Repair of DNA Damage Produced by Gamma-radiation in *Escherichia coli* K-12 and a Radiation- sensitive *exrA* Derivative during Inhibition of Protein Synthesis and Normal DNA Replication by Chloramphenicol

By M. H. L. GREEN, W. J. H. GRAY, S. G. SEDGWICK AND
B. A. BRIDGES

*MRC Cell Mutation Unit and School of Biological Sciences,
University of Sussex, Falmer, Brighton BN1 9QH, Sussex*

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SUMMARY

Exponentially growing *Escherichia coli* PAM5717 *exrA* is radiation-sensitive, does not carry out 'slow' repair of single-strand DNA breaks and shows substantial DNA degradation after gamma-irradiation. When chloramphenicol is present for 90 min before and after gamma-irradiation, survival is enhanced, DNA degradation is minimal and single-strand DNA breaks are repaired both in PAM5717 *exrA* and the radiation-resistant parental strain AB1157. Thus, both radiation-resistant and *exrA* bacteria can repair single-strand breaks in the absence of normal DNA replication. Similar repair was observed in a *polA* strain suggesting that DNA polymerase I is not involved. Both radiation-resistant and *exrA* bacteria also show a small amount of gamma-ray stimulated DNA synthesis under these conditions. It is suggested that neither the *exrA*⁺ gene nor protein synthesis are required for repair of single-strand DNA breaks in the absence of an active DNA replication fork.

INTRODUCTION

Most single-strand breaks produced in bacterial DNA by ionizing radiation are repaired by a 'rapid' process involving DNA polymerase I (Town, Smith & Kaplan, 1971). In all except *polA* strains these breaks are normally sealed before the DNA can be isolated from the bacteria. About 15 to 20 % of breaks, however, are repairable only by a 'slow' process requiring the *recA*⁺, *recB*⁺ and *exrA*⁺ gene products (McGrath & Williams, 1966; Morimyo, Horii & Suzuki, 1968; Kapp & Smith, 1970; Sedgwick & Bridges, 1972). To distinguish between the action of the gene products necessary for 'slow' repair, Gray, Green & Bridges (1972) determined the sensitivity of the initial rate of DNA synthesis to gamma-irradiation in various strains of *Escherichia coli* deficient in one or more of these genes. The initial rate of DNA synthesis in *recA* strains was found to be extremely sensitive to gamma-irradiation whereas in *exrA* strains the initial rate showed nearly wild-type resistance. They concluded that the *recA*⁺ gene product acts immediately, certainly before DNA replication, in the repair of gamma-induced lesions and that the *exrA*⁺ gene product might act at a later stage, possibly at or after the arrival of the lesion at the DNA replication complex. If this were so, one would predict that in the absence of DNA replication there would be no 'slow' repair and that the pathological effects of the *exrA* allele would not be apparent.

We have found, however, that 'slow' repair can occur in the absence of active DNA replication. Furthermore, when active replication is inhibited by treatment with chloram-

phenicol, the *exrA* genotype does not markedly affect DNA breakdown, gamma-ray-stimulated DNA synthesis or 'slow' repair of single-strand breaks. This is in contrast to the situation in growing bacteria where the *exrA* genotype results in substantial DNA degradation and failure to carry out 'slow' repair of single-strand breaks. We conclude that the *exrA*⁺ gene is needed for 'slow' repair of single-strand breaks only in the presence of an active replication fork but that the repair process does not necessarily occur at the time that DNA containing the lesion is replicated.

METHODS

Bacterial strains. *Escherichia coli* K-12 AB1157 (radiation-resistant) and PAM5717 *exrA* were used. PAM5717 was produced by transduction into AB1157 of the *exrA* gene of strain B₃₂ (Donch, Green & Greenberg, 1968). In one experiment strains AB2463 *recA*, AB2470 *recB*, JC5495 *recArecB* and CM293 *recAexrA* (all derived from AB1157) were used (Gray, Green & Bridges, 1972). In another experiment *E. coli* p3478 *thypolAr* was used (de Lucia & Cairns, 1969).

Cultural conditions. Cultures were grown at 37 °C with shaking in M-9 minimal medium supplemented with 0.4 % (w/v) glucose, 1 % (w/v) Difco Casamino acids and 0.25 µg thiamine/ml. For *Escherichia coli* p3478 the medium was supplemented with 20 µg thymine/ml. Viable counts were determined after dilution with phage buffer (Boyle & Symonds, 1969), on nutrient agar (Oxoid Nutrient Broth no. 2, solidified with 1.5 % New Zealand Agar).

Irradiation. Samples were irradiated at room temperature (with certain exceptions) and with aeration in a ⁶⁰Co 'hot spot' source at a dose rate of approximately 4 krad per min.

Radiochemicals. [³H-methyl]Thymine (specific radioactivity 23.6 Ci/mM), [³H-methyl]-thymidine (5 Ci/mM) and [³H-methyl]thymidine monophosphate (12 Ci/mM) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Measurement of DNA breakdown. Degradation of DNA was estimated by observing the loss from labelled cultures of radioactivity precipitable by trichloroacetic acid (Green, Gray, Murden & Bridges, 1971).

Cultures were randomly labelled by diluting stationary overnight cultures into fresh medium containing 50 µM-thymidine monophosphate carrier and 1 µCi [³H-methyl]thymidine monophosphate/ml and incubating at 37 °C for 150 min. After filtering, the bacteria were resuspended in fresh medium and incubated for a further 30 min to allow self-chasing to occur. Where appropriate, chloramphenicol (Sigma Chemical Co., Ltd, London) was added to a final concentration of 200 µg/ml after 90 min. The antibiotic was added as a concentrated ethanolic solution.

Cultures were pulse-labelled after 180 min growth by adding 1 µCi [³H-methyl]thymidine/ml and filtering after a further 5 min of incubation. Following resuspension in fresh medium a fraction of the culture was immediately irradiated at 4 °C. The rest was incubated further and after 20, 40 and 60 min samples were removed and irradiated at 4 °C.

Measurement of DNA synthesis. Overnight stationary cultures were diluted into fresh medium and grown for 120 min. Chloramphenicol (200 µg/ml) was added and incubation continued for a further 180 min after which the cultures were divided into two samples one of which was irradiated with 4 krads. Immediately after irradiation [³H-methyl]thymidine (1 µCi/ml) was added and portions were removed at intervals up to one hour and precipitated with trichloroacetic acid (Green, *et al.* 1971).

Alkaline sucrose gradient sedimentation. A modification (Sedgwick & Bridges, 1972) of

Fig. 1. Uptake of [^3H]thymidine monophosphate into trichloroacetic acid precipitable material in strain PAM5717 *exrA*. Open symbols, no chloramphenicol added; closed symbols, 200 μg chloramphenicol/ml added at 0 min.

the method of McGrath & Williams (1966) was used. Bacteria were labelled by growing in a 1 ml culture supplemented with 50 μg deoxyadenosine, 0.5 μg thymidine and 10 μCi [^3H -methyl]thymidine. For strain p3478 the culture was supplemented with 5 μCi [^3H -methyl]thymine. Prior to irradiation the cultures were filtered and resuspended in fresh medium without label. Samples (0.08 ml $\sim 10^7$ bacteria) were taken immediately before irradiation, 2 min after irradiation and after 60 min of postirradiation incubation at 37 $^\circ\text{C}$. Each sample was lysed in a 0.1 ml band containing 1 % Sarkosyl (Geigy Ltd, Manchester), 0.5 N-NaOH, 0.25 N-NaCl, 0.01 M-tris and 0.001 M-EDTA floating on the surface of a 5 ml linear gradient of 5 % to 20 % (w/v) sucrose in 0.2 N-NaOH, 0.5 N-NaCl and 0.001 M-EDTA. The gradients were centrifuged at 27000 rev./min at 20 $^\circ\text{C}$ either in a Spinco SW 39 rotor for 105 min or in a Spinco SW 50-1 rotor for 90 min. Approximately 35 fractions of 8 drops were collected on strips of Whatman 17 chromatography paper (Carrier & Setlow, 1971) and assayed for acid insoluble radioactivity.

RESULTS

The experiments were designed to measure DNA repair and breakdown in the absence of active DNA replication. Fig. 1 shows that DNA synthesis in strain PAM5717 *exrA* (as in wild-type bacteria) was reduced to a minimal rate 70 to 80 min after the addition of 200 μg

Fig. 2. Alkaline sucrose gradient sedimentation profiles of DNA from strain AB1157 (radiation-resistant). (a) No chloramphenicol; (b) 200 μ g chloramphenicol/ml present from 90 min before irradiation. $\circ$, No irradiation; $\triangle$, 20 krad aerobic gamma-irradiation, 2 min of incubation; $\blacksquare$, 20 krad aerobic gamma irradiation, 60 min of incubation.

chloramphenicol/ml, the bacteria presumably being brought to the end of a round of DNA replication (Ward & Glaser, 1969). To prevent active DNA replication we therefore routinely treated cells with 200 μ g chloramphenicol/ml for 90 min prior to irradiation.

We examined next the ability of bacteria to repair gamma-ray-induced DNA strand breaks in the absence of active DNA replication. Fig. 2(a) is an alkaline sucrose sedimentation profile of DNA from the radiation-resistant strain AB1157. It can be seen that a dose of 20 krad aerobic radiation induced strand breaks which caused the DNA to sediment more slowly and to appear nearer the top of the gradient. When the bacteria were incubated at 37 $^{\circ}$ C in growth medium for 60 min after irradiation the strand breaks were repaired and the DNA sedimented in the same position as in the unirradiated control (McGrath & Williams, 1966; Kapp & Smith, 1970). Fig. 2(b) is a profile of a similar experiment in which chloramphenicol (200 μ g/ml) was added 90 min before irradiation and kept there throughout the experimental period. It can be seen that repair of strand breaks occurred to almost the same extent as in Fig. 2(a). Thus neither protein synthesis nor normal DNA replication appear to be necessary for repair of strand breaks. This result refutes the hypothesis which led us to undertake the experiments, namely that DNA replication would be necessary for repair of strand breaks to occur.

The ability of strain PAM5717 *exrA* to repair strand breaks was studied under the same conditions. Fig. 3(a) shows that with no chloramphenicol treatment there was extensive postirradiation DNA breakdown and little evidence of strand rejoining. However, when chloramphenicol was present (Fig. 3b), DNA breakdown was abolished and extensive strand rejoining occurred. Thus not only can 'slow' repair of strand breaks occur in the absence of DNA replication but repair under these conditions does not require the *exrA*⁻ gene.

The possibility existed that the repair we observed in Fig. 2(b) and 3(b) was mediated by

Fig. 3. Alkaline sucrose gradient sedimentation profiles of DNA from strain PAM5717 *exrA*. (a) No chloramphenicol; (b) 200 µg chloramphenicol/ml present from 90 min before irradiation. Symbols as for Fig. 2.

Fig. 4. Alkaline sucrose gradient profiles of DNA from strain p3478 *polA*. (a) No chloramphenicol; (b) 200 µg chloramphenicol/ml present from 90 min before irradiation. Symbols as for Fig. 2. The gamma-irradiation dose was 10 krad.

Kornberg polymerase. Repair was therefore examined in strain p3478 *polA* in the presence of chloramphenicol (Fig. 4a, b). There were, as expected, more strand breaks observed per unit of radiation dose (see Town, Smith & Kaplan, 1971), and repair occurred to about an equal extent in the presence and absence of chloramphenicol. In view of this observation and the known rapidity of *polA* repair (Town *et al.* 1971), it would seem unlikely that the repair which we observe in the presence of chloramphenicol in the *pol⁺* strains is mediated by Kornberg polymerase.

Fig. 5. Loss of trichloroacetic acid precipitable activity from a randomly labelled culture of strain PAM5717 *exrA* following aerobic gamma-irradiation. Open symbols, 15 krad irradiation; closed symbols, no irradiation; ○, ●, no chloramphenicol present; △, ▲, 200 µg chloramphenicol/ml added immediately before irradiation; □, ■, 200 µg chloramphenicol/ml added 90 min before irradiation.

That treatment with 200 µg chloramphenicol/ml for 90 min before irradiation eliminated the excessive DNA breakdown normally found in PAM5717 *exrA* after gamma-irradiation is shown more clearly in Fig. 5. In contrast, treatment with chloramphenicol immediately before irradiation did not prevent breakdown. Although this result does not actually prove that chloramphenicol prevents breakdown by preventing DNA replication, it excludes the possibility that chloramphenicol is acting by preventing induction of an enzyme, such as a nuclease, after irradiation.

If gamma-ray-induced DNA breakdown in an *exrA* strain only occurs when an active DNA replication fork is present, the simplest explanation would be that breakdown occurs when the replication fork encounters strand breaks. In this case one would expect DNA breakdown to be greatest and immediate in the region of the replication point. As a test of this hypothesis cultures were pulse-labelled for a short period with [^3H]thymidine and either irradiated at once or chased for various periods of time prior to irradiation (see Methods). The loss of acid-precipitable radioactivity was followed as a function of time after irradiation (Fig. 6). No significant difference in stability between various regions of the chromosome was apparent since the small differences between various treatments can be explained by residual incorporation of radioactivity after removal of the labelled precursor.

Fig. 6. Loss of trichloroacetic acid precipitable activity from a pulse-labelled culture of strain PAM5717 *exrA* following gamma-irradiation. ●, No irradiation, no chase; ○, 15 krad irradiation, no chase; ▲, 15 krad, 20 min chase; △, 15 krad, 40 min chase; ■, 15 krad, 60 min chase.

Table 1. *Gamma-ray induced DNA synthesis in chloramphenicol-treated cultures*

Uptake of [^3H]thymidine into the trichloroacetic acid precipitable fraction was measured. Values based on three to seven experiments for each strain.

Strain	^3H Uptake over 60 min with 4 krad irradiation/ without irradiation (ratio)
AB1157 radiation resistant	4.57
PAM5717 <i>exrA</i>	1.74
AB2470 <i>recB</i>	0.95
AB2463 <i>recA</i>	0.42
JC5495 <i>recArecB</i>	0.93
CM293 <i>recAexrA</i>	0.23

If taken at face value, these results suggest that the degradation of DNA after gamma-irradiation of *exrA* strains occurs randomly around the chromosome but is probably dependent upon normal DNA replication. It may be argued that there is an interaction involving the *exrA* gene between the replication fork and lesions remote from it. Gray *et al.* (1972) gave evidence for an analogous interaction involving the *recA*⁺ gene product in the maintenance of DNA synthesis after irradiation.

Table 2. *Effect of chloramphenicol treatment on survival of Escherichia coli* AB1157 (radiation-resistant) and PAM5717 *exrA* after aerobic gamma-irradiation

Chloramphenicol (200 µg/ml) was present in growing cultures from 90 min before irradiation to 60 min afterwards, when it was removed by dilution before the determination of viabilities. In the cultures without chloramphenicol viabilities were determined immediately after irradiation. Values based on three to four experiments.

		Radiation dose (krads)		
AB1157		0	20	50
(a)	Surviving fraction	1	3.07×10^{-2}	8.02×10^{-4}
(b)	Surviving fraction with chloramphenicol treatment	1	1.11×10^{-1}	6.56×10^{-3}
	Ratio (b)/(a)		3.6	8.2
		Radiation dose (krads)		
PAM5717		0	10	20
(a)	Surviving fraction	1	1.09×10^{-3}	8.34×10^{-6}
(b)	Surviving fraction with chloramphenicol treatment	1	1.65×10^{-1}	5.77×10^{-3}
	Ratio (b)/(a)		151.4	691.8

In another experiment wild-type, Rec and Exr strains were treated with chloramphenicol (200 µg/ml) for 180 min prior to irradiation. After irradiation the rate of DNA synthesis was estimated as described in Methods. In the radiation-resistant strain gamma-irradiation stimulated DNA synthesis four- to sixfold compared with an unirradiated control (see Emmerson & Kohiyama, 1971) and in the *exrA* strain 1.5- to twofold (Table 1). No stimulation was observed in the repair-deficient strains *recA*, *recB*, *recArecB* and *recAexrA*. Thus in the absence of active DNA replication the *exrA* gene does not abolish gamma-ray-induced initiation of DNA synthesis, and this synthesis apparently does not lead to breakdown.

Table 2 shows that pretreatment with chloramphenicol increases the survival of both strain PAM5717 *exrA* and strain AB1157 following aerobic gamma-irradiation. The effect is particularly large with the *exrA* strain although full radiation resistance is not restored. It is not known whether the additional repair occurs during the treatment with chloramphenicol, or after the chloramphenicol is removed.

DISCUSSION

The repair of single-strand breaks in the radiation-resistant strain *Escherichia coli* AB1157 can occur in the absence of normal DNA replication. This finding has not to our knowledge been reported previously, and indeed experiments with direct inhibition of DNA synthesis, suggest that some DNA synthesis is required for repair (see Fangman & Russel, 1971; Smith, 1971). If the 'slow' repair of single-strand breaks can occur without the damaged regions being replicated, it suggests that Rec⁺-dependent repair may occur prereplicatively as well as postreplicatively as found after u.v. irradiation. In this connexion Cole (1972) has demonstrated repair of psoralen:DNA cross-links in a prereplicative Rec⁺-dependent process and Cooper & Hanawalt (1972) have presented evidence that the repair of a fraction of u.v. excision gaps is Rec⁺-mediated. Nevertheless our results do not exclude the possibility of postreplicative repair of ionizing radiation damage in other circumstances.

The fact that repair of strand breaks was also observed in chloramphenicol-treated *polA* bacteria appears to exclude the possibility that DNA polymerase I repair, rather than 'slow'

Rec⁺ repair is involved. This was, in any case, unlikely in view of the known rapidity of polymerase repair (Town *et al.* 1971).

The repair of DNA that we have observed in both *exrA* and *exrA*⁺ bacteria would seem to be biologically significant since in both strains an increase in survival was observed. More complete viability and mutagenesis studies will be published elsewhere. Although the repair of strand breaks does not appear to require that the breaks be replicated, our data suggest that repair is subject to an interaction between the lesion and the existing replication fork elsewhere on the chromosome. When the *exrA* strain was treated with a high concentration of chloramphenicol for 90 min prior to irradiation, it did not degrade its DNA excessively, it repaired DNA breaks via the 'slow' pathway and it showed gamma-ray-stimulated DNA synthesis. Thus when normal DNA replication was prevented, strain PAM5717 *exrA* appeared to behave similarly to the radiation-resistant strain AB1157.

Just as Gray *et al.* (1972) showed that in a *recA* strain DNA replication could not proceed when a lesion was present elsewhere on the chromosome, we suggest that, in an *exrA* strain, repair of a lesion cannot occur if there is a normal active replication point elsewhere on the chromosome. We favour this hypothesis not only because repair of strand breaks in both *exrA* and *exrA*⁺ strains occurs in the absence of a replication fork, but also because the kinetics of DNA breakdown after gamma-irradiation show little delay in an *exrA* strain (suggesting breakdown does not require actual replication of the lesion) and because little difference in susceptibility to breakdown can be demonstrated between different regions of the chromosome.

The role (if any) of the gamma-radiation stimulated DNA synthesis observed in both wild-type and *exrA* strains is open to question. It clearly does not lead to DNA breakdown but we are unable at present to say unequivocally whether it is required for successful repair or is pathological.

Ganesan & Smith (1972) have recently concluded that inhibiting protein synthesis may interfere with some biochemical step in Rec⁺-dependent repair, having observed that chloramphenicol added after X-irradiation partially inhibited repair of strand breaks in radiation resistant K-12 bacteria. We have confirmed this (unpublished observations) but the present results show that chloramphenicol has little effect if the bacteria are also pre-treated with the antibiotic before irradiation. In addition to bringing DNA replication to a halt this pretreatment has the (probably related) effect of greatly reducing DNA degradation after irradiation. It may be that the effect observed by Ganesan & Smith (1972) is caused indirectly by an enhancement of DNA degradation rather than directly by inhibition of repair (see Town *et al.* 1970). Alternatively, only repair in the presence of an active replication fork may require protein synthesis.

Finally, our results do not account for the increase in survival of an *exrA* strain that Marshall & Gillies (1972) obtained by treatment with a low concentration of chloramphenicol after gamma-irradiation. In our hands treatment of a growing culture of PAM5717 *exrA* with a high level of chloramphenicol at the time of irradiation did not prevent DNA breakdown, but it is known that high and low concentrations of chloramphenicol may have different effects.

S. G. Sedgwick is a Medical Research Council scholar.

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