REACTIVE DERIVATIVES OF BENZO(A)PYRENE AND 7,12-DIMETHYLBENZ(A)ANTHRACENE CAUSE S_1 NUCLEASE SENSITIVE SITES IN DNA AND "UV-LIKE" REPAIR

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SUMMARY: Normally excising human fibroblasts and two strains of DNA repair deficient cells from xeroderma pigmentosum (XP) patients were exposed to the following: 7-bromomethylbenz(a)anthracene, benzo(a)pyrene 4,5-oxide, UV radiation, two 7,8-diol-9,10-epoxide isomers of benzo(a)pyrene, and N-methyl-N'-nitro-N-nitrosoguanidine. Except for the latter, each agent exhibited greater cytotoxicity in the XP strains than in the normal and the loss of cloning ability was greatest in the strain most deficient in excision and intermediate in the one with an intermediate rate of repair. Each agent which caused this differential survival also produced sites in native DNA which were susceptible to the action of the single-strand specific endonuclease S_1. These data suggest that each agent produces distortions (i.e., localized denaturation) in the DNA helix and that these distortions are recognized by the excision process operating in normal cells but deficient in XP.

INTRODUCTION:

We recently reported that normal human fibroblasts and cells from a series of classical XP patients exhibit sensitivity to the killing action of UV which is correlated with their capacity for excision repair of UV-induced DNA damage (1,2). This differential survival of the colony-forming capacity is also seen when these strains are exposed to reactive derivatives of a series of carcinogenic aromatic amides (3) and the "K-region" epoxides of BP, DMBA, DBA, and BA (4). Stich and his associates have shown that the abnormal sensitivity of the colony-forming capacity of another strain of XP cells to the latter epoxide (5)

Abbreviations: XP, xeroderma pigmentosum; UV, ultraviolet radiation; BP, benzo(a)pyrene; BP diol epoxide I, (±)-7α,8β,-dihydroxy-9β,10β,-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene; BP diol epoxide II, (±)-7β,8α-dihydroxy-9β,10β-tetrahydrobenzo(a) pyrene; 7-BrMeBA, 7-bromethylbenz(a)anthracene; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine, N-AcO-AAF, N-acetoxy-2-acetylaminofluorene; SSC, standard saline citrate (0.15 M NaCl, 0.017 M NaCitrate, pH 7.2).

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as well as to derivatives of 4NQO (6) is correlated with an inability to repair DNA damage caused by these agents, but that no such differential survival occurs with MNNG, a carcinogenic agent which XP cells can repair at the normal rate (7,8). X-ray damage is also repaired with equal efficiency in normal and XP cells (8).

The fact that XP cells are deficient in excision repair of damage introduced into DNA by certain carcinogens, but not by others, suggests that the former agents cause lesions in DNA which have a common property. Hayes et al. showed that UV radiation causes single-stranded regions in DNA approximately 14 nucleotides in length (9). Fuchs presented evidence of single-stranded regions in DNA to which large numbers of AAF residues had been covalently bound (10). Data by Fornace et al. (11) showed using their alkaline elution technique that classical XP cells, in contrast to normal cells, lack the ability to cleave large molecular weight DNA which contains UV-induced lesions into smaller molecular weight molecules or do so at a rate very much slower than normal, suggesting that XP cells are unable to recognize such distortions. It was, therefore, of interest to determine whether a series of agents which evoke a differential cytotoxicity response between normal and XP cells would cause distortions in the DNA helix (i.e., localized denaturation; single-stranded regions) which would be recognized by a single-strand specific endonuclease S_1.

MATERIALS AND METHODS:

Carcinogens: MNNG was purchased from Pfaltz and Bauer. N-AcO-AAF, BP 4,5-oxide, and the diol epoxides of BP were given to us by the National Cancer Institute. 7-BrMeBA was provided by Dr. Anthony Dipple, Frederick Cancer Research Center.

Cell Culture: The cell strains and culture conditions used have been described previously (3).

Cytotoxicity Studies: The cytotoxicity of the chemical carcinogens and UV radiation in normal and XP fibroblasts was determined from percent survival of colony-forming ability as described previously (3).

Labeling of DNA from Human Cells: Normal human fibroblasts (~1.5×10^6) were seeded into a 75 cm^2 culture vessel, allowed to attach, and grown for 48 hr in Eagle's MEM supplemented with 10% fetal calf serum and 10 μCi/ml [^{3}H]Tdr. The cells were detached by trypsinization, resuspended in 2.5 ml SSC and lysed in the presence of 2.5% diethyl pyrocarbonate by making the suspension 0.4% in

sodium lauryl sarcosine and incubating 10 min at 60°C. After a single extraction with chloroform/butanol (24:1), the DNA was treated with 1.25 µg pancreatic RNAse (Worthington, Freehold, NJ) and 50 units T_1 RNAse (Sigma, St. Louis, MO) for 30 min at 37°C, followed by incubation with 250 µg proteinase K (EM Laboratories, Elmsford, NY) for 60 min at 37°C. The DNA was extracted with chloroform/butanol until no material was found at the interface between the aqueous and organic phases. The DNA solution was dialyzed at 4°C for 24 hr against 3 changes of SSC. DNA concentration was determined by absorbance at 260 nm and adjusted to 0.5 µg/ml with SSC.

Carcinogen Treatment of DNA: Carcinogens, dissolved in 10 µl of freshly distilled acetone, were added to 100 µl aliquots of DNA solution and allowed to react for 30 min at 37°C. DNA was treated with UV radiation by slowly rocking 250 µl of DNA solution in a 35 mm culture dish under a model UVS12 254 nm UV source (Ultraviolet Products, San Gabriel, CA). The dose of UV radiation or chemical selected was ca. 150 times the amount required to reduce the cloning ability of normal cells from 50% down to 25% of untreated populations.

S_1 Nuclease Assay: To the treated DNA solution was added an equal volume of 3.4 mM $ZnCl_2$, 0.1 M NaCl, 0.06 M Na acetate buffer, pH 4.5, containing double stranded salmon sperm DNA (Sigma) (50 µg/ml) and heat denatured salmon sperm - DNA (10 µg/ml) and S_1 nuclease (Sigma) (20 units/ml). The mixture was incubated 2 hr at 50°C. At the end of the incubation, the size of the labeled DNA was determined by velocity sedimentation through neutral sucrose (5% to 20% in 50 mM Na phosphate buffer, pH 7.0). Labeled T7 phage DNA (M.W. 25.2 x 10^6 daltons) was included as a marker.

Calculation of Number of Double-Strand Breaks: DNA molecular weights were estimated using the expression, $D_1/D_2 = (M_1/M_2)^{0.38}$ (cf. refs. 12 and 13). The number of double-strand breaks induced by S_1 digestion of carcinogen treated DNA was calculated from the formula:

$$\text{No. of breaks} = \frac{\text{molecular weight untreated DNA}}{\text{molecular weight treated DNA}} - 1$$

RESULTS AND DISCUSSION:

Comparing the Cytotoxicity of the Agents in Normal and XP Strains: The percent survival of the colony-forming ability of normal human cells and two strains of XP cells, XP12BE with little or no measurable capacity for excision repair of UV-induced damage to DNA (complementation group A) and XP2BE with intermediate excision repair capacity (excision at 16% the normal rate, group C), were compared following exposure to low doses of a series of reactive derivatives or metabolites of BP and DMBA. The results are shown in Fig. 1. For comparative purposes, the survival curves for these particular strains following exposure to UV radiation and MNNG are also presented. It is evident that each of these agents, except MNNG, causes much greater cytotoxicity in the XP cells than in the normal. More significantly, the percent survival for these agents is correlated with the strain's capacity for excision repair of UV-induced damage.

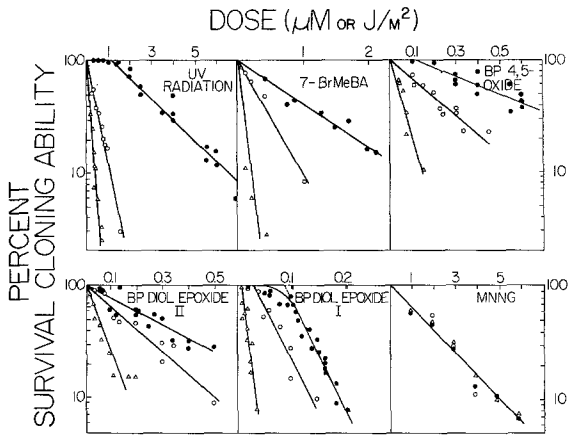

Fig. 1. Comparison of the cytotoxic effect of reactive derivatives of carcinogenic agents in normal human cells (●), XP2BE (○), and XP12BE (△). Cells were plated, allowed to attach, rinsed and irradiated, or exposed to carcinogen in serum-free medium for 3 hr, and then refed with culture medium 3 times weekly until macroscopic colonies developed. The cloning efficiency of the treated cells divided by that of the untreated controls receiving solvent only is expressed as a percent. Lines were fitted by eye on the shoulder of the curves and by the method of least squares for the curves lacking shoulders. Each symbol represents survival averaged from 8 to 10 duplicate dishes.

No such differential survival was found with exposure to the methylating agent, MNNG. It will be seen that there is a shoulder on the normal cell survival curve for UV, BP 4,5-oxide, and diol epoxide I. The presence of this shoulder can be indicative of repair, but its absence does not indicate lack of repair. For example, evidence of excision repair by normal human cells has been presented for 7-BrMeBA (14) and for N-AcO-AAF (3,15-17), an agent which causes differential killing in these strains (3) and yields survival curves very similar to those shown in Fig. 1 for 7-BrMeBA.

Evidence of S_1 Nuclease Susceptible Sites in DNA: Purified [^{3}H]Tdr-labeled human cell DNA was exposed to doses of each of the above agents and to N-AcO-AAF at doses which were ca. 150 times that which caused an equal cytotoxic effect on the cloning capacity of the normal cells. These doses were selected in an attempt to introduce approximately similar amounts of damage to the DNA. Such a choice assumes that the cytotoxic effect of exposure to each of these agents

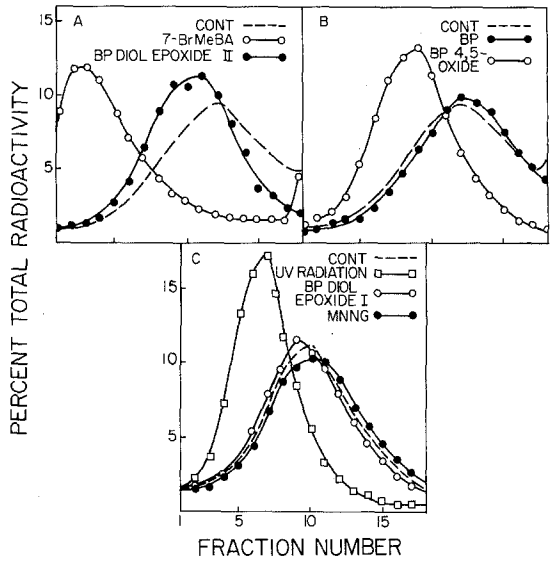

Fig. 2. Neutral sucrose gradient centrifugation profiles of [^{3}H]-labeled human cell DNA which has been exposed to the indicated agents and then treated with S_1 nuclease (see Materials and Methods). The gradients were centrifuged in a SW 50.1 rotor at 20°C for 16 hr at 22,000 rpm. Centrifugation is from left to right. Control DNA was exposed to all of the conditions of the assay, but without exposure to the radiation or chemical agent.

will reflect, at least to an approximation, damage to DNA. The treated DNA was then subjected to the enzymatic action of single-strand specific S_1 nuclease. As shown in Figure 2, treatment with 7-BrMeBA, BP 4,5-oxide, UV radiation, and BP diol epoxides I and II, each of which shows a greater cytotoxic effect in XP cells than in normally excising cells, caused S_1 sensitive sites in DNA, resulting in smaller-sized DNA. Table 1 compares the number of double-strand breaks produced by S_1 nuclease in DNA exposed to each of these agents and to N-AcO-AAF. Treatment of the DNA with MNNG and BP (at the highest concentration that will remain in solution) did not alter DNA size upon S_1 digestion. (The parent compound BP does not covalently bind to DNA and we have previously shown (4) that it does not cause a cytotoxic effect in normal or XP cells.) The effect of N-AcO-AAF was very similar to that produced by exposure to UV or BP 4,5-oxide. It is evident from Fig. 2 and Table 1 that the two diol epoxides

TABLE 1

Number of Double-strand Breaks Produced by S_1 Nuclease in Human DNA Exposed to Carcinogenic Radiation or Chemicals.

Treatment	Breaks per 12,000 base pairs
None	0.0
BP	0.0
MNNG	0.0
BP diol epoxide I	0.4
BP diol epoxide II	0.5
BP 4,5-oxide	1.7
N-AcO-AAF	2.2
UV radiation	2.4
7-BrMeBA	86.5

of BP do not cause as significant a change in the size of the DNA exposed to S_1 nuclease digestion as do 7-BrMeBA, UV, N-AcO-AAF, or BP 4,5-oxide. However, the slight S_1 sensitivity for diol epoxide I-treated DNA was reproducible and could be increased by using higher doses of the compound. No evidence of single-strand regions caused by exposure to MNNG could be detected and an increase in MNNG concentration did not alter the sensitivity of the DNA to nuclease attack.

The presence of areas of local denaturation in DNA treated with UV radiation has been well documented (9,18-20) and these denatured areas have been shown to be sensitive to the single-strand specific nuclease of N. crassa (20) and S_1 (21). N-AcO-AAF, an aromatic amide carcinogen, has also been shown to produce denatured areas in DNA (22-27) and S_1 susceptible sites (10,28). We have previously suggested (4) that the excision repair of UV-radiation induced pyrimidine dimers and polycyclic aromatic hydrocarbon DNA adducts is accomplished, at least in part, by a common pathway which is defective in XP cells and that the damage in DNA induced these agents causes a distortion in the DNA helix which is recognized by a common endonuclease or other protein involved in excision repair which is defective in XP cells. In this present work, we have demonstrated that treatments which result in differential cytotoxicity between normal and XP cells create S_1 susceptible sites in DNA and treatments which do

not result in differential cytotoxicity, do not produce S_1 susceptible sites. While the qualitative agreement is good, it should be noted that there is a wide variation in the number of S_1 susceptible sites produced by the various agents, even though an attempt was made to intorduce an equal number of DNA lesions. For example, 7-BrMeBA, causes a greater number of S_1 susceptible sites than might be predicted from its cytotoxic effect. This result may simply reflect differences in the number and types of DNA lesions produced by these agents at high concentrations. We are carrying out experiments utilizing radioactively-labeled carcinogen derivatives to quantitate the number and kind of lesions produced in DNA and recognized by S_1 nuclease.

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