

10.1 *Deinococcus radiodurans*: Revising the Molecular Basis for Radiation Effects on Cells

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Abstract: The field of radiobiology was built on the premise that radiation is dangerous because of its damaging effects on DNA, where only a few events, or even a single event, at the molecular level can inactivate cells (Hutchinson 1966). The discordance of modern radiation toxicity models with results spanning nearly 5 decades of research on the extremely radiation-resistant bacterium *Deinococcus radiodurans* is reviewed. Much of the early data implicating DNA itself were for bacterial systems. However, recent studies show that extreme resistance to gamma radiation among bacteria consistently coincides with a greatly diminished susceptibility to protein oxidation but with similar DNA lesion-yields as other organisms. A growing body of experimental evidence now supports that naturally sensitive bacteria are killed by radiation mainly owing to protein oxidation, whereas extreme resistance in bacteria is achieved by protecting enzymes and the repair functions they catalyze. Based on new insights, the prospects for exporting the radioprotective mechanisms outside of *D. radiodurans* for practical purposes are considered.

Introduction

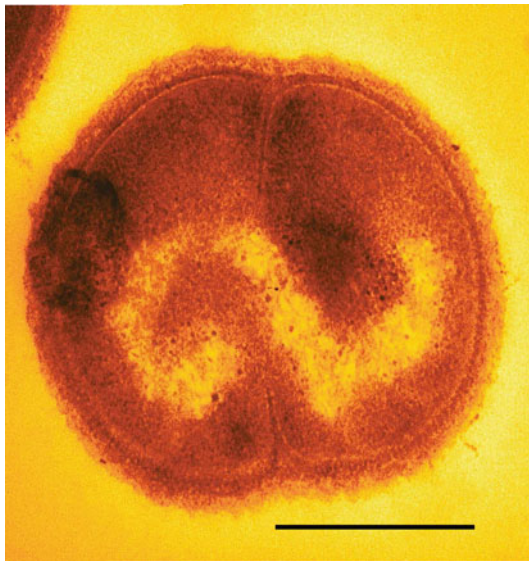
At the end of the nineteenth century, the greater scientific community had little faith in the existence of entities that could not be seen. All this began to change on November 8, 1895 when Wilhelm C. Röntgen was studying the passage of an electric current through a vacuum tube. At the Physical Institute of the University of Würzburg, Germany, he noticed that, if the Crookes tube was wrapped in black cardboard in a dark room, a barium platinocyanide screen located a few feet away glowed softly (Glasser 1993). Because the nature of the invisible light emanating from the Crookes tube was then unknown, Röntgen gave them the name X-rays. Within weeks, his discovery was an international news story; within months, Röntgen's original experiment was being treated as a novelty (Kevles 1997). Thomas A. Edison arranged a special exhibit on Röntgen rays at the annual Electrical Exhibition in New York City's Grand Central Palace in May 1896. This exhibit was a public sensation, mainly due to his demonstrating on a fluorescent screen the shadows of the bones of the hands of visitors. Thousands accessed the fluoroscopic room circuitously along galleries, which contrived to establish a supernatural atmosphere and which ended in a restaurant where the visitors could discuss their experiences (Kevles 1997). The early success and acceptance in the practical use of the X-ray in medicine was facilitated by such public displays. Unfortunately, the dangers of X-rays were not recognized until too late.

Radiation burns were recorded within a month of Röntgen's announcement of his discovery of X-rays (Glasser 1993). Edison had noticed some reddening around his own eyes and stopped experimenting with X-rays himself. About the same time, Marie and Pierre Curie found that pitchblende, a common ore of uranium, also emitted invisible light, gamma rays, even more penetrating than X-rays (Glasser 1993). From 1896 to 1903, hundreds reported slowly developing burns, and discharge tube operators in both medical and novelty settings died from overexposure. Reports on the lethal effects of X-rays and gamma rays followed, with safety guidelines for medical workers finally published by Britain's Röntgen Society in 1913. In 1928, 5 years after Röntgen's death, scientific units to quantify ionizing radiation (IR) were defined as the roentgen (R), the amount of energy deposited in air (Mould 1993). In 1953, a more practical unit of radiation was introduced. Named the "rad," it was an abbreviation for "radiation absorbed dose," the amount of radiation received by a person or object exposed to X-rays, gamma rays, alpha particles (helium nuclei), or beta particles (electrons) (Mould 1993). In 1975, the standard dose unit was modified once again, with the adoption of the

“gray,” this time named after the British radiobiologist Louis Harold Gray, where 100 rad is the same as 1 gray (Gy) (Mould 1993).

Just 10 Gy of radiation will kill most vertebrate animals including humans, a dose delivered by conventional ^{60}Co irradiators in a few seconds. In general, the vast majority of bacteria will not survive 500 Gy (Thornley 1963), which is key to the widespread success of industrial IR-based sterilization technologies. Yet, a few representatives from the three domains of life display remarkably high levels of resistance. For instance, baker's yeast can survive 300 Gy; many insects, which are largely post-mitotic, can survive 500 Gy; the roundworm *Caenorhabditis elegans*, the water bear *Milnesium tardigradum*, and the freshwater invertebrate animal *Philodina roseola* can tolerate 1,000–3,000 Gy but are rendered sterile (Gladyshev and Meselson 2008), and as a diploid, the basidiomycete fungus *Ustilago maydis* can withstand 6,000 Gy (Holloman et al. 2007). The archaeon *Halobacterium* sp. NRC-1 can resist 5,000 Gy (Kottemann et al. 2005), and a few spore-forming bacteria (e.g., *Bacillus megaterium*) and vegetative cyanobacteria (e.g., *Chroococidiopsis*) can withstand 10,000 Gy (Levinson and Hyatt 1960; Thornley 1963; Billi et al. 2000; Daly 2009).

By far, the most radiation-resistant group of organisms yet discovered belongs to the bacterial family *Deinococcaceae*, which typically displays near 100% survival following acute exposures to 12,000 Gy or 1,000 J/m² (254 nm) ultraviolet (UV) light (Battista 1997; Makarova et al. 2001) and can grow under extremely harsh conditions of chronic irradiation (60 Gy/h) (Daly 2000). The most characterized member of this group of extremophiles is *Deinococcus*



■ Fig. 10.1.1

Transmission electron micrograph of *D. radiodurans*. The cell was harvested from a mid-logarithmic culture in rich medium (Daly et al. 2004). The cell envelope consists of the plasma and outer membranes, which are separated by a 14- to 20-nm peptidoglycan layer (Makarova et al. 2001). The central, light-staining (∞ -shaped) structure is the genome-containing nucleoid, which is being replicated and partitioned into two daughter cells. When division is complete, the diplococcus contains approximately four haploid genome copies per cell. Scale bar 0.5 μm

radiodurans (● Fig. 10.1.1), first isolated from irradiated meat in 1956 by Arthur (Andy) Anderson and colleagues at the Oregon Agricultural Experimental Station in Corvallis, Oregon, USA (Anderson et al. 1956). The picture that has emerged for the life cycle of most *Deinococcus* species is one comprised of a cell-replication phase that requires nutrient-rich conditions, such as in the gut of an animal, followed by release, drying, and dispersal (Makarova et al. 2001; Daly 2009). Desiccated deinococci can endure for years, and if blown by winds through the atmosphere would be expected to survive and land worldwide. As reported, some become encased in ice (Zhang et al. 2009), and some entombed in dry desert soils (de Groot et al. 2005). High temperatures also are not an obstacle to the survival of some deinococcal species. *Deinococcus geothermalis* and *Deinococcus murrayi* were originally isolated from hot springs in Italy and Portugal, respectively (Ferreira et al. 1997). The prospects of harnessing the protective systems of *D. radiodurans* for practical purposes have captured the imagination of four generations of scientists. Perhaps if we understood why *D. radiodurans* is so resistant, we could find ways to protect people from atomic radiation, clean up radioactive waste sites, and live longer.

Cellular Targets of Ionizing Radiation

Classical models of radiation toxicity are built on the tacit assumption that ionizing radiation (IR) indiscriminately damages cellular macromolecules (Scott 1937; Hutchinson 1966). As individual proteins in a cell typically exist at much higher levels than their corresponding genes, IR-induced cell death has been attributed mainly to DNA damage (Hutchinson 1966; Daly 2009); long before the structure of DNA was solved, the genetic effects of IR were attributed directly to nuclear injury and chromosomal damage (Scott 1937). However, extreme IR resistance among bacteria consistently coincides with a greatly diminished susceptibility to IR-induced protein oxidation but with similar IR-induced DNA lesion-yields as other organisms (Daly et al. 2007). It has been proposed recently that naturally sensitive bacteria are killed by IR mainly owing to protein oxidation, whereas manganese complexes in extremely resistant bacteria protect enzymes needed to repair DNA and allow survival (Daly 2009; Daly et al. 2010).

The argument that damaged proteins may be responsible for the lethal action of IR was first developed by Walter M. Dale in the early 1940s (Dale 1940, 1942, 1943). This early view of IR toxicity was based on findings that enzymes in aqueous solution could be inactivated by relatively small doses of X-rays (less than 50 Gy). The possibility that resistance to IR could be increased was supported by studies which showed that the radiosensitivity of an enzyme is not a fixed entity but a variable, where inactivation could be prevented by the addition of nucleotides, sugars, amino acids, and a variety of other organic compounds. Dale's lasting contribution to radiobiology was to establish that the major route to radiation injury was reactive molecular species derived from the ionization of water (Barron et al. 1949), which generates hydroxyl radicals (HO^\bullet), superoxide ($\text{O}_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) as the major products (von Sonntag 1987); In contrast, in the 1930s, the lethal action of IR had been attributed mainly to direct damage (Scott 1937). Dale's idea that damaged proteins might be most responsible for toxicity in irradiated cells, however, was supplanted in the 1960s by radiobiology's dogmatic theme "death by DNA damage" (Hutchinson 1966).

The discordance of modern radiation toxicity models with results spanning nearly 5 decades of *Deinococcus* research illustrates the extent to which entrenched ideas have stymied progress. Experimental evidence favoring a transition away from DNA-centric models of

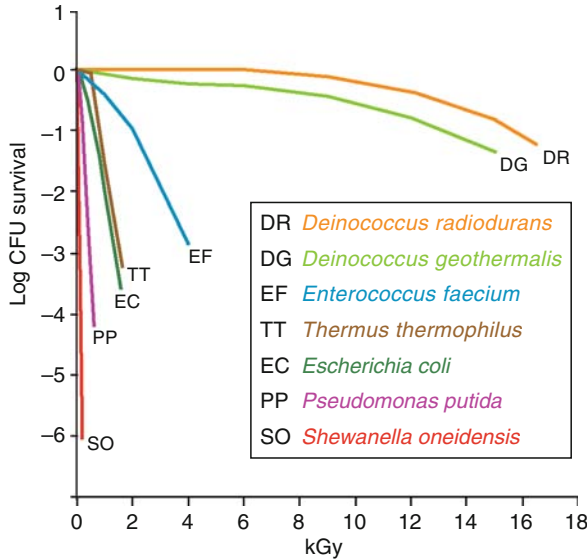
radiation toxicity has been mounting since the 1960s. Numerous lines of evidence have converged on the conclusion that damage to DNA and lipids is a secondary process, and that proteins are more probable initial targets of cellular radiation damage and should be placed at the top in the hierarchy of radiation-induced lesions most responsible for cell death (Du and Gebicki 2004; Daly et al. 2007; Kriško and Radman 2010). Certainly, DNA is a critical target in all irradiated cells (Scott 1937; Hutchinson 1966), but their survival ultimately rests on the ability of proteins to repair the damage (Daly et al. 2010).

When You Have Eliminated the Impossible, Whatever Remains, However Improbable, Must Be the Truth

The nature of the “target” molecules in cells – the alteration of which by ionizing radiation (IR) leads to the eventual damage – was first studied in bacteria, and the implications were broadly applied to formulating models of radiation toxicity (Hutchinson 1966; Blok and Loman 1973). However, it was evident to a few scientists in the 1930s that many improbable assumptions and false implications had been applied to the biological actions of IR (Scott 1937). Yet, many radiation biologists today remain beholden to the idea that only a few primary IR-induced events are required to inactivate a cell. By postulating a clear-cut all-or-none effect instead of a gradual build-up of toxic radiation products, target theory was able to explain the exponential (or nearly exponential) survival curves of the cells being studied (Blok and Loman 1973). Typical survival curves for *Escherichia coli*, and other IR-sensitive bacteria, seemed to correspond to the requirement that only a few events are necessary to produce inactivation (▶ Fig. 10.1.2). Because the survival of an irradiated cell ultimately depends on whether its genome is repaired, the inactivation events were ascribed to single genes as IR damage to a few proteins was not likely to be a lethal event (Daly 2009). In contrast, survival curves for *D. radiodurans*, and other extremely IR-resistant bacteria, display very large shoulders in their dependence on dose, which are attributed to very efficient DNA repair (▶ Fig. 10.1.2) (Daly et al. 2004). Neither DNA protection, unusual DNA repair proteins nor genome multiplicity, alignment, and structure provide an explanation for the extended shoulders in *Deinococcus* dose–response relations that distinguish them from radiosensitive organisms (▶ Fig. 10.1.2) (Daly 2009). The picture that emerges from the considerations listed below is that the major defense against radiation damage in *Deinococcus* bacteria is an enhanced capacity for scavenging the reactive molecular species generated by IR. The fate of irradiated bacteria that contain multiple genomes appears to reside not on the level of DNA damage but instead on their capacity to protect proteins (Daly et al. 2007).

DNA Protection

Early on, it was shown that the number of IR-induced double strand breaks (DSBs) inflicted per unit length of DNA in diverse organisms was similar and increased linearly with dose (Blok and Loman 1973; Gérard et al. 2001; Daly et al. 2004; Rothkamm and Löbrich 2003); DSBs are the most severe form of DNA damage (Daly 2009). The yield of IR-induced DSBs in a wide variety of cell-types was originally determined by sedimentation distances after ultracentrifugation of their DNA through sucrose gradients (Blok and Loman 1973). Later, IR-induced DSB



■ Fig. 10.1.2

Gamma-radiation survival curves for multi-genomic bacterial species that encode a similar repertoire of DNA-repair proteins (Daly et al. 2004). Bacteria were grown in rich medium to the late-logarithmic phase and irradiated (^{60}Co) on ice. Survival frequencies were determined by dilution of irradiated cultures and colony forming unit (CFU) assays on nutrient agar plates (Daly et al. 2004; Omelchenko et al. 2005). 1 kGy = 1,000 Gy = 100,000 rad

studies on bacteria used pulsed field gel electrophoresis (PFGE) (Daly et al. 2004), and studies on irradiated mammalian cells used immunofluorescence techniques based on quantifying DSB-dependent gamma-H2AX foci (Rothkamm and Löbrich 2003; Goodarzi et al. 2009). Values approximating 0.005 ± 0.002 DSB Gy $^{-1}$ Mbp $^{-1}$ were reported for IR-sensitive bacteria and *D. radiodurans*. Most bacteria can tolerate only 1–5 IR-induced DSBs per haploid genome, whereas *D. radiodurans* can survive nearly 200 IR-induced DSBs per haploid genome (Daly and Minton 1995; Lin et al. 1999). The value of 0.005 ± 0.002 DSB Gy $^{-1}$ Mbp $^{-1}$ is similar to those for IR-resistant archaea (e.g., *Pyrococcus* sp.), for simple eukaryotes (e.g., yeast), for simple animals (e.g., rotifers), human cells in liquid culture (e.g., NIH 3T3), and for viruses (e.g., SV40) (Blok and Loman 1973; Krisch et al. 1991; Gérard et al. 2001; Gladyshev and Meselson 2008; Rothkamm and Löbrich 2003; Daly 2009). Thus, the level of DNA protection in irradiated *D. radiodurans* is not appreciably different than in other organisms.

DNA Repair Proteins

The first clue that a highly specialized set of DNA repair proteins was not needed for extreme IR resistance, surprisingly, came from *E. coli*. In 1961, Erdman and colleagues reported the directed evolution of IR-resistant *E. coli* by the repeated passage of survivors through successive sublethal doses of ^{60}Co irradiation (Erdman et al. 1961). This work was followed in 1973 by

similar studies and results published by Davies and Sinskey for *Salmonella typhimurium* (Davies and Sinskey 1973) and then in 1974, by Parisi and Antoine for *Bacillus pumilus* (Parisi and Antoine 1974). The stepwise approach to selecting exceptionally high levels of radioresistance in *E. coli* was validated once again in 2009 by John R. Battista and colleagues, followed by genome sequencing and analysis of the most radiation-resistant mutants, which revealed surprisingly few mutations (Harris et al. 2009). A subsequent study showed that the radiation-resistant *E. coli* mutants were significantly less susceptible to IR- and UV-induced protein oxidation than the wild-type parent strain (Kriško and Radman 2010). Collectively, these experimental results support that a relatively conventional set of DNA repair genes is sufficient for extreme radiation resistance (Daly 2009).

Over the years, genetic and molecular studies reinforced this conclusion. In 1971, Moseley and Mattingly reported the first mutant analyses for *D. radiodurans* which showed that its recovery from radiation is dependent on DNA repair (Moseley and Mattingly 1971). Subsequent research confirmed that DNA repair enzymes, which are central to recovery of irradiated bacteria in general, were key to *D. radiodurans* survival. However, several highly radiation-sensitive *D. radiodurans* DNA repair mutants were fully complemented by the expression of orthologous genes from *E. coli* (Daly 2009). For many IR-sensitive cell-types, the frequency of the induction of a given mutation is directly proportional to the dose of IR, but not for *D. radiodurans*, which displays the same low levels of IR-induced mutation irrespective of dose (Minton 1994). Thus, the extreme resistance phenotype appears to be dependent, at least in part, on a conventional set of DNA repair proteins, which *D. radiodurans* uses extremely efficiently (Daly 2009).

The whole-genome sequence of *D. radiodurans* was one of the first to be published. The international news stories covering this milestone accurately reported the most significant finding: *D. radiodurans* encoded just about the same number and types of DNA repair proteins as radiation-sensitive bacteria. *Deinococcus* researchers were flummoxed. Perhaps there were novel DNA repair genes lurking unrecognized within the 3,284,156 base pairs of its genome (Makarova et al. 2001). This possibility was addressed systematically using functional genomic techniques based on whole-genome microarrays and whole-proteome approaches to study RNA and protein expression patterns in *D. radiodurans* cells recovering from high-dose irradiation (Daly 2009). Alas, the mystery deepened further with the finding that hundreds of genes were induced in *D. radiodurans* recovering from IR, and most of the upregulated novel genes had no effect on resistance when disrupted. Since then, two other *Deinococcus* species have been sequenced, *Deinococcus geothermalis* and *Deinococcus deserti*, which share only approximately 150 uncharacterized genes with *D. radiodurans* (Makarova and Daly 2010). Among the shared novel genes induced in irradiated *D. radiodurans*, only a few have a discernible functional relevance to the preservation of genome integrity. One moderately IR-sensitive *D. radiodurans* mutant which has been constructed is *pprA*⁻, which is a putative DNA-binding protein (Kota and Misra 2006). Another is a moderately IR-sensitive *D. radiodurans* mutant *ddrB*⁻, which encodes an extremely diverged single-strand DNA-binding protein (Norais et al. 2009). However, for most of the mutants derived from this subset of novel deinococcal genes, there was no significant change in the level of IR resistance, indicating that few of the putative resistance proteins, at least individually, contribute to recovery (Daly 2009). This large body of functional genomics research was conducted in numerous laboratories and funded mainly by the US Department of Energy (DOE) from 1997 to 2007. Thus, genetic evidence supporting the existence of novel genes responsible for extreme IR resistance in deinococci has grown progressively weaker.

Genome Multiplicity, Alignment, and Structure

Until the 1970s, bacteria were generally considered haploid organisms with only one copy of their genome in resting cells. This possibility might have explained the great sensitivity to IR of most bacteria as one DSB in a bacterial genome would have been lethal. However, subsequent studies revealed that IR-resistant and IR-sensitive bacteria are multi-genomic. For example, *D. radiodurans* and *E. coli* have four to eight haploid genomes per cell during logarithmic growth (Hansen 1978; Akerlund et al. 1995), but only *D. radiodurans* is IR-resistant (▶ Fig. 10.1.2). Other notable examples are *Micrococcus luteus*, *Micrococcus sodonensis*, and *Azotobacter vinelandii*, which contain at least ten haploid genomes per cell but are IR-sensitive (Minton 1996).

In an early repair model, the alignment of *D. radiodurans*' four to eight genomes per cell was taken as the launching point for DSB repair (Minton and Daly 1995). This model made two major predictions: first, *recA*-dependent recombination between homologous DSB fragments originating from widely separated genomic locations should show strong positional effects upon irradiation, and second, transmission electron microscopy (TEM) of chromosomal DNA in *D. radiodurans* should reveal evidence of structures linking chromosomes. Both predictions were tested and refuted: molecular studies showed high levels of recombination between homologous DSB fragments irrespective of their genomic origin (Daly and Minton 1996, 1997), and no linking structures were observed by TEM-based optical mapping (Lin et al. 1999). Another model proposed that high levels of chromosomal condensation observed in *D. radiodurans* grown in rich medium (▶ Fig. 10.1.1) facilitated repair by holding proximal DSB ends together, and that manganese promoted the condensation of its nucleoids into ring-like structures. This model is also generally discounted: *D. radiodurans* grown in defined minimal medium did not display condensed nucleoids but remained extremely IR-resistant, and *D. radiodurans* which was depleted in manganese displayed condensed ring-like nucleoids but was rendered IR-sensitive (Daly et al. 2004; Ghosal et al. 2005). Thus, IR-induced DSB fragments in irradiated *D. radiodurans* are not immobilized, and the structural form of its nucleoids does not play a deciding role in radioresistance. *D. radiodurans* contains numerous, unusual, mosaic-type small nuclear repeats (SNRs) and G-quadruplex sequences (Makarova et al. 2001); both types of sequence potentially could contribute to genome structure and reassembly. However, SNRs and G-quadruplex sequences were not identified in the genomes of *D. geothermalis* or *D. deserti* (Makarova and Daly 2010). In summary, no distinctly unusual features based on genome structure, sequence or multiplicity are shared between *D. radiodurans*, *D. geothermalis*, and *D. deserti*, to the exclusion of IR-sensitive bacteria, which establish an unequivocal molecular basis of radioresistance.

A Recipe for Radiation Resistance?

Manganese in *D. radiodurans*

Defined minimal media compositions for *D. radiodurans*, all list Mn as an essential ingredient. In 1976, Alan K. Bruce and colleagues first reported a large depot of Mn in *D. radiodurans*, which contained approximately 100 times more Mn than *E. coli*, and that Mn depletion decreased the UV resistance of *D. radiodurans* (Leibowitz et al. 1976). Using neutron activation analysis (NAA), they showed that *D. radiodurans* normally accumulated about 5 mM Mn. In

2004, the NAA results were corroborated using inductively coupled plasma mass spectrometry (Daly et al. 2004), and other studies showed that when *D. radiodurans* was incubated in defined minimal medium containing the radioisotope ^{54}Mn , the cells accumulated about 2 mM Mn (Daly et al. 2004). More recently, X-ray fluorescence (XRF) microspectroscopy revealed that Mn is distributed throughout *D. radiodurans* cells but with regional intracellular Mn concentrations ranging from 0.3 to 3 mM (Fig. 10.1.3) (Daly et al. 2007). In contrast, most of the Fe in *D. radiodurans* was sequestered outside of the cytosol, in the septa of dividing cells (Fig. 10.1.3). Based on electron paramagnetic resonance (EPR) spectroscopy and X-ray-absorption near-edge structure (XANES) analyses, the dominant form of manganese in normal *D. radiodurans* cells is Mn^{2+} , with no significant levels of Mn^{3+} detected (Daly et al. 2004, 2007). When *D. radiodurans* cells were grown in conditions that limited Mn accumulation, their cellular Mn concentration decreased together with their IR resistance (Daly et al. 2010). Since the concentration of Mn in bacteria does not affect the level of IR-induced DSBs, this left the question, what does Mn protect in *D. radiodurans*? It has been proposed that Mn^{2+} ions functionally replace Fe^{2+} and Mg^{2+} ions as mononuclear cofactors in enzymes, thereby protecting the active sites from oxidative damage (Daly 2009), and that Mn complexes are

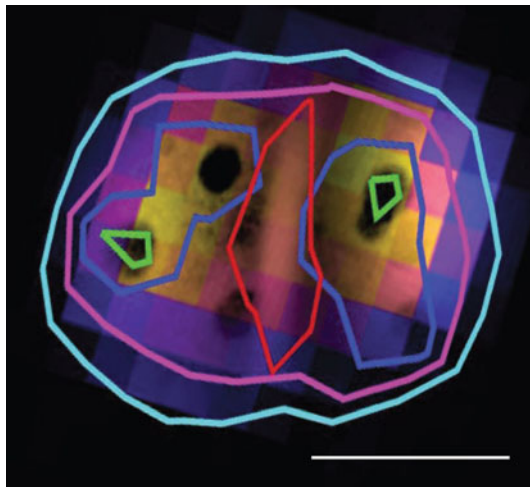


Fig. 10.1.3

X-ray fluorescence maps of the qualitative distribution and concentration gradients of manganese and iron in *D. radiodurans*. Transparent image overlay assembled from transmission electron microscopy, light microscopy, and X-ray fluorescence (XRF) microspectroscopy analyses of a single desiccated *D. radiodurans* diplococcus (Daly et al. 2007). Approximate depth-average abundance of Mn (green, 3 mM; dark blue, 2 mM; mauve, 1 mM; light blue, 0.3 mM) and Fe (red, 0.5 mM) are shown. A mathematical model of the original morphology of the diplococcus was constructed to determine the distribution and approximate concentration of Mn and Fe. As the diplococcus was dried out during preparation for XRF, the regional concentrations of Mn and Fe are predicted to be lower in hydrated *D. radiodurans* cells. XRF analysis measurements were made using the hard X-ray microprobe beamline 2ID-D at the Advanced Photon source, Argonne National Laboratory, Chicago, USA, under previously described conditions (Daly et al. 2007). Scale bar 0.5 μm

formed which defend against $\text{O}_2^{\bullet-}$ and H_2O_2 (Ghosal et al. 2005; Daly et al. 2010). The most consequential damage by $\text{O}_2^{\bullet-}$ and H_2O_2 in cells is to proteins which contain exposed iron-sulfur or heme groups, to proteins which contain cysteine residues, and to proteins containing cation-binding sites, where an iron-catalyzed site-specific oxidation occurs (Ghosal et al. 2005; Daly 2009).

Proteins as Targets of Radiation

The effect of IR on enzyme systems was the subject of in vitro investigations in the 1930s (Scott 1937). In those experiments, the amount of radiation necessary to produce inhibition was so high (>400 Gy) that researchers quite reasonably concluded that X-rays and gamma rays only influence enzymes when the dose is enormous. Failure to see enzyme inactivation at high doses turned out to be an artifact of using large amounts of enzyme and impure preparations (Dale 1940). In the 1940s, Walter M. Dale and his small group at the Holt Radium Institute in Manchester, England were the first to study the in vitro effects of IR on purified enzymes, which were inactivated by doses of less than 20 Gy (Dale 1940, 1942, 1943). He noted that when the purified enzymes were irradiated at low concentrations, their activity was lost at doses below those used in radiotherapy. Dale went on to show that the addition of an enzyme's substrate or other small organic compounds (e.g., nucleotides, amino acids, and sugars) greatly increased their IR resistance. It seemed that radiation acted indirectly on the protein moiety of the enzymes and on their prosthetic groups. Within several years, Thomas P. Singer's group demonstrated that sulfhydryl enzymes were even more sensitive than the enzymes studied by Dale, inactivated by just 5 Gy (Barron et al. 1949), which is in the same range as doses which kill most organisms. It took another 65 years before the dose-dependent relationship between protein oxidation and survival was first examined in vivo. In 2007, Michael J. Daly and colleagues showed that for a given dose of IR, the level of protein oxidation in IR-resistant and IR-sensitive bacteria was very different and quantitatively related to their survival (► Fig. 10.1.4) (Daly et al. 2007). For a group of well-characterized bacteria at the opposite ends of IR resistance, when intracellular Mn to Fe concentration ratios were high, bacteria were extremely resistant to protein oxidation and IR; when intracellular Mn to Fe concentration ratios were low, bacteria were hypersensitive to protein oxidation and IR (► Fig. 10.1.4) (Daly et al. 2004; Daly 2009). A mathematical model of radiogenic oxidative stress by Igor Shuryak and David J. Brenner is consistent with those data and can potentially be generalized to other organisms and lower radiation doses (Shuryak and Brenner 2009).

Manganese Complexes

The idea that Mn might somehow impart protection to proteins exposed to reactive oxygen species (ROS) is not new. Irwin Fridovich and colleagues discovered that Mn^{2+} and orthophosphate spontaneously form complexes, which catalytically remove $\text{O}_2^{\bullet-}$ from solutions via a disproportionation mechanism (Archibald and Fridovich 1982), and Earl Stadtman and colleagues discovered an unexpected property of complexes consisting of Mn^{2+} and amino acids or peptides, namely their ability to catalytically decompose H_2O_2 (Berlett et al. 1990). Since $\text{O}_2^{\bullet-}$ and H_2O_2 do not react with purified DNA (Blok and Loman 1973; Daly 2009), proteins were the putative targets of protection by Mn complexes (Daly et al. 2004). The first

Species	SO		PP		EC		EF		DG		DR	
kGy	0	4	0	4	0	4	0	4	0	4	0	4
DNPH	-	+	-	+	-	+	-	+	-	+	-	+
Coomassie												
Carbonyl												
Overlay of coomassie and carbonyl												
D ₁₀ (kGy)	0.07		0.25		0.7		2		12		12	
Mn/Fe	0.0005		<0.0001		0.0072		0.17		0.46		0.24	

■ Fig. 10.1.4

Western blot immunoassay of protein-bound carbonyl groups in cell extracts prepared from bacteria irradiated to 4,000 Gy (⁶⁰Co at 0°C); 20 μg of a protein sample (soluble fraction) was loaded per lane and assayed for oxidation using 2,4-dinitrophenylhydrazine (DNPH) under previously described conditions (Daly et al. 2007). Coomassie represents a Coomassie-stained polyacrylamide denaturing gel of the soluble proteins, whereas carbonyl represents the corresponding western blot, which reveals the presence (*black*) or absence of protein oxidation (*no signal*). Carbonyl groups (aldehydes and ketones) are widely used as markers of irreversible protein damage. The transparent overlay of the Coomassie and carbonyl images shows that not every protein in IR-sensitive bacteria is oxidized during irradiation. Abbreviations – bacterial species, as listed in ▶ Fig. 10.1.2. D₁₀, the ionizing radiation dose that reduces the number of viable cells by 90%; note, the D₁₀ cell survival value for *D. radiodurans* is slightly less than the D₁₀ CFU survival value (▶ Fig. 10.1.2) because *D. radiodurans* typically clusters as groups of two cells (diplococci) or four cells (tetrads) (Daly et al. 2004). Mn/Fe, intracellular Mn to Fe concentration ratios, was determined by inductively coupled plasma mass spectrometry as described previously (Daly et al. 2004). For all data sets, bacteria were grown in the same medium to the same stage of growth (late-logarithmic phase).

evidence that small radioprotective molecules existed in *D. radiodurans* was published by Alan K. Bruce in 1964 (Bruce 1964). He reported that low molecular weight agents (<15 kDa) in protein-free extracts prepared from *D. radiodurans* protected sensitive bacteria against the lethal effects of IR. Based on whole-genome comparisons, there is a remarkable abundance in *Deinococcus* species of genes encoding phosphatases, proteases, and peptide transporters, which might give rise to precursors for ROS-scavenging Mn²⁺ complexes needed for recovery

(Makarova et al. 2001; Ghosal et al. 2005). In 2010, such Mn complexes were identified and characterized in *D. radiodurans* (Daly et al. 2010), and protein damage in *E. coli* exposed to IR or UV was shown to be causative and not merely correlative in radiation toxicity (Kriško and Radman, 2010).

Deinococcus Prospects

Since the 1960s, the goal of exporting the radioprotective processes of *D. radiodurans* outside of the host cell for practical purposes has eluded researchers. The conceptual framework presented here, that protein oxidation in irradiated cells is not the consequence of cell death but its major probable cause, carries with it important theoretical and practical implications (Daly et al. 2010).

Mn-Dependent Chemical Antioxidants

Based on experimental analyses in *D. radiodurans*, Mn²⁺ accumulation trumps enzymatic ROS defense systems by far; the genes that encode the constitutively and highly expressed ROS-scavenging enzymes SodA (superoxide dismutase) and KatA (catalase) can be knocked out in *D. radiodurans* with almost no loss in IR resistance, but its Mn²⁺ transporter gene (*nramp*, DR1709) is essential (Daly 2009). Given that protein-free cell extracts of *D. radiodurans* are highly protective of irradiated *E. coli* and human cells in liquid culture (Bruce 1964; Daly et al. 2010), this bodes well that reconstituted chemical antioxidants of *D. radiodurans* could be delivered to widely differing cell-types without toxicity. In diverse practical settings, this could facilitate the development of bioremediation strategies aimed at the cleanup of radioactive mixed wastes, or sites containing other strong oxidants (Daly 2000), and approaches to protect humans from radiation (Daly et al. 2010). For example, several naturally IR-sensitive bacteria are known to express a suite of functions that can deal with toxic organic mixtures and heavy metals but are not an option at radioactive sites. Biostimulation with Mn complexes may present an opportunity to increase the resistance of bacteria without the need for genetic engineering (Daly 2000). In another setting, targeted delivery of Mn complexes to patients undergoing radiotherapy may help prevent side effects such as alopecia.

Metabolic Routes to Radiation Resistance

Survival of *D. radiodurans* exposed to extreme doses of ionizing radiation (IR) is highly dependent on a rich source of peptides and amino acids during recovery (Ghosal et al. 2005). The possibility that Mn-dependent chemical antioxidants in *D. radiodurans* are based on common metabolites raises the possibility that equivalent synergistic processes promoted by Mn²⁺ may be acting similarly in other organisms (Daly 2009). Over the last 2 decades, dozens of genes from IR-sensitive bacteria have been cloned and functionally expressed in *D. radiodurans* growing under high-level chronic irradiation or recovering from high-dose acute irradiation (Daly 2000; Daly et al. 2004). Similarly, many *D. radiodurans* proteins have been functionally expressed in IR-sensitive bacteria without problems. When purified from *D. radiodurans*, proteins are neither inherently radiation-resistant nor do they have unusual requirements for activity (Daly et al. 2007). A direct route to IR resistance appears to be via

metabolite regulation. For example, the development of exceptional radioresistance in naturally sensitive bacteria is typically accompanied by a progressive loss of metabolic functions, which appear to promote the accumulation of secondary metabolites. The heterotrophic nutritional modes reported in IR-resistant mutants of *E. coli*, *S. typhimurium*, and *B. pumilus* resemble those of wild-type *Deinococcus* species (Davies and Sinskey 1973; Parisi and Antoine 1974; Ghosal et al. 2005). The radioprotective benefits of Mn accumulation in cells which are unable to amass small organic molecules together with orthophosphate may be limited, and similarly for cells which accumulate small molecules without Mn. Several extremely IR-resistant cell-types are known to accumulate precursors of Mn complexes. For example, dormant spores of *Bacillus* species, both monogenomic (*Bacillus subtilis*) and digenomic (*Bacillus megaterium*), are IR-resistant and accumulate high levels of Mn and dipicolinic acid as well as a large depot of small, acid-soluble proteins (SASP) (Levinson and Hyatt 1960; Eisenstadt et al. 1973; Setlow 2007), Mn-rich cyanobacteria stockpile trehalose (Shirkey et al. 2003; Billi et al. 2000), and radiation resistant fungi accumulate melanin (Chapter 10.3, Melanin and Resistance to Ionizing Radiation in Fungi). It is conceivable that metabolic interventions in mammalian cells in G2 (tetraploid) could facilitate the accumulation of small organic compounds, Mn²⁺, and orthophosphate, and thereby prevent oxygen radical-mediated protein damage during irradiation or aging (Levine and Stadtman 2001).

Irradiated Vaccines

All proteins in *D. radiodurans* cells are enormously resistant to IR-induced oxidation (► Fig. 10.1.4) (Daly et al. 2007). In contrast, many proteins in IR-sensitive bacteria are readily and irreversibly oxidized (Daly et al. 2007) (◀ Fig. 10.1.4). One tangible application of Mn-dependent chemical antioxidants of *D. radiodurans* could be the preparation of IR-sterilized whole bacteria, whole virus, or protein vaccines with only nominal loss in immunogenicity. Others have shown that bacteria attenuated by exposure to 8,000 Gy are able to trigger long-lasting immunity (Datta et al. 2006). However, the anticipated levels of IR required to inactivate bacteria without any risk of infection would be massive and render vaccines with greatly diminished or no immunogenicity due to oxidation of their antigenic determinants. Similar drawbacks apply to viruses, which require even higher IR doses than bacteria for inactivation because of the small size of their genomes. It is tantalizing to consider that the epitopes of cells or viruses irradiated in the presence of reconstituted deinococcal Mn complexes might survive IR doses which obliterate their genomes. This could expedite vaccine production and the deployment during epidemic outbreaks, bioterrorist attacks, and other biothreats (Datta et al. 2006; Daly et al. 2010).

Summary

The vast majority of known organisms are extremely sensitive to the cytotoxic effects of X-rays and gamma rays, killed by doses far below 200 Gy; hence, the global concerns over the impact of environmental radiation on human health, including from medical devices, and growing concerns over the emerging threat of radiological terrorism acts involving potential mass casualty incidents. There are, however, bacteria which are remarkably resistant, capable of surviving immense doses of acute IR (12,000 Gy), and growing under high-level chronic IR (60 Gy/h) (Daly et al. 2004). The most characterized member of this group of extremophiles is

D. radiodurans (Makarova et al. 2001). As the lethal effects of radiation are mediated principally through reactive oxygen species (ROS) generated from water in irradiated cells, the essential protective role of accumulated manganese in *D. radiodurans* has been attributed to the formation of small ROS-scavenging Mn complexes (Ghosal et al. 2005; Daly et al. 2007; Daly 2009; Daly et al. 2010) and the replacement of Fe^{2+} and other divalent cations with Mn^{2+} as mononuclear cofactors in enzymes, thereby preventing metal-catalyzed reactions which proliferate ROS (Anjem et al. 2009; Daly 2009). Based on the insights from physiology, comparative genomics, and biochemical studies on *D. radiodurans*, the prime candidates for the complexes include orthophosphate and Mn^{2+} , which together catalytically remove $\text{O}_2^{\bullet-}$ (Barnese et al. 2008; Daly et al. 2010) and amino acids and peptides bound to Mn^{2+} , which catalytically decompose H_2O_2 and stoichiometrically scavenge hydroxyl radicals (Berlett et al. 1990; Daly et al. 2010).

During the Duck-and-Cover Times of the Cold War, the overriding goal of the field radiobiology was to develop medical countermeasures against atomic radiation released from nuclear bombs. Yet, 50 years later, almost no clinically relevant radioprotective pharmaceuticals have been developed. Early studies on radiation-sensitive bacteria implicated DNA as the principal radiosensitive target, which became a working hypothesis around which scientists began searching for a unified theory of radiation toxicity (Hutchinson 1966). Unfortunately, the DNA double strand break (DSB) assumed the role of the most lethal event in irradiated cells (Blok and Loman 1973). As a result, early studies on the effects of irradiated proteins (Dale 1942) were largely ignored, and the phenomenal ability of *D. radiodurans* to survive hundreds of IR-induced DSBs was dismissed as a biological curiosity. Today, *D. radiodurans* stands poised to reform approaches for radioprotection based on preventing protein oxidation, ranging from pre-exposure prophylactic interventions to post-exposure therapeutics (Daly et al. 2010). It has been 115 years since Röntgen's discovery of X-rays, and it is sobering to think that the main target of IR in cells remains ambiguous. Given the delayed, but profound impact, *D. radiodurans* could have in revising the molecular basis for radiation effects on cells (Daly 2009; Daly et al. 2010; Kriško and Radman 2010), the immediate lesson to the greater scientific community may be a warning – ignore extremophiles at your peril.

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Cross-References

- ▶ 10.2 Ecological Stress: Melanization as a Response in Fungi to Radiation
- ▶ 10.3 Melanin and Resistance to Ionizing Radiation in Fungi

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