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Using Accelerator Electron Beams To Induce Massive Cell Killing In The Extremophilic Deinococcus Radiodurans

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Abstract

Background: The extraordinary ability of *Deinococcus radiodurans* to withstand lethal radiation effects has been verified with gammas but not with electron. Results show that electrons are able to severely damage plasmid DNA, shattering it into myriads of fragments, thus motivating the performing of *D. radiodurans* irradiation with electrons to test the limits of its radioresistance *vis-à-vis* new concepts on proteome radiation protection for this bacterium.

Methods: Cells of *D. radiodurans* in stationary growth phase were irradiated with electron of 1.174 MeV and gammas from a ^{60}Co facility. Evaluation of viable colony-forming units of cells was performed by optical cell density measurements at 600 nm, performed in triplicate up to 12 kGy. Samples of *Escherichia coli* were irradiated under the same conditions (positive control). Genomic DNA of *D. radiodurans* irradiated with electrons was extracted, and images of DNA fragments were obtained by Atomic Force Microscopy (AFM).

Results:

1. The 8kGy wide *shoulder* of *D. radiodurans* survival curve, observed in irradiations with gammas, was eliminated by electrons.

2. At electron doses over 2 kGy the number of colonies dropped below the detection limit.

3. Comparison between survival curves shows that *D. radiodurans* and *E. coli* exhibit equal yet low radiosensitivity when exposed to electron beams, and that both also are nearly 100% non-viable at 1.5–2 kGy.

4. *E. coli* is equally highly radiosensitive to gammas and electrons.

5. Dose rates play no role in the massive cell killing of *D. radiodurans* by electrons.

6. State-of-the-art radiological calculations showed that electron beams produce intense fluxes of secondary electrons, a severe radiogenic stress.

7. AFM imaging of *D. radiodurans* shows that its genome is shattered by electrons into large pieces.

Conclusions:

1. Suppression of repairing shoulder demonstrates that electrons are highly cytotoxic to *D. radiodurans*.

2. Intense fluxes of secondary electrons produced by electron beams hamper *D. radiodurans* recovery.

3. *D. radiodurans* is as radiosensitive to electrons as *E. coli*, compelling evidence that protection mechanisms for proteins and genome no longer prevail under this radiogenic stress.

Keywords: *Deinococcus radiodurans*; electron beams; gamma radiation; Atomic Force Microscopy; DNA fragment sizes; clonogenic death; repairing shoulder depletion

Introduction

Deinococcus radiodurans has puzzled biologists since its discovery as a contaminant in corned beef treated with sterilizing radiation [1]. For over a half century its extreme radiation resistance has been associated with its equally high capacity to repair massive DNA damage. Actually, *D. radiodurans* exhibits an extraordinary ability to withstand lethal and mutagenic effects of DNA damaging agents, particularly those from exposure to ionizing radiation producing over a thousand DSB (double-strand breaks) in each cell [2], [3].

Dose-response relationships for clonogenic survival are generally obtained from cell survival data, usually represented by shouldered (sigmoid) survival curves. The conceptual significance of the shoulder in mammalian cells goes back to 1959, as revealed by the seminal work of Elkind and Sutton [4]. According to them, the shoulder is indicative of repair of damage to whatever the radiation sensitive system in the cell is – this is to date crucial for the understanding of radiation biology. Since the survival of irradiated cells would crucially depend on their capacity to repair radiation induced DSB, this shoulder is sometimes referred to as *repairing shoulder*. In this sense, the 8 kGy-wide *repairing shoulder* of *D. radiodurans* would point to the

repair mechanism of this bacterium being highly proficient, thus explaining its extremely high radioresistance.

However, the conceptual scenario in radiation biology has drastically been changing. In this regard, a new paradigm for all species was proposed by Krisko and Radman, as lucidly outlined in a recent review [5]. According to them, the proteome rather than the genome is the prime target in radiation-induced cell death. The reason being the fact that cell survival itself depends primarily on vital functions performed by the proteome, while genome integrity is necessary for the perpetuation of the surviving cells. These concepts have been copiously justified elsewhere [Ref. 5 and references therein].

Regarding the bacterium *Deinococcus radiodurans*, more specifically, one is led to conclude that its very high resistance to radiation, and to other sources of oxidative damage, is a consequence of efficient protection against proteome damage. In fact, there has to be in *D. radiodurans* a regulatory interplay between different processes associated with oxidative stress response pathways [3], [6], [7]. These functions, therefore, would manifest themselves as protein protection, preserving the high efficiency of DNA repair enzymes [3], [8].

Thus, survival curves must be interpreted *cum grano salis*, an attempt pursued in the present work. Typical *D. radiodurans* survival curves for acute irradiation with ions of different LET (linear energy transfer), as distinct as He, O and Ar, are shown in Figure 2 of Ref. 9. The curves are characterized by an initial shoulder followed by a relatively abrupt transition to an exponential terminal slope at higher doses (modulated by radiation type). This basic shape is the same as for the low-LET gamma-radiation. The shoulder is nearly insensitive to the radiation-LET, an indication of very efficient DNA damage repair in *D. radiodurans*. This is consistent with current knowledge on both protein protection [Ref. 5 and references therein] and DNA repair in this organism. The latter is homology based, utilizing multiple genome copies, and is in principle error free, resulting in essentially 100% cell survival and absence of mutagenesis up to certain doses [9], [10], [11].

Because of these intriguing characteristics, *D. radiodurans* is high on the agenda of many radiobiological applications and as pointed out elsewhere, the use of this microorganism as a bacterial model for oncology [12] and long-lived non-dividing neurons processes [10] is an appealing possibility. Moreover, exploring mechanisms of *Deinococcal* robustness may well also inspire approaches in anti-ageing research and regenerative medicine [6].

The extraordinary radioresistance of *D. radiodurans* has been verified mostly with the low-LET gamma radiation from ^{60}Co-facilities and Ultra-Violet-C radiation, but not with electron beams from linear electron accelerators (LINAC), another low-LET radiation also widely used in radiotherapy. The reason could be the fact that both radiations have comparable LET.

However, an experiment on fragmentation profiles of plasmid DNA irradiated with gammas and electrons, recently carried out at this Laboratory using Atomic Force Microscopy to determine fragment lengths, showed that the DNA strands were highly shattered when irradiated with electrons [13]. This unexpected finding served as motivation to examine the limits of *D. radiodurans* radioresistance by carrying out viability measurements after irradiation with electrons.

As shown in this work, electron beams from a LINAC facility produce intense fluxes of secondary electrons, constituting thus a severe radiogenic stress to both proteome and genome of D. radiodurans – also addressed in this work.

Finally, a possible extraterrestrial origin of *D. radiodurans* has been conjectured elsewhere [14]. In fact, *D. radiodurans* is one of the most investigated organisms in the experimental test of the *panspermia* hypothesis. According to this hypothesis, bacterial cells are able to be transferred across large distances of interplanetary space and thus ‘‘seed’’ planetary bodies. Any viable life-form putatively traveling from one inhabited planet to another would, therefore, have to cope with cosmic radiation of the interplanetary space, which is constituted mostly by protons and electrons (≈ 92%, in roughly equal numbers). The electron distribution functions in the solar wind approximate Maxwellian energy distribution with average energies in the order of 10 eV [15]. Although these are low energy electrons, the amount of radiation imparted to *D. radiodurans*, while hypothetically traveling from one planet to another, would be very high (order of several kGy) given the long lasting time of exposure.

Materials And Methods

Cultures of *Deinococcus radiodurans*, GY 9613 (R1) wild-type strain, were obtained from a stock kept at the Institute for Radioprotection and Dosimetry/(IRD/RJ), Rio de Janeiro, Brazil. The cells in freezer stocks (glycerol 10%, -80°C) were streaked in solid TGY

medium (1% tryptone, 0.2% glucose, 0.6% yeast extract, 1.5% agar) (Oxoid LTD, Basingstoke, Hampshire, England) and incubated for 24 hours (30 ± 1°C). Ten colonies from this agar plate were used to inoculate liquid TGY medium and incubated for 24 hours in a rotary shaker (30 ± 1°C, 200 rpm) (model TE 420, from Tecnal, São Paulo, Brazil). The culture of *D. radiodurans* is axenic (pure) and as a consequence, monoclonal. Therefore, the collection of 10 colonies is only a standard procedure to inoculate a controlled amount of cells. A volume of this culture, corresponding to 10% of the final volume, was used to inoculate fresh liquid TGY medium and incubated in a rotary shaker (30 ± 1°C, 200 rpm). Samples of this culture were harvested during the stationary growth phase. Cells were pelleted by centrifugation (10,000xg, 5 min., 5° C), washed with physiological saline solution (NaCl 0.85%, pH equal to 6.8), re-suspended in the same volume of physiological solution and distributed in 2 mL aliquots in micro-centrifuge tubes.

Cells in the stationary growth phase were irradiated with electron beams delivered by a Linear Electron Accelerator (Dynamitron, model JJOB 188, RDI RADIATION DYNAMICS, INC) operating with an energy of 1.174 MeV and average beam intensity of 0.5 mA. The samples were accommodated in a conveyor belt loop, exposing the samples to electron beams at intervals of 3 minutes (conveyor belt lap time), each exposure imparting a dose of 0.5 kGy during 0.223 seconds (Figure 1). The corresponding average dose rate was 10 kGy/h. Cells were also irradiated with gammas from a ^{60}Co facility (Gammabeam, model 650 from MSD Nordion, Otawa, Canada), with doses in the interval 0 – 12 kGy at a rate of 2.4 kGy/h. Cells irradiations with electrons and with gammas were independent experiments.

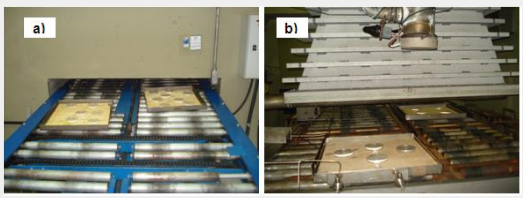

Figure 1-(a) Conveyor belt of samples at the Linac entrance to the irradiation site. (b) Linac irradiation site showing the beam exit pipe (top) and the samples set

One vial containing *D. radiodurans* was used for all experiments. Three independent irradiations were performed for each dose in the interval 0 – 12 kGy, and at different days. Saline solution (0.5 M NaCl) was added to the cultures in order to irreversibly fixe potentially lethal injury so that it cannot be repaired during the irradiation process – survival was then measured.

In order to avoid possible thermal effects, all exposures were performed at controlled temperature, continuously remote monitored by a Digital Thermometer TH-1200 C model (INSTRUTHERM, USA), equipped with Chromel – Alumel sensors (HOMIS, USA).

Samples of *Escherichia coli* (ATCC 11229 strain), as a positive control were also prepared and irradiated under the same conditions.

To evaluate the concentration of viable cells (CFU/mL – colony-forming units per milliliter), treated and untreated samples were submitted to serial decimal dilutions and plated on solid TGY medium and the number of colonies was counted after 36 hours of incubation (30 ± 1°C). Optical cell density (OD) was measured at 600 nm (Cary 50 Bio UV Visible Spectrophotometer, from Varian, Mulgrave, Australia). Measuring OD values during cell growth is a rapid and current method used to monitor cell growth phases during cultivation. The initial number of cells (D = 0 kGy) in all irradiations was maintained between $1.0x10^9$ and $1.4x10^9$.

Error bars appearing in figures represent external standard deviations. Data handling procedure consisted solely of data averaging; thus only the external standard deviation of the averaged values was calculated, a simple and conventional parametric statistic in the normal model [16]. We would like to point out that ANOVA-like analysis of variance is recommended only in situations where differences between sets of data are small and/or difficult to perceive, making objective conclusions an uncertain task, a circumstance not verified in this work.

Genomic DNA of *D. radiodurans* R1 was extracted with the Bacterial DNA kit (D3350-02, OMEGA bio-tek, USA). It should be noted that its genome is composed of two large 3.06 Mb DNAs (chromosomes) and two smaller 223 kb DNAs (plasmids) [17]. The samples were quantified in a 1% agarose gel and the lambda HinDIII (Invitrogen, Brasil) marker was used as DNA standard. The samples were stained with ethidium bromide. Measurement of DNA fragment sizes was carried out by Atomic Force Microscopy (AFM). Several modes of operation can be used, depending on the type of material and even microscope type. The microscope used in this work is the Agilent-5500, and data was analyzed with software provided by the AFM manufacturer allowing for segmented measurements. For DNA imaging this AFM was operated with conical silicon tips (covered with gold) in air and at

non-tapping mode. Sample preparation for AFM imaging consisted of the deposition of aqueous DNA solution on an atomically flat mica surface to assure electrostatic adhesion of DNA molecules (more details in Nieto el al. [13]). Large pieces of the genomic DNA are imaged once the linearization of the DNA molecule is not feasible due to bacterial genome size (more in Results and Discussion).

Results and Discussion

In Figure 2 typical temperature behavior is shown, all along a time-consuming irradiation of 4 h, where temperatures were comfortably maintained within 6.5 and 7.0 ^{0}C. *D. radiodurans* is highly resistant not only to radiation but to heat treatment too, as reported by Imamura and collaborators [12], where *D. radiodurans* cells were effectively killed when using acid heating, wherein the mechanism was assumed to be based on depurination of DNA.

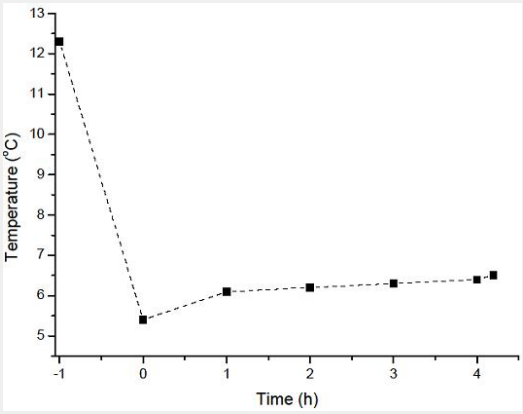

Figure 2– Typical temperature behavior of a D. radiodurans culture during 4h irradiation. The average low-dispersion temperature of 6.5 oC is within the optimum 5 – 10 oC temperature range for this bacterium.

The growth curves for *D. radiodurans* and *E. coli* are displayed in Figure 3. Regarding the OD issue, one reached DO_{600nm} = 14 in the stationary phase corresponding to 2.2x10^{9} UFC/ml. All results obtained for viability are consistent with this DO_{600nm} and are reproducible. Interestingly, *E. coli* grows slightly faster than *D. radiodurans* in the exponential phase.

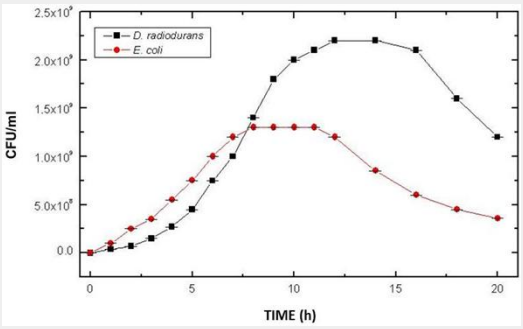

Figure 3 – Growing curves D. radiodurans and E. coli expressed as colony formation unities per ml (CFU/ml) versus growth time in hours (T).

1. Survival curves following irradiation with gammas and electrons

The survival curves of *D. radiodurans*, S(%), obtained by irradiation with electrons and gammas are displayed in Figure 4. The characteristic and well-known *repairing shoulder* observed in irradiation with gammas, extending from 0 to 8 kGy, is nearly eliminated when *D. radiodurans* is irradiated with electron beams. Such a high toxicity of *D. radiodurans* to electrons is discussed in the following Sections, particularly with the concept that a survival curve could be interpreted as one of the cell protein protection signatures.

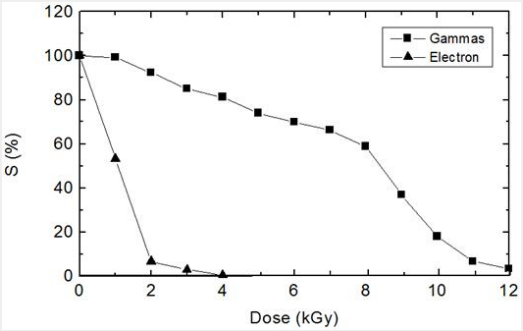

Figure 4– Survival curves of D. radiodurans after irradiation with gammas and electrons, obtained from samples in the stationary growth phase. The curves serve only to guide the eyes, that is, they were not generated from fitting or modeling procedures.

In Figure 5 is shown the corresponding results for *E. coli*. Given its much lower radioresistance *vis-à-vis D. radiodurans* the dose range investigated was limited to 0 – 2 kGy. Despite the relatively higher experimental uncertainties (expressed by the error bars in Figure 5), it is evident the following features associated with S(e) and S(γ), the survival curves of *E. coli* for irradiations with electrons and gammas, respectively: (i) these two

survival curves converge to zero (that is, the detection limit) as they approach a dose of 2 kGy, approximately; (ii) S(e) > S(γ) systematically up to at least 1.5 kGy; (iii) at higher doses the *E. coli* viability is nearly zero for both electrons and gammas irradiations.

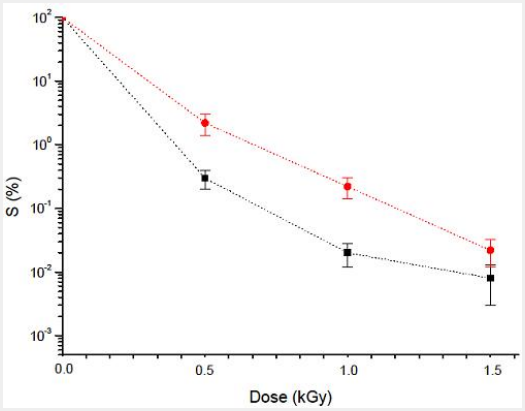

Figure 5– E. coli survival curves after irradiation with gammas and electrons, obtained with samples in the stationary growth phase too. The curves serve only to guide the eyes.

Interestingly, on the one hand, there is reported evidence showing that there is no particular DNA protection mechanism responsible for the radiation resistance of this key molecule to gammas (see e.g. [18] and [19]). On the other hand, the extent of gamma radiation-induced DSBs in *D. radiodurans* DNA is the same as in very radiosensitive bacteria (as the presently studied *E. coli*) and mammalian cells. The results shown in Figure 5 demonstrate that *E. coli* is highly radiosensitive to gammas and electrons equally. This is a compelling indication that a protein protection system, against gamma radiation injury, is present at much higher levels of activity in *D. radiodurans* than in *E. coli*, as previously reported in the literature [8]. Actually, 10% of *D. radiodurans* cells survive 12,000 Gy, a dose that induces 120 DSBs per genome [3], but 10% of *E. coli* survive 700 Gy (see Figure 5), a dose that induces 7 DSBs per genome [20]. While this is the case for gamma radiation, our results show that both *D. radiodurans* and *E. coli* exhibit the same low radiosensitivity when exposed to electron beams (see Figures 4 and 5). This intriguing finding is discussed and addressed below.

2. Physical aspects of gammas and electrons – the role played by dose rates

Gamma Irradiation: In every case of a photon scattering process, an energetic electron is one of the products. The high kinetic energy electrons produced in this primary process are the key element in the transfer of energy from the scattered photon to the molecular systems of the living cell. The Compton process is the most significant contributor to energy absorption in photon irradiations. The corresponding cross sections are known since the seminal calculations carried out by Hubbell [21]. Given their importance for the present study, *secondary electrons* are separately discussed in the next section.

Electron Beams: Energetic electrons, as all energetic charged particles, lose their kinetic energy by means of coulombic interactions with other charged particles (mostly other electrons from their environment). Energy loss is proportional to $1/v^2$, where v is projectile-electron velocity. The directions of these projectiles are, in precise physical parlance, stochastically distributed around the scattering center. However, the momentum transfer per collision is small for individual events, so that only small changes in the direction of the incoming electron take place. But overall, after multiple collisions, the change is significant. Therefore, the multiple collision process is of paramount importance for energy deposition in molecular species.

Dose rate: Dose imparted by a given radiation, or projectile, is the amount of energy absorbed per mass unit of the sample (Joules per kg, or, Gray). The energy absorption process has the signature of the radiation physical characteristics. In this sense, distinct radiations imparting the same dose could induce different effects, as dramatically observed in the present study with gammas and electrons.

Dose rate, on the other hand, simply is the amount of dose imparted per time interval unit (Gray per second). It is clear that the higher or lower magnitude of dose rates does not change the radiation physical characteristics. In fact, times associated with physical processes are infinitely smaller than irradiation times. For example, an electron is ejected from an atom, in the photoelectric effect, 10^{-10} seconds after completion of the incoming photon interaction with the atom. Therefore, the total number of damages inflicted by radiation, as e.g. the total amount of DSB, is directly proportional to the time integrated dose rate, that is, the total dose. Dose rates are particularly important in radiotherapy for the planning of tumor fractioned irradiations, a treatment strategy to deal with cellular damaging recover (details in [22]).

We note that the average electron dose rate in this study was 10 kGy/h, while in all experiments conducted by Pang *et al.* with electrons [23] the dose rate was 2.9 kGy/h. However, despite these quite different electron dose rates the results of the two Laboratories are highly comparable, particularly when referring to the fragmentation ability of electrons (see

e.g. Figure 3-b in [23]). Therefore, the plasmids fragmentation patterns observed with electron beams are nearly unaffected by different dose rates, all exhibiting an exponential-like behavior.

Regarding irradiation with gammas, survival curves of *D. radiodurans* were obtained by Zahradka et al. [10] and Slade et al. [24] at dose rates of 39.6 kGy/h and 1.8 kGy/h respectively, a 22-fold dose rate difference, and yet the survival curves were the same. The Co-60 gamma source facility of this study delivered 2.4 kGy/h (see Materials and Methods).

There are two additional and compelling biological circumstances indicating that there is no role played by dose rates in the massive cell killing of *D. radiodurans* by electrons (Figure 4). These are,

(i) *D. radiodurans* cannot repair its DSBs during irradiation in a buffer (see Materials and Methods). In this sense, it is irrelevant if the *final number of DSBs* was obtained in a smaller or bigger irradiation time.

(ii) Moreover, a quite fundamental study on reassembly of shattered chromosomes in *D. radiodurans* revealed that neither repair nor synthesis was visible for 1.5 h after irradiation with 7 kGy of gamma radiation but, in the following 1.5 h, chromosomal DNA appeared fully reassembled at 80–90% survival [10].

3. Secondary electrons

The precise mechanism by which radiation causes breaks in DNA strands has not completely been established. There is recent evidence showing that DNA damage can also be caused by low-energy (below about 20 eV) secondary electrons generated by the incident radiation. The implications of this are potentially significant as secondary electrons represent the most abundant species formed along a radiation track in condensed matter (roughly $5x10^4$ secondary electrons are produced per 1 MeV *primary energy deposited*) [25], [26], [27]. These studies have demonstrated that a large fraction of these electrons are generated by a relatively unusual auto-ionization process known as intermolecular Coulombic decay [28], [29].

However, matter is quite transparent to gammas. For instance, attenuation in water (the predominant cellular milieu) of 1.5 MeV photons to half of its total primary energy requires a track length of 15cm, approximately. On the other hand, 1.5 MeV electrons transfer their ***total*** primary energy to a few centimeters of water [21], [30]. Therefore, the *primary energy deposited* by electrons on *D. radiodurans* samples is at least two orders of magnitude higher than the energy deposited by gammas. Likewise, the flux of secondary electrons produced in irradiations of *D. radiodurans* with electrons is also two to three orders of magnitude higher. It is evident that besides the DNA molecule, repair-related proteins are also equally and intensively “illuminated” by these copious fluxes of secondary electrons, therefore constituting a severe radiogenic stress negatively contributing to the recovering of *D. radiodurans*.

4. The role played by DNA fragmentation induced by gammas and electrons

Although gamma radiation shatters the genomic DNA of *D. radiodurans* its reassembly is accurate (see Figure 1-*a,b* of Ref. 10). On the other hand, recent results obtained at this Laboratory for fragment-size distributions of plasmid DNA, using Atomic Force Microscopy, showed that in irradiation with electrons size distributions were constituted by much smaller fragments. Actually, irradiation with electrons shatters the plasmid DNA into myriads of small fragments (see Figures 3 and 5 in Ref. 13), an indication that a much greater number of DSB is produced. This is consistent with the fact that copious fluxes of secondary electrons are produced in irradiation with electrons (see details above in paragraph *3 –Secondary electrons*).

Although these findings were obtained with plasmid DNA, it is quite important to emphasize that the shattering ability of electrons could also be verified in the case of genomic DNA. It is a well-known physical fact that radiation senses only the density and atomic composition of the molecule building blocks, interacting similarly with nucleotides from either plasmid or genomic DNA as to produce, e.g., DSB [22].

As pointed out elsewhere [10], if there is an unusually large number of DSB, then an unusually long homology, and thus a higher single-strand exposure, is required for an accurate reassembly, which could render DNA repair synthesis uncertain or unperformed. Since the repair synthesis should occur before cell division, the non accomplishment of this process leads to cell lethality, highly consistent with the findings of this study, where irradiation with electron beams eliminated the *D. radiodurans repairing shoulder* (Figure 4). Such a possibility is strongly reinforced by the fact that high intracellular levels of Mn(II) in *D. radiodurans* protect proteins, thus allowing for fast repair of damaged DNA after irradiation with gammas [20], [31], but not after irradiation with electron beams (present work).

Measurement of genomic DNA fragment sizes by means of AFM images is a rather difficult and uncertain task. For instance, linearization of the DNA molecule is not feasible due to bacterial genome size.

Difficulties are aggravated by the genome structure of *D. radiodurans*, which is complex for a bacterium (see *Materials and Methods*), and the high compaction of its DNA [32]. The present AFM imaging results of *D. radiodurans* show large pieces of its genome (Figure 6). This is an indication that electrons, by producing a great number of DSBs, are able to disrupt the compact DNA of this bacterium (see discussion above on *secondary electrons*).

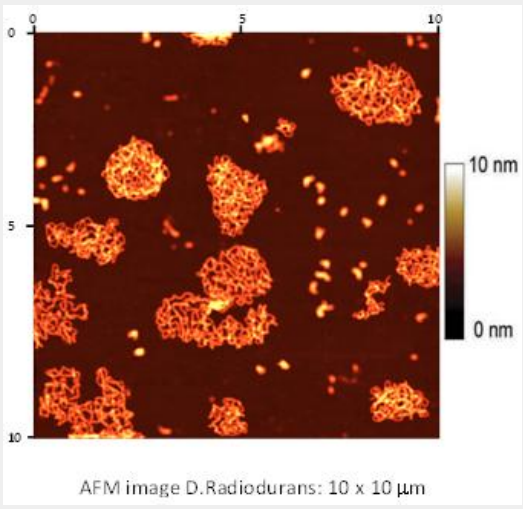

Figure 6– AFM image of D. radiodurans genome after 5 kGy irradiation with electrons – experimental procedure and details in Materials and Methods.

Also, the present results for *D. radiodurans* would cast some doubts on the correctness of the *panspermia* hypothesis (see *Introduction*), that is: since doses imparted by electrons from cosmic radiation to microorganisms amount several kGy (given a hypothetical travel between planets), their survival chances could be very low.

Finally, the intriguing and novel findings here presented and discussed could stimulate further *ad-hoc* studies of energetic electrons effects on repair-related mechanisms.

Conclusions

1. The repairing shoulder of the D. radiodurans survival curve is suppressed in irradiations with electron beams, but it is maintained when exposed to gamma radiation (Figure 4), indicating that electrons are highly cytotoxic to D. radiodurans.
2. Both E. coli, which worked as a positive control, and D. radiodurans are equally very radiosensitive to electrons, and both also are nearly 100% non-viable at doses equal to and higher than 1.5–2 kGy (Figures 4 and 5).
3. Electron beams from a LINAC facility (a) produce intense fluxes of secondary electrons, thus constituting severe radiogenic stress, and (b) shatter DNA into small fragments giving rise to a greater number of DSB.
4. It is therefore very likely that D. radiodurans recovery is hampered in irradiation with electrons because intense fluxes of secondary electrons generate a greater number of DSB.
5. This last conclusion is reinforced by the fact that high intracellular levels of Mn(II) in D. radiodurans protect, in principle, repair proteins [20], [31].

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