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Repair and Mutagenesis in *Escherichia coli* K-12 After Exposure to Various Alkyl-Nitrosoguanidines

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The mutagenic and toxic effects of a series of *N*-alkyl-*N'*-nitro-*N*-nitrosoguanidines were examined in *Escherichia coli* K-12. The role of nucleotide excision repair, the SOS response, and the adaptive response in both the reduction and the production of the biological effects of these chemicals was tested. The effects of ethyl-nitrosoguanidine are similar in nucleotide excision repair-proficient and -deficient strains, but both the mutagenicity and the toxicity of alkyl groups larger than two carbons are significantly reduced by the presence of this repair system. Similarly, when alkyl groups are larger than two carbons, the *umuC* gene product is essential for the production of a fraction of the mutations that these lesions produce. The induction of the adaptive response had a significant effect on the toxicity of all of the chemicals tested, but its effect on mutagenicity was less uniform, having a larger effect on ethylating and propylating agents than on butylating and amylating agents.

The mutagenic effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) on *Escherichia coli* have been recognized for many years (12). The spectrum of DNA lesions that this alkylating agent produces and the enzyme systems involved in both the repair of these lesions and the production of mutations in response to them have been thoroughly investigated. This work has shown that the major mutagenic lesion produced by MNNG is O^6-methylguanine (O^6-MeGua) (11, 19). Replication errors in copying this modified base lead to guanine-cytosine to adenine-thymine transitions, which are the major type of mutation observed (1). The production of these mutations requires neither an active *umuC* gene product nor the induction of the SOS system (16). This is in sharp contrast to the mechanism which leads to mutations in response to bulky lesions (9).

The repair of O^6-MeGua is performed by a methyltransferase which directly demethylates O^6-MeGua (4, 13). This enzyme is induced by treating cells with sublethal doses of MNNG (4). This treatment also leads to the induction of several other enzymes (3, 8; P. F. Schendel, B. V. Edington, J. G. McCarthy, and M. L. Todd, *in* E. C. Friedberg and B. R. Bridges, ed., *Cellular Response to DNA Damage, UCLA Symposia on Molecular and Cellular Biology, vol. 11*, in press). When taken together, these MNNG-inducible functions are called the adaptive response (7). The induction of the adaptive response substantially reduces both the mutagenic and the cytotoxic effects of MNNG (15). It also interferes with the induction of the SOS response (2). Neither the mechanism by which the adaptive response reduces cytotoxicity nor the mechanism by which it interferes with SOS induction is understood fully, but it is likely that both may be in part mediated through the induction of a second methyltransferase which removes alkyl groups from the phosphotriesters on the DNA backbone (Schendel et al., in press).

Nucleotide excision repair is a system which provides protection against many kinds of agents which produce bulky lesions that can disrupt DNA structure. It does not seem to play an important role in the repair of MNNG damage, however, since *E. coli* cells deficient in nucleotide excision repair due to mutations in gene *uvrA*, *uvrB*, or *uvrC* are neither hypersensitive to nor hypermutable by MNNG. This suggests that cells do not sense most of the damage produced by MNNG as bulky lesions.

The biological effects of other alkyl-nitrosoguanidines have been studied less thoroughly. The treatment which induces resistance to MNNG has been reported to reduce the mutagenic and cytotoxic effects of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) (7), and the O^6-MeGua methyltransferase has been shown to repair O^6-ethylguanine, albeit at a slower rate

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than O^6-MeGua (20). Beyond this, little is known about the repair of larger alkyl groups.

This paper describes a study in which the biological consequences of ENNG and *N*-propyl-, *N*-butyl-, and *N*-amyl-*N'*-nitro-*N*-nitrosoguanidine (PNNG, BNNG, and ANNG, respectively) on *E. coli* K-12 were determined. The impact of various repair systems on the magnitude of these consequences and the role of various mutagenic processes in the production of mutations in response to these chemicals were measured. These results have led to several generalizations about the way *E. coli* deals with alkylation damage.

MATERIALS AND METHODS

Bacterial strains. Strain AB1157 is a multiply auxotropic strain of *E. coli* K-12 which has an ochre mutation in its *argE* gene (6). Strain AB1886 is a derivative of AB1157 carrying the *uvrA6* mutation (6). JC3890 is a derivative of AB1157 carrying a *uvrB* deletion (9), whereas TK501 was derived from JC3890 and carries the *umuC36* mutation (9). CON48AW is a Met^- strain which carries the *uvrB*::Mu d1-48 insertion (18), whereas CON48AW(pDR1494) is this same strain carrying a wild-type *uvrB* gene on a multicopy plasmid (18).

Chemical reagents. MNNG, ENNG, PNNG, BNNG, and ANNG, were purchased from Aldrich Chemical Co., Milwaukee, Wis. Tetracycline, *o*-nitrophenyl-β-D-galactopyranoside, and all necessary amino acids, sugars, and vitamins used in the growth of cultures were from Sigma Chemical Co., St. Louis, Mo. L-[^{3}H]methionine was from New England Nuclear Corp., Boston, Mass.

Methods. The conditions for growth of cells (7), induction of the adaptive response with MNNG (7), and measurement of mutation frequencies and survival (7, 17) have all been described previously. For all experiments in which mutation frequency was measured, doses were chosen which gave >10% survival of the culture. The doses of mutagen to which cells were exposed are expressed as the product of the concentration of the mutagen (micromolar) times the time of exposure (minutes). For ENNG and PNNG, the time of exposure was 10 min. For BNNG and ANNG, the time of exposure was 20 min. The induction of the *uvrB* operon was determined by the method previously described (18). L-[^{3}H]methionine incorporation was used to measure total protein synthesis.

RESULTS

Mutagenic effects of alkyl-nitrosoguanidines. The comparative mutagenicity of the series of *N*-alkyl-*N'*-nitro-*N*-nitrosoguanidines was examined. Figure 1 shows that in both repair-proficient and -deficient cells, alkyl groups up to five carbons in length can be detectably mutagenic; however, as the size of the alkyl group increases, much higher concentrations of mutagen and longer exposures to the doses are necessary to produce similar biological effects. ENNG is equally mutagenic to *uvr*$^+$ and *uvrA* cells. As the groups get larger, however, the ability of cells to carry out nucleotide excision repair has an increasing impact on the mutation frequency. This suggests that the larger alkyl groups are recognized as bulky lesions, whereas one- and two-carbon alkyl groups are not.

Role of *umuC* in alkylation mutagenesis. It has been shown previously that *umuC*$^+$ and *umuC*$^-$ strains are equally mutable by MNNG (16). This is also true for ENNG (Fig. 2). As the size of the alkyl groups increases, a significant fraction of the mutants arises through the action of the *umuC* product. The *umuC*$^+$ control in this experiment (JC3890) grows less well and develops fewer mutants per dose than other *umuC*$^+$ *uvr*$^-$ strains. We have no explanation for this behavior, but it has been seen consistently. Despite this complication, it seems appropriate to draw the general conclusion that *umuC* is involved in a significant amount of the mutagenesis produced by propylating, butylating, and amylating agents.

Effect of induction of the adaptive response. The adaptive response in *E. coli* K-12 can be induced by exposing the culture to a sublethal dose of MNNG for a period of 20 min or more

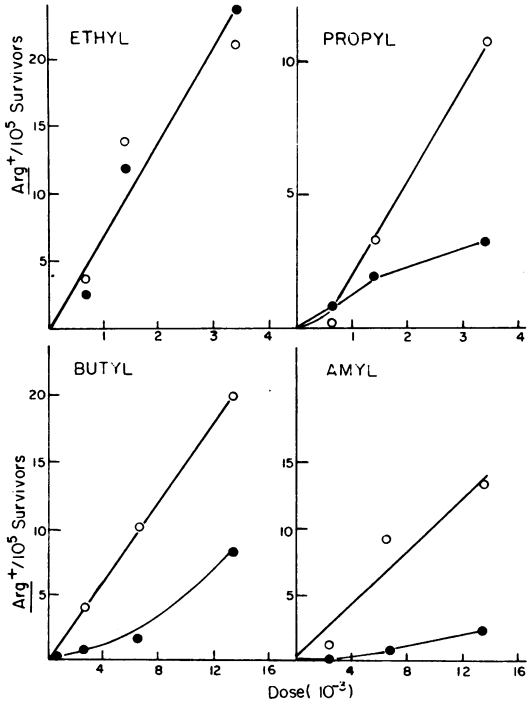

FIG. 1. Effect of nucleotide excision repair on the frequency of Arg$^+$ revertants found among the survivors in cultures challenged with various alkyl-nitrosoguanidines. The doses are expressed as described in the text. Symbols: ●, AB1157 (*uvr*$^+$) cultures; ○, AB1886 (*uvrA6*) cultures.

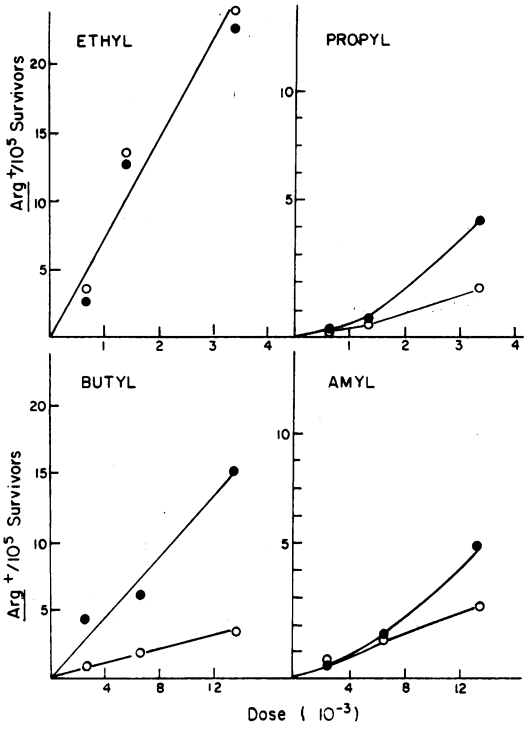

FIG. 2. Effect of *umuC* on the frequency of Arg^+ revertants found among the survivors in cultures exposed to various alkyl-nitrosoguanidines. Symbols: ●, JC3890 (*uvrB*$^-$ *umuC*$^+$) cultures; ○, TK501 (*uvrB*$^-$ *umuC36*) cultures.

(17). Cultures were exposed to 3.3 μM MNNG for 90 min, at which time they were challenged with one of the other alkyl-nitrosoguanidines. Figures 3 and 4 show the effect of this pretreatment on the mutant yields in *uvr*$^+$ and *uvrA* cultures, respectively. In AB1157 (*uvr*$^+$), pretreatment with MNNG substantially reduced the mutant yield in all cases. In AB1886 (*uvrA*), the same pretreatment reduced the mutants produced by ENNG and PNNG but had only a small effect on BNNG and ANNG mutagenesis. This suggests that pretreatment with MNNG has several effects, at least one of which requires a Uvr^+ phenotype to be manifest.

Figure 5 shows the effect of MNNG pretreatment on *umuC*$^-$ *uvrB*$^-$ cells. Once again the pretreatment reduced the frequency of Arg^+ revertants produced by ENNG and PNNG, but it had no effect on the mutagenicity of BNNG and ANNG. This argues that the *umuC*-independent mutagenesis induced by alkyl-nitrosoguanidines is mediated through a lesion that can be repaired by the adaptive response if it is an ethyl or a propyl derivative, but it cannot be repaired if it is a butyl or amyl derivative.

Effect of excision repair and MNNG pretreatment on the toxicity of alkyl-nitrosoguanidines. Figures 6 and 7 demonstrate the toxic effects of the series of alkylating agents under study. ENNG is very toxic and is about equally so in both *uvr*$^+$ and *uvrA* cells. PNNG is less toxic, but again the presence of excision repair does not seem to be of great importance. BNNG and ANNG, on the other hand, are not very toxic to *uvr*$^+$ cells but continue to show large effects in *uvrA* strains. This again suggests that butyl and amyl groups are processed like large bulky lesions rather than being treated like methyl groups.

Pretreatment with MNNG to induce the adaptive response has a substantial effect on reducing toxicity of all of the alkyl-nitrosoguanidines. Unlike the results with mutagenesis, this reduction is seen in both *uvr*$^+$ and *uvrA* cultures.

Induction of the SOS response by alkyl-nitrosoguanidines. The demonstration that pretreatment with MNNG substantially reduced the mutagenic effects of BNNG and ANNG in *uvr*$^+$ cells and that an active *umuC* gene product is essential for the production of mutations with the larger alkyl groups suggested the possibility

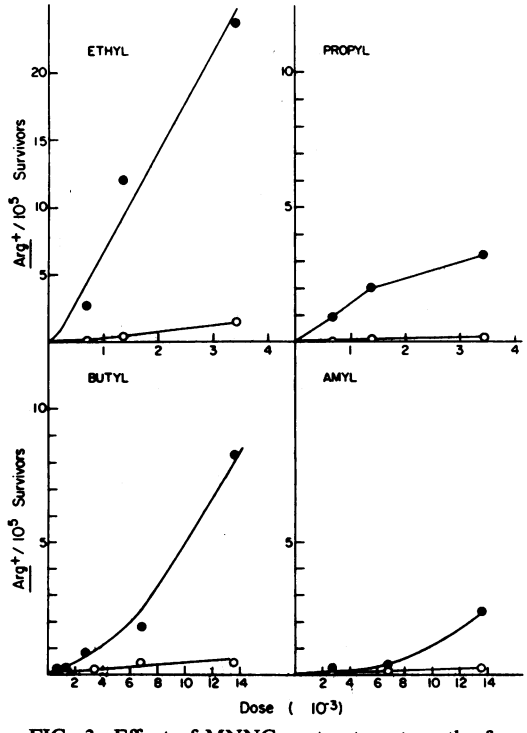

FIG. 3. Effect of MNNG pretreatment on the frequency of Arg^+ revertants found among the survivors in cultures of AB1157 (*uvr*$^+$) exposed to various alkyl-nitrosoguanidines. Symbols: ●, control cultures; ○, cultures treated with 3.3 μM MNNG for 90 min before challenge.

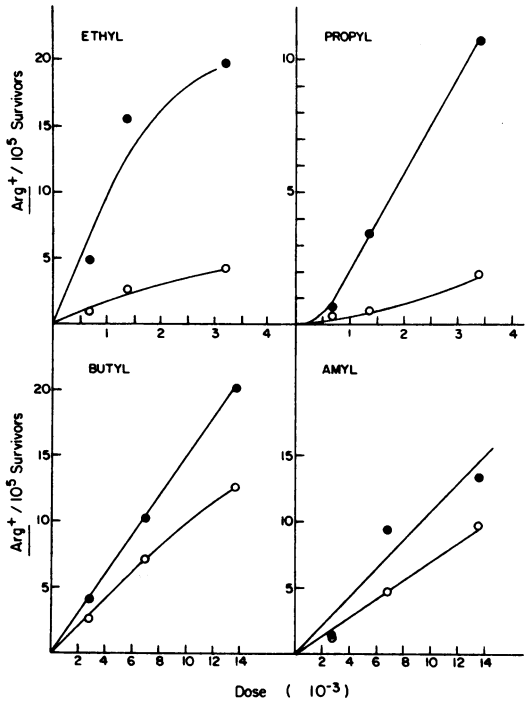

FIG. 4. Effect of MNNG pretreatment on the frequency of Arg^+ revertants found among the survivors in cultures of AB1886 (*uvrA6*) exposed to various alkyl-nitrosoguanidines. Symbols: ●, control cultures; ○, cultures treated with 3.3 μM MNNG for 90 min before challenge.

that pretreatment was suppressing the induction of *umuC* and was in this way limiting mutagenesis. This seemed plausible since the induction of the adaptive response is known to substantially reduce the ability of MNNG to induce Weigle reactivation and bacteriophage lambda (2). The hypothesis was tested by monitoring β-galactosidase production from a *uvrB*::Mu d(Ap *lac*) operon fusion. This operon fusion is under *lexA* and *recA* control, and the rate of β-galactosidase production in this strain is a measure of SOS induction (18). Table 1 shows the amount of β-galactosidase synthesized per micromole of methionine incorporated into phenotypically uvr^+ [CON48AW(pDR1494)] and uvr^- (CON48AW) strains challenged with the various alkyl-nitrosoguanidines. Several general conclusions can be reached from these data. First, all of the alkylating agents are capable of inducing *uvrB*. Second, the extent of induction is greater in uvr^- cells than in uvr^+ strains. In fact, ANNG is unable to produce significant induction of *uvr*B in uvr^+ cells at the doses used. Third, the pretreatment with MNNG does reduce the subsequent amount of SOS induction by all of the alkylating agents, although the effect on ENNG and PNNG induction is somewhat larger than the effect on BNNG and ANNG induction. Unlike the case of Weigle reactivation, where the adaptive response totally blocks MNNG induction of this phenomenon (2), *uvrB* could be induced to some extent even in adapted cells. This was true for all of the alkylating agents reported in Table 1, and it is also true for MNNG (data not shown). This difference probably reflects the fact that some SOS operons are more easily activated than others, and the *uvrB* operon is one of the more readily induced members of the SOS response (18). Thus, the suppression of *umuC* induction by MNNG pretreatment is likely to be greater than the effect shown in Table 1.

DISCUSSION

The following general features of alkylation mutagenesis and repair of alkylated DNA have emerged from the results just presented. (i) As the size of an alkyl group transferred to DNA increases, the nucleotide excision repair system plays an increasing role in the repair of the damage. (ii) For alkyl groups larger than two

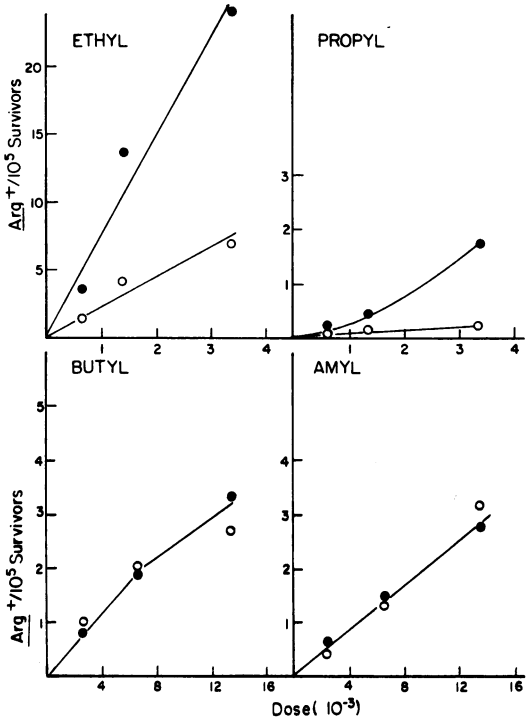

FIG. 5. Effect of MNNG pretreatment on the frequency of Arg^+ revertants found among the survivors in cultures of TK501 (*uvrB*$^-$ *umuC36*) exposed to various alkyl-nitrosoguanidines. Symbols: ●, control cultures; ○, cultures treated with 3.3 μM MNNG for 90 min before challenge.

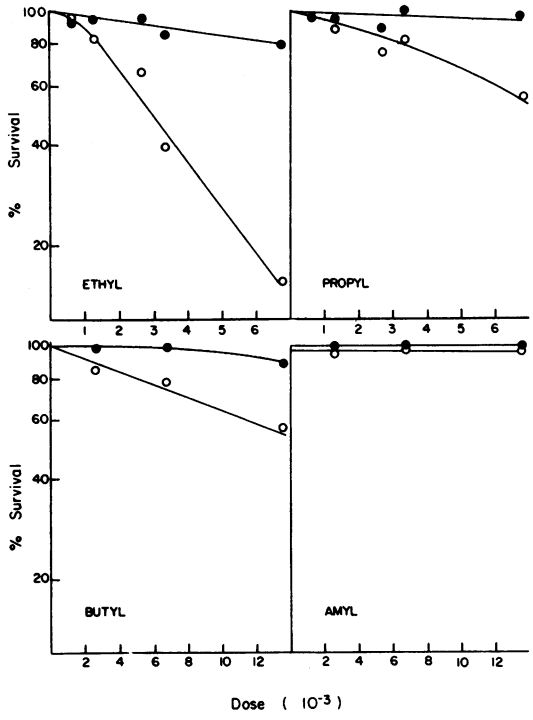

FIG. 6. Effect of MNNG pretreatment on the survival of cultures of AB1157 (*uvr*$^+$) exposed to various alkyl-nitrosoguanidines. Symbols: O, control cultures, ●, cultures treated with 3.3 μM MNNG for 90 min before challenge.

carbons, a significant amount of the mutagenesis that they produce is mediated by the action of the *umuC* gene product. (iii) Treatments which induce the adaptive response cause a significant reduction in the mutagenic effects of all of the alkylating agents tested. Nevertheless, the methyltransferase which repairs O^6-alkylguanine lesions is probably only active on alkyl groups up to three carbons in length. Most of the reduction in mutagenicity of butylating and amylating agents brought about by MNNG pretreatment requires a *uvr*$^+$ genotype. (iv) Pretreatment with MNNG causes a reduction in the toxic effects of all of the alkylating agents. This effect is independent of nucleotide excision repair.

Before discussing what these results suggest about the biochemistry of alkylation repair and mutagenesis, some consideration of the chemistry of the various alkyl groups seems appropriate. As far as is known, all alkyl-nitrosoguanidines alkylate via the production of an alkyldiazonium ion which decomposes to the alkonium ion and then reacts with DNA (10). As the sizes of the alkyl groups increase, the alkonium ions should be slightly more stable. This leads to an increased amount of alkylation on sites such as the phosphates of the DNA backbone (21). Second, once the number of carbons in the alkyl group is greater than two, the primary alkonium ion has the possibility of shifting to the more stable secondary alkonium ion before reacting with the DNA. When this occurs, the alkyl group on the DNA is branched. Such branched chains may well be repaired differently than are straight chains and, in fact, may resemble bulky lesions.

The biochemistry of alkylation repair appears to be quite complex, and the kinds of general mutagenicity and toxicity data presented in this paper can only give an indication as to the molecular events which are occurring in the cells. Nevertheless, they can serve as a source of testable hypotheses which can be pursued by more analytical methods. To this end, the following ideas are presented.

We propose that many kinds of branched-chain alkylations cause sufficient disruption to the DNA structure to be sensed as bulky lesions. These alkylations are repaired by nucleotide excision repair. Since MNNG and ENNG cannot produce branched-chain alkylation, the bio-

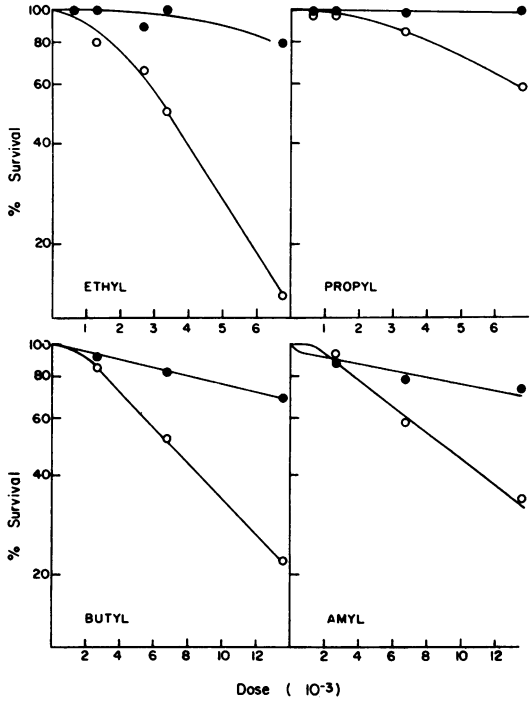

FIG. 7. Effect of MNNG pretreatment on the survival of cultures of AB1886 (*uvrA6*) exposed to various alkyl-nitrosoguanidines. Symbols: O, control cultures; ●, cultures treated with 3.3 μM MNNG for 90 min before challenge.

TABLE 1. Specific synthesis of β-galactosidase

Strain (phenotype)	Alkylating agent	Synthesis of β-galactosidase					
		Control (no challenge)	+ Challenge[a]	Fold induction	Pretreated with MNNG (no challenge)[b]	+ Challenge[a]	Fold induction
CON498AW (Uvr⁻)	ENNG	209[c]	757[c]	3.6	250[c]	394[c]	1.6
	PNNG	255	756	3.0	257	502	2.0
	BNNG	161	577	3.5	210	566	2.7
	ANNG	147	490	3.3	152	343	2.3
CON48AW(pDR1494) (Uvr⁺)	ENNG	158	318	2.0	193	230	1.2
	PNNG	270	642	2.4	260	435	1.7
	BNNG	270	763	2.8	273	446	1.6
	ANNG	317	366	1.1	313	301	0.95

[a] Dose used in challenge was the highest one used in Fig. 1.
[b] Pretreated for 90 min with 3.3 μM MNNG.
[c] Units of β-galactosidase synthesized per micromole of methionine incorporated into strain.

logical effects of these agents are similar in *uvr*⁺ and *uvr*⁻ cultures. As the alkyl groups get longer, the probability of producing branched-chain alkylations increases and so does the role of excision repair in reducing their biological effects.

In contrast, we propose that the O^6-alkylguanine methyltransferase acts mainly on straight-chain alkyl groups. It thus can remove all methyl and ethyl groups from the O^6-position of guanine and, presumably, can remove many of the propyl groups as well. By the time the alkyl groups reach four carbons, most of the O^6-alkylguanine produced is branched chain and unrepairable. Since the *umuC* gene product is necessary for BNNG and ANNG mutagenicity, the reduced mutation frequency seen with MNNG-pretreated cultures challenged with these chemicals is likely due to the suppression of *umuC* induction which occurs when the adaptive response is induced.

An explanation of the lethal effects of the various alkylating agents is more difficult. For the smaller alkyl groups, the lethality may result from the disruptive effects of certain alkylated bases and from the production of double-strand breaks. These lethal breaks develop as a result of the simultaneous repair of both DNA strands within the same region (5). The probability of producing lethal breaks is increased by things which slow gap filling and extend the time between initiation of repair and ligation of the DNA. Phosphotriesters should have such an effect. Since gaps can also serve as sites for the activation of *recA* protein (14), the presence of phosphotriesters may also increase the probability of SOS induction. The induction of the adaptive response reduces these problems by providing a glycosylase which can repair at least one disruptive alkylated base (3, 8) and by providing a methyltransferase to remove triesters from the DNA backbone (Schendel et al., in press). The repair of triesters would be expected to facilitate excision repair and reduce SOS induction.

For the larger alkyl groups, the situation may change slightly. Now, branched-chain alkyl groups at a variety of sites could well become the dominant lethal lesions. In *uvr*⁺ cells, most of these lesions are efficiently repaired; in *uvr*⁻ cells, they are not and the cells die.

The effect of MNNG pretreatment on the lethality of BNNG and ANNG is difficult to interpret in any straightforward manner. The simplest possibility is that the inducible methyltransferase which repairs phosphotriesters removes all kinds of alkyl groups. In so doing, it allows gaps to be filled quickly and thus avoids double-strand breaks and SOS induction. This requires both a very broad substrate specificity for the triester repair activity and glycosylases that act on lesions containing various-sized alkyl groups. At present, it is not known whether either of these conditions is met in adapted *E. coli* cells. Alternatively, repair systems that we have not yet discovered may be induced by MNNG pretreatment.

The results presented in this paper clearly demonstrate the interlocking and overlapping nature of DNA repair systems. Often, subtle changes in the chemistry and in the amount of the DNA damage have profound effects on the extent of involvement of various systems in the repair process. In other cases, the functioning of one system is vital for the efficient functioning of a second genetically unrelated system. In yet other cases, one system is involved in regulating the expression of another. All of this allows an extraordinary amount of fine control over a process vital to the genetic stability and survival of the cell.

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