Discriminated Induction of SOS Functions in *Escherichia coli* by Alkylating Agents

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(Received 24 November 1982)

Treatment of *Escherichia coli* with the alkylating agents diethyl sulphate, ethyl methane-sulphonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produces a different pattern of expression of SOS functions. There is a full induction of *recA*-dependent inhibition of cell respiration, a slight induction of lambda prophage, and no inhibition of cellular division. In a comparative study with bleomycin, an agent which is able to induce these three SOS functions, we have also shown that the differences in expression of SOS functions are not due to any variation in the pattern of DNA synthesis, or DNA degradation after treatment with alkylating agents. These results suggest that the kind of damage induced in the DNA may be important in determining which SOS function is expressed.

INTRODUCTION

Structural damage to cellular DNA by UV and ionizing radiations and chemical mutagens induces a set of cellular activities. These inducible functions, called SOS functions, include prophage induction, DNA repair and mutagenesis, filamentous cell growth, inhibition of cell respiration and massive synthesis of the RecA protein (Radman, 1975; Witkin, 1976). This pleiotropic response depends on the RecA⁺ and LexA⁺ functions. Genetical and biochemical studies have established that LexA protein is the repressor of the *recA* gene and certain SOS operons. RecA protein is a protease which is activated by an inducing signal after DNA damage. In these conditions, RecA protein inactivates, by cleaving, a whole series of sensitive repressor proteins such as lambda prophage repressor (Roberts *et al.*, 1978) and the product of the *lexA* gene (Little *et al.*, 1980). Recently it has been shown that genes *uvrA* and *uvrB*, involved with gene *uvrC* in the nucleotide excision repair, are controlled by the genes *lexA* and *recA* (Kenyon & Walker, 1981; Schendel *et al.*, 1982). In addition to the action of physical and chemical agents upon DNA replication, the SOS system is induced at the restrictive temperature in both temperature-sensitive *dnaB* and temperature-sensitive ligase mutants (Caillet-Fauquet & Defais, 1977; Condra & Pauling, 1982). Oishi & Smith (1978) have also shown that elevated dNTP concentrations can induce ϕ 80 prophage in permeabilized cells. Several authors (Little & Hanawalt, 1977; Bockrath & Hanawalt, 1980) have reported that no causal relationship exists between the production of DNA fragments by degradation and induction of SOS functions after UV radiation. Nevertheless, it has also been shown that induction of the SOS system by some inducing agent such as bleomycin (Gudas & Pardee, 1976) or nalidixic acid (Gudas & Pardee, 1976; Crowl *et al.*, 1978) is dependent on DNA degradation products. Thus, the question of the importance of DNA degradation in expression of the SOS system remains open.

Treatment of nucleic acids with the alkylating agents ethyl methanesulphonate (EMS), diethyl sulphate (DES) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) produces a wide variety of modifications of the bases (Singer, 1976). The large number of products, many of which are unstable, hampers the identification of the specific modification responsible for mutagenesis. Many current studies have focused on the miscoding properties of *O*⁶-alkylguanine

which was predicted to play a role in G : C $\rightarrow$ A : T transitions (Loveless, 1969), and on *O*⁴-alkylthymine, which has been predicted to play a role in A : T $\rightarrow$ G : C transitions (Lawley *et al.*, 1973). Nevertheless, recent studies of the mutagenic specificity of EMS (Coulondre & Miller, 1977) have indicated a 10- to 100-fold higher frequency of G : C $\rightarrow$ A : T transitions than A : T $\rightarrow$ G : C transitions, and although other interpretations are possible, this asymmetry is consistent with the view that guanine is the base most likely to mispair. Similar results have been found with DES (Singer, 1975) and MNNG (Lawley, 1974). Up to the present the effect of DES, EMS and MNNG in the induction of SOS response has not been extensively studied: the mutagenic properties of EMS are largely independent of *recA* gene, whereas those of MNNG depend partly upon this same gene (Kondo *et al.*, 1970). The capacity of DES for inducing lambda prophage is higher than that of EMS, even though it is still very weak (Moreau & Devoret, 1977; Hussain & Ehrenberg, 1975). These observations led us to study the expression in *Escherichia coli* of three SOS functions: induction of prophages, inhibition of cell division and inhibition of cell respiration after treatment with DES, EMS and MNNG. We chose these compounds because they produce mainly ethylations (DES) (Lawley, 1966), ethylations and methylations (EMS) (Lawley, 1974) and methylations (MNNG) (Lawley & Thatcher, 1970). Our results show the existence of a discrimination in the expression of three SOS functions tested after treatment with these alkylating agents. The possible causes and implications of this phenomenon are discussed.

METHODS

Bacteria and phage. Bacterial strains used in this work are listed in Table 1. They are all *Escherichia coli* K12 derivatives. The phage used was the temperature sensitive mutant λ cIts857 (Sussman & Jacob, 1962).

Media and growth conditions. Cultures were grown at 32 °C or 37 °C with shaking. The rich medium used was LB (Miller, 1972). The minimal medium was AB (Clark & Maaløe, 1967) supplemented with thiamin (10 µg ml⁻¹), glucose (0.2%, w/v), and required amino acids (20 µg ml⁻¹). When necessary 25 µg thymidine ml⁻¹ was added.

UV irradiation and mutagenic treatment. Cultures were grown to 2×10^8 cells ml⁻¹ in supplemented AB medium; at this point, the mutagen was added and incubation was continued at 37 °C for 15 or 30 min. The cells were pelleted by centrifugation washed in 10 mM-MgSO₄, resuspended in the same volume of AB supplemented medium, and incubated again at 37 °C. For UV treatment, cultures were grown to 2×10^8 cells ml⁻¹ in supplemented AB medium, and were irradiated in a glass Petri dish (10 cm diameter) with a General Electric GY1578 germicidal lamp, at a rate of 0.5 J m⁻² s⁻¹ (determined with a Latarjet dosimeter). After irradiation cells were centrifuged, resuspended in the same volume of supplemented AB medium, and incubated at 37 °C. At regular intervals filamentation and oxygen consumption were determined. Cultures treated with MNNG were grown in AB supplemented medium and adjusted to pH 5.5, since this pH is optimal for MNNG action (Miller, 1972). When EMS or DES were used, 0.1 ml dimethyl sulphoxide (DMSO), containing a sufficient volume of the mutagen to give the desired final concentration, was added to 4.9 ml of bacterial suspension. To make the results comparable, concentrations of each compound and UV radiation fluence used were those causing a 90% decrease in cell viability in RecA⁺ strain 30 min after the product was added.

Determination of cell size. Cell size was determined with a Coulter counter model ZBI equipped with a 30 µm orifice. An attenuation setting of 1, aperture current setting of $\frac{1}{4}$ and a lower threshold of 5 were used for all counts. Periodically, culture samples of 0.5 ml were added to 25 ml of filtered Isoton II (Coultronics). These samples were appropriately diluted in minimal medium to keep the particle counts between 7000 and 50000 per 50 µl.

Respiration. After treatment with each compound, oxygen consumption of a 2 ml sample of cells in AB supplemented medium was measured with a Gilson differential respirometer, at 37 °C.

Measurement of lambda induction. Strain UA2651 was grown in supplemented medium to 2×10^8 cells ml⁻¹. At this point, the culture was diluted to 2×10^4 cells ml⁻¹ and 200 µl samples of this dilution were distributed into tubes containing 5 µl DMSO (for DES and EMS) or phosphate buffer (for MNNG and bleomycin) with increasing amounts of the compound being tested. Each preparation was gently agitated at 32 °C for 25 min and poured on to LB plates plus ampicillin with 3 ml of soft agar and 0.2 ml of GY4015 indicator bacteria. For determination of UV induction, the cells, after suitable dilution, were irradiated with various doses of UV radiation and plated in the same way. To evaluate the toxic effect of the treatment on lambda induction, a parallel set of cell samples, following UV irradiation or mutagen addition, were incubated before and after plating at 42 °C since at this temperature cells lysogenic for λ cIts857 are induced. Total induction was calculated with respect to the phage survival at 42 °C after each mutagen treatment (Moreau & Devoret, 1977). Plates were counted after 12 h incubation at 32 ° or 42 °C. Total viable cells were also determined for the untreated controls by plating samples on LB-plates and incubation at 32 °C. These viable counts were used to calculate the percentage of cells induced to produce infective centres.

Table 1. *Strains of Escherichia coli* used

Strain	Genotype	Source and reference
AB1157	<i>argE3 his4 thr1 proA2 leu6 thi1 supE44 strA</i>	M. Blanco (Howard-Flanders & Theriot, 1962)
AB2497	as AB1157, <i>thyA</i>	M. Blanco (Howard-Flanders <i>et al.</i> , 1966)
AB2463	as AB1157, <i>recA13</i>	M. Blanco (Howard-Flanders, 1967)
UA2651	as AB1157, lysogenic for λ Its857	This work
GY4015	<i>thr1 leu6 thi1 supE44 lacY ampA</i>	R. Devoret (Moreau <i>et al.</i> , 1976)

DNA degradation. Overnight cultures of strain AB2497 were diluted 1:50 into fresh supplemented AB medium and grown to exponential phase. Then DNA was labelled over several generations by the addition of [3 H]thymidine [$3 \mu\text{Ci ml}^{-1}$, specific activity 26 Ci mmol^{-1} ($960 \text{ GBq mmol}^{-1}$)]. Bacteria were washed with AB medium and grown in non-radioactive AB supplemented medium for one doubling time. At this point, the mutagen was added, and 1 ml samples were removed at various times and TCA soluble material was measured according to Buttin & Wright (1968).

DNA synthesis. Incorporation of [3 H]thymidine ($3 \mu\text{Ci ml}^{-1}$, specific activity 26 Ci mmol^{-1}) by strain AB2497 was used to measure continuous DNA synthesis after the addition of mutagenic compounds. In experiments to measure the rate of DNA synthesis following product addition, 1 ml samples were withdrawn at the times indicated and were pulse-labelled for 3 min with $3 \mu\text{Ci}$ [3 H]thymidine.

Chemicals. Thymidine, EMS, DES and MNNG were obtained from Sigma and bleomycin from Almirall Laboratories (Barcelona, Spain). Amino acids, vitamins and mineral salts were purchased from Merck, and tryptone, yeast extract and agar from Oxoid. Radiochemicals were obtained from Amersham. Ampicillin was a gift from Antibióticos de León (León, Spain).

RESULTS

Inhibition of cell division

Changes of cell volume of RecA^+ and RecA^- bacteria after UV irradiation and 15 or 30 min incubation with bleomycin are shown in Figs 1 and 2. Under these conditions, an increase in cell

Fig. 1. Distribution of cell size of a non-irradiated RecA^+ culture (a), of a RecA^+ culture (b) and of a RecA^- culture (c) after UV irradiation at 60 J m^{-2} . Measurements were made at intervals after the treatment.

Fig. 2. Distribution of cell size after bleomycin treatment in *RecA*⁺ (a, b) and *RecA*⁻ (c) strains of *E. coli*. Cells were treated with bleomycin for 15 min (a) or 30 min (b, c). After this, cultures were washed and resuspended in fresh supplemented AB medium, taking this moment as *t* = 0. Concentration of bleomycin was 20 $\mu\text{g ml}^{-1}$ in both *RecA*⁺ and *RecA*⁻ strains.

volume of the *RecA*⁺ cultures occurred, but neither UV radiation (Fig. 1c) nor bleomycin (Fig. 2c) gave rise to any variation in the cell volume of *RecA*⁻ cultures, as might be expected for a SOS function. However, the *RecA*⁺ culture treated with bleomycin showed a progressive recovery towards normal cell volumes. This recovery occurred earlier in cultures treated with bleomycin for 15 min rather than 30 min.

In contrast with these results, 15 or 30 min treatment with the alkylating agents DES, EMS and MNNG did not induce any filamentation (data not shown). Even after continuous exposure to these compounds there was no division inhibition (Fig. 3). Under the same conditions, bleomycin caused a progressive increase in cell volume (Fig. 3d).

Inhibition of respiration

Cessation of respiration after UV irradiation in *E. coli* is an induced response dependent upon the *recA* and *lexA* gene products (Swenson & Schenley, 1974), although the effect of alkylating agents on this SOS function has not been reported. Respiration of cultures of *RecA*⁺ and *RecA*⁻ strains after 15 min or 30 min treatment with DES, EMS, MNNG and bleomycin, and using UV radiation as a positive control, is shown in Fig. 4. In the *RecA*⁻ bacteria the cessation of respiration was not induced either after UV irradiation, or by the four chemicals, whereas in the *RecA*⁺ strain all treatments inhibited respiration. Furthermore, this inhibition of respiration was reversible, since in cultures treated with the chemicals for both 15 min and 30 min there was a progressive recovery of respiration about 120 and 150 min, respectively, after the compounds were removed. Respiration also recovered about 120 min after irradiation in UV-exposed cultures.

When treatment with bleomycin and alkylating agents was continued, there was a prolonged inhibition of respiration in *RecA*⁺ bacteria, but not in *RecA*⁻ mutants (Fig. 5), for at least 3 h after these products were added. These results show that EMS, DES and MNNG were able to induce the *recA*-dependent inhibition of cell respiration.

Fig. 3. Distribution of cell size of a *RecA*⁺ strain of *E. coli* after continuous treatment with DES (a), EMS (b), MNNG (c) and bleomycin (d). Concentrations of each product were: DES, 3 $\mu\text{l ml}^{-1}$; EMS, 30 $\mu\text{l ml}^{-1}$; MNNG, 20 $\mu\text{g ml}^{-1}$; bleomycin, 20 $\mu\text{g ml}^{-1}$.

Lambda prophage induction

DES produced a weak induction of prophages in *RecA*⁺ lysogens although it was greater than the response elicited by MNNG and EMS, which induced practically no lambda prophages (Fig. 6). In contrast bleomycin and UV radiation gave rise to an increase in prophage induction, showing that the alkylating agents were very poor inducers of lambda prophage in *RecA*⁺ strains. This cannot be due to any inhibition of replication and/or maturation of the bacteriophage, since with a lambda temperature sensitive *cI* mutant, *λcIts857*, we did not find significant variations in temperature-dependent induction regardless of the presence or absence of mutagens.

Synthesis and degradation of DNA

We have tested the possibility that the discrimination in the *recA*-dependent expression of respiration inhibition, filamentation and induction of prophages produced by DES, EMS and MNNG was due to a differential effect of these alkylating agents on the replication and degradation of chromosomal DNA. DNA degradation was measured by solubilization of DNA in TCA, after treatment of strain AB2497 with EMS, DES, MNNG and bleomycin (Fig. 7). The four agents gave rise to similar levels of DNA degradation (about 40%) 60 min after addition of the products, showing that the discrimination in the induction of three SOS functions was not due to a different degree of DNA degradation.

Fig. 4. Effect of treatment with bleomycin (a), DES (b), EMS (c) and MNNG (d) for 15 or 30 min on the inhibition of respiration of RecA⁺ and RecA⁻ strains of *E. coli*. In both RecA⁺ and RecA⁻ strains concentrations of each product were: bleomycin, 20 μg ml⁻¹; DES, 3 μl ml⁻¹; EMS, 30 μl ml⁻¹; MNNG, 20 μg ml⁻¹. Open symbols represent 15 min of treatment and filled symbols 30 min. The RecA⁻ strain is represented with the same symbol (▲) in all cases. As a control, respiration of RecA⁺ (▼) and RecA⁻ (▽) strains after UV irradiation at 60 J m⁻² and RecA⁺ strain (□) without any treatment is shown in panel (a). Further details are given in Methods.

Fig. 5. Effect of continuous treatment with bleomycin (a), DES (b), EMS (c) and MNNG (d) on the inhibition of respiration of RecA⁺ and RecA⁻ strains of *E. coli*. In both RecA⁺ and RecA⁻ strains concentrations of each product were: bleomycin, 20 μg ml⁻¹; DES, 3 μl ml⁻¹; EMS, 30 μl ml⁻¹; MNNG, 20 μg ml⁻¹. Open symbols represent the RecA⁻ strain and filled symbols the RecA⁺ strain. Respiration of a culture without treatment (■) is shown in panel (a).

DNA synthesis was measured in the same AB2497 strain by two methods, continuous synthesis (Fig. 8) and pulse labelling (Fig. 9). Both figures show that bleomycin and EMS produced a stronger inhibition of DNA synthesis than did DES and MNNG, suggesting that the cause of the discrimination in the expression of SOS functions is not to be found in a different level of inhibition of DNA synthesis. Although the effect of EMS on chromosome replication was greater than that of DES and MNNG, all three compounds presented the same pattern of SOS induction.

Fig. 6. Induction of lambda prophage by DES, EMS, MNNG, bleomycin and UV radiation. Bacteria of strain UA2651 were treated as described in Methods with increasing doses of: (a) bleomycin (●) and MNNG (▽); or (b) UV radiation (□), DES (▲), and EMS (○).

Fig. 7. DNA degradation following treatment with 20 µg bleomycin ml⁻¹ (△), 3 µl DES ml⁻¹ (●), 30 µl EMS ml⁻¹ (□) and 20 µg MNNG ml⁻¹ (○); a control culture without treatment (▽).

DISCUSSION

We have presented evidence to indicate that the alkylating agents EMS, DES and MNNG can trigger *recA*-dependent inhibition of respiration but not inhibition of division and only a very slight induction of the lambda prophage. The *recA*-dependent inhibition of respiration induced by the alkylating agents tested was reversible when the mutagen had been present for 15 or 30 min (Fig. 4). The same occurred for filamentation (Fig. 2) and inhibition of respiration (Fig. 4) induced by bleomycin. This finding might be attributed to repair of damage produced in the DNA and, consequently, to loss of the signal that triggers the expression of these SOS functions. This could be analogous to the sequence of events taking place after UV irradiation (Swenson & Schenley, 1974).

Defais *et al.* (1980) have reported that exposing *E. coli* cells to sublethal doses of alkylating agents (a process called adaptation) inhibits to some extent the UV and *tif-1* mediated induction of SOS functions, such as Weigle reactivation and lambda prophage induction. Furthermore, they showed that unadapted cells do not express as much Weigle reactivating activity after MNNG treatment as after UV irradiation. Their results, together with data presented in this work, suggest the existence of a discrimination in the expression of SOS functions. This

Fig. 8. Continuous DNA synthesis in *E. coli* AB2497 growing in AB supplemented medium during treatment with 3 μ l DES ml^{-1} ($\blacktriangle$), 20 μ g MNNG ml^{-1} ($\square$), 20 μ g bleomycin ml^{-1} ($\triangle$) and 30 μ l EMS ml^{-1} ($\blacksquare$). Cells were labelled with [^3H]thymidine (3 $\mu\text{Ci ml}^{-1}$; specific activity 26 Ci mmol^{-1}) and mutagens were added at $t = 0$. Radioactivity incorporation in a control culture without treatment is also shown ($\bullet$).

Fig. 9. Rate of incorporation of [^3H]thymidine (3 $\mu\text{Ci ml}^{-1}$, specific activity 26 Ci mmol^{-1}) during treatment of *E. coli* AB2497 with 3 μ l DES ml^{-1} ($\bullet$), 20 μ g MNNG ml^{-1} ($\circ$), 20 μ g bleomycin ml^{-1} ($\square$) and 30 μ l EMS ml^{-1} ($\triangle$). Mutagens were added to exponentially growing cultures in AB supplemented medium at $t = 0$. Samples (1 ml) were withdrawn at the times indicated and pulse-labelled with [^3H]thymidine.

phenomenon is in agreement with our previous study (Guerrero & Barbé, 1982) where we showed that, in *Salmonella typhimurium*, thymine starvation can induce prophages and filamentation but not inhibition of respiration. In a related study, Hutchinson & Stein (1980) found that thymine starvation did not produce Weigle mutagenesis in *E. coli*.

Smith & Oishi (1978) have reported that several SOS inducing agents or treatments produce different kinetic pattern of inactivation of $\phi 80$ prophage repressor molecules, showing that the time needed for $\phi 80$ repressor inactivation is correlated with the timing of appearance of treatment-induced DNA degradation. Nevertheless, the data presented here show that DES, EMS and MNNG produce the same level and kinetics of DNA degradation as bleomycin, for a similar degree of cell survival after addition of the compound (Fig. 7). Since bleomycin is an efficient SOS inducer, and DES, EMS and MNNG are not, we conclude that the discrimination reported here is not due to different levels of DNA degradation.

Recently, Casaregola *et al.* (1982) have shown that DNA replication antagonizes SOS induction. So it would be possible to think that the discrimination in the expression of SOS functions after DES, EMS and MNNG treatment could be due to a pattern of DNA synthesis different from that produced by bleomycin, but Fig. 8 and Fig. 9 show that EMS and bleomycin inhibit DNA synthesis to the same extent in both continuous and pulse synthesis experiments.

Therefore our data show that, independent of both DNA degradation and DNA synthesis inhibition levels, there must exist a pathway of expression of some SOS functions while other SOS responses remain repressed. We suggest that the molecular basis for this discrimination could be based on the kind of lesion produced in the DNA. Craig & Roberts (1980) have proposed that the RecA protein could be activated to bind at single-stranded regions (gaps) of the chromosomal DNA originated by interruption of replication due to pyrimidine dimers caused by UV irradiation. If the gaps on the DNA were generated by the action of alkylating agents, correct linkage between the single-strand fragment and the RecA protein could be hindered by the alkylations still present in those fragments. This might then prevent the normal activation of the proteolytic function of the RecA protein and consequently explain the expression of only *recA*-dependent inhibition of respiration induced by EMS, DES and MNNG. Using antipain (a protease inhibitor), Swenson & Schenley (1978) have shown that this SOS function is independent of the proteolytic activity of the RecA protein, while lambda induction, UV mutagenesis, and filamentous growth are dependent (Meyn *et al.*, 1977). There are at least two systems in which inhibitory effects of DNA modification on DNA-protein interactions have been unambiguously demonstrated: (i) at sequences specifically modified by DNA methyltransferases, where restriction endonucleases are not able to cleave double-stranded DNA (Arber, 1974); (ii) when *lac* repressor binding to DNA is affected by alterations in the major groove of DNA, e.g. by 5-bromodeoxyuridine (Lin & Riggs, 1972). Thus, there cannot be any doubt that modified bases at strategic positions in specific DNA sequences can have an important effect on DNA-protein interactions. Irbe *et al.* (1981) found that only oligodeoxyribonucleotides composed of deoxyguanilates are able to trigger induction of prophage $\phi 80$ in a permeable *E. coli* cell preparation. Their results are in agreement with our hypothesis, because it is known that the major biological effects of EMS, DES and MNNG on DNA are alkylations in guanine residues (for a review, see Hoffman, 1980). However, further studies of the interaction between RecA protein and DNA affected by several kinds of lesions, as well as the activation of RecA protein under these conditions, are needed to test this hypothesis for the mechanism of the differing patterns of SOS functions expression induced by the alkylating agents EMS, DES and MNNG.

We thank Dr M. Blanco for his generous gift of several strains. We acknowledge the help of the *Fundació M.F. de Roviralta* for a grant for the purchase of equipment. This work was supported by a grant from the *Comisión Asesora de Investigación Científica y Técnica*, Spain.

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