Sensitization to Radiation by Loss of Recombination Ability in a Temperature-sensitive DNA Mutant of *Micrococcus radiodurans* Held at Its Restrictive Temperature

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SUMMARY

Two temperature-sensitive mutants of *Micrococcus radiodurans* defective in DNA synthesis, which were very resistant to the lethal effect of ultraviolet and ionizing radiation at permissive temperatures, became sensitive to radiation and also to the action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine when held at the restrictive temperature of 39 °C. With *M. radiodurans* ts1 the sensitization began soon after transfer to 39 °C and reached a maximum 4 h later. During this period there was no loss of viability. After 4 h the shoulders of the ultraviolet and ionizing radiation survival curves had almost completely disappeared and the exponential part of the curves had doubled in slope. The size of the shoulder fell exponentially with the time the bacteria were held at 39 °C. Sensitization occurred in the presence of chloramphenicol. During the period the bacteria were held at 39 °C their ability to effect recombination as measured by transformation fell exponentially and was correlated with the rate of loss of the shoulder. This suggests that the repair which gives rise to the large shoulders of the radiation survival curves is of the post replication recombination type.

The recovery of radiation resistance at 30 °C in bacteria which had been exposed to 39 °C for 75 min did not begin immediately. For 55 min there was no measurable increase in resistance but after 75 min substantial recovery had occurred and by 105 min was complete. Recovery of resistance did not occur in the presence of chloramphenicol even when the chloramphenicol was added 30 min after the bacteria had been at 30 °C.

The sensitization to radiation was not a general property of temperature-sensitive (ts) mutants.

INTRODUCTION

The vegetative bacterium *Micrococcus radiodurans* is very resistant to the lethal action of ultraviolet (u.v.) and ionizing radiation (Anderson *et al.* 1956; Duggan, Anderson, Elliker & Cain, 1959) because it is able to repair radiation damage to its DNA very efficiently. It possesses a mechanism for the removal of u.v.-induced pyrimidine dimers from its DNA (Boling & Setlow, 1966) and on the basis of the isolation of a u.v.-sensitive mutant, *M. radiodurans* UV17 (Moseley, 1967), which has the characteristics of a *lex*[−] or *exr*[−] mutant of *Escherichia coli* (Witkin, 1969), it has been suggested that it also possesses a postreplication recombination repair system (Moseley & Mattingly, 1971) of the type described originally for *E. coli* (Rupp & Howard-Flanders, 1968). Since the mutant UV17 is about five times as sensitive as the wild-type to both u.v. and ionizing radiation (Moseley & Mattingly, 1971) it follows that the recombination repair may account for a considerable part of the total radiation resistance.

Temperature-sensitive (ts) mutants of *Micrococcus radiodurans* have been isolated which,

unlike the wild-type, stop DNA synthesis immediately they are raised to 39 °C, although synthesis of RNA and protein continues and the bacteria become enlarged (Moseley, Mattingly & Shimmin, 1972). The mutants are similar to the wild-type in being very resistant to the lethal action of u.v. and ionizing radiation, and can carry out excision repair of u.v.-induced pyrimidine dimers at the restrictive temperature, although at a slower rate than in the wild-type. This paper describes the phenomenon of these mutants becoming increasingly sensitive to radiation as they are held at 39 °C.

METHODS

Bacteria. *Micrococcus radiodurans* wild-type; *M. radiodurans* ts1 and ts2, temperature-sensitive mutants defective in DNA synthesis at 39 °C; *M. radiodurans* ts9 and ts11, temperature-sensitive mutants defective in cell wall synthesis at 39 °C.

Media. TGY medium for growth contained Bactotryptone (Difco), 5 g; glucose, 1 g; yeast extract (Difco), 3 g; distilled water, 1 l. 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 per 1 l of distilled water.

Growth of bacteria. Bacteria were grown in 20 ml quantities of TGY medium in 250 ml conical flasks which were swirled at 30 °C until the optical density of the culture was between 0.25 and 0.30 on a nephelometer (Evans Electroselenium Ltd, Halstead, Essex) with an orange filter (equivalent to about 8 to 9 × 10⁷ viable units/ml). To raise the bacteria to their restrictive temperature the flasks were transferred to a reciprocating shaker immersed in a water bath at 39 °C.

Irradiation of bacteria. Ten ml samples of the bacteria were washed and resuspended in phosphate buffer at a concentration of about 10⁸ viable units/ml. For u.v. irradiation a 5 ml sample of the washed 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 22.5 ergs/mm²/s). At intervals 0.1 ml samples were removed, suitably diluted in TGY and 0.1 ml quantities spread on TGY plates. Colonies were counted after incubation for 2 days at 30 °C.

Gamma irradiation was carried out in a ⁶⁰Co source at a dose rate of 7 to 8 krad/min. Three ml volumes of the washed bacterial suspension were irradiated, oxygen being bubbled during the irradiation. Samples were removed at intervals and viability measured in the same way as for u.v. irradiation.

Resistance to the lethal action of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). To 9 ml quantities of bacteria in growth medium at 30 °C were added 1 ml amounts of a sterile NTG solution (1 mg/ml phosphate buffer) to give a final concentration of 100 µg/ml. Samples were removed at intervals, diluted and colony counts made on TGY agar.

Procedure for transformation. No competent stage has been found for genetic transformation in *Micrococcus radiodurans* and so it is possible to use any culture of bacteria for transformation. To 0.2 ml quantities of culture were added 0.05 ml DNA (from an acriflavin-resistant mutant) and 0.05 ml of an autoclaved extract of the wild-type (Moseley & Setlow, 1968). The resulting cultures were shaken gently at 30 °C for 3 h, diluted appropriately with chilled TGY and 1 ml samples plated in 10 ml of TGY agar which had been melted and cooled to about 45 °C before being mixed with the bacteria. The plates were incubated for 90 min at 30 °C and then overlaid with 10 ml of the same melted agar containing 5 µg acriflavin/ml. Transformant colonies were counted after incubation at 30 °C for at least 4 days.

Fig. 1. Ultraviolet irradiation survival curves of *Micrococcus radiodurans* ts1 after 0 (○), 75 (●), 150 (×), and 240 (△) min of incubation at 39 °C.

Measurement of DNA uptake by irradiated bacteria. To 0.2 ml bacteria as used in the transformation assay was added 7 μ g tritiated DNA from *Micrococcus radiodurans* (specific activity, 1.25×10^4 c.p.m./ μ g DNA) and the bacteria were incubated at 30 °C for 3 h. Deoxyribonuclease (0.1 ml of 0.5 mg/ml) was added to each culture for an additional 30 min and the bacteria collected by centrifuging. They were washed four times with phosphate buffer and then lysed with hot 98% formic acid in scintillation vials. The formic acid was boiled off and 1 ml of distilled water was added while the vials were still hot. Dioxan scintillation fluid was added after the vials cooled, and the radioactivity in each vial was measured. In order to get significant counts of radioactivity the autoclaved extract was not added in this experiment because it inhibits the uptake of labelled DNA by at least an order of magnitude. This is partly a result of competition between the denatured DNA in the extract and the transforming DNA (Moseley & Setlow, 1968). Nevertheless it is assumed that this method adequately measures the relative amounts of DNA taken up by the organism.

RESULTS

Radiation inactivation of Micrococcus radiodurans ts1 and ts2 as a function of time at 39 °C. The increasing sensitivity to u.v. radiation of a culture of *M. radiodurans* ts1 held at 39 °C is shown in Fig. 1. The u.v.-survival curve of log phase bacteria grown to a concentration of about 9×10^7 viable units/ml showed an intercept value of 11600 ergs/mm 2 and a $1/e$ value of 1430 ergs/mm 2 (average values from 10 experiments). On incubating the bacteria at their restrictive temperature of 39 °C they became increasingly sensitive to u.v. radiation and after 4 h the intercept and $1/e$ values were reduced to 270 and 750 ergs/mm 2 respectively, i.e. the shoulder of the initial survival curve was almost eliminated and the $1/e$ value reduced

Table 1. *Analysis of ultraviolet radiation survival curves of Micrococcus radiodurans ts1 after incubation at 39 °C*

The $1/e$ value is the dose required to decrease the viability of a culture by 63 % on the exponential part of the survival curve and is the dose required to give an average of one lethal event/bacterium. The intercept value, in terms of dose, is attained by extrapolating the exponential part of the curve to unit survival.

Time at 39 °C (min)	Intercept (ergs/mm ²)	Ratio*	$1/e$ Dose (ergs/mm ²)	Ratio*
0	11 600	1	1 430	1
20	11 400	0.98	1 300	0.90
40	9 200	0.80	860	0.70
75	5 500	0.47	1 190	0.84
150	2 100	0.18	750	0.52
240	270	0.025	750	0.52

* Intercept or $1/e$ value at given time: value at time zero.

by a factor of about two. The sensitivities to u.v. radiation at intermediate times are given in Table 1 and a plot of the intercept values against time at 39 °C is shown in Fig. 7. There appeared to be an exponential reduction in the intercept value as a function of time at the restrictive temperature. The sensitization began soon after the temperature was raised to 39 °C, was easily measurable by 20 min, and reflected a sensitization of the population of bacteria as a whole since no evidence of a heterogeneous response could be observed in the survival curves. Up to 4 h at 39 °C there was no loss of bacterial viability. The only measurements made on cultures held at 39 °C in excess of 4 h was at 7 h when the viability of the culture was reduced to about 40 % of the original or the bacteria were aggregating sufficiently to give such an impression. However no further sensitization to radiation was observed.

Similar results were obtained for *ts2* (Fig. 2). When it was held at 39 °C for 3 h the intercept value of its u.v. survival curve was reduced from 10000 to 2900 ergs/mm² and the $1/e$ value from 1420 to 940 ergs/mm².

The increased sensitivity of *ts1* to u.v. radiation was associated with a correspondingly increased sensitivity to ionizing radiation (Fig. 3). The intercept value for a culture prior to exposure to 39 °C was 600 krad with a $1/e$ dose of 45 krad and after incubation at the restrictive temperature for 4 h the intercept and $1/e$ values were reduced to 10 and 22.5 krad respectively. Again the shoulder of the survival curve was almost eliminated and the $1/e$ dose reduced by a factor of two.

Inactivation of Micrococcus radiodurans ts1 by NTG. The effect of NTG on *M. radiodurans ts1* both before and after exposure to a temperature of 39 °C is shown in Fig. 4. The bacteria which were held at the restrictive temperature were more sensitive to the lethal action of NTG than those tested just prior to exposure, the shoulder of the survival curve having been eliminated although the exponential rate of inactivation did not appear to be affected.

Radiation inactivation of Micrococcus radiodurans ts9 and ts11 as a function of time at 39 °C. These two *ts* mutants were not defective in DNA, RNA or general protein synthesis at their restrictive temperature (unpublished observation). They were unusual in that at growth temperatures just above 30 °C the culture contained many 'membranous bodies' which seemed to burst out from the cell in the region of the division septum during division. It was concluded that *ts9* and *ts11* were either cell wall or division mutants. The results of holding these bacteria at 39 °C on their radiation sensitivity are shown in Fig. 5. With *M. radiodurans ts9* there was a reduction in the shoulder of the u.v. survival curve after 2 h of incubation at 39 °C, from 11 100 to 9300 ergs/mm² but the $1/e$ value did not alter. When the culture was

Fig. 2. Ultraviolet irradiation survival curves of *Micrococcus radiodurans* ts2 after 0 (O) and 180 (●) min of incubation at 39 °C.

Fig. 3. γ -Irradiation survival curves of *Micrococcus radiodurans* ts1 after 0 (O) and 240 (●) min of incubation at 39 °C.

Fig. 4. Resistance of *Micrococcus radiodurans* ts1 to the lethal action of 100 μ g NTG/ml after 0 (○) and 240 (●) min of incubation at 39 °C.

Fig. 5. Ultraviolet irradiation survival curves of *Micrococcus radiodurans* ts9 after 0 (×) and 120 (Δ) min of incubation at 39 °C and ts11 after 0 (○) and 120 (●) min of incubation at 39 °C.

Fig. 6. Ultraviolet irradiation survival curves of *Micrococcus radiodurans* ts1 after 0 (○) and 75 (●) min of incubation at 39 °C and after 75 min of incubation at 39 °C in the presence of 15 µg chloramphenicol/ml (×).

Table 2. Frequency of transformation in *Micrococcus radiodurans* ts1 after incubation at 39 °C

Time at 39 °C (min)	Frequency transformation	DNA uptake c.p.m. ³ H
0	2.0×10^{-8}	2 600
30	2.0×10^{-8}	—
60	1.0×10^{-8}	—
90	3.5×10^{-4}	—
120	3.4×10^{-4}	—
150	1.0×10^{-4}	—
180	7.8×10^{-5}	—
210	4.0×10^{-5}	704

incubated for another 2 h there was no further increase in sensitivity to u.v. radiation. For *M. radiodurans* ts11 the culture became more resistant when incubated at 39 °C.

Effect of chloramphenicol on sensitization to radiation. *Micrococcus radiodurans* is sensitive to the action of chloramphenicol at a concentration of 3 to 4 µg/ml (Hawiger & Jeljaszewicz, 1967). The presence of 15 µg chloramphenicol/ml, added at the time the bacteria were raised to the restrictive temperature, did not affect the sensitization to u.v. radiation (Fig. 6). The optical density of a culture of ts1 held at 39 °C increases as RNA and protein synthesis continue and the bacteria become enlarged. This, of course, did not occur in the presence of chloramphenicol but nevertheless the bacteria became sensitive to radiation.

Effect of incubating Micrococcus radiodurans ts1 at 39 °C on the transformation frequency. A culture of ts1 was raised to 39 °C and 0.2 ml samples removed at intervals and used as recipients in transformation assays. The transformation frequency to acriflavine resistance fell by about 98 % over the time during which the bacteria became sensitive to u.v. radiation (Table 2). This could not be accounted for in terms of a reduced uptake of transforming DNA since experiments indicated that during the same time the DNA uptake was reduced

Fig. 7. Comparison of the reduction in the intercept value (O) and the reduction in the frequency of transformation (x) as a function of the time of incubation at 39 °C of *Micrococcus radiodurans* ts1. Values at time zero were made equal to 1. Allowance has been made for the reduced DNA uptake in the transformation results.

by 73%. The transformation frequency as a function of time at 39 °C is plotted in Fig. 7, allowance having been made for the reduction in DNA uptake (which was assumed to be linear with time), together with the reduction in intercept value as a function of time 39 °C.

Recovery of radiation resistance and the effect of chloramphenicol. Since ts1 became sensitised to u.v. radiation by incubation at 39 °C for 75 min it was possible to study the recovery of u.v. resistance when the cells were subsequently incubated at 30 °C before irradiation. After 55 min at 30 °C there was no increase in the resistance of the bacteria to u.v. radiation but after 75 min there was a considerable recovery which was completed by 105 min. Recovery did not take place when chloramphenicol was added to the sensitized bacteria either before they were returned to 30 °C, or 30 min later.

DISCUSSION

Micrococcus radiodurans ts1 is unable to synthesize DNA at 39 °C probably as a result of a single mutation of the wild-type (Moseley *et al.* 1972). The increasing sensitivity to u.v. radiation when a log phase culture of this mutant was transferred from 30 °C to 39 °C was a property of the entire bacterial population, i.e. sensitivity was solely a function of the time at 39 °C and did not depend upon a particular stage of the cell cycle being reached; nor did it appear to be a result of the bacteria enlarging because of an imbalance of synthesis, since the bacteria became sensitive to radiation at 39 °C in the presence of chloramphenicol when

the optical density of the culture did not change. Temperature-sensitive mutants defective in some aspect of wall or membrane synthesis at 39 °C did not become sensitized to radiation on incubation at 39 °C even though they also swelled considerably.

For the restoration of the repair capacity lost when *Micrococcus radiodurans* ts1 was incubated at 39 °C for 75 min, protein synthesis was required when the bacteria were returned to 30 °C. The reappearance of the lost repair function occurred between 55 and 75 min and was complete by 105 min. Since synthesis of DNA began immediately the bacteria were returned to 30 °C (unpublished observation) the protein synthesis was not that required for the initiation of new rounds of DNA synthesis (Maaløe & Hanawalt, 1961) because the restoration of function was prevented even when protein synthesis was inhibited 30 min after DNA synthesis had begun. Under these circumstances at least some new rounds of replication would be expected to have been initiated and would have shown up in the survival curves had resistance returned in such cells.

The radiation response curve of a bacterial culture can be described by its intercept value (a measure of the size of the shoulder of the curve) and a $1/e$ value which defines the slope of the exponential part of the curve in terms of an average 1-hit dose. The parameter most affected by incubating *Micrococcus radiodurans* ts1 at its restrictive temperature was the intercept value which decreased exponentially with time until almost eliminated after 4 h. In the same period the $1/e$ value was reduced only twofold.

During the time the bacteria were held at 39 °C their capacity to be transformed fell exponentially at the same rate as the loss of shoulder of the survival curve. This fall in transformation frequency could not be accounted for in terms of a reduced uptake of DNA and probably indicated a reduced recombination level in the bacterial host. Thus the exponential loss of both transformation, which requires a recombination event, and the shoulder of the survival curve appear to be connected and it is probable that recombination repair is responsible for the shoulder of the radiation survival curve.

Since the radiation-sensitive mutant *Micrococcus radiodurans* UV17 which is five times more sensitive to u.v. radiation than wild-type is equivalent to an *exr*⁻ mutant of *Escherichia coli* (Moseley & Mattingly, 1971) it follows that a large fraction of the resistance of wild-type *M. radiodurans* must be of the postreplication recombination type. However an *exr*⁻ mutant still has some recombination ability, albeit reduced compared with wild-type, and this is reflected in the intercept value of 1630 ergs/mm² for UV17. Thus the loss of recombination function in *M. radiodurans* ts1 held at 39 °C is not caused by the decay of the *exr* gene product (since the intercept value falls to almost zero) but of some other function involved in the recombination event.

Assuming that recombination repair accounts for the large shoulder of the survival curve then what is the contribution of the very efficient excision repair mechanism in *Micrococcus radiodurans* (Boling & Setlow, 1966; Moseley, 1968)? It may be that the large $1/e$ values in the wild-type can be accounted for in terms of excision repair. For example the $1/e$ value for *M. radiodurans* is 1200 and for *Escherichia coli* B/r is about 120 ergs/mm². An explanation of the fact that all the u.v.-sensitive mutants of *M. radiodurans* so far obtained (unpublished observations) are also sensitive to ionizing radiation may follow from this. All mutants defective in recombination repair would be expected to be sensitive to ionizing radiation, whereas mutants defective in excision repair would be expected to be u.v.-sensitive but show wild-type resistance to ionizing radiation. If the contribution of excision repair to the total repair capacity of *M. radiodurans* were of a minor nature the selective procedures employed (Moseley, 1967) would not be expected to detect excision defective mutants of *M. radiodurans* if the recombination repair system was still operating. Attempts are being made to isolate

an *hcr*⁻ (excision-deficient) mutant of *M. radiodurans* to measure the contribution of the *hcr* system to the total repair capacity of the bacterium.

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