviously, radiation mitigators that would prevent late chronic conditions would be of great value in the clinic, especially for survivors of childhood cancers, of bone marrow transplantation, of any intense anti-cancer regime, and possibly many other chronic inflammatory diseases.

Importantly, the experimental evidence suggests that radiation doses that are insufficient to cause ARS or subacute pneumonitis are sufficient to cause late disease, the incidence of which is exaggerated at low dose rate (7). Because late effects can occur after lower radiation doses than those that cause ARS, mitigation of one condition might actually allow another radiation-induced disease to become critical with time, while mitigation can also allow previously unrecognized syndromes that were masked by early lethality to emerge.

One major question that arises with respect to late disease is when is intervention indicated. The current work with countermeasures is focused on mitigators being given shortly after radiation exposure, which, in our hands, results in efficacy against both ARS and late disease. However, late disease occurs rather more capriciously than ARS and there will be a temptation to leave the until the first signs and symptoms and it is possible that, at this late stage, symptom management is the only option. Biomarkers for chronic inflammation such as IL-6 and C-reactive protein may help define those at

risk of late complications, as might myeloid cell profiling, but early changes in biomarkers may not be informative.

Unlike ARS, there is a degree of heterogeneity in which individuals develop chronic radiation disease that is not fully understood. Our data suggest that late disease is associated with radiation-induced skewing of the immune system towards myeloid cell expansion and activation, but it is also possible that MODS may be precipitated by certain post-radiation events, such as infections, physical damage, or other stresses that might tip the balance in individuals who have depleted reserves in terms of their ability to control inflammation. The microbiome is particularly interesting and targetable potential drivers of these responses. Even non-pathogenic microbes can shape the response of a previously irradiated intestine, acting perhaps through Toll-like receptors. Recently, radiation has been shown to influence the composition of the microbiome (37), and it seems likely that the microbiome that is generated will play a role in both acute and late radiation disease. The exact impact of this has yet to be evaluated, but it is crystal clear that the microbiome can no longer be ignored. As such, an understanding of its influence needs to be incorporated into countermeasure development.

In conclusion, countermeasure development can not only be concerned with ARS, but must take into account the factors that influence the long-term consequences of radiation disease and its temporal evolution. Late radiation effects are in many ways more insidious than ARS and can result in serious debilitating morbidities and a shorter life. The underlying pathology is suggestive of chronic inflammation linked to an imbalanced immune system that fails to control in particular myeloid cell expansion and activation, but no doubt other immune components play roles; many aspects of the etiopathogenesis of these late effects are still unclear. The challenge to mitigator development is to take such late effects into account even when ARS is the initial concern. So far, the evidence suggests that these late effects can be mitigated to an extent by delivery of mitigators soon after exposure, but this may not be the case for all mitigators or all aspects of the disease.

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