LET Effects in *Micrococcus radiodurans* Irradiated with Alpha Particles in the Liquid Phase

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From viewpoint of the study on LET effects on bacteria, log- and stationary-phase cells of *M. radiodurans*, an extremely radioresistant vegetative bacterium, suspended in the phosphate buffer were exposed to 26 MeV α-particles from the IPCR cyclotron. The results obtained were compared with those on γ-irradiation. On such liquid-phase irradiation, survival curves of *M. radiodurans* for α-particles were found exponential while they were of sigmoidal or cumulative type for γ-rays. In addition, the dose-rate effect was observed with α-bombardment, although it was not found with γ-irradiation. For a given dose and in a system where the effective volume was sufficiently small as compared with the total sample volume, the relation of the surviving fraction S to the beam intensity I was represented by an empirical equation, $S = \exp\left[\left(-\frac{It}{q_0}\right)\left(1-\exp\left(-kq_0/I\right)p\right)\right]$ where t is irradiation time, $q_0$ is $D_{37}$, and k and p are constant. By means of sedimentation analysis, double-strand scissions in DNA caused by α-particles were found repairable during the postirradiation incubation similarly to the case of γ-irradiation. The findings observed here with *M. radiodurans* do not support the view that DNA is an only primary target for cell killing by α-particles.

It has been indicated by many investigators that the relative biological effectiveness (RBE) of ionizing radiations for inactivation of viruses, bacteria and yeasts varies as functions of linear energy transfer (LET). In the interpretation for such LET effect on the radiolethality in these biological systems, some different mechanisms have been considered. Munson et al. determined the LET dependence of radiosensitivity for several strains of *E. coli* having different capacities for the repair of DNA damages and analysed the data from viewpoint of the target theory in terms of two types of lethal damages to DNA, i.e. single- and double-strand scissions in DNA. The fact that differential sensitivity among various bacteria declines with increasing LET has been also explained by the conception of increase in irrepairable fraction of DNA lesions with increasing LETs. Nevertheless, an alternative possibility that there might be the sensitive target other than DNA for cell inactivation by high-LET radiations still remains to be examined.
Thus, the studies on the radiosensitivity of biological cells to ionizing radiations of different LETs may provide unique informations on the mechanism for their lethal action. From this point of view, a series of studies on LET effects on bacterial cells has been attempted in this research group using ionizing particles of different LETs from the IP CR cyclotron. As the first part of these studies, the present paper will describe the results obtained with a radioresistant bacterium, Micrococcus radiodurans by means of liquid-phase irradiation with 26 MeV α-particles in respects of the kinetics for radiolethality and the repair or DNA lesions.

MATERIALS AND METHODS

Throughout this study, M. radiodurans R, which was kindly supplied by Dr. Anderson, Oregon University, U. S. A., was used. Log- and stationary-phase cells grown in the B-broth was harvested by centrifugation, washed twice with 0.067 M phosphate buffer (pH 7.0) and resuspended in the same buffer at the cell concentration of 2×10⁷–9×10⁷ cells/ml, except when otherwise noted. The cell suspension was exposed to α-particles and survivors were determined by counting visible colonies developed on the B-broth agar after 4-day incubation at 30°C. Such irradiation procedure for bacterial cells suspended in a liquid medium will be denoted as the liquid-phase irradiation.

Alpha-bombardment was carried out using the IP CR cyclotron. This machine is an ordinary-type variable energy cyclotron. A 3.5-ml aliquot of the cell suspension was transferred into a quartz irradiation vessel and exposed to α-particles through a thin quartz window (~52 µ in thickness). The incident energy of α-particles used were 29 MeV and their energy was reduced to 26 MeV on passing through the quartz window of an irradiation vessel. The cross section of the beam flux at the quartz window was 4.5 mm in diameter. For stirring and maintaining the aerobic condition during α-bombardment, the cell suspension was bubbled with air through a capillary tube at a constant rate of 45 ml/min during irradiation. Irradiation was made at the room temperature (18–20°C). For dosimetry, two determination methods were employed as will be described in details elsewhere. First, the beam current absorbed in the cell suspension was read from a platinum wire sealed into the suspension and connected to ground through a current integrator. The beam current was also monitored by the nuclear electronic method, with which α-particles elastically scattered by a thin gold foil were counted using a solid state detector. Consideration for so-called “charge displacement effect” was taken in the dosimetry. The arrangement is illustrated in Fig. 1. In this system, most of α-particles passed through a gold scattering foil, while only a small fraction of α-particles were elastically scattered and counted as linear function of the beam current. The energy spectrum was displayed on a multi-channel pulse height analyzer. The elastic peak was integrated by summing all counts by a scaler so to adjust by discriminator that peaks above the first excited state.

were counted. The ratio of the number of α-particles elastically scattered into the detector to their number passed through a gold scattering foil and entered into the cell suspension was accurately determined by replacing the cell suspension with a Faraday cup. The determination of this ratio was done before and after each experiment and found independent of the beam intensity over the wide range.

α-Particles from the cyclotron were injected into the cell suspension after passing through a gold scattering foil 2 μ thick, an aluminum vacuum window 20 μ thick, the air layer (~10 mm) and then a quartz window of the irradiation vessel (32 μ in thickness). The energy of α-particles accelerated in the cyclotron was determined with a beam analyzing magnet or from a radio frequency of the cyclotron. The energy of α-particles entering into the cell suspension was calculated from the range-energy relation considering the energy loss in their passage. These methods described above ensure to determine the absorbed dose with an over-all error less than 3%. Under the experimental condition employed here for the liquid-phase irradiation, the absorbed dose in rad for 26 MeV α-particles is calculated as 371 rads/nC.

Gamma irradiation was performed using a 60Co irradiation unit (1000 Ci) with a dose rate of about 9.9 × 10^4 rads/hr at room temperature (~20°C). The air was bubbled through a capillary tube during γ-irradiation.

The sedimentation analysis on DNA was carried out by the lysozyme-SDS method as described previously.15) Log-phase cells labeled with 3H-thymidine was lysed on a 5–20% sucrose gradient (pH 7.6) and centrifugation was carried out at 30,000 rpm for 80 min at 5°C in a Hitachi 55P ultracentrifuge using a RPS-40 rotor. After centrifugation, each 10-drop fraction was taken on a filter paper disk, extracted with 10% cold TCA and dried. Radioactivity in TCA-insoluble tritiated materials was determined by a Beckman LS-150 liquid scintillation counter.

RESULTS AND DISCUSSION

Kinetics of cell death on the liquid-phase irradiation

When bacterial cells suspended in the phosphate buffer were irradiated with α-particles, i.e. on liquid-phase irradiation, only a small fraction of the bacterial population just behind the window of the irradiation vessel (denoted as an effective volume) was subjected to the α-bombardment because of the very short range of 26 MeV α-particles in an aqueous system (0.56 mm). In this study, an effective volume for α-bombardment was estimated as ~0.009 ml corresponding to about 1/400 of the total sample volume. Therefore, being different from the case of γ-irradiation, the efficiency of stirring and beam intensity should influence seriously upon the effect of the liquid-phase irradiation with α-particles. In fact, as indicated in Table I, surviving fractions with the same dose were obviously found to depend on the efficiency of stirring or the rate of aeration in this experiment. Therefore, the rate of aeration was fixed at 45 ml/min throughout this study. No effect of the cell concentration during α-bombardment was found on the lethal action on bacterial cells as shown in Table I.

The survival curves for log- and stationary-phase cells at some different beam intensities are shown in Fig. 2 together with those for 60Co γ-rays. It is noteworthy that the survival curves are of exponential type for α-particles while sigmoidal or of the cumulative type

| Table I. Effects of Cell Concentration and Rate of Aeration During the Liquid-Phase Irradiation with 26 MeV α-Particles on Surviving Fraction of M. radiodurans |
|---|---|---|---|---|
| Dose (nC) | Beam intensity (nA) | Rate of air-bubbling (ml/min) | Initial visible counts (cells/ml) | Surviving fraction (%) |
| 8,000 | 28 | 45 | 1.21 × 10^6 | 8.7 |
| 8,000 | 28 | 45 | 1.28 × 10^6 | 8.5 |
| 8,000 | 12 | 45 | 2.24 × 10^6 | 0.26 |
| 8,000 | 12 | 45 | 2.09 × 10^6 | 0.28 |
| Experiment 1 (Effect of cell concentration) |
| 20,000 | 30 | 7.5 | 1.21 × 10^6 | 31.5 |
| 20,000 | 30 | 45 | 1.21 × 10^6 | 3.97 |
| 20,000 | 30 | 75 | 1.21 × 10^6 | 0.43 |
| Experiment 2 (Effect of the rate of aeration) |

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![Survival Curves of *M. radiodurans* for the Liquid-phase Irradiation.](image)

**FIG. 2.** Survival Curves of *M. radiodurans* for the Liquid-phase Irradiation.

For 26 MeV α-particles
- A: log-phase cells, 16 nA
- B: log-phase cells, 78 nA
- C: stationary-phase cells, 16 nA
- D: stationary-phase cells, 78 nA

For 60Co γ-rays
- E: log-phase cells
- F: stationary-phase cells

**TABLE II. RELATIVE RADIOSENSITIVITY OF LOG- AND STATIONARY PHASE CELLS OF *M. radiodurans***

<table>
<thead>
<tr>
<th>Radiation</th>
<th>Parameter of radiosensitivity</th>
<th>Phase of growth cycle</th>
<th>Ratio B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 MeV α, 78 nA</td>
<td>D&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Log A</td>
<td>Stationary B</td>
</tr>
<tr>
<td>26 MeV α, 16 nA</td>
<td>D&lt;sub&gt;0&lt;/sub&gt;</td>
<td>7.6</td>
<td>8.9</td>
</tr>
<tr>
<td>60Co γ</td>
<td>Induction dose</td>
<td>1.8</td>
<td>2.5</td>
</tr>
<tr>
<td>60Co γ</td>
<td>D&lt;sub&gt;0&lt;/sub&gt;</td>
<td>Mrad</td>
<td>Mrad</td>
</tr>
</tbody>
</table>

| 60Co γ | D<sub>0</sub> | 0.33 | 0.87 | 2.6 |
| 60Co γ | D<sub>0</sub> | 0.063 | 0.11 | 1.7 |

-<sup>a</sup> Calculated from data in Fig. 2.
-<sup>b</sup> The value for the exponential part of a sigmoidal survival curve at higher doses.

The effect was found for γ-irradiation. The stationary-phase cells were somewhat resistant more than log-phase cells to α-bombardment similarly to γ-rays, but the differential radiosensitivity between them was declined with α-bombardment, as compared with γ-irradiation (Table II).

In order to examine the more detailed relation of the beam intensity to radiolethality on the liquid-phase irradiation, determinations of surviving fractions for a given dose were made with different beam intensities under a constant rate of aeration. All survival curves of *M. radiodurans* for α-particles obtained over the whole range of the beam intensities examined were exponential. The relationship between the beam intensity and surviving fraction at 4000 nC for 26 MeV α-particles is illustrated in Fig. 3. When a beam current was sufficiently small, the identical surviving fraction S<sub>i</sub> was given with the constant dose of 4000 nC, the surviving fraction being independent of the beam intensity. However, above such critical beam current, the surviving fraction at a given dose was increased with increasing the beam intensity.
FIG. 3. Relationship between Beam Intensity and Surviving Fraction of Log-phase Cells of M. radiodurans with a Constant Dose of 4000 nC. 

Real line: theoretical curve for eq. (1) (see text) when log S1 = -1.95, k = 0.0104 and p = 0.969. The value of log S1 was graphically estimated.

Current, approaching to the level of ~100%.* From the value S1 of the surviving fraction obtained at the sufficiently small beam current, the D37 (a dose required for 1/e, i.e. 37% survival) on the liquid-phase irradiation was calculated as 3.31 × 10^5 rads for 26 MeV α-particles. Then the target mass would be given as 2.9 × 10^-18 g on the basis of 60 eV per energy-loss event.16) Since the mass of DNA per cell of M. radiodurans has been found to be 3.5 × 10^-14 g,12) the target mass corresponds to 0.83 × 10^-4, that is, about 1/10,000 of DNA.* This suggests that the target for α-particles is not the whole but a small fraction of DNA and/or other cytoplasmic macromolecular particles.

If the liquid-phase irradiation is done with a given dose, the kinetics of the dose-rate effect may be represented by an empirical equation:

\[ S = e^{-It/q_0(1-e^{-kq_0/I})} \]  

(1)

where S is the surviving fraction, q0 is D37 in nC, t is irradiation time in sec, I is the beam current in nA, k is a constant having a dimension of sec^-1 and p is a dimensionless constant. For sufficiently small I, S1 is given by

\[ S_1 = e^{-It/q_0} \]  

(2)

For curve fitting, equation (1) is modified into a linear relation,

\[ \ln (\ln 1/x) = p(\ln k - \ln T) \]  

(3)

where

\[ x = 1 - \frac{\ln S}{\ln S_1} \]

\[ T = \frac{I}{q_0} \]

Since log (2.3 log 1/x) is found as a linear function of log T, as shown in Fig. 4, the dose rate-surviving fraction curve can be expressed by equation (1). By the least squares method, k and p are found as 0.0104 and 0.969, respectively. A parameter k would depend upon the efficiency of stirring and the size of the effective volume. Further determinations are now under way to make clear the meaning of these parameters k and p using different bacterial strains and irradiation vessels.

* Discussion on the kinetics of the dose-rate effect in this paper is restricted to the system in which the effective volume is the sufficiently small fraction of the sample irradiated. The percentage of the ratio (total sample volume-effective volume)/(total sample volume) is approximately 100 here.

Sedimentation analysis on DNA after α-bombardment

The DNA molecule has been regarded as a principal target in bacterial cells for radiation killing. On X- or γ-irradiation, it has been assumed that double-strand scissions in DNA are not repairable and lead to cell death in bacteria. If the DNA would be the target molecule even for α-particles, sigmoidal survival curves should be obtained with *Micrococcus radiodurans*, because this bacterium has four separated nuclear materials in a tetrad cell. However, the survival curve obtained for α-bombardment was of the exponential type. Thus, criticism for the view that DNA is an only target molecule is still opened for high-LET radiations.

In the previous communication, it was evidenced by the sedimentation analysis that double-strand scissions in DNA caused by γ-rays can be repaired during postirradiation incubation in this extremely radioresistant vegetative bacterium *M. radiodurans*. To investigate DNA damages and their repair after α-bombardment in comparison with the radiolethality, sedimentation analysis on DNA was performed on neutral sucrose gradients with log-phase cells labeled with 3H-thymidine and exposed to α-bombardment with 3000 nC at a beam intensity of 10 nA (~10% colony survival). The sedimentation patterns immediately after irradiation and during postirradiation incubation at 30°C with shaking are shown in Fig. 5, which can be interpreted as a reflection of changes in the molecular size of the double-stranded DNA. Immediately after irradiation, the main peak was shifted to the top. Although some fraction of two peaks for the unirradiated control and irradiated sample (Fig. 5A and B) is overlapped, the complete separation has been observed on γ-irradiation. From this fact, it appears that there is no significant role of cellular radioprotecting systems or of the polyploidy in radiation resistance of this bacterium. As will be reported elsewhere, the almost same probability of double-strand scissions (DSS) caused by γ-rays was determined with *M. radiodurans* (2.7 × 10⁴ rads/DSS), as compared with that in *E. coli* (2.7 × 10⁴ rads/DSS).

The decreased sedimentation rate by α-bombardment was found to be restored during postirradiation incubation. After 7 hr of reincubation in the B-broth, 46% of the whole tritiated DNA restored its sedimentation rate to the control value (Fig. 5E). From this observation, it is likely that double-strand scissions in DNA caused by α-particles are well repaired in *M. radiodurans*. However, attention should be paid towards the possibility of occurring secondary double-strand scissions at the sites of single-strand scissions in DNA during the experimental procedure. By estimation from the data of Munson et al., the relative probability of single-strand scissions to primary double-strand scissions is about 3 in average for the whole range along the track of α-particles with an initial energy of 26 MeV. If at the most one-fifth of single-
strand scissions might produce such secondary double-strand scissions as estimated in the previous communication, the ratio of secondary double-strand scissions to primary double-strand scissions become 0.6. Thus primary double-strand scissions would be about 63% (=1/1.6) of the whole observed as apparent double-strand scissions, single-strand
scissions being 37%. Since the fraction of DNA restored the sedimentation rate on neutral gradients is 46% of the whole DNA as mentioned above, the repair of double-strand scissions caused by α-particles is still plausible with *M. radiodurans* even if the experimental error resulted from secondary double-strand scissions is taken in mind. Further considerations described in the previous short communication\(^{18}\) lead us to the view that the value 63% mentioned above is the underestimate for the fraction of primary double-strand scissions by the following reasons: (1) the single-strand scissions would be restored well during postirradiation incubation of 7 hr because of the efficient repair capacity in this bacterium, (2) considerable amounts of radioactivity are released into the acid-soluble fraction and the medium (about 14% with 330 kR according to Dean *et al.*\(^{18}\)), and such release of radioactivity is brought about by the excision on the DNA strand to be restored.

In respect of another question arising from the possibility of reincorporation of \(^{3}H\)-radioactivity excreted from DNA by the excision mechanism, the number of viable cells and DNA synthesis during postirradiation incubation were measured (Fig. 6). The DNA synthesis was determined by the incorporation of \(^{3}H\)-thymidine. The surviving fraction after irradiation with 26 MeV α-particles of 3000 nC was 22% in this run, which was a little higher perhaps because of the smaller diameter of a beam flux used. Since the reincorporation of \(^{3}H\)-radioactivity excreted into the medium should be insignificant in the present discussion, the problem could be restricted to the reincorporation into the daughter DNA synthesized in the same cell. After 7 hr of postirradiation incubation, viable counts were increased with about 30% of the initial unirradiated-control counts. Subsequently, the number of cells which reincorporate the \(^{3}H\)-radioactivity into the daughter DNA might not exceed 30% of the initial total counts. Even if one assumes that the whole \(^{3}H\)-radioactivity excreted from the radiation-damaged DNA may be reincorporated into the daughter DNA in the same cell, although this assumption appears unlikely, less than 30% of the total \(^{3}H\)-radioactivity in the 7 hr reincubated sample might be resulted from the reincorporation. Since the appreciable amount of loss in \(^{3}H\)-radioactivity from DNA of irradiated cells was found as mentioned above, the reincorporated \(^{3}H\)-radioactivity, if any, would be much less than 30%. In addition, almost no increase in viable counts and DNA synthesis were found after 5 hr reincubation, while the restoration of the sedimentation rate was observed with the sedimentation patterns of 3 and 5 hr reincubated samples. From these results, the possibility of the reincorporation seems not to be significant for the present sedimentation analysis.

Thus it is concluded that the repair of DNA during reincubation after α-bombardment of *M. radiodurans* involves restoration of double-strand scissions. However, it can not

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**Fig. 6.** Viable Counts (A) and DNA Synthesis (B) during Reincubation of *M. radiodurans* Irradiated with 26 MeV α-Particles (3000 nC) in the Liquid Phase.

Open circle, unirradiated control; closed circle, irradiated sample.
be described that DNA is the only primary site for radiation killing in *M. radiodurans*, because (1) survival curves for α-particles obtained in this study were of the exponential type, that is, one-hit type, (2) the target mass estimated from D₃7 was appreciably smaller than DNA and (3) the restored fraction (46%) of DNA exceeds the surviving fraction (10%). In another experiment on macromolecular synthesis in irradiated *M. radiodurans*, protein and RNA syntheses were found considerably inhibited by high-doses of γ-rays and the similar extent of inhibition of their syntheses was indicated, as compared with the inhibition of DNA synthesis. Furthermore, the inhibition of protein synthesis after γ-irradiation was found to bring about the decrease in survivals, i.e. radiosensitization. These facts may suggest that the cause of cell death in this radio-resistant bacterium by radiations involves non-DNA damages. Further investigations are now undertaken to elucidate the basic mechanisms for cell death in *M. radiodurans*, including the irradiation with ionizing particles of different LETs.

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