RESPONSE TO BLEOMYCIN OF *ESCHERICHIA COLI* MUTANTS DEFICIENT IN DNA REPAIR

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The effect of bleomycin on the colony forming ability of *Escherichia coli* K12 strains in exponential growth at 37°C was not affected by introducing *recA13*, *lexA1*, *polA1* and *uvrA6* mutations. For cells starved for amino acids, wild type strains became ten-fold more resistant to bleomycin, but again introducing *lexA1*, *polA1* and *uvrA6* strains did not change the effect on colony forming ability; however, starved *recA13* cells were now four-fold more sensitive. Strains with *recA13*, *lexA1* and *polA1* mutations were always more sensitive than wild type to gamma rays under the same conditions as used for the bleomycin treatment. It is suggested that bleomycin-induced lesions may be concentrated in that part of the bacterial genomes at the cell wall, near the replication forks.

The antitumor drug, bleomycin, produces effects on living cells which resemble those produced by ionizing radiations: loss of ability to form colonies for mammalian and bacterial cells[1], production of chromosomal aberrations[2], degradation of intracellular DNA to acid-soluble form[3], inhibition of DNA synthesis[4], release of DNA from a complex with membrane[5], induction of prophages in bacteria[6], and induction of synthesis of *recA* protein in *Escherichia coli* cells[7]. On DNA in solution, bleomycin produces single-strand[8] and double-strand breaks[4,9], as do ionizing radiations. Bleomycin releases bases from the DNA[10,11], and so do ionizing radiations[12]. The similarity of biological effects may be a consequence of similarities in the DNA lesions. However, it has been reported that bleomycin reduces the colony forming ability of *lexA*[13] and *polA1*[14] *E. coli* cells in exponential growth at 37°C to the same level as for wild type cells, although both mutants are more sensitive to X-rays. *E. coli recA* cells, quite sensitive to X-rays, have been reported to be more sensitive to phleomycin[14], or equally sensitive to bleomycin[13].

The experiments reported in this paper show that the colony forming ability of *E. coli* cells depends strongly on the conditions of treatment with bleomycin. *RecA* mutants are more sensitive than wild type under some conditions, and have the same sensitivity under other conditions. *LexA*, *polA* and *uvrA* cells respond to bleomycin in the same way as do wild type strains under the conditions used.

Materials and Methods

Chemicals

Bleomycin A2 (Lot 33), the generous gift of the Developmental Therapeutics Program, Chemotherapy, N.C.I., was dissolved in double distilled water at 2 mg/ml and kept frozen at −20°C in small quantities until used. All other chemicals were obtained from commercial sources.

Bacterial Strains

Those used, and the relevant genotypes, were: *E. coli* K12 AB2497 *rec*$^+$*arg*$^-$*his*$^-$ and its almost isogenic derivatives AB2487 *recA13 arg*$^-$*his*$^-$, AB2500 *uvrA6 arg*$^-$*his*$^-$ (all from P. Howard-Flanders);

Z7 *lexA1 arg*$^+$*his*$^-$ (derived from AB2494 *lexA1 arg*$^+$*his*$^-$); m147 *polA*$^+$, m22 *polA1* (derived from P3478, both strains a gift of R. MOSES) have an unknown amino acid requirement.

Culture Media

K medium (M9 buffer + 1% glucose + decolorized, vitamin-free 1% casamino acids) containing 10 μg/ml thymine were used for cell growth. M9 buffer used in this work was 18.7 mM NH_4Cl, 41.5 mM Na_2HPO_4, 22 mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM $CaCl_2$, and 0.1 μg/ml thiamine. Starvation medium (M9 buffer + 1% glucose + 10 μg/ml thymine) was used for preparation of amino acid starved *E. coli*. Minimum agar plates were made from medium 56 agar (see below) with addition of 2 g/liter glucose, 83 μg/ml DL-threonine, 83 μg/ml DL-leucine, 333 μg/ml L-proline, 0.17 μg/ml thiamine, and 17 μg/ml thymine. To measure survival of *arg*$^-$*his*$^+$ cells, we used minimum agar supplemented with 147 μg/ml L-arginine. For *arg*$^+$*his*$^-$ cells, we used minimum agar plates supplemented with 87 μg/ml DL-histidine. Medium 56 agar contains: 2.56 g/liter KH_2PO_4, 4.25 g/liter Na_2HPO_4, 1 g/liter $(NH_4)_2SO_4$, 100 mg/liter $MgSO_4 \cdot 7H_2O$, 5 mg/liter $Ca(NO_3)_2$, 0.25 mg/liter $FeSO_4 \cdot 7H_2O$, and 1.5% Difco Bactoagar. L agar plates contain: 1% tryptone, 0.5% yeast extract, 0.05% NaCl, adjusted to pH 7.0, and 1.5% Difco Bactoagar. Lambda buffer used for cell dilution was: 0.03 M Tris (hydroxymethyl) aminomethane-HCl pH 7.2 containing 0.05 M $MgSO_4 \cdot 7H_2O$ and 0.25 g/liter gelatin.

Effect of Bleomycin on Cells

An exponentially growing culture of the test strain ($2 \sim 5 \times 10^7$/ml) was washed two times in cold M9 buffer at 4°C by low speed centrifugation, resuspended in cold buffer and held in ice until used.

Amino acid-starved bacteria were prepared by incubating exponentially growing cells for 60 minutes at 37°C in the starvation medium. Cells were exposed to bleomycin at 37°C for 10 minutes or 0°C for 30 minutes, then diluted 100-fold in λ buffer and plated on L agar, or minimum agar plates, as described in the figure legend.

Effect of Gamma Rays on Cells

Cells were prepared in exactly the same way as for treatment with bleomycin (see above). After suspension in the media as described in the caption to Fig. 1, they were warmed to 37°C and exposed at that temperature to Co^{60} gamma rays at 400 rads/min for $0 \sim 10$ minutes; after removal from the gamma ray source they were put in a 37°C bath and held there until the total time of exposure to gamma rays plus postirradiation incubation was 10 minutes (the time of incubation with bleomycin). They were then chilled until they could be dilated for assay for colony forming ability.

Results

In our hands, the action of bleomycin on *E. coli* cells was erratic, with substantial differences in the fraction of cells able to form colonies from exposure to the same amount of bleomycin under the same conditions in experiments done at various times. The sources of this irreproducibility are not known; we have found that 10^{-5} M metal ions can increase (for Co^{++}, Fe^{++}) or decrease (for Cu^{++}, Zn^{++}) the amount of bleomycin needed, by factors up to 3, to give the same decrease in colony forming ability of *E. coli*.

To obtain the most reproducible results, in most of our experiments the isogenic wild type and repair-deficient mutants were mixed in the same tube, treated together with bleomycin, and the effects of the bleomycin on each strain sorted out by the use of selective genetic markers. For example, from the strain AB2497 *rec*$^+$*arg*$^-$*his*$^-$, we selected spontaneous revertants AB2497-1 *arg*$^-$*his*$^+$ and AB2497-2 *arg*$^+$*his*$^-$; from almost isogenic strain AB2487 *recA13 arg*$^-$*his*$^-$, we prepared AB2487-1 *arg*$^-$*his*$^+$ and AB2487-2 *arg*$^+$*his*$^-$. When the two wild type strains were mixed together, treated with bleomycin and plated on selective agars, the sensitivities of the two substrains to bleomycin were identical; the same was true for the two *recA* strains (data not shown).

These strains have been used to show the effects of the physiological state of the cell on the action

of bleomycin. Strain AB2497-1 *rec*$^+$*arg*$^-$*his*$^+$ in exponential growth was mixed with AB2497-2 *arg*$^+$ *his*$^-$ which had been starved for amino acids. They were treated with bleomycin in starvation medium with arginine (but not histidine) added, so that the cells in exponential growth phase grew exponentially, whereas the starved cells, lacking histidine, would stay in the starved state. The starved cells were much more resistant to bleomycin, having the same colony forming ability as exponentially growing cells treated with eight- to ten-fold more bleomycin (data not shown). This is in agreement with results

Fig. 1. The effect of bleomycin and gamma rays on mutant *E. coli* strains.

--- Strains, wild type in repair ability. — Strains, mutant in repair capability, as designated. Open symbols (○, □, ◇) cells in exponential growth. Filled symbols (●, ■, ◆) cells starved for amino acids for 60 minutes.

a Exponentially growing AB2497-1 *arg*$^-$*his*$^+$ (---○---) and AB2487 *recA*$^-$*arg*$^+$*his*$^-$ (—○—) were mixed in K medium. Amino acid-starved AB2497-1 *arg*$^-$*his*$^+$ (---■---) and AB2487-2 *recA*$^-$*arg*$^+$*his*$^-$ (—■—) were mixed in starvation medium. Mixed cells were treated with bleomycin at 37°C for 10 min. Cells were plated on selective minimum agar after appropriate dilutions.

b Exponentially growing AB2497-1 *his*$^+$ (---○---) and amino acid-starved AB2587-2 *recA his*$^-$ (—●—) were mixed in starvation medium with every needed amino acid except histidine, and exposed to γ rays for 0~10 min at 37°C. Amino acid-starved AB2497-1 *arg*$^-$ (---■---) were mixed with exponential AB2487-2 *recA arg*$^+$ (—□—) in starvation medium containing all required amino acids except arginine, irradiated with γ rays, and plated on selective agar.

c Exponentially growing AB2497-1 *arg*$^-$*his*$^+$ (---○---) and Z7 *lexA*$^-$*arg*$^+$*his*$^-$ (—○—) were mixed in K medium. Amino acid-starved AB2497-1 *arg*$^-$*his*$^+$ (---■---) and Z7 *lexA*$^-$*arg*$^+$*his*$^-$ (—■—) were mixed in starvation medium. Bleomycin treatment and plating were the same as Panel (a).

d The same as Panel (c) except the cells were irradiated with γ rays.

e Exponentially growing m147 *polA*$^+$ (---○---) and m22 *polA*$^-$ (—◇—) were suspended in K medium, each strain in a separate tube, and treated with bleomycin. Amino acid-starved m147 *polA*$^+$ (---●---) and m22 *polA*$^-$ (—◆—) were suspended in starvation medium, each strain in a separate tube, and treated with bleomycin. An appropriate dilution was plated on L agar.

f The same as Panel (e) except the cells were irradiated with γ rays.

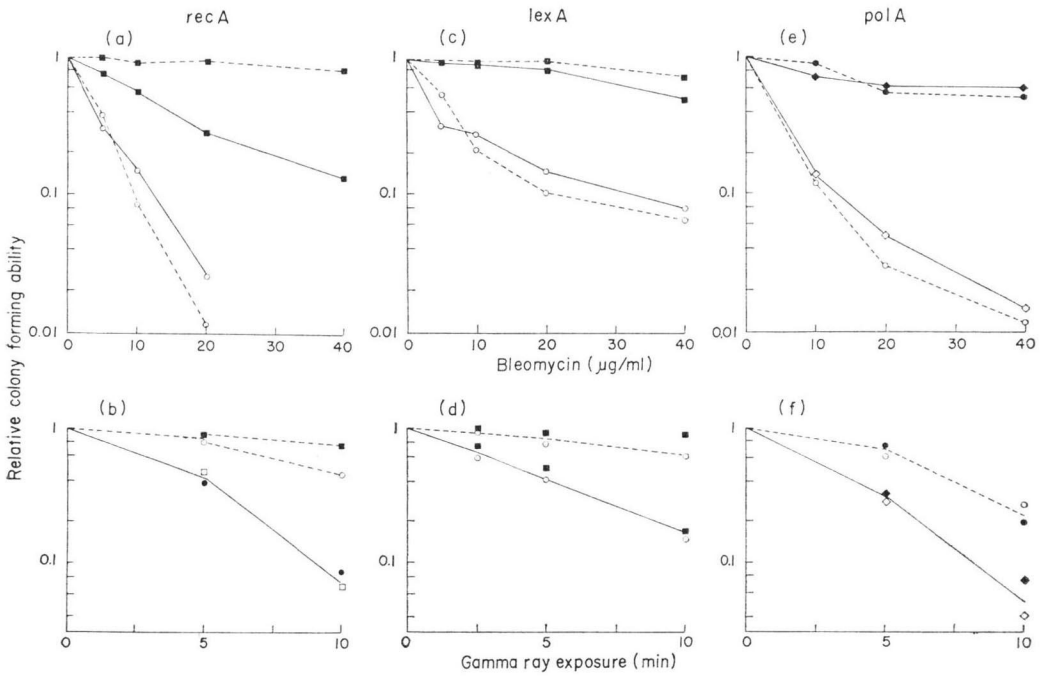

Table 1. Response of *E. coli* repair mutants to bleomycin and gamma rays.

Strain	Exponential cells / Exponential wild type	Starved cells / Exponential wild type	Starved cells / Starved wild type
AB2497 wild type	1.0 (1)	8.0±2	1.0 (1)
AB2487 *recA13*	1.0±0.3 (0.2)	2.0±0.2 (0.26)	0.2±0.02 (0.35)
Z-7 *lexA1*	1.0±0.3 (0.15)	7.2±3	0.9±0.4 (0.11)
m22 *polA1*	1.1±0.14 (0.35)	8.0±2	1.0±0.2 (0.46)
AB2500 *uvrA6*	0.8±0.2 (1)	6.4±2	0.8±0.2 (1)

The unbracketed figure given in the Table is the amount of bleomycin which reduces the colony forming ability of the cell to the same level as does one unit of bleomycin acting on the wild type strain, either exponentially growing or starved for amino acids as shown. The figure in brackets is the experimental value for the gamma ray exposure of the mutant at 37°C which reduced colony forming ability to the same level as one unit of exposure of the wild type. Note that sensitivity to X-rays is the same for cells in exponential growth and starved for amino acids, so the bracketed figure for any particular mutant, *i.e. recA*, should be about the same for all three columns.

previously published for the effect on *E. coli* cells not synthesizing protein of bleomycin[15], or phleomycin[14,16]. Starved AB2487 *recA* cells, however, had the same colony forming ability as exponentially growing *recA* cells treated with only twice the amount of bleomycin.

If cells in exponential growth were treated with bleomycin in a medium lacking an essential amino acid, the curves of colony forming ability against bleomycin concentration tended to bend up, showing the presence of a resistant fraction, presumably cells going into a starved phase. Starved cells, treated with bleomycin in medium in which they could grow, developed a sharp initial drop in the survival curve, presumably from cells which had gone into growth during the bleomycin treatment.

Fig. 1 shows the effects of bleomycin on cells which are defective in DNA repair. In exponential growth, *recA, lexA* and *polA* cells had the same sensitivity to bleomycin as did wild type cells. *LexA* and *polA* cells, starved for amino acids, responded to bleomycin as did starved wild type, but starved *recA* cells were about five-fold more sensitive.

Because of the similarities between many effects of ionizing radiations and of bleomycin as noted in the Introduction, the results for *recA*, *lexA* and *polA* cells were puzzling. Therefore, *recA, lexA* and *polA* mutant strains, together with the corresponding wild type strains, were exposed to gamma rays under conditions as nearly as possible those used for exposure to bleomycin (Fig. 1), instead of the usual conditions, 0°C in buffer. These results show that the mutants are all more sensitive to ionizing radiations than the corresponding wild type, and that, for each strain, cells in exponential growth have about the same sensitivity as those starved for amino acids.

The results of the experiments shown in Fig. 1, and of a number of other experiments for which the data are not given, are summarized in Table 1. The temperature at which the cells are exposed to bleomycin is important. For exposure at 0°C for 30 minutes, the colony forming ability of *recA*$^-$ cells is about four-fold more sensitive to bleomycin than is that of wild type, and cells in exponential growth have similar sensitivities to those which have been starved for amino acids (data not shown).

Discussion

The bleomycin results summarized in Table 1 are in good agreement with published reports except that of NAKAYAMA[14]. The responses of various repair mutants of *E. coli* to bleomycin clearly

differ from those to ionizing radiations. It is possible that bleomycin acts on cell constituents other than DNA, but in view of the many similarities between the effects of bleomycin and X-rays, and the remarkable activity of bleomycin on DNA, such an hypothesis is not appealing. If the effects of both bleomycin and ionizing radiations are ascribed to DNA strand breaks and to bases released from the DNA, some significant difference in the two agents must be found. An obvious one is that the drug must pass through the cell wall to get to most of the cell DNA. YAMAGAMI, ISHIZAWA and ENDO[17] found a mutant *E. coli* strain quite sensitive to bleomycin in which the exponentially growing *recA* derivative is four-fold more sensitive than the *rec*$^+$ genotype; a bleomycin-resistant revertant had the same drug sensitivity for both the *rec*$^+$ and *recA* derivatives. These authors suggested that the cell wall of the mutant might have increased permeability to bleomycin.

Our results and the difficulties expected in passing a molecule as big as bleomycin (molecular weight about 1,500) through a cell wall, suggest that much of the activity of bleomycin on bacteria may be from drug adsorbed to the cell wall acting on that part of the DNA attached to the wall. DNA damage near the replication fork, or confined to a very small part of the genome, may well act differently than damage from X-rays randomly distributed through the DNA.

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