The EMBO Journal vol.7 no.7 pp.2261 - 2267, 1988

Alternative pathways for the *in vivo* repair of O^6-alkylguanine and O^4-alkylthymine in *Escherichia coli*: the adaptive response and nucleotide excision repair

Leona Samson, Jürgen Thomale[1] and Manfred F.Rajewsky[1]

Charles A.Dana Laboratory of Toxicology, Harvard School of Public Health, Boston, MA 02115, USA, and [1]Institut für Zellbiologie (Tumorforschung), Universität Essen (GH), D-4300 Essen 1, FRG

Communicated by J.Cairns

The *in vivo* removal of three different *O*-alkylated bases from DNA was measured in *Escherichia coli*. Using monoclonal antibodies specific for O^6-methylguanine, O^6-ethylguanine and O^4-ethylthymine we have monitored the removal of these lesions from six different strains to assess the relative contributions of the adaptive response and of nucleotide excision repair. During the first hour after DNA alkylation, O^6-methylguanine, O^6-ethylguanine and O^4-ethylthymine lesions were repaired almost exclusively by nucleotide excision, except when the adaptive response was being constitutively expressed. In wild-type *E.coli* the adaptive response began to contribute to O^6-methylguanine repair about one hour after alkylation, the time required for the full induction of the *ada* DNA methyltransferase. In contrast, the adaptive response did not play such a large role in the repair of O^6-ethylguanine and O^4-ethylthymine in wild-type *E.coli*, presumably because DNA ethylation damage is a poor inducer of the adaptive response; possible reasons for this poor induction are discussed. The repair of all three *O*-alkylated lesions was virtually absent in *ada*$^-$ *uvr*$^-$ bacteria suggesting that no alternative pathway is available for their repair, at least during the first two hours after alkylation. When the repair of *O*-alkylated bases was compromised by an *ada*$^-$ or by a *uvr*$^-$ mutation, the bacteria became more sensitive to alkylation induced killing and mutation.

Key words: DNA alkylation/repair/*ada*/*uvr*/immunoanalysis/monoclonal antibodies

Introduction

The repair of DNA alkylation damage in *Escherichia coli* is achieved in a number of different ways and the choice of repair mechanism appears to depend in part upon the chemical nature of the specific DNA alkylation product. Genetic and biochemical evidence has led to the identification and characterization of four enzymes specific for the repair of DNA methylation damage. The *tag* gene product, N^3-methyladenine (N^3MeA) DNA glycosylase I, excises N^3MeA from DNA (Karran *et al.*, 1980), and the *alk*A gene product, N^3MeA DNA glycosylase II, excises N^3-methylpurines and O^2-methylpyrimidines (Karran *et al.*, 1982; McCarthy *et al.*, 1984); the *ada* gene product, DNA methyltransferase (MT), transfers methyl groups from O^6-methylguanine (O^6-MeG), O^4-methylthymine (O^4MeT) and methylphosphotriesters (MePT) in DNA to two specific cysteine residues in the Ada protein itself (Schendel and Robins, 1978; Olsson and Lindahl, 1980; Foote *et al.*, 1980; Teo *et al.*, 1984; McCarthy and Lindahl, 1985); once the cysteines are methylated the enzyme is inactive, i.e. it is a suicide enzyme (Robins and Cairns, 1979; Lindahl *et al.*, 1982; Demple *et al.*, 1982). Lastly, another suicide DNA MT has been identified in *E.coli* that repairs O^6MeG and O^4MeT but not MePT lesions; this enzyme has been called DNA MT II (Potter *et al.*, 1987; Rebeck *et al.*, 1988). The *tag* gene and the DNA MT II gene are expressed constitutively in *E.coli*, but the *ada* and *alk*A genes are induced as part of the adaptive response to alkylating agents. Exposure of *E.coli* to non-toxic levels of *N*-methyl-*N'*- nitro-*N*-nitrosoguanidine (MNNG) induces the adaptive response which confers resistance to the killing and mutagenic effects of MNNG (Samson and Cairns, 1977) by inducing the expression of *ada* and *alk*A along with at least two other genes, *alk*B and *aid*B (Teo *et al.*, 1986; Nakabeppu and Sekiguchi, 1986; Volkert and Nguyen, 1984). The functions of the *alk*B and *aid*B gene products are not yet known. The Ada protein plays a central role in the regulation of the adaptive response. After methyl-transfer from MePT lesions to the active site near the amino-terminal, the methylated Ada protein binds efficiently to the operator region of the *ada*–*alk*B operon, the *alk*A gene and probably also the *aid*B gene, to stimulate transcription (Teo *et al.*, 1986; Nakabeppu and Sekiguchi, 1986). The repair of O^6MeG and O^4MeT by the Ada protein provides resistance to MMNG-induced mutation (Schendel and Robins, 1978; Jeggo, 1979; Evensen and Seeberg, 1982), and the repair of N^3-methylpurines and O^2-methylpyrimidines by N^3MeA DNA glycosylase II provides resistance to MNNG-induced cell killing (Karran *et al.*, 1982; McCarthy *et al.*, 1984; Evensen and Seeberg, 1982).

It is generally thought that the repair of methylated DNA is mediated, for the most part, by the DNA MTs and DNA glycosylases described above (Lindahl, 1982, 1988). However, recent evidence has shown that methylated DNA can, in fact, serve as substrate *in vitro* for the *uvr*ABC excinuclease (van Houten and Sancar, 1987). Here we show directly that when the *E.coli* genome contains a high level of methylation damage a large proportion of O^6MeG is repaired by the *uvr* nucleotide excision repair pathway *in vivo*. Despite this, the *uvr* repair pathway only provides a moderate amount of protection against methylation induced mutation; possible reasons for this are discussed.

Purified *E.coli* DNA MT is also able to accept ethyl groups from O^6EtG containing DNA *in vitro* (Sedgwick and Lindahl, 1982), and adapted *E.coli* have been shown to be resistant to killing and mutation induced by ethylating agents (Jeggo *et al.*, 1977; Sedgwick and Lindahl, 1982). However, Warren and Lawley (1980) have presented evidence that the

uvr system is responsible for O^6EtG repair *in vivo*. The results of the present study show that wild-type *E.coli* repair O^6EtG and O^4EtT, and are protected against ethylation-induced mutation and cell death, almost exclusively by the *uvr* repair pathway. The adaptive response plays a role in the repair of O^6EtG and O^4EtT only in bacteria that have been adapted by prior exposure to methylating agents or are constitutively adapted.

Results

The role of the adaptive response in the repair of O^6EtG and O^4EtT

The adaptation of *E.coli* induces an increased resistance to the mutagenic effects of ethylating agents (Jeggo *et al.*, 1977; Sedgwick and Lindahl, 1982) suggesting that the *ada* DNA MT can repair O^6EtG and O^4EtT. In support of this notion it was shown that ethyl groups can be transferred from O^6EtG in DNA to the purified Ada protein *in vitro* (Sedgwick and Lindahl, 1982). To determine whether this reaction actually occurs *in vivo*, we monitored the removal of O^6EtG and O^4EtT from *E.coli* DNA during a 2-h period following challenge with *N-ethyl-N*-nitrosourea (EtNU), in various strains of *E.coli* that express widely different levels of the *ada* DNA MT. Three strains were used for the initial study: *E.coli* B strain F26 (*his*$^-$ *thy*$^-$ *ada*$^+$) which is wild-type for the adaptive response to alkylating agents, and its derivative strains BS23 (*ada*$^-$*alkB*$^-$) which is unable to induce the adaptive response, and BS21 (*ada*^{c}) which constitutively expresses the adaptive response. *E.coli* F26 has a basal level of ~40 DNA MT molecules per cell (presumably a mixture of both the Ada protein and DNA MT II), which is raised ~200-fold during adaptation (Mitra *et al.*, 1982); *E.coli* BS23 bears a deletion of the *ada* gene and has a basal level of ~30 DNA MT molecules per cell (presumably comprised entirely of DNA MT II) that cannot be elevated by adaptation (Mitra *et al.*, 1982; Rebeck *et al.*, 1988); *E.coli* BS21 has a basal level of about 8000 DNA MT molecules per cell (Mitra *et al.*, 1982). All three strains were challenged with 10 mg/ml of EtNU for 10 min and the levels of O^6EtG and O^4EtT in DNA were measured using specific monoclonal antibodies (mAbs) (Adamkiewicz *et al.*, 1982, 1985). Figure 1 shows that the *ada*$^-$ and unadapted *ada*$^+$ strains repair O^6EtG and O^4EtT at the same rate, which implies that under these conditions the adaptive response is not making a significant contribution. In 2 h *ada*$^-$ bacteria repairs >400 O^6EtG and ~25 O^4EtT molecules per cell. Since *ada*$^-$ *E.coli* have an uninducible level of only 30 DNA MT II molecules per cell, which can only act once, we infer that O^6EtG and O^4EtT are repaired via another DNA repair pathway. Since *ada*$^+$ and *ada*$^-$ bacteria repair O^6EtG and O^4EtT at exactly the same rate it seems likely that these lesions are repaired via the same pathway in the two strains. By contrast, the repair of O^6EtG and O^4EtT was much more rapid in the constitutively adapted strain (BS21), presumably because the Ada protein can act upon both of these lesions *in vivo*.

The role of the uvr pathway in the repair of O^6EtG and O^4EtT

Since Warren and Lawley (1980) presented evidence that the *uvr* excision repair pathway plays a role in the repair of O^6EtG, we constructed *uvrB*$^-$ derivatives of F26, BS23 and BS21 and have tested their ability to repair O^6EtG and O^4EtT. Figure 2 shows that in each instance the absence of a functional *uvr* repair pathway decreased the rate of O^6EtG and O^4EtT removal *in vivo*. The decrease was more marked in *ada*$^+$ and *ada*$^-$ strains than in the *ada*^{c} strain, and there was hardly any repair in the *ada*$^-$ *uvr*$^-$ strain, indicating that no major alternative pathway exists for the repair of these lesions (at least for a 2-h period following alkylation).

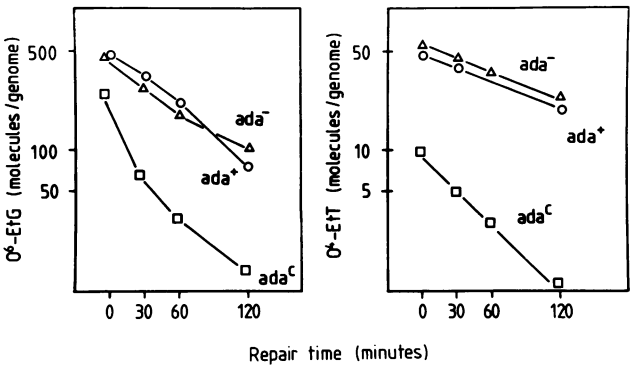

Fig. 1. Repair of O^6EtG and O^4EtT after ethylation of *ada*$^+$, *ada*$^-$ and *ada*^{c} *E.coli*. *E.coli* F26 *ada*$^+$ (○), BS23 *ada*$^-$ (△) and BS21 *ada*^{c} (□), were exposed to 10 mg/ml EtNU for 10 min. At various times after exposure cells were harvested and the number of O^6EtG residues (**panel A**) and O^4EtT residues (**panel B**) per genome equivalent determined using the ISB method.

The contributions of the adaptive response and the uvr repair pathway to the repair of O^6MeG

The rate of O^6MeG repair *in vivo* in *ada*$^+$, *ada*$^-$ and *ada*^{c} bacteria after treatment with *N*-methyl-*N*-nitrosourea (MeNU) is shown in Figure 3A. As expected, repair was extremely rapid in *ada*^{c} cells and was somewhat higher in *ada*$^+$ than *ada*$^-$ bacteria. The difference in rate of O^6MeG repair between *ada*$^+$ and *ada*$^-$ became apparent only after the first hour and was presumably due to the induction of Ada DNA MT in *ada*$^+$ bacteria.

The repair of O^6MeG *in vivo* in *uvrB*$^-$ derivatives of the *ada*$^+$, *ada*$^-$ and *ada*^{c} strains is shown in Figure 3B–D. The absence of a functional *uvr* repair pathway clearly decreased O^6MeG removal in the *ada*$^+$ and *ada*$^-$ backgrounds, and repair was almost completely eliminated in the *ada*$^-$ *uvr*B$^-$ strain; the contribution to O^6MeG repair by 30 molecules per cell of the DNA methyltransferase II enzyme would be negligible at the doses of MNU used in these experiments. The effect of eliminating the *uvr* pathway was small in an *ada*^{c} background.

The repair of ethylphosphotriesters (EtPT) by the E.coli Ada protein

The increased rate of O^6MeG repair in *ada*$^+$ bacteria (compared to *ada*$^-$) at longer repair times is presumably due to the induction of Ada protein after MeNU exposure. The absence of a similar increase in the repair of O^6EtG in EtNU treated *ada*$^+$ cells suggests that EtNU does not efficiently induce significant levels of the Ada protein, and this agrees with the observation that ethylating agents are inefficient at inducing the expression of an *ada'–lacZ'* gene fusion (Nakabeppu *et al.*, 1985). Regulation of *ada* in

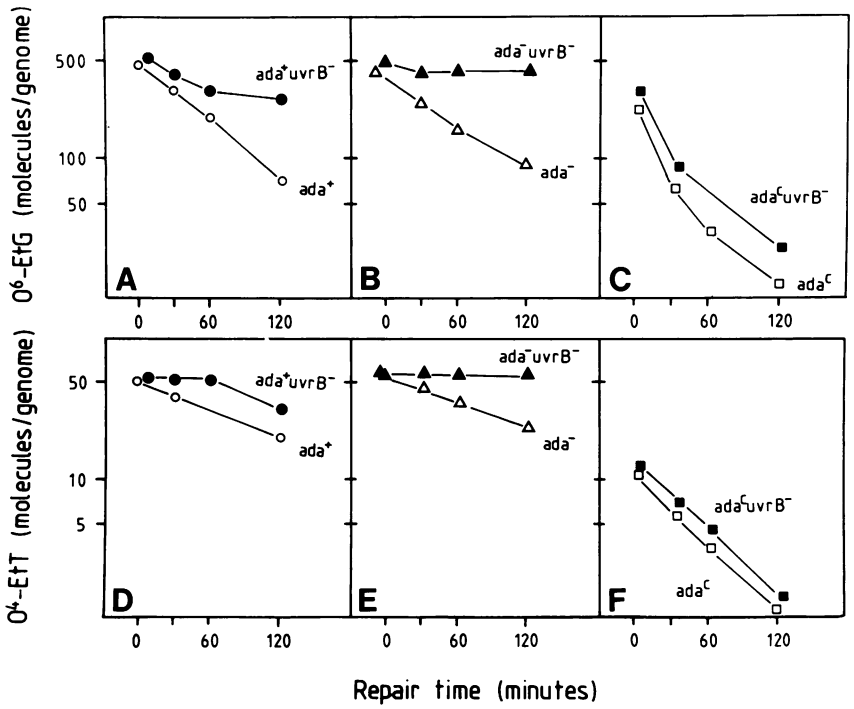

Fig. 2. Repair of O^6EtG and O^4EtT after ethylation of *uvr*B$^-$ bacterial strains. *E. coli* F26 *ada*$^+$ (○), F26 *ada*$^+$ *uvrB*$^-$(●), BS23 *ada*$^-$ (△), BS23 *ada*$^-$ *uvr*B$^-$ (▲), BS21 *ada*^{c} (□) and BS21 *ada*^{c} *uvr*B$^-$ (■) were exposed to 10 mg/ml EtNU for 10 min and the repair of O^6EtG (**top panel**) and O^4EtT (**bottom panel**) was monitored as in Figure 1. Data for F26, BS23 and BS21 were taken from Figure 1.

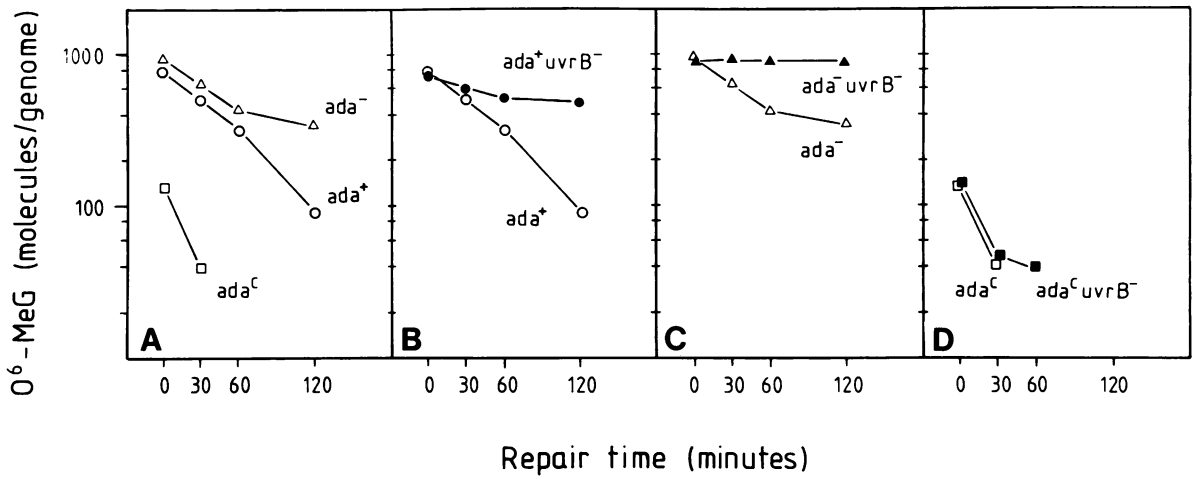

Fig. 3. Repair of O^6MeG after methylation of *uvr*B$^-$ bacterial strains. The same set of *E.coli* strains as in Figure 2, represented by the same symbols, were exposed to 4 mg/ml MeNU for 10 min and the repair of O^6MeG monitored as in Figure 1. Data for F26, BS23 and BS21 in panels B, C and D were taken from panel A.

response to methylation damage depends on methyl tranfer from MePT lesions to the Ada protein which then becomes an inducer for *ada–alkB* expression. The failure of ethylating agents to induce the *ada* gene could be because the Ada protein cannot accept ethyl groups from EtPT lesions. We therefore incubated extracts containing the 39 kd *E.coli* Ada protein with DNA containing tritium labelled EtPT or MePT (but no O^6AlkylG or O^4AlkylT). As Figure 4 shows, the Ada protein accepted alkyl groups from both MePT and EtPT *in vitro*. It remains to be determined whether it is only the methylated, and not the ethylated, Ada protein that can act as an inducer of the *ada* operon.

The role of the uvr pathway in the protection of E.coli against killing and mutation by DNA ethylation and methylation damage

Since both ethylation and methylation produce DNA substrates for repair by the *uvr* nucleotide excision repair pathway, we determined how much the *uvr* pathway contributes towards mutation and killing resistance. The response of *ada*$^-$, *ada*$^+$ and *ada*^{c} cells and their *uvrB* derivatives to killing by EtNU and MeNU is shown in Figures 5 and 6. The *uvrB* mutation made all three strains more sensitive to EtNU-induced cell killing and made the *ada*^{c} and *ada*$^+$ strains more sensitive to MeNU-induced

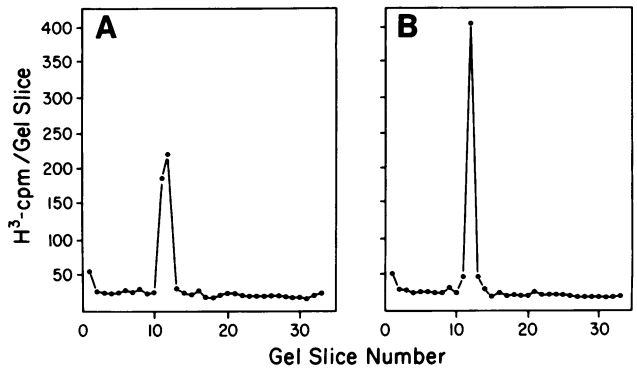

Fig. 4. Transfer of alkyl groups from MePT and EtPT DNA lesions to the Ada protein *in vitro*. HeLa S3-9 cell extract proteins were incubated with MePT (**panel A**) or EtPT (**panel B**) containing DNA, then separated in a 12% polyacrylamide gel. Proteins that accepted labelled alkyl groups were located by counting 2 mm gel slices; slice number 1 is from the top of the gel.

killing, but it made little difference to the sensitivity of *ada*$^-$ cells to MeNU-induced killing. With the *uvrB* mutation all three strains became more sensitive to the mutagenic effects of both EtNU and MeNU (Tables I and II), but the sensitization was more profound for EtNU. In a wild-type *uvrB*$^+$ background the *ada* pathway plays a very small role in protecting *E.coli* against EtNU-induced mutation, but in a *uvr*B$^-$ background the *ada* pathway clearly provides substantial protection (Table I). From this we infer that the *ada* pathway can, in fact, eventually be induced in response to DNA ethylation damage to provide protection against EtNU induced mutation.

Discussion

The demonstration that a particular DNA repair enzyme is able to repair a DNA alkylation product *in vitro* does not necessarily mean that this enzyme is responsible for *in vivo* repair. Recent developments for the use of mAbs in highly sensitive immunological assays to detect femtomole amounts of alkyldeoxynucleosides (Adamkiewicz *et al.*, 1982, 1985; Nehls *et al.*, 1984) have allowed us to study the *in vivo* repair of three DNA alkylation products in *E.coli*. We have thus monitored the repair of O^6MeG, O^6EtG and O^4EtT in six strains of *E.coli* that were chosen to show the relative contributions of the adaptive response and the *uvr* nucleotide excision repair pathway.

Our results show that when wild-type *E.coli* are suddenly exposed to EtNU or MeNU, most of the repair of O^6AkylG and O^4AlkylT is via the *uvr* nucleotide excision repair pathway; in the absence of the *uvr* pathway the *ada* pathway is apparently slowly induced and achieves some repair after a considerable delay, and this serves to protect *uvr*$^-$ strains from the long term consequences of DNA damage (i.e. mutation and killing). If, however, the *ada* pathway has already been induced (as it is in *E.coli ada*^{c}), the lesions are repaired rapidly and there is a much greater protection against killing and mutation. Thus, the *uvr* pathway seems to provide the main protection against transient exposure to these alkylating agents, and the *ada* pathway seems to provide the protection against chronic exposure. The kinetics of repair by these pathways are very different: *ada*, once induced, acts very quickly but is consumed in the repair

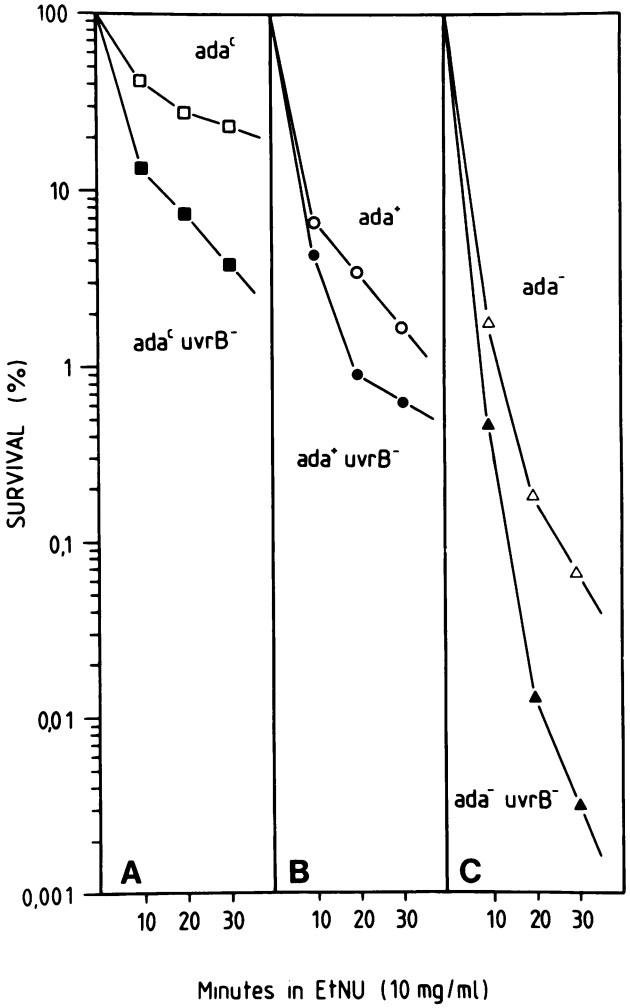

Fig. 5. EtNU-induced cell killing of *uvr*B$^-$ bacterial strains. The same set of *E.coli* strains as in Figure 2, represented by the same symbols, were exposed to 10 mg/ml EtNU for the times indicated. Cell killing was measured as described in Materials and methods.

process; *uvr* acts more slowly but can ultimately repair many lesions because, as a classical enzyme, it is not consumed in the repair process. It remains to be determined whether similar pairs of pathways to deal with transient and chronic exposures exist for the repair of other kinds of DNA damage.

It seems likely that the *uvr*ABC excinuclease initiates repair at other sites of DNA ethylation damage (e.g. N^3-ethylpurines) and, therefore, that *uvr*$^-$ bacteria are deficient in the repair of lesions other than O^6AlkylG and O^4AlkylT. It is thus not possible to say exactly which unrepaired alkylated DNA products are responsible for cell death and which are responsible for mutation. However, the decreased repair of O^6AlkylG and O^4AlkylT is probably responsible for at least part of the increased mutation in *uvr*$^-$ *E.coli* since both of these DNA alkylation products are known to mispair during replication (Loechler *et al.*, 1984; Preston *et al.*, 1986, 1987).

EtNU appears to induce the adaptive response very slowly. The failure of the ethylated Ada protein to act efficiently as a positive regulator for *ada* expression might be because its binding affinity for the *ada* promoter is not as strong as the binding affinity of the methylated Ada protein. Alternatively, both alkylated proteins may bind equally well to

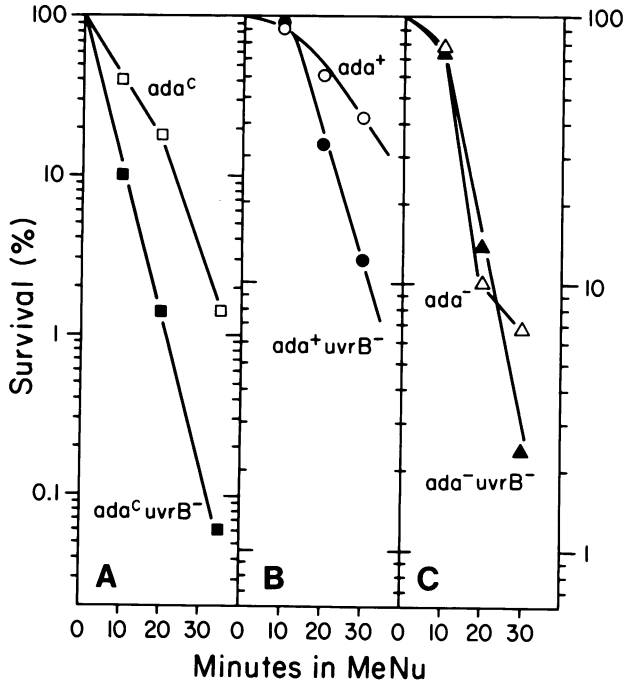

Fig. 6. MeNU-induced cell killing of *uvr*B$^-$ bacterial strains. The same set of *E.coli* strains as in Figure 2, represented by the same symbols, were exposed to 2 mg/ml MeNU (**panel A**) or 0.5 mg/ml MeNU (**panels B** and **C**) for the times indicated. Cell killing was measured as described in Materials and methods.

Table I. EtNU-induced mutation in *uvr*B$^-$ derivatives of *ada*$^+$, *ada*$^-$ and *ada*^{c} *E.coli* strains

Relevant genotype	*His*$^+$ revertants/10^8 survivors after EtNU (5 mg/ml) for 10 min
ada^c	7.34
ada^c *uvr*B$^-$	21.69
ada$^+$	942
ada$^+$ *uvr*B$^-$	1777
ada$^-$	1303
ada$^-$ *uvr*B$^-$	10 861

Table II. MeNU-induced mutations in *uvrB*$^-$ derivatives of *ada*$^+$, ada$^-$ and *ada*^{c} *E.coli* strains

Relevant genotype	MeNu dose	*His*$^+$ revertants/10^8 survivors after MeNU for 10 min
ada^c	2mg /ml	9.7
ada^c *uvr*B$^-$	2 mg/ml	128.9
ada$^+$	0.5 mg/ml	733
ada$^+$ *uvr*B$^-$	0.5 mg/ml	1104
ada$^-$	0.5 mg/ml	7717
ada$^-$ *uvr*B$^-$	0.5 mg/ml	10 250

the *ada* promotor but differ in their subsequent ability to stimulate transcription.

The broad specificity of *uvr* mediated DNA repair has been attributed to the ability of the *uvr*ABC excinuclease to recognize major distortions of the phosphodiester backbone in the DNA duplex (Weiss and Grossman, 1987). It was, therefore, surprising to find that the *uvr* pathway plays a major role in the *in vivo* repair of O^6MeG since DNA methylation is not usually thought to generate major DNA distortions (van Houten and Sankar, 1987), and since it was generally believed that this lesion is repaired, for the most part, by the Ada protein (Lindahl, 1982, 1988). The O^6MeG lesion, when paired with any one of the four possible bases, does in fact cause some helix destabilization (as detected by a decrease in T_m and by ^{1}H-NMR spectra), and the O^6MeG:T base pair causes the greatest helix destabilization (Gaffney *et al.*, 1984; Patel *et al.*, 1985, 1986a,b,c). In contrast, ^{31}P-NMR spectra have shown that while the O^6MeG:C base pair causes a slight distortion in the phosphodiester backbone of duplex DNA, the O^6MeG:T base pair does not (Patel *et al.*, 1985, 1986a,b,c). Since it is not known exactly which features of damaged DNA the *uvr*ABC excinuclease recognizes one cannot say whether nucleotide excision occurs at the O^6MeG:C base pair, the O^6MeG:T base pair, or both. It is curious that, for the bacterial strains used in this study, being proficient in excision repair only provides a moderate level of resistance to MeNU induced mutation despite the fact that it provides a significant level of O^6MeG repair. However, it should be noted that mutation was only measured at one locus and that it is possible that this particular site may be somewhat refractory to nucleotide excision repair.

It has now been shown that the O^6MeG DNA lesion is subject to repair by a number of different DNA repair systems which include the Ada protein (Lindahl, 1982), DNA methyltransferase II (Rebeck *et al.*, 1988) and the nucleotide excision DNA repair system. Presumably, the choice of repair mechanism depends upon enzyme availability. At very low levels of DNA methylation damage DNA methyltransferase II (present constitutively at ~30 molecules per cell) probably assumes the responsibility for O^6MeG repair. As the level of DNA methylation damage increases the adaptive response will be induced and the Ada protein presumably takes over the responsibility for O^6MeG repair. However, following a transient exposure to high doses of MeNU (such as those used in this study), which inhibit DNA replication and which saturate the DNA methyltransferases, the nucleotide excision repair pathway plays a major role in the repair of this highly mutagenic DNA lesion.

Materials and methods

Bacterial strains

All strains were derived from *E.coli* F26, a *his*$^-$ *thy*$^-$ derivative of *E.coli* B/r. BS21 is an *ada*^{c} derivative and BS23 an *ada*$^-$ *alk*B$^-$ derivative of F26 (Sedgwick and Lindahl, 1982; Rebeck *et al.*, 1988). A *uvr*B5 mutation (Backendorf *et al.*, 1986) was introduced into F26, BS21 and BS23 by P1 transduction from *E.coli* (Miller, 1972).

Cell growth and exposure to MeNU and EtNU

All strains were grown with aeration at 37°C in M9 minimal medium (Miller, 1972) supplemented with histidine (40 μg/ml), thymine (4 μg/ml) and glucose (0.2%). For each DNA repair kinetics, four batches of 150 ml log-phase cells (5 × 10^8 cells/ml) were harvested by centrifugation at room temperature (4000 *g* for 5 min), resuspended in 13.5 ml prewarmed growth medium and exposed to EtNU (10 mg/ml) or MeNU (4 mg/ml) for 10 min at 37°C with aeration. MeNU (40 mg/ml) and EtNU (100 mg/ml) were dissolved fresh each time in 50% ethanol. The cells were removed from the *N*-nitrosamide containing medium by centrifugation, resuspended in 150 ml prewarmed M9 growth medium and incubated with aeration for

0, 30, 60 and 120 min. At these times each 150 ml batch of cells was chilled on ice and the cells harvested by centrifugation at 4°C. The cell pellets were quickly frozen in liquid nitrogen and stored at −20°C until DNA isolation.

Cell killing and mutation induction

At 0, 10, 20 and 30 min after the addition of MeNU or EtNU to log-phase bacteria (5×10^8/ml), 0.1 ml aliquots were withdrawn, diluted in M9 salts (without supplements) and plated on supplemented M9 plates, to measure cell killing. At the 0 and 10 min time points, 10 ml aliquots were removed, cells concentrated by centrifugation (2 min in an Eppendorf microfuge) and resuspension in 0.5 ml M9 salts (unsupplemented). The frequency of *his*$^-$ revertants was determined by plating the appropriate dilutions on supplemented M9 plates and supplemented M9 plates lacking histidine.

Isolation of high mol. wt E.coli DNA

DNA isolation was by a modified procedure of Karran *et al.* (1980). Bacterial cells were resuspended in 400 μl saline−EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0) at 0°C. The cell suspension was incubated for 20 min at 0°C in lysozyme (1 mg/ml), then sarcosyl was added to 0.5% and Tris−HCl (pH 9.0) added to 50 mM. Incubation at 0°C continued for a further 15 min, $NaClO_4$ was added to 0.5 M, and the mixture was extracted with an equal volume of chloroform:isoamyl-alcohol (24:1). The organic phase was re-extracted with 600 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Nucleic acids were ethanol precipitated from the combined aqueous phases and dissolved in 250 μl TE buffer. The RNA in this mixture was digested for 1 h at 37°C by RNase A (0.2 mg/ml) and RNase T_1 (200 U/ml) in the presence of 10 μM coroformycin (Calbiochem). DNA was precipitated from the mixture by the addition of sodium acetate (to 300 mM) and 180 μl ice cold isopropanol, redissolved in 150 μl sodium acetate (300 mM in TE buffer) and the DNA was reprecipitated with 90 μl isopropanol. The DNA pellet was washed twice with ice cold 70% ethanol, and dissolved in 100 μl TE buffer.

Immunological quantitation of O^6MeG, O^6EtG and O^4EtT residues in ssDNA

The concentration and purity of the isolated DNA samples was first determined by HPLC analysis after enzymatic hydrolysis to deoxynucleosides as described previously (Huh and Rajewsky, 1986). The concentrations of O^6EtG, O^6MeG and O^4EtT in the isolated DNA were determined using the immuno-slot-blot (ISB) technique described by Nehls *et al.* (1984). Briefly, standard curves were generated by blotting 3 μg of calf thymus DNA containing various known amounts of ethylated or methylated DNA onto nitrocellulose (NC) filters. The levels of DNA alkylation products in these standard DNAs were prevously determined by radioimmunoassay (RIA; Muller and Rajewsky, 1980). In this way a series of slots were blotted with DNA containing 1−50 fmol of O^6EtG, 0.5−20 fmol of O^4EtT and 50−250 fmol of O^6MeG. The particular mAbs used were ER-6 to detect O^6EtG (Rajewsky *et al.*, 1980), ER-01 to detect O^4EtT (Adamkiewicz *et al.*, 1982), and ER-7 to detect O^6MeG (Adamkiewicz *et al.*, 1984). Various dilutions of the *E.coli* DNA samples were blotted in the same way. Filters were first incubated with the specific mAbs, then with ^{125}I-labelled sheep anti-rat IgF(ab')$_2$ (Amersham, 1500 Ci/mmol). The NC filters were washed, dried and exposed to Kodak X-O mat AR film. After development the films were analysed by densitometry using an integrating TLC-Scanner (SC-930, Shimadzu). The concentrations of O^6EtG, O^6MeG and O^4EtT in DNA were calculated using calibration standards blotted onto the same NC filter. Determinations were made at least twice for each DNA sample. Corrections were made for the dilution of adducts in DNA due to DNA replication, as judged by the increase in cell number during the 2-h period of incubation. Concentrations of specific DNA alkylation products were expressed as the number of a given alkylation product per bacterial genome equivalent (assuming 4×10^{-15} g DNA per genome).

Measurement of in vitro transfer of alkyl residues from DNA alkylphosphotriesters to the E.coli Ada protein

Poly(dT) (175 μg) in 0.2 M EPPS (pH 8.6) was alkylated for 2 h at 37°C with either [^{3}H]MeNU (Amersham, 1 mCi/ml, 1.75 Ci/mmol) or [^{3}H]-EtNU (NEN, 1 mCi/ml, 2.58 Ci/mmol) as described by Yarosh *et al.* (1985). O^4AlkylT was removed by 30 min hydrolysis at 70°C in 0.1 N HCl, leaving O^4-alkylphosphotriesters intact (Teo *et al.*, 1986). The poly(dT) was then annealed to poly(dA) and used as substrate in the methyltransfer reactions. All O^4AlkylT residues had been removed from the alkylated poly(dT) as confirmed by the lack of transfer of alkyl groups from the substrate to the purified 19 kd fragment of the Ada protein which bears only the active site for the methyl group transfer from O^6MeG and O^4MeT but not from MePT (Teo *et al.*, 1986); these data are not shown. We had previously shown that the intact 39 kd Ada protein is quite stable when expressed in HeLa S3-9 cells in cell culture (Samson *et al.*, 1986). Because the Ada protein is, in our hands, more stable in HeLa S3-9 cell extracts than in *E.coli* extracts, HeLa S3-9 extracts were used as a source of the intact Ada protein. Extract proteins (3 mg) were incubated for 10 min with ethylated (1000 c.p.m.) or methylated (800 c.p.m.) DNA substrate. Cell extract proteins were then separated by 12% PAGE, and the gel was cut into 2 mm slices. The gel slices were incubated overnight at 55°C in non-aqueous scintillation fluid containing 5% Protosol (NEN) and then assayed for tritium radioactivity by liquid scintillation spectrometry. Note that there is no transfer of alkyl groups to any protein species in extracts of HeLa S3 cells that do not express the Ada protein (Samson *et al.*, 1986).

Acknowledgements

We thank John Cairns and Eric Eisenstadt for helpful discussion, and Sarah Crane who performed some initial experiments in this study. This work was supported by American Cancer Society Research Grant NP448 and National Institute of Environmental Health Science Grant 1-P01-ES03926. L.S. was supported by an American Cancer Society Scholar Award and then an American Cancer Society Faculty Research Award. J.T. and M.F.R. were supported by the Deutsche Forschungsgemeinshafft (SFB 102/A7).

References

Adamkiewicz,J., Drosdziok,W., Eberhardt,W., Langenberg,U. and Rajewsky,M.F. (1982) In Bridges,B.A., Butterworth,B.E. and Weinstein,I.B. (eds), *Indicators of Genotoxic Exposure. Banbury Report 13.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 265−276.

Adamkiewicz,J., Ahrens,O., Huh,N., Nehls,P., Rajewesky,M.F. and Speiss,E. (1984) In *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer*, IARC Scientific Publication No. 57. IARC, Lyon, pp. 581−587.

Adamkiewicz,J., Eberle,G., Huh,N., Nehls,P. and Rajewsky,M.F. (1985) *Environ. Health Persp.*, **62**, 49−56.

Backendorf,C., Spaink,H., Barbeiro,A.P. and van der Putte,P. (1986) *Nucleic Acids Res.*, **14**, 2877−2890.

Demple,B., Jacobsson,A., Olsson,M., Robins,P. and Lindahl,T. (1982) *J. Biol. Chem.*, **257**, 13776−13780.

Evensen,G. and Seeberg,E. (1982) *Nature*, **296**, 773−775.

Foote,R.S., Mitra,S. and Pal,B.C. (1980) *Biochem. Biophys. Res. Commun.*, **97**, 654−659.

Friedberg,E. (1985) *DNA Repair*. W.H.Freeman Co., NY.

Gaffney,B.L., Marky,L.A. and Jones,R.A. (1984) *Biochemistry*, **23**, 5686−5691.

Huh,N. and Rajewsky,M.F. (1986) *Carcinogenesis*, **7**, 435−439.

Jeggo,P. (1979) *J. Bacteriol.*, **139**, 783−791.

Jeggo,P., Defais,M., Samson,L. and Schendel,P. (1977) *Mol. Gen. Genet.*, **157**, 1−9.

Karran,P., Hjelmgren,T. and Lindahl,T. (19827 *Nature*, **296**, 770−773.

Karran,P., Lindahl,T., Ofsteng,I., Evensen,G.B. and Seeberg,E. (1980) *J. Mol. Biol.*, **140**, 101−127.

Lindahl,T. (1982) *Annu. Rev. Biochem.*, **51**, 61−87.

Lindahl,T. (1988) *Annu. Rev. Biochem.*, in press.

Lindahl,T., Demple,B. and Robins,P. (1982) *EMBO J.*, **1**, 1359−1363.

Loechler,E.L., Green,C.L. and Essigman,J.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6271−6275.

McCarthy,T.V. and Lindahl,T. (1985) *Nucleic Acids Res.*, **13**, 2683−2698.

McCarthy,T.V, Karran,P. and Lindahl,T. (1984) *EMBO J.*, **3**, 545−550.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, NY.

Mitra,S., Pal,B.C. and Foote,R.S. (1982) *J. Bacteriol.*, **152**, 534−537.

Muller,R. and Rajewsky,M.F. (1980) *Cancer Res.*, **40**, 887−896.

Muller,R. and Rajewsky,M.F. (1981) *J. Cancer Res. Clin. Oncol.*, **102**, 99−113.

Nakabeppu,Y. and Sekiguchi,M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6297−6301.

Nakabeppu,Y., Mine,Y. and Sekiguchi,M. (1985) *Mutat. Res.*, **146**, 155−167.

Nehls,P., Adamkiewicz,J. and Rajewsky,M.F. (1984) *J. Cancer Res. Clin. Oncol.*, **108**, 23−29.

Olsson,M. and Lindahl,T. (1980) *J. Biol. Chem.*, **255**, 10569−10571.

Patel,D.J., Shapiro,L., Kozlowski,S.A., Gaffney,B.L. and Jones,R.A.

(1985) *Biochemie*, **67**, 861–886.
Patel,D.J., Shapiro,L., Kozlowski,S.A., Gaffney,B.L. and Jones,R.A. (1986a) *Biochemistry*, **25**, 1027–1036.
Patel,D.J., Shapiro,L., Kozlowski,S.A., Gaffney,B.L. and Jones,R.A. (1986b) *Biochemistry*, **25**, 1036–1042.
Patel,D.J., Shapiro,L., Kozlowski,S.A., Gaffney,B.L. and Jones,R.A. (1986c) *J. Mol. Biol.*, **188**, 677–692.
Potter,,P.M., Wilkinson,M.C., Fritton,J., Carr,F.J., Brennabd,J., Cooper,D.P. and Margison,G.P. (1987) *Nucleic Acids Res.*, **15**, 9177–9193.
Preston,B.D., Singer,B. and Loeb,L.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8501–8505.
Preston,B.D,. Singer,B. and Loeb,L.A. (1987) *J. Biol. Chem.*, **262**, 13821–13827.
Rajewsky,M.F., Muller,R., Adamkiewicz,J. and Drosdziok,W. (1980) In Pullman,B., Ts'o,P.O.P. and Gelboin,H. (eds), *Carcinogenesis: Fundamental Mechanisms and Environmental Effects*. D.Reidel, Dordrecht, pp. 207–218.
Rebeck,G.W., Coons,S., Carroll,P. and Samson,L. (1988) *Proc. Natl. Acad. Sci. USA*, in press.
Robins,P. and Cairns,J. (1979) *Nature*, **280**, 74–76.
Samson,L. and Cairns,J. (1977) *Nature*, **367**, 281–283.
Samson,L., Derfler,B. and Waldstein,E.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5607–5610.
Sancar,A. and Rupp,W.D. (1983) *Cell*, **33**, 249–260.
Schendel,P.F. and Robins,P.E. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 6017–6020.
Sedgwick,B. and Lindahl,T. (1982) *J. Mol. Biol.*, **154**, 169–175.
Teo,I., Sedgwick,B., Demple,B., Li,B. and Lindahl,T. (1984) *EMBO J.*, **3**, 2151–2157.
Teo,I., Sedgwick,B., Kilpatrick,M.W., McCarthy,T.V. and Lindahl,T. (1986) *Cell*, **45**, 315–324.
Van Houten,B. and Sancar,A. (1987) *J. Bacteriol.*, **169**, 540–545.
Volkert,M.R. and Nguyen,D.C. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4110–4114.
Warren,W. and Lawley,P.D. (1980) *Carcinogenesis*, **1**, 67–78.
Weiss,B. and Grossman,L. (1987) *Adv. Enzymol.*, in press.
Yarosh,D.B., Fornace,A.J. and Day,R.S. (1985) *Carcinogenesis*, **6**, 949–953.

Received on March 4, 1988; revised on April 26, 1988