EFFECT OF ELECTRON BEAM DOSE RATE ON MICROBIAL SURVIVAL

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Electron beam accelerators can be used to neutralize a wide variety of biological agents with minimal collateral damage. However, the response of cellular systems to a wide range of dose-rates is not well understood. While it is generally accepted that low dose-rate is less detrimental to biological systems, the effect of doses delivered in time frames on the order of chemical and physical responses is largely unexplored. We have investigated microbial survival at two vastly different dose rates. 18-20 MeV electrons from a medical-grade pulsed linear accelerator were delivered at an average dose rate of 30 Gy/s, at a repetition rate of 60 pulses per second, with a pulse width of 2 μs. At the higher dose rates, 3 MeV electrons were delivered from a spiral line induction accelerator (ISIS) is one 35 ns pulse, resulting in dose rates close to 10^{12} Gy/s. For vegetative cells, the LD_{50} of electrons delivered from the ISIS accelerator was consistently lower than comparable doses delivered from the LINAC, whereas the opposite was true for bacterial spores. In metabolizing cells, the time of dose delivery using the LINAC may allow repair activity to take place, resulting in increased survival after comparable doses.

I. INTRODUCTION

Biological weapons development continues to be a major threat for the United States, and neutralization techniques must evolve with the technology. Basic research into effective methods for such neutralization efforts is necessary from a practical standpoint of understanding the limits of current methods to allow changes to be made that will increase their effectiveness, while maintaining cost, portability, safety and applicability. From a scientific standpoint, understanding the basic biological mechanisms behind the responses will allow more effective strategies for neutralization to be developed, and may provide critical information applicable to a wide range of disciplines, from medicine to astrobiology.

I.A. Dose and Dose Rates

Ionizing radiation has long been used for neutralization of microbial pathogens, in applications as varied as medicine, food industries and planetary protection. Microbes in general require significantly larger doses for killing than do cells of higher organisms; however, there is an extremely broad range of LD_{50} (dose resulting in 50% survival) values across the kingdoms eubacteria and archaea. The LD_{50} for *Escherichia coli*, a common laboratory organism, is on the order 100 Gy[1, 2]. In contrast, we have confirmed the reported LD_{50} value for the highly radiation-resistant bacterium *Deinococcus radiodurans* at approximately 8000 Gy[3]. Desiccation-resistance also been linked with radiation-resistance in *D. radiodurans*[4]. The halophilic archaeon *Halobacterium* sp. NRC-1, which is resistant to desiccation as well as high salt, has an LD_{50} between 2500 and 5000 Gy, depending on the metabolic state of growth[3, 5]. We have recently reported the isolation of mutants of *Halobacterium* with increased resistance to ionizing radiation, making these organisms the most radiation-resistant known[3]. Although certain microbes are naturally resistant to DNA damage during all stages of growth, others, such as the *Bacillus* sp., form inert chemical and radiation-resistant spores that allow survival, or regeneration of growth, after prolonged exposure to a variety of physical and chemical stresses[6].

In order to effectively neutralize microbial cells using ionizing radiation, the dose must be delivered in a time frame sufficiently short to circumvent any cell-mediated repair processes. Typical sterilizing radiation exposures take place in time frames of minutes to hours, depending on the source of radiation. Numerous studies have indicated that longer exposure times allow greater survival, presumably because biological processes, such as DNA damage repair, have sufficient time to occur. In addition, cellular adaptation to lower chronic doses allows increased long-term survival. Despite the abundance of

research on low dose rate effects, there is little known about the effect of acute doses delivered at extremely high dose rates on cellular survival.

Cellular sensitivity to radiation is a function of many factors. Across species, cells with larger genomes generally display greater sensitivity, as a larger target is available for damage[7]. Within a given cell type, (genetic background) mutations in genes responsible for DNA damage repair confer sensitivity. Microorganisms, like higher cells, generally display increased sensitivity to radiation effects when actively growing (exponential phase). The lower level of resistance during exponential phase has been attributed to saturation of the repair machinery during rapid growth [8]. Although most microbial species tested, including *D. radiodurans*, have been reported to show higher resistance during stationary phase, there are examples of the reverse situation [9, 10]. *Halobacterium* NRC-1, as well as its radiation-resistant mutants, show maximum resistance to γ-radiation when in the exponential phase [3, 5]. Microbial spores represent a very different state of existence from vegetative (cellular metabolic) growth, in which virtually all cellular function has ceased. The nature of the environment (aqueous or desiccated) may be important in determining survival in all cases.

I.B. Model Organisms

I.B.1. Deinococcus radiodurans

Deinococcus radiodurans is a gram-positive staining eubacterium of the family Deinococcaceae [11]. The distinguishing characteristic of this organism is the ability of vegetative cells to withstand levels of ionizing radiation lethal to most other organisms. This extreme resistance to ionizing radiation has been linked to desiccation resistance, and may be a result of repair systems evolved to combat the damage inflicted during drying [12].

I.B.2. Halobacterium

Representing a different kingdom from *D. radiodurans*, the archaeon *Halobacterium* sp. NRC-1 thrives in near-saturating salt concentrations, and has been isolated from brine pools where its natural pigmentation imparts a pink color to the surroundings. Like *D. radiodurans*, vegetative cells of this non-spore forming microbes show high levels of resistance to desiccation, as well as both ionizing and non-ionizing radiation [5, 13, 14]. The mechanisms behind this resistance are less well explored than in *D. radiodurans*, and may involve repair mechanisms more similar to those seen in higher cells (eukaryotes) than in bacteria (prokaryotes) [15].

I.B.3. Bacillus subtilis

Bacillus subtilis is a gram-positive, endospore-forming eubacterium. The non-metabolizing spores formed from vegetative cells of this bacterium are remarkably hardy to a wide range of environmental conditions, displaying resistance to multiple stresses including heat and ionizing radiation. Passage through the spore state is non-reproductive, in that each cell can only produce one spore. Addition of proper nutrients stimulates the emergence of vegetative growth from spores. Spores themselves do not reproduce, but resume normal growth upon germination into vegetative cells.

II. MATERIALS AND METHODS

II.A. Strains and growth conditions

Halobacterium strain NRC-1[3] was obtained through our collaboration with Shiladitya DasSarma at UMBI/COMB. *Deinococcus radiodurans* R1 (ATCC 13939) was a gift from John Battista at Louisiana State University. *Bacillus subtilis* strain BAL218 was a gift from Beth Lazzazara at UCLA. *Halobacterium* cultures were grown in CM^+ medium[16] at 42°C with shaking, without supplemental illumination. For solid medium, 2% agar was added. *D. radiodurans* was grown in TGY (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) at 30°C with aeration [17]. *B. subtilis* was grown in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37°C with aeration. Spores were isolated from *B. subtilis* following the procedure of Niebuhr and Dickson [18]. Briefly, cells were grown quiescently in LB supplemented with 1% starch and 0.01% $MnSO_4$ for 96 hours at 32°C. Cells were harvested by centrifugation, and lysed in cold 100% ethanol for 4 hours. For solid medium, agar was added to TGY and LB to 1.5%. *Halobacterium* plates were incubated at 42°C for 7 days, then removed and continued to grow at room temperature until no new colonies appeared. *D. radiodurans* plates were incubated at 30°C for 3-5 days. *B. subtilis* plates were incubated at 37°C overnight. Liquid cultures for irradiation were inoculated from purified isolates on solid medium. Stock cultures were maintained in glycerol at -80°C. For short-term use, purified cultures were maintained on stock plates at 4°C. For survival determination, irradiated liquid cultures or spore suspensions were immediately diluted and spread on plates. Survival after irradiation was determined by comparing surviving colony-forming units to an unirradiated control.

II.B. Accelerators and Facilities

All cell irradiations were carried out at the Idaho Accelerator Center at Idaho State University.

II.B.1. 25 MeV LINAC

Linac-based irradiations were done with an S-band 25 MeV linac. It has a maximum pulse width of 2 μs, a peak beam current of 80 mA and a repetition rate from single pulse to 60 Hz.

II.B.2. Induction Accelerator

Single-pulse irradiations were done with the Idaho State Induction accelerator System (ISIS). It is capable of unusually high beam currents (~10-20 kA). These irradiations were delivered in one 35 ns-long electron pulse at 3.5 MeV and 15 kA.

II.C. Irradiation and dosimetry.

The 25 MeV pulsed electron LINAC delivered 18-20 MeV electrons at a peak current of 80 mA, with a peak dose rate of 2.5 x 10^5 Gy/s and an average dose rate of 30 Gy/s. The pulse width was 2 microseconds and the repetition rate was 60 Hz. Samples were irradiated at room temperature in 0.2 ml thin-walled PCR tubes held 2.5 m from the beam port. At this distance, the uniformity of the beam was within 10% of the peak dose over a circular area of 10 cm diameter, which encompassed the sample holder size. Samples were located on isocontours of dose with sample dose variations less than 1% as measured with PIN diode scans and a real-time "Faraday" cup array. Beam location was determined before irradiation and monitored during and after irradiations with the Faraday cup array and PIN diodes, and the sample doses delivered were measured using a GEX Corporation (Centennial, CO) thin film dosimetry system and GEX B3 radiochromic film[19].

The ISIS accelerator delivered 3.0 MeV electrons at approximately 10 kA. The beam spot was large enough to provide uniformity to the samples within 10%. Liquid samples were irradiated in custom-designed aluminum coupons sealed with sterile aluminum tape. These coupons were held at a fixed distance from the exit window, which varied from 1" to 6", depending on the dose/ dose-rate. Since each dose was delivered in a single 35 ns pulse, dose rates varied with total dose. For the doses used in these experiments, dose rates varied from 3 x 10^{10} Gy/s at 1 kGy to 8.6 x 10^{11} Gy/s at 30 kGy. Total dose delivered was measured with radiochromic and Gafchromic film. Pulse width was measured using inductive current monitors.

II.D. Cell Irradiation and Survival Measurement.

Each strain was grown in liquid culture to stationary phase, and, if applicable, spores were isolated. In an individual experiment, a culture or spore suspension was divided into aliquots and subjected to a pre-determined set of doses, including no irradiation. The aliquots were diluted in growth medium or water, for spores, immediately after irradiation and plated in duplicate or triplicate. In a given experiment, the multiple platings of the unirradiated (0 Gy) aliquot were counted and averaged to give the initial cell density in colony-forming units per ml (cfu/ml). This value represented 100% survival, and was used as the basis of comparison for all irradiated aliquots of that culture. The duplicate or triplicate platings of each irradiated aliquot were averaged to determine cfu/ml at that dose. The surviving fraction for that dose was determined by dividing the cfu/ml by the cfu/ml value representing 100% survival (0 Gy, or initial cell density) of that culture. For each strain, multiple experiments (independent cultures or spore isolations) were performed on at least two different days, and the surviving fractions were combined.

II.E. Survival Curve fitting.

For the two vegetative strains, surviving fraction values from multiple irradiations were combined and plotted in OriginPro 7 (OriginLab Corporation, Northampton, MA). The resulting data were fitted to a sigmoidal Boltzmann function (1)

$$\left[\frac{(A_1 + A_2)}{1 + e^{\left(\frac{X - X_0}{dx}\right)}}\right] + (A_2) \qquad (1)$$

with two parameters fixed at A_2=0 and A_1=1. Here X_0 = LD_{50} and dx = the width of the transition from surviving fraction = 1 to surviving fraction = 0. Individual (unbinned) values from multiple irradiations for each strain were combined, and used for curve fitting. These values were binned by dose in Figure 1 and 2 for clarity of presentation. The y-error was calculated as the error on the mean surviving fraction of each bin. Binned data for the two vegetative cells are presented with the curves generated from the fit of the unbinned data points.

Spore survival data were fitted to a first-order exponential decay in OriginPro. All data points are presented without binning by dose.

III. RESULTS

The purpose of this study was to compare the effect of electron beam radiation delivered in vastly different

time frames, ranging from ~ 10^{-8} to 10^{3} seconds, on survival of model organisms. The average dose-rates varied by 10 orders of magnitude. The wild type strain of the extremely radiation-resistant eubacterium *Deinococcus radiodurans* (R1), the type strain of the halophilic archaeon *Halobacterium* (NRC-1), and spores isolated from a wild type laboratory strain of *B. subtilis* were irradiated with electrons from two different accelerators. In one case, dose was delivered in 35 ns, resulting in dose rates on the order of 1 x 10^{11} Gy/s. In the other case, average dose rates were 30 Gy/s. Survival after each treatment was measured by growth into colonies on appropriate media, and comparison with the unirradiated control.

III.A. *Deinococcus radiodurans*

Cultures of *Deinococcus radiodurans* were grown to stationary phase in the appropriate medium, and irradiated with either 18-20 MeV electrons (LINAC) at a dose rate of 30 Gy/s, or with 3 MeV electrons in a single 35 ns pulse (ISIS) with dose rates ~ 10^{10} – 10^{11} Gy/s. Each experiment was performed on multiple independent cultures. The results of these combined experiments are presented in Figure 1.

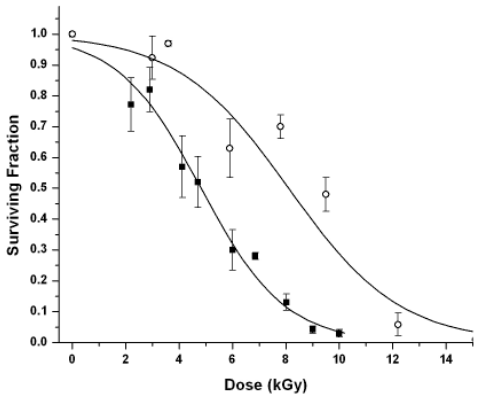

Fig. 1. Survival of *Deinococcus radiodurans* after electron beam irradiation from two different accelerators. Values from multiple experiments were binned according to dose. Error bars represent the error on the mean survival per bin. LINAC (○) and ISIS (■). Individual data points were fit to the Boltzmann curves shown.

The LD_{50}, determined from the individual data points, for irradiation with the lower dose rate (30 Gy/s) from the LINAC was 8.1 +/- 0.3 kGy. In contrast, the LD_{50} for irradiation with ISIS (10^{10}-10^{11} Gy/s) was 4.8 +/- 0.2 kGy.

III.B. *Halobacterium*

NRC-1, the type strain of the model archaeon *Halobacterium*, was also irradiated. In contrast to most cells, this microbe has been shown to exhibit greater resistance to radiation when actively growing (exponential growth) compared to stasis (stationary phase)[3, 5].

NRC-1 was grown to stationary phase and irradiated in growth medium. The combined results from multiple irradiations on the two accelerators are shown in Figure 2. The LD_{50} difference (2.9 +/- 0.2 kGy for the LINAC and 3.5 +/- 0.2 kGy for ISIS) is markedly less than that for *D. radiodurans.*

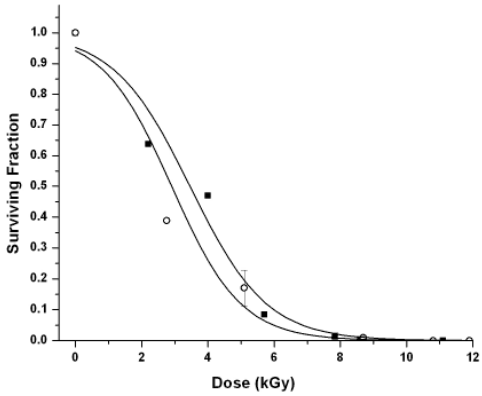

Fig. 2. Survival of *Halobacterium* sp. NRC-1 after electron beam irradiation from two different accelerators. Values from multiple experiments were binned according to dose. Error bars represent the error on the mean survival per bin. LINAC (○) and ISIS (■). Individual data points were fit to the Boltzmann curves shown.

III.C. *Bacillus subtilis* spores

In the previous two model systems described, vegetative cells were exposed. In this example, vegetative cells were allowed to produce non-metabolizing spores, which were purified and subsequently irradiated in water suspensions. Following irradiation, the spore suspensions were diluted in water, and plated on appropriate medium to allow germination and growth. The survival results after irradiation at the two different dose rates are shown in Figure 3.

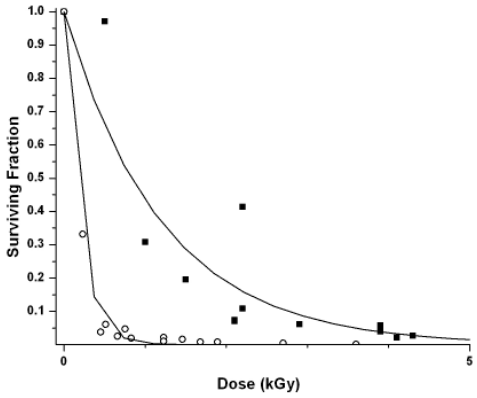

Fig. 3. Survival of *Bacillus subtilis* spores after electron beam irradiation from two different accelerators, LINAC (○) or ISIS (■). All data points are shown, and survival is plotted as a logarithmic function on the y-axis for clarity.

Unlike the results in the previous examples using vegetative cells, the survival of *Bacillus subitilis* spores is greater after irradiation with the extremely high dose rates (10^{10}-10^{11}) produced by the ISIS accelerator (LD_{50} 1 kGy) than after irradiation at 30 Gy/s (LD_{50} 0.2 kGy).

IV. CONCLUSIONS

We have compared survival of microbial cells and spores at two vastly different dose rates, ~30 Gy/s vs. ~ 10^{11} Gy/s. The organisms used represent two domains of life, the eubacteria and the archaea. In two microbes, *Deinococcus radiodurans* and *Halobacterium* sp. NRC-1, cells in stationary phase of growth were irradiated and their survival measured by their ability to form colonies on plates. Unlike these microbes, in the third species tested, *Bacillus subtilis*, vegetative cells naturally form non-metabolizing spores when conditions are less than optimal. Purified spores were irradiated, and survival was measured once by the ability of the spores to germinate and form viable colonies on a plate.

In the eubacterium *Deinococcus radiodurans*, irradiation with extremely high dose rates from the ISIS accelerator was markedly more effective than irradiation at the lower dose rate (Figure 1). The LD_{50} we obtained using the LINAC at 30 Gy/s was comparable to that seen by others using gamma sources at much lower dose rates [11] In contrast, irradiation of the archaeon *Halobacterium* sp. NRC-1 was unaffected by the dose rate (Figure 2). The LD_{50} values we report are comparable to those obtained with gamma sources reported by others[5] using dose rates approximately four times higher (121 Gy/s) than our lower dose rate of 30 Gy/s. That increased dose rate affected one organism but not the other may reflect fundamental differences between the two.

The two organisms come from two domains of life, and are evolutionarily distant. Although DNA repair mechanisms are evolutionarily conserved, individual organisms differ in ability to cope with DNA damage, reflecting adaptations peculiar to each. *Halobacterium* is also adapted to extremely saline environments. The high salt conditions necessary for its growth may contribute to the difference in response, by affecting the type and extent of damage.

The physiological state of the cells may be important for response to dose rate. Both cell types were grown to the stationary phase of growth. For most cell types, radiation exposure is less effective during this period, as cells are not rapidly dividing, and there is more time between divisions for repair of damage to occur [7]. Unlike other microorganisms, however, *Halobacterium*, shows lower resistance during stationary phase, and greater resistance while rapidly dividing [3,5] The mechanism for this peculiarity is not known, but may be associated with the response to different dose rates reported here. Further investigation using specific mutants with increased resistance to radiation may shed light on the mechanisms involved[3]

In contrast to the results from the vegetative cells of *Deinococcus radiodurans* and *Halobacterium*, spores of the gram-positive eubacterium *Bacillus subtilis* showed higher survival after irradiation at extremely high dose rates. Since spores are not metabolically active, repair of any DNA damage to the spores necessarily occurs during germination and outgrowth, not during the irradiation [6]. In addition, spores contain very little water and DNA is stabilized by the presence of specific proteins. The difference in response to the two dose rates, then, may reflect fundamental differences in types of damage inflicted and the response of subsequent repair of this existing damage during outgrowth. These differences may be addressed by utilizing spores containing specific mutations in these repair genes, to determine the role of each.

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