The Rate of Recombination Repair and its Relationship to the Radiation-induced Delay in DNA Synthesis in *Micrococcus radiodurans*

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SUMMARY

The measurement of the time at which normal colony-forming ability returns in irradiated cultures of *Micrococcus radiodurans* ts1 held at 30 °C can be used to estimate the time of completion of recombination repair. By comparing the times to complete such repair in populations given increasing radiation doses it is possible to calculate the rate of recombination repair. The rate was independent of the radiation dose; recombination could repair in one minute the damage caused either by 1.2 krad gamma radiation or 4×10^{-6} J mm⁻² u.v. radiation.

The time taken for the normal rate of DNA synthesis to return in irradiated *M. radiodurans* ts1 was measured under conditions identical to those used to measure recombination repair. The delay in DNA synthesis was 1.0 min per 1.2 krad gamma radiation and 1.0 min per 5.6×10^{-6} J mm⁻² u.v. radiation. The data suggest that the normal rate of DNA synthesis resumes immediately after the completion of recombination repair of gamma-induced damage, but before the completion of recombination repair of u.v.-induced damage. It is postulated that cell death at the lethal dose of u.v. radiation is caused by a second round of replication of DNA which is still being repaired by recombination.

INTRODUCTION

The vegetative bacterium *Micrococcus radiodurans* is extremely resistant to the lethal effect of ionizing and u.v. radiation (Anderson *et al.*, 1956; Duggan *et al.*, 1959). This property is dependent on a very efficient recombination repair mechanism (Moseley, Mattingly & Copland, 1972*a*; Moseley & Copland, 1975*a*) and, to a lesser extent, an excision process (Boling & Setlow, 1966) which between them cope with extensive γ radiation-induced DNA strand breakage and u.v. radiation-induced pyrimidine dimer formation.

The temperature-sensitive mutant *M. radiodurans* ts1 has the same resistance to radiation as the wild type at 30 °C, indicating that it has wild-type repair capacity at the permissive temperature. However, if it is raised to its restrictive temperature of 39 °C, DNA synthesis stops immediately (Moseley, Mattingly & Shimmin, 1972*b*) and it gradually becomes sensitized to both ionizing and u.v. radiation. The rate of loss of radiation resistance has been correlated with the rate of loss of a recombination function when the culture is held at 39 °C (Moseley *et al.*, 1972*a*).

Sublethally-irradiated populations of *M. radiodurans* ts1 give rise to colonies identical to those derived from unirradiated populations if they are incubated at 30 °C. However, if they are exposed to the restrictive temperature of 39 °C for 3 h (restrictive temperature holding) before incubation at 30 °C, more than 99 % of the irradiated bacteria produce

small, grossly abnormal colonies which, in their early development, are composed entirely of giant cells. It appears, therefore, that the recombination function which is depleted at 39 °C, is required not only for the repair of radiation damage, but also for the regulation of cell division (Moseley & Copland, 1975*b*).

Irradiated populations of *M. radiodurans* ts1 only give rise to a high frequency of abnormal colonies if they are subjected to restrictive temperature holding when the recombination function is actively involved in the repair of the radiation-induced damage. This provides a method of timing recombination repair, since the population will only become resistant to the effects of restrictive temperature holding when recombination repair is complete. The time taken for recombination to occur at 30 °C has been measured for a range of non-lethal doses of ionizing and u.v. radiation, and a rate for recombination repair calculated. This has been compared with another parameter of DNA repair, namely the radiation-induced delay in DNA synthesis.

METHODS

Organism. *Micrococcus radiodurans* ts1 (Moseley *et al.*, 1972*b*).

Media. TGY medium for growth contained (g/l distilled water): Bacto-tryptone (Difco), 5; glucose, 1; yeast extract (Difco), 3. TGY agar was made by solidifying this medium with 15 g Bacto-agar/l. The 0.067 M-phosphate buffer pH 7.0 contained 4.73 g Na₂HPO₄ and 4.54 g KH₂PO₄/l distilled water.

Growth of bacteria. Bacteria were grown in 20 ml quantities of TGY broth in 250 ml conical flasks which were swirled at 30 °C until the extinction was between 0.25 and 0.30 on an EEL nephelometer with an orange filter. This was equivalent to a concentration of 8×10^7 to 9×10^7 viable units/ml.

Irradiation of bacteria. Samples (10 ml) of the bacteria were washed and resuspended in phosphate buffer at a concentration of about 10^8 viable units/ml. For u.v. irradiation a 5 ml sample of the washed bacterial suspension in a 9 cm Petri dish was agitated with a magnetic stirrer at a distance of 40 cm from a Hanovia model 12 germicidal lamp (incident dose rate 2.25×10^{-6} J mm⁻² s⁻¹). Gamma irradiation was carried out in a ⁶⁰Co source at a dose rate of 5 to 6 krad min⁻¹. Portions (3 ml) of the washed suspension were irradiated, oxygen being bubbled during the irradiation.

The effect of restrictive temperature holding on irradiated populations of M. radiodurans ts1 after increasing times of incubation at 30 °C. After exposure to a single dose of radiation ten samples of the irradiated suspension were diluted immediately into fresh TGY medium at 30 °C. Two dilution factors were used, 100-fold (0.1 ml into 9.9 ml TGY) and fivefold (2 ml into 8 ml). Details are given in the Figure legends and in Results. These cultures were incubated at 30 °C on a reciprocating shaker immersed in a water bath for times ranging from 0 to 240 min after irradiation, when they were shifted to 39 °C. When each culture had been incubated at 39 °C for 3 h, suitable dilutions were made in TGY medium and 0.1 ml amounts of each dilution were spread on the surface of TGY agar plates. Normal colonies were counted after incubation for two days and abnormal colonies after four days at 30 °C. A graph was then plotted on semi-logarithmic paper of the return of normal colony-forming ability as a function of time at 30 °C.

Delay in the recovery of normal colony-forming ability as a function of the radiation dose. The above treatment was carried out on populations given increasing doses of u.v. and γ radiation and the results plotted. The times taken for 10 % of each irradiated population to recover normal colony-forming ability were obtained from the graphs. The difference in

time for two cultures given different radiation doses to recover normal colony-forming ability in 10 % of the population was taken to be the time required for the recombination repair of the extra radiation damage in the more heavily irradiated culture.

Delay in DNA synthesis as a function of the radiation dose. Samples of unirradiated and irradiated suspensions, exposed to increasing non-lethal doses of radiation, were diluted fivefold into 0.2 ml TGY containing 10 μ Ci [$6\text{-}^3\text{H}$]thymidine (26 Ci/mmol) in test tubes. These were incubated at 30 °C on a reciprocating shaker, and at suitable intervals 10 μ l samples were removed and assayed for radioactivity in the trichloroacetic acid (TCA)-insoluble fraction of the cell, as described previously (Moseley, 1969).

RESULTS

*Recovery of normal colony-forming ability in irradiated populations of *M. radiodurans* ts1*

The effect on colony formation of u.v.-irradiating a population of *M. radiodurans* ts1 and holding it in TGY medium at 30 °C for increasing lengths of time before subjecting it to restrictive temperature holding is shown in Fig. 1. When a culture given a non-lethal dose of 2.64×10^{-4} J mm $^{-2}$ was subjected to restrictive temperature holding immediately after irradiation, 1.4 % of the population gave rise to normal colonies and 98.6 % to abnormal colonies. During the first 60 min after irradiation the proportion of the population giving rise to normal colonies fell to 0.6 %, but after 70 min it started to increase exponentially to a maximum value of about 40 % of the total population, attained approximately 180 min after irradiation. The proportion did not rise above 40 % because the restrictive temperature holding procedure caused 60 % of all cultures, including unirradiated control populations, to form abnormal colonies. No explanation has been sought for the initial drop in the proportion of normal colonies during the first 60 min after irradiation.

Delay in recovery of normal colony-forming ability as a function of dose

The time of recovery of normal colony-forming ability was dose-dependent. In Fig. 1 the effect of a u.v. dose of 1.32×10^{-4} J mm $^{-2}$ on normal colony formation is compared with a u.v. dose of 2.64×10^{-4} J mm $^{-2}$. The ability of 10 % of the irradiated bacteria to give rise to normal colonies was recovered 33 min earlier when the u.v. dose was 1.32×10^{-4} J mm $^{-2}$ than when it was 2.64×10^{-4} J mm $^{-2}$, i.e. the delay in recovery time was 1.0 min per 4.0×10^{-6} J mm $^{-2}$ u.v. radiation. The effect on normal colony formation of 30 and 100 krad γ radiation is shown in Fig. 2. There was a similar dose dependence. The recovery of the ability to give rise to normal colonies in 10 % of the population occurred 57 min earlier in bacteria given 30 krad than in those given 100 krad, i.e. 1.0 min per 1.2 krad. These results were obtained in identical experiments, the irradiated culture having been diluted initially 100-fold.

To make the results directly comparable with those measuring the radiation-induced delay in DNA synthesis, similar experiments were performed when the irradiated cultures were diluted fivefold. The time differences between the curves obtained with different doses were unaltered. In a series of experiments, we constructed sets of curves similar to those of Figs. 1 and 2, and compared the times for 10 % of irradiated populations to recover their ability to give normal colonies following increasing radiation doses. It was not possible to study doses below 1.32×10^{-4} J mm $^{-2}$ u.v. radiation or 30 krad γ radiation because there was an inadequate loss of normal colony-forming ability. These doses were used, therefore, as the minimum comparative doses. The results of several experiments are summarized in Table 1. The difference in the times for 10 % of the irradiated population to recover normal

Fig. 1

Fig. 2

Fig. 1. The effect of u.v. irradiation doses of 1.32×10^{-4} (□) and 2.64×10^{-4} (○) J mm^{-2} on the return of normal colony-forming ability in cultures of *M. radiodurans* ts1 held at 30 °C following irradiation. The ability to give rise to normal colonies was tested by incubating the bacteria at 39 °C for 3 h before plating. The total number of colonies, normal and abnormal, for the $2.64 \times 10^{-4} \text{ J mm}^{-2}$ dose is included (●). The irradiated cultures were diluted 100-fold.

Fig. 2. The effect of γ radiation doses of 30 (○) and 100 (□) krad on the return of normal colony-forming ability in cultures of *M. radiodurans* ts1 held at 30 °C following irradiation. The ability to give rise to normal colonies was tested by incubating the bacteria at 39 °C for 3 h before plating. The total number of colonies, normal and abnormal, for the 30 krad dose is included (●). The irradiated cultures were diluted 100-fold.

Fig. 3. The effect of a fivefold (□) or 100-fold (○) dilution on the return of normal colony-forming ability in *M. radiodurans* ts1 given an initial dose of $2.64 \times 10^{-4} \text{ J mm}^{-2}$ and held at 30 °C. The ability to give rise to normal colonies was tested by incubating the bacteria at 39 °C for 3 h before plating. The derivation of the theoretical line (---) is given in the text.

Table 1. *Differences in the recovery times of normal colony-forming ability in M. radiodurans ts1 cultures given different irradiation doses*

Radiation doses compared	Difference in time for recovery of normal colony-forming ability in 10 % of the population	Radiation dose inducing damage repaired per minute
$10^{-4} \times \text{u.v. radiation}$ (J mm ⁻²)	(min)	($10^{-6} \times \text{J mm}^{-2}$)
1.32, 2.64	33.0	4.0
1.32, 1.98	18.5	3.7
1.98, 2.64	16.5	4.0
1.32, 2.64	31.0	4.3
2.64, 3.96	36.0	3.7
1.32, 2.64	30.0	4.4
2.64, 3.96	37.0	3.6
γ Radiation (krad)	(min)	(krad)
30, 100	57.0	1.23
30, 75	40.0	1.13
75, 120	40.0	1.13

colony-forming ability was 1.0 min for either 4.0×10^{-6} J mm⁻² u.v. radiation or 1.2 krad of γ radiation, these values being constant over the dose ranges studied.

Although the time difference for the delays in the return of normal colony formation in two populations of *ts1* given different irradiation doses was identical whether the bacteria were diluted 100-fold or fivefold, normal colony formation returned about 40 min earlier in an irradiated culture diluted fivefold than when diluted 100-fold (Fig. 3). This dilution effect was independent of dose and applied to both u.v. and ionizing radiation.

Because of limitations imposed by the method, the rate of recombination repair can only be shown to be constant once minimum doses of 1.32×10^{-4} J mm⁻² u.v. radiation or 30 krad γ radiation have been given to cultures. However, if rate of recombination repair of u.v.-induced damage, derived from higher doses, is such that the damage induced by 4.0×10^{-6} J mm⁻² is repaired per min, and we assume this rate to be constant from time zero, then the recombination repair of the damage induced by 2.64×10^{-4} J mm⁻² u.v. radiation should be completed in 66 min. If this time is subtracted from the experimental line for the return of normal colony-forming ability in a culture given a u.v. dose of 2.64×10^{-4} J mm⁻² and diluted only fivefold, the theoretical line shown in Fig. 3 is obtained. It indicates that recombination repair began immediately after irradiation, since the theoretical line rises from time zero. The intercept value on the ordinate suggests that some repair may have occurred before dilution. However, in the culture diluted 100-fold, either repair began 30 to 40 min after irradiation and then proceeded at a constant rate or it began at a slower rate and was only able to attain the rate at which the damage induced by 4.0×10^{-6} J mm⁻² u.v. radiation was repaired per minute by about 70 min.

Radiation-induced delay in DNA synthesis

Following the sublethal irradiation of an exponentially-growing culture of *M. radiodurans ts1*, there is a reduction in the rate of DNA synthesis to almost zero, but this gradually increases and reaches the control rate of an unirradiated culture. The delay in DNA synthesis is taken to be the difference in time for an unirradiated and irradiated population to incorporate the same amount of [³H]thymidine (1000 c p.m.) into the TCA-insoluble

Fig. 4

Fig. 5

Fig. 4. Radiation-induced delay of DNA synthesis in *M. radiodurans* ts1 following u.v. doses of 0 (○), 1.32×10^{-4} (△), 2.64×10^{-4} (●), 3.96×10^{-4} (▲) and 5.28×10^{-4} (□) J mm⁻².

Fig. 5. Radiation-induced delay of DNA synthesis in *M. radiodurans* ts1 following γ radiation doses of 0 (○), 50 (△), 100 (●) and 150 (▲) krad.

fraction of the cells, provided that the irradiated culture has reached the same incorporation rate as the unirradiated. Thus the radiation-induced delay is the time taken for the normal rate of DNA synthesis to return. With both u.v. and γ radiation the delay in DNA synthesis is directly proportional to the radiation dose. For u.v. radiation, the delay in DNA synthesis caused by sublethal doses of 5.28×10^{-4} J mm⁻² or less is shown in Fig. 4. There was a delay of 24 min per 1.32×10^{-4} J mm⁻² u.v. radiation, equivalent to 1.0 min per 5.6×10^6 J mm⁻² u.v. radiation. This linear relationship between the u.v. radiation dose and delay in DNA synthesis is valid for doses as high as 1.05×10^{-3} J mm⁻². Data for the delay in DNA synthesis following γ irradiation are in Fig. 5. For every 50 krad increase in γ radiation dose, the delay in DNA synthesis increased by 42.5 min, this being equivalent to a delay of 1.0 min per 1.2 krad.

DISCUSSION

The time of recovery of normal colony-forming ability in irradiated populations of *M. radiodurans* ts1 is dose-dependent and provides a biological end-point for measuring the rate of recombination repair of radiation-induced damage. The time of recovery being measured is not necessarily the completion of recombination repair, since the recombination function defective in ts1 has not been identified. Indirect evidence suggests that it may be similar in function to the *recA* gene product of *Escherichia coli* (Moseley & Copland, 1975*b*). Therefore, the method identifies the end of the involvement of the recombination function in the repair of radiation damage, and the difference in time for this step to be completed in populations given increasing radiation doses indicates the rate of recombination repair, namely that recombination repairs in one minute the damage induced by 4.0×10^{-6} J mm⁻² u.v. radiation or 1.2 krad γ radiation. It has been demonstrated that this rate of repair is constant for sublethal doses up to 3.96×10^{-4} J mm⁻² u.v. radiation and 120 krad γ radiation. Using this method, it is not possible to demonstrate directly whether this rate of recombination operates from time zero after irradiation. However, Lett *et al.* (1967) showed that in [³H]thymidine-labelled wild-type *M. radiodurans* the repair of sublethal damage induced by X-rays was associated with the partial breakdown of DNA into perchloric acid-soluble material. The extent of the breakdown was dose-dependent but its rate was dose-independent,

being constant at 0.12 % of the DNA label per minute. The breakdown started immediately after irradiation and continued until maximum release was reached, and only then did DNA synthesis resume. Lett *et al.* (1967) were presumably studying the breakdown of DNA associated with the activity of recombination-dependent repair, and it is reasonable to assume from this that the rate of recombination repair of damage induced by ionizing radiation is constant from time zero. The evidence for recombination repair of u.v. radiation-induced damage beginning at time zero and continuing at a constant rate is less direct, but from a consideration of the theoretical line in Fig. 3 it is not unreasonable to assume that it does.

To measure DNA synthesis at short times after irradiation we used initially unlabelled bacteria, so that small amounts of synthesis would give relatively large increases in radioactive counts (Setlow & Setlow, 1970). The results confirmed previous findings that there is a delay in DNA synthesis which is linearly related to the radiation dose (Setlow & Boling, 1965; Dean, Feldschreiber & Lett, 1966). Under comparable conditions to those used to measure the rate of recombination repair the delay was 1.0 min per 5.6×10^{-6} J mm⁻² u.v. radiation and 1.0 min per 1.2 krad γ radiation. Since for γ radiation, recombination repaired the damage induced by 1.2 krad per min the data suggest that DNA synthesis resumes immediately recombination repair is complete. A similar situation exists in *E. coli*, in which it has been demonstrated that the *recA* gene product required in the slow repair of DNA single strand breaks introduced by γ irradiation (McGrath & Williams, 1966; Kapp & Smith, 1970) acts before DNA replication (Gray, Green & Bridges, 1972).

For u.v. radiation, however, recombination repairs the damage induced by 4.0×10^{-6} J mm⁻² in one minute while the delay in DNA synthesis is only 1.0 min per 5.6×10^{-6} J mm⁻², indicating that DNA synthesis resumes before recombination is complete. Resumption of DNA synthesis before the completion of excision repair leads to the formation of DNA strand gaps which are the substrate for recombination repair enzymes (Rupp & Howard-Flanders, 1968). It is possible that cell death following doses of more than 1.22×10^{-3} J mm⁻² u.v. radiation is caused by a second round of replication of DNA which is still being repaired by recombination.

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