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RESISTANCE AND CROSS-RESISTANCE OF *ESCHERICHIA COLI* S MUTANTS TO THE RADIOMIMETIC AGENT PROFLAVINE

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ABSTRACT

WOODY-KARRER, PEARL (Palo Alto Medical Research Foundation, Palo Alto, Calif.), AND JOSEPH GREENBERG. Resistance and cross-resistance of *Escherichia coli* S mutants to the radiomimetic agent proflavine. J. Bacteriol. 87:536-542. 1964.—All 50 of the first-step mutants of *Escherichia coli* S selected for resistance to proflavine were resistant to ultraviolet light and each of five different radiomimetic chemicals. The mutants were classified into eight types on the basis of their relative resistance to six different radiomimetic drugs and on the basis of the shape of their ultraviolet survival curves. Three of these types are identical to types previously isolated with other radiomimetic drugs; five of the types are new. A high proportion of the clones surviving proflavine treatment were phenotypically but not genetically resistant, and no strains were isolated which were resistant to proflavine but were not resistant to radiation.

Most of the mutants of *Escherichia coli* S selected for resistance to alkynitrosoguanidines (Mandell, Woody, and Greenberg, 1961) or azaserine (Greenberg, Mandell, and Woody, 1961*a*), nitrogen mustard or nitromin (Woody, Mandell, and Greenberg, 1961), or mitomycin C (Greenberg et al., 1961*b*) were resistant to the other chemicals in this group and to ultraviolet and X-radiation (Greenberg and Woody-Karrer, J. Gen. Microbiol. *in press*). These chemicals are mutagenic in bacteria, induce filaments or "snakes," and are alkylating agents. Recently, nitrofurazone (5-nitro-2-furaldehyde semicarbazone), which is not an alkylating agent, was found to belong to the group of agents exhibiting cross-resistance relationships with radiation (Woody-Karrer and Greenberg, 1963), indicating that alkylation is not essential for membership in the group. On the other hand, a broad spectrum of other bactericidal agents was found not to belong to this class.

Acridine dyes were reported mutagenic for *E. coli* (Witkin, 1947; De Mars et al., 1953) as well as other organisms. Acriflavine, a mixture containing proflavine as a constituent, preferentially inhibited cell division (Loveless, Spoerl, and Weisman, 1954). Mutants of *E. coli* B selected for resistance to ultraviolet radiation displayed concomitant resistance to proflavine (Greenberg and Woody-Karrer, *in press*). The data of Lerman (1961) and Luzzatti, Masson, and Lerman (1961) indicate that acridines are bound to deoxyribonucleic acid (DNA) by intercalation between normally adjacent base pairs in a plane perpendicular to the helix axis. Such a binding is accommodated by a localized untwisting and extension of the DNA helix. On the basis of this model, Lerman (1963) has proposed a possible explanation for proflavine mutagenesis: that intercalation of acridines displaces the homology of paired chromosomes by one or more nucleotide pairs resulting in replication errors such as insertions and deletions of nucleotides.

Since a great deal is known about the effects of proflavine on biological systems, it would be of interest if a common mechanism of action could be established for proflavine and ultraviolet radiation. Cross-resistance relationships would imply a common mode of action. It was already shown that the majority of survivors of *E. coli* S after treatment with any one radiomimetic agent are concomitantly resistant both to other radiomimetic compounds and to ultraviolet radiation. Moreover, on the basis of cross-resistance patterns, i.e., the degree of resistance to ultraviolet light and radiomimetic agents, 14 different radiation-resistant mutants of *E. coli* S were distinguished; these are referred to as types R_1 through R_{14} (Woody-Karrer and Greenberg, 1963). Regardless of the agent used to select them, at least 50% of the radioresistant mutants were type R_4 and 20 to 30% were type R_3; the remaining R-types occurred at very low frequencies, the types varying somewhat with the selecting agent used. Most, but not all, of the R-types displayed

an identical degree of resistance to ultraviolet radiation. Differences between parent strain S and the radioresistant mutants, and also among the latter, disappeared after irradiation when minimal glucose-salts-agar rather than Tryptone-agar was used as the plating medium (plating medium response).

Mutants of *E. coli* S, occurring with low frequencies, have also been isolated which are significantly resistant only to the selecting agent and closely related compounds. These have been termed chemoresistant, indicating resistance to a specific chemical structure and not to the class of radiomimetic agents.

The results to be presented will show that all of the first-step mutants of *E. coli* S resistant to proflavine were cross-resistant to other radiomimetic agents and to ultraviolet radiation. Of these, 50% were type R_4, 20% were type R_3, and 2% were type R_6. The remainder were distributed among five previously undescribed R-types, similar to type R_6 in being less resistant than types R_3 or R_4 to ultraviolet radiation and radiomimetic chemicals, and in showing some plating medium response. A high proportion of the clones surviving proflavine treatment were only phenotypically resistant to proflavine and were classified as sensitives. No genetically stable chemoresistant strains were isolated.

MATERIALS AND METHODS

Bacterial strains. E. coli S, obtained from A. D. Hershey, was the parent strain.

Compounds. Proflavine hydrochloride was obtained from Allied Chemical and Dye Corp., New York, N.Y. The sources of nitrofurazone (5-nitro-2-furaldehyde semicarbazone), 1-methyl-3-nitro-1-nitrosoguanidine, mitomycin C, nitrogen mustard, and sodium penicillin G were reported in a recent publication (Woody-Karrer and Greenberg, 1963). All compounds were prepared in sterile distilled water immediately before use.

Media. The Tryptone agar (adjusted to pH 5.5 with hydrochloric acid or to pH 7.0 and 7.8 with sodium hydroxide), M9 (minimal glucose-salts) agar, peptone broth, and 0.02 M phosphate-buffered saline (pH 6.8) were described previously (Woody-Karrer and Greenberg, 1963). Cultures were stored on Tryptone Glucose Extract Agar (Difco).

Isolation of resistant mutants. Resistant mutants were isolated from plates of Tryptone agar (pH 7.8) containing 3.0, 3.5, or 4.0 μg of proflavine per ml, spread with 3.5×10^7 cells of *E. coli* S (log phase in peptone broth), and incubated overnight at 37 C.

Measurement of resistance to chemical agents. Overnight broth cultures containing 3.5×10^8 cells per ml were streaked on gradient plates as previously described (Woody-Karrer and Greenberg, 1963; Szybalski and Bryson, 1952). Gradient plates were made with Tryptone agar at pH 5.5, except that pH 7.8 was used in tests involving proflavine and M9 agar was used for penicillin. Controls used were *E. coli* S and prototypic strains of all previously isolated R-types.

Sensitivity to ultraviolet radiation. To determine the radiation resistance of survivors of *E. coli* S after proflavine treatment, overnight peptone broth cultures (2 ml) were streaked with cotton swabs on square plates of Tryptone agar, pH 7.0 (25 cultures per plate). Each plate was exposed to 77 ergs/mm^2 of ultraviolet radiation, incubated 2.5 hr at 37 C, and then re-exposed to a dose of 230 ergs/mm^2. After overnight incubation, streaks with no growth indicated hypersensitivity to radiation; streaks with sparsely isolated colonies, sensitivity equivalent to the parent strain; streaks ranging from many isolated colonies to granular growth, intermediate degrees of radioresistance; streaks with confluent growth, high degree of radioresistance (Woody-Karrer, *in preparation*). These preliminary classifications were confirmed for representative strains by conventional survival curves to ultraviolet radiation. The ultraviolet radiation source and calibration, and the method of cell preparation, exposure, and plating on both Tryptone agar (pH 7.0) and glucose-salts medium were as previously described (Woody-Karrer and Greenberg, 1963).

RESULTS

All previously isolated radioresistant mutants of *E. coli* S (R_1 through R_{14}) were tested for resistance to nitrofurazone, and all were found to be 2.5-fold resistant relative to the parent strain.

The results of a survey of 100 (10^{-6}) surviving colonies of *E. coli* S selected from Tryptone agar (pH 7.8) containing 3.0, 3.5, and 4.0 μg/ml of proflavine are shown in Tables 1 and 2. Broth cultures of *E. coli* S used in experiments 1 and 2 were inoculated from the same stock culture. The clones picked as possible resistant mutants grew

TABLE 1. *Classification of survivors after treatment of Escherichia coli S with proflavine*

Expt no.	Proflavine (μg/ml)	No. of normal-sized colonies after 18 hr*	No. of colonies tested	Sensitive to proflavine	No. of radioresistant type							
					R_3	R_4	R_6	R_{15}	R_{16}	R_{17}	R_{18}	R_{19}
1	3.0	49	34	25	0	5	0			2	2	
1	3.5	28	28	22	1	2			1		2	
1	4.0	8	2	0								2
2	3.0	80	31	3	9	15	1	1		2		
2	3.5	11	5	0		3						2
Totals			100	50	10	25	1	1	1	4	4	4

* Number of cells plated was 3.5×10^7.

TABLE 2. *Cross-resistance relationships among first-step proflavine-resistant mutants of Escherichia coli strain S*

Bacterial strain	Radioresistant designation	Test compound*						Ultraviolet treatment	
		PF (1.5 ± 0.2)	NF (0.2 ± 0.05)	NG (0.055 ± 0.005)	MC (0.045 ± 0.005)	NM (16 ± −1)	PN (2.5 ± 0.1)	31†	616†
S/Pf 1a	R_3	2.5‡	11	22	9	12	1	20	20
S/Pf 1b	R_4	2.5	11	35	16	24	1	20	20
S/Pf 1c	R_6	2.5	11	4	2	2.3	1	11	20
S/Pf 1d	R_{15}	2.5	11	3.1	2	2.3	1	7.5	20
S/Pf 1e	R_{16}	3.9	11	4.4	9	2.8	2.9	11	20
S/Pf 1f	R_{17}	2.5	11	5	2.2	3	1	15	20
S/Pf 1g	R_{18}	1.7	2	1.8	1.2	1.2	1	3.5	20
S/Pf 1h	R_{19}	4.5	3.3	2.2	3.6	1.8	1	3.5	20

* PF = proflavine; NF = 5-nitro-2-furaldehyde semicarbazone; NG = 1-methyl-3-nitro-1-nitrosoguanidine; MC = mitomycin C; NM = nitrogen mustard; PN = penicillin. Numbers in parentheses indicate the minimal inhibitory concentrations for *E. coli* S in μg/ml estimated from gradient plates (Szybalski and Bryson, 1952).

† Ergs/mm² to give 10% survival plated on Tryptone or glucose-salts medium.

‡ Figures represent the factor of resistance (fold-increase) compared with the parent strain set at unity.

as medium-sized colonies against a background of pinpoint colonies. It is not clear why a large proportion of surviving clones were found to be sensitive in experiment 1. Of the 50 survivors found to be resistant to proflavine, all were resistant to ultraviolet radiation and, on the basis of their cross-resistance patterns (Table 2) or ultraviolet survival curves (Fig. 1), could be divided into eight distinct types. The majority of mutants were indistinguishable from previously described radioresistant mutants of *E. coli* S: 50% (represented by S/Pf 1b) were classed as R_4; 20% (S/Pf 1a) as R_3; 2% (S/Pf 1c) as R_6. The remaining mutants had cross-resistance patterns not previously encountered: 2% (S/Pf 1d) were designated as R_{15}; 2% (S/Pf 1e) as R_{16}; 8% (S/Pf 1f) as R_{17}; 8% (S/Pf 1g) as R_{18}; and 8% (S/Pf 1h) as R_{19}. No stable chemoresistant mutants were found. In general, all of the new radioresistant types, as well as R_6, displayed low degrees of resistance to all the chemicals except nitrofurazone. Moreover, like R_6 and sensitive parent S, types R_{15} through R_{19} underwent plating medium recovery when plated on glucose-salts medium after ultraviolet radiation (Fig. 1). Because of this recovery, the survival of irradiated *E. coli* S, R_6, and R_{15} through R_{19} on glucose-salts medium was indistinguishable from that of the more radioresistant types R_3 and R_4. The response of R_3 and R_4 to ultraviolet radiation

was identical and unchanged by the postirradiation plating medium.

Figure 1 also shows that five different levels of radiation resistance were identified among proflavine mutants. Comparing the dose to give 10% survival, R_{18} and R_{19} (S/Pf lg and S/Pf lh) were 3.5-fold more resistant to ultraviolet radiation than was *E. coli* S; R_{15} (S/Pf ld), 7.5-fold; R_6 and R_{16} (S/Pf lc and S/Pf le), 11-fold; R_{17} (S/Pf lf), 15-fold; and R_3 and R_4 (S/Pf la and S/Pf lb), 20-fold. The survival curves displayed by S/Pf lg, S/Pf ld, and S/Pf lf were not previously encountered for radioresistant mutants of *E. coli* S.

Furthermore, strains with identical ultraviolet survival curves when assayed on Tryptone agar did not have identical cross-resistance patterns with respect to radiomimetic drugs. R_{16} (S/Pf le) and R_6 (S/Pf lc) had the same response to ultraviolet radiation, but R_{16} was significantly more resistant to proflavine, mitomycin C, and penicillin than was R_6. R_{18} (S/Pf lg) and R_{19} (S/Pf lh) were also indistinguishable from each other after exposure to ultraviolet radiation, but R_{18} displayed less resistance to proflavine, nitrofurazone, and mitomycin C than did R_{19}. There are instances of the converse; R_6, R_{15}, and R_{17} all had similar cross-resistance patterns to radiomimetic agents (no differences greater than twofold), yet their ultraviolet survival curves on Tryptone medium differed in shape.

It should also be noted that although the previously described radioresistant mutants of *E. coli* S (R_1 to R_{14}) all displayed 2.5-fold resistance to proflavine (Greenberg and Woody-Karrer, *in press*), there were several levels of proflavine resistance expressed by first-step radioresistant mutants isolated after proflavine treatment. Types R_{15} and R_{17} displayed the 2.5-fold degree of resistance, but R_{18} was 1.7-fold as resistant as S; R_{16}, 3.9-fold; and R_{19}, 4.5-fold.

DISCUSSION

There is a group of chemicals related in such a way that mutants of *E. coli* S selected for resistance to any one chemical are resistant to all other chemicals in the group and to radiation, both X- and ultraviolet. These compounds are also related in that they are mutagens and preferentially inhibit cell division. Cross-resistance among bactericidal agents implies a common mechanism of action which the resistant cell in some way is

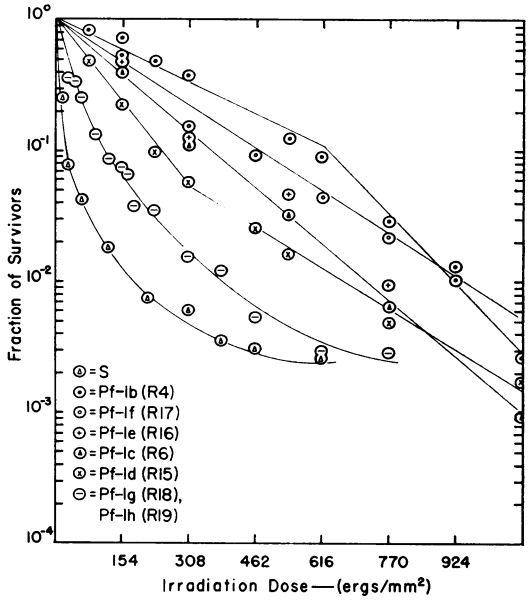

FIG. 1. *Survival of Escherichia coli S and radioresistant strains S/Pf 1b, S/Pf 1f, S/Pf 1e, S/Pf 1c, S/Pf 1d, S/Pf 1g, and S/Pf 1h exposed to ultraviolet radiation and plated on Tryptone agar (pH 7.0).*

able to neutralize. Yet, the members of the group of agents which exhibit this cross-resistance relationship are physically and chemically quite different. Proflavine, for example, which has now been shown to belong to the group, is quite different from the alkylating agents (which comprise the majority of radiomimetic compounds) both in its structure and in what is presumably its mechanism of biological action. The presumed primary target of both is DNA. Yet, alkylating agents alkylate ring nitrogens of purines, mostly guanine, and, if bifunctional, cross-link DNA (Brookes and Lawley, 1961), whereas evidence suggests that proflavine intercalates itself between adjacent nucleotides causing replication errors of insertions or deletions of nucleotides (Lerman, 1963). Thymine dimers have been shown to be produced by ultraviolet light (Beukers and Berends, 1961) and to account for much of the biological activity of ultraviolet light (Setlow and Setlow, 1963); yet, alkylating agents, proflavine, and X-radiation have not been shown to produce such dimers.

Two possible explanations for the disparity of the chemical effects and the community of the biological effects present themselves. The first

is that all the radiomimetic agents and radiation have a common chemical effect, presumably on DNA, which has not yet been discovered. Second, the initial and diverse chemical effects are translated through one or more subsequent steps into a common lesion which the resistant cell can repair or otherwise neutralize.

Whatever the explanation for this kind of cross-resistance, it is apparent that *E. coli* S can become resistant through a large number of different stable mutations. This, at least, would be an interpretation of the fact that we have identified 19 types of radioresistant mutants of S, characterized chiefly, but not entirely, by their cross-resistance patterns, i.e., their degree of resistance to each of five or six radiomimetic chemicals. Most of these resistant mutants (both type and absolute number) have identical survival curves when plated on Tryptone medium after ultraviolet irradiation. However, seven mutants have intermediate levels of resistance to ultraviolet radiation. We have sufficient experience with one of these intermediate types (R_6) to be able to state with confidence ($> 90\%$) that its curve is as shown in Fig. 1: that it is more resistant to ultraviolet radiation than *E. coli* S and less resistant than the majority of mutants. Furthermore, it is differentiated by two other properties from most of the other R-types of strain S. (i) It exhibits plating medium recovery, i.e., like *E. coli* S it is more resistant to ultraviolet radiation when plated on minimal glucose-salts medium than when plated on Tryptone medium. (ii) It has a relatively low degree of resistance to the radiomimetic chemicals. (Only the rare type R_1 is as sensitive as R_6 to the chemical agents and yet responds as type R_4 after ultraviolet radiation.)

Limited experience does not permit us to state with 90% confidence that the survival curves of R_{15}, R_{16}, R_{17}, R_{18}, and R_{19} are precisely as drawn in Fig. 1. However, all five mutants exhibit plating medium response and all have a very low degree of resistance to radiomimetic chemicals. They seem, therefore, to form a family of mutants characterized by intermediate resistance to ultraviolet radiation, by a low degree of resistance to radiomimetic chemicals, and by plating medium recovery. The shape of the survival curve of types R_{18} and R_{19} strongly suggest that they are quite different from R_6. On the other hand, both type R_{17} and type R_6 have exponential survival curves but with different slopes so that after an exposure of 924 ergs there were almost ten times as many R_{17} survivors as R_6. The survival curve of R_{15} differed from that of R_6 only in being slightly convex upward rather than exponential.

The multiplicity of mutants with various degrees of radioresistance is not limited to *E. coli* S. At least six different radioresistant mutants of *E. coli* B (Greenberg and Woody-Karrer, *in press*), including B/r (Witkin, 1947) and Bpr5 (Alper and Gillies, 1960), have been described. Bpr5 was found to resemble the R_6 radioresistant mutant of *E. coli* S (Greenberg and Woody-Karrer, *in press*). In addition, three radiosensitive mutants of B have been isolated: B_{s-1} (Hill, 1958), B_{s-2} (Hill and Simson, 1961), and B_{III} (Rörsch et al., 1962). B_{s-1} and B_{III} are similar (and may be identical) in that neither has the ability to reactivate ultraviolet-irradiated T_1 phage. Radiosensitivity, irradiated T_1 phage recovery, and other properties of B_{III} have been attributed by Rörsch, Edelman, and Cohen (1963) to a gene (*syn*) located between the loci for streptomycin resistance and xylose fermentation. A radiosensitive mutant of K-12 (K-12 is approximately as radioresistant as B/r) was isolated by Howard-Flanders et al. (1962) by enrichment of the population of bacteria in which irradiated T_1 phage failed to recover. This strain, AB1886, which has many properties in common with B_{s-1} (Greenberg and Woody-Karrer, *in press*), was shown to be the result of a mutation of a gene (*UV*) located between the loci for arabinose fermentation and arginine synthesis. Mutations at presumably two different loci, *UV* and *syn*, therefore, produce almost identical phenotypes. In addition, in crosses between *E. coli* K-12 and S or B it was found (Cook and Greenberg, *in preparation*) that there is a gene, *RA*, closely linked to the locus for phage T_6 resistance which determines radioresistance in K-12 (Adler, 1963). This is a region of the chromosome in which Adler and Copeland (1962) also found determinants for radioresistance in K-12. It was also shown (Greenberg and Woody-Karrer, *in press*), that a radiosensitive mutant of K12 isolated in our laboratory is almost identical to *E. coli* S and B with respect to the shape of its ultraviolet survival curve, plating medium response, sensitivity to radiomimetic chemicals, and ability to reactivate irradiated T_1 phage. Finally, data

from crosses of K-12 and B_{s-2} in this laboratory indicate that the radiation sensitivity of B_{s-2} is controlled by a gene very closely linked to a locus for maltose fermentation. Therefore, there have been mapped more or less rigorously at least four different genes, a mutation in any one of which will alter the sensitivity of *E. coli* to radiation as well as to radiomimetic chemicals. This, of course, represents only the lower limits of the number of sites determining radiosensitivity on the chromosome of *E. coli*, and the phenotypic expression of all of these have been described in only one of two states of the gene, + or −. What genetic changes and which, if any of the known genes, are involved in mutation of strain S and B to the various types of radioresistance is not known. These types of radioresistance could be phenotypic expressions of multiple alleles at one or more known sites, or they could be mutations of modifier genes at as yet undetermined sites.

The problem, then, is to determine the actual functions which the genes controlling radiosensitivity modify, how these functions are interrelated, and how they modify a presumed common lesion produced by a number of different chemical and physical insults.

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