Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 5933–5937, October 1980
Cell Biology

Error-free excision of the cytotoxic, mutagenic N^2-deoxyguanosine DNA adduct formed in human fibroblasts by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

(benzo[*a*]pyrene-diol epoxide/6-thioguanine-resistant mutants/xeroderma pigmentosum/DNA repair/diploid cells)

LI L. YANG, VERONICA M. MAHER, AND J. JUSTIN MCCORMICK

Carcinogenesis Laboratory—Fee Hall, Department of Microbiology and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Communicated by Elizabeth C. Miller, June 12, 1980

ABSTRACT **The ability of normal diploid human fibroblasts and excision repair-deficient xeroderma pigmentosum cells (XP12BE, complementation group A) to excise potentially cytotoxic or mutagenic lesions induced in DNA by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Bz*a*P-diol epoxide) was determined. Large populations of cells were prevented from replicating by being grown to confluence; after 3 days they were exposed to tritiated Bz*a*P-diol epoxide for 2 hr. One set of cultures was immediately released and assayed for the number of residues covalently bound to DNA, percent survival of colony-forming ability, and frequency of induced mutations. After various periods of time in confluence, other sets were similarly released and assayed. The normal cells exhibited a gradual increase in survival with time held in confluence (recovery from potentially cytotoxic lesions) which was directly correlated with a gradual loss of radioactivity from their DNA and a gradual decrease in the frequency of induced mutations. In contrast, no loss of radioactively labeled carcinogen from the DNA of the XP12BE cells could be detected during a 6-day period and their percent survival and frequency of induced mutations did not change. DNA from normal cells harvested immediately after treatment or after 2, 4, or 8 days in confluence was enzymatically hydrolyzed and analyzed by high-pressure liquid chromatography. Only a single peak was detected that cochromatographed with a standard prepared from deoxyguanosine treated with Bz*a*P-diol epoxide. The kinetics of decrease of tritium label in this specific peak corresponded to the decrease in radioactivity of the total DNA with time and with the kinetics of recovery of the cells from the potentially cytotoxic and mutagenic effects of Bz*a*P-diol epoxide. These results suggest that the N^2-deoxyguanosine adduct is responsible for these biological effects and indicate that excision repair of this lesion by the normal human cells is "error free."**

We have shown previously that diploid human skin fibroblasts derived from normal individuals are more resistant to the killing action of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (Bz*a*P-diol epoxide) than are cells derived from xeroderma pigmentosum (XP) patients (1, 2). A comparison of the frequency of mutations to 6-thioguanine resistance induced by Bz*a*P-diol epoxide indicates that normal fibroblasts are also significantly more resistant than XP cells to the mutagenic action of this compound (unpublished data). Indirect evidence suggests that this resistance results from the ability of normal fibroblasts to remove potentially cytotoxic and mutagenic adducts from their DNA at a much faster rate than XP cells (3–6). To investigate this question and to determine which Bz*a*P-diol epoxide–DNA adducts are responsible for these effects, we compared density-inhibited cultures of normal fibroblasts and XP cells for the kinetics of removal of these DNA adducts and of recovery from the potentially cytotoxic and mutagenic lesions induced. In addition, we determined the nature of the Bz*a*P-diol epoxide–DNA adducts formed in these cells.

MATERIALS AND METHODS

Chemicals. Bz*a*P-diol epoxide, obtained as an unlabeled solid or generally tritiated (270 or 400 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) and dissolved at 1.1 mg/ml in tetrahydrofuran/triethylamine, 19:1 (vol/vol), was provided by the Cancer Research Program of the National Cancer Institute and stored at −20°C in a desiccator. The labeled compound was evaporated to dryness by a gentle stream of N_2 just prior to use. The enzymes used to hydrolyze DNA (7) were purchased from Sigma; Sephadex LH-20 was from Pharmacia; CsCl was from ICN; and glass-distilled acetone, methanol, and ethyl acetate were from Burdick and Jackson (Muskegon, MI).

Cells and Culture Medium. Fibroblasts were derived from foreskin material or were obtained from the American Type Culture Collection (XP cells from patient XP12BE and 6-thioguanine-resistant cells from a Lesch Nyhan patient, CRL1112). Culture medium, medium depleted of mitogens ("spent medium"), and medium for selecting 6-thioguanine-resistant cells have been described (6).

Exposure of Cells to Bz*a*P-diol Epoxide. Cells were treated as confluent monolayers in 250-ml plastic flasks (Corning). The culture medium was first replaced with serum-free medium, pH ≈ 7.3. Bz*a*P-diol epoxide, freshly dissolved in anhydrous acetone (stored in the dark over molecular sieves), was delivered into the serum-free medium by micropipette and the medium was immediately inverted and allowed to cover the cells. After 2 hr this medium was removed and replaced with spent medium.

Cytotoxicity Assay. Immediately or after various periods of time in confluence, treated and untreated cell cultures were released by gentle trypsinization (0.25% trypsin, GIBCO), resuspended in fresh culture medium, diluted appropriately, plated at cloning densities (100–2000 cells per dish), and allowed to develop into colonies (6).

Mutagenicity Assay. At various times after exposure, confluent cultures of treated and untreated cells were trypsinized, resuspended in fresh culture medium, pooled, and plated into 150-mm-diameter dishes to allow expression of mutations. For each determination, sufficient numbers of dishes were used to ensure a minimum of 10^6 *surviving* target cells at the beginning of the expression period. The cells were maintained in exponential growth during expression by trypsinizing the popula-

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Abbreviations: Bz*a*P-diol epoxide, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; XP, xeroderma pigmentosum.

tion, pooling the total progeny, and replating at lower density as necessary. The number of population doublings was monitored with an electronic cell counter (Coulter), and the length of the expression period was adjusted for each determination so that the cells underwent a total of 5.5 population doublings before selection. At that time the progeny cells were trypsinized and pooled, and 2.5×10^6 cells were plated into selective medium at a density of 500 cells per cm^2 (≈90 dishes per point) or further diluted and plated at cloning densities in nonselective medium to determine cloning efficiency at the time of selection. Reconstruction experiments (6) with Lesch Nyhan cells, which accompanied each individual determination, indicated that the efficiency of recovery of resistant cells exceeded 70%.

Determination of Radioactivity Remaining Covalently Bound to Cellular DNA. At the same time that cells treated with labeled Bz*a*P-diol epoxide were released from confluence and assayed for survival or mutation frequency, a corresponding set of cells (≈$35–80 \times 10^6$) was lysed in sodium lauroyl sarcosine and the DNA was extracted (1) and purified by CsCl centrifugation (3). The $A_{260\,nm}$ profile of the gradient was determined, the DNA fractions were pooled and dialyzed against three changes of 0.15 M NaCl/20 mM sodium citrate, pH 7.4, and the number of Bz*a*P-diol epoxide residues bound to DNA was determined from the specific activity. Each value represents the average of two to six determinations.

High-Pressure Liquid Chromatography Analysis of Bz*a*P-Diol Epoxide–DNA Adducts. Samples of this purified DNA were enzymatically hydrolyzed (7) and the modified nucleosides obtained were separated from nonmodified nucleosides by chromatography on a Sephadex LH-20 column (1.5 × 25 cm) with a discontinuous gradient of H_2O and methanol (7). The modified nucleosides from the methanol fractions were concentrated by evaporation under N_2, dissolved in methanol, and separated with an 80-min linear gradient of 30–80% methanol in water on a Spectra-Physics SP 8000 high-pressure liquid chromatograph equipped with a Waters Associates μ Bondapak C^{18} column (3.9 × 300 mm) maintained at 40°C with a flow rate of 1.5 ml/min. Fractions were collected at 2-min intervals and mixed with 10 ml of aqueous counting scintillant (Amersham), and radioactivity was measured on a Beckman LS 9000 Liquid Scintillation Spectrometer equipped with dpm calculation capacity.

RESULTS

Kinetics of Recovery from Potentially Cytotoxic Lesions. Normal human fibroblasts are significantly more resistant than XP12BE cells to the killing action of UV radiation or of a series of polycyclic aromatic chemical carcinogens that distort the DNA helix when they bind to this macromolecule (1–3, 6). We showed that this is also true if these cells are exposed to Bz*a*P-diol epoxide (1, 2). Our studies on the rate of recovery of density-inhibited, nonreplicating cultures of diploid human fibroblasts from the potentially cytotoxic lesions induced by UV radiation (6, 8) indicate that normal fibroblasts can reduce the DNA damage from an initial equivalent of ≈7 J/m^2 (i.e., a dose yielding ≈20% survival) to an equivalent of <2.5 J/m^2 (i.e., a dose yielding ≈100% survival) in less than 24 hr. Recovery from comparatively low doses of UV radiation was directly correlated with the rate of repair replication induced in these cells and, by inference, with the rate of pyrimidine dimer excision (8). Therefore, we wanted to compare the rate of recovery of these human cells from the potentially lethal effect of Bz*a*P-diol epoxide with their recovery from UV-induced damage and to determine whether such recovery was correlated with the rate of removal of covalently bound Bz*a*P-diol epoxide residues from cellular DNA.

In preparation for a large study with tritiated Bz*a*P-diol epoxide, we exposed a series of confluent cultures of normal cells to various concentrations of nonlabeled compound and assayed them for recovery from the potentially cytotoxic effects of this compound over 3–8 days (Fig. 1*A*). The data, which are indistinguishable from results obtained with tritium-labeled Bz*a*P-diol epoxide, indicated that recovery was much slower than after UV radiation. For example, when cells exposed to a concentration of Bz*a*P-diol epoxide that yielded an initial survival of ≈27% were held for 24 hr, they did not exhibit 100% survival as they do after a UV dose giving that initial survival (6, 8). Instead, it required 4–5 days in confluence after treatment with Bz*a*P-diol epoxide to reduce the cytotoxic lesions sufficiently for the cells to exhibit 80% survival upon release from confluence. Similarly, cells given a concentration of Bz*a*P-diol epoxide that initially yielded ≈12% survival required 7–8 days to reduce the potentially cytotoxic damage sufficiently to exhibit 72% survival when released and plated at cloning densities. Furthermore, the data indicated that, in contrast to their recovery from UV radiation (6, 8), the normal fibroblasts held in the nonreplicating state were not capable of removing sufficient numbers of potentially cytotoxic lesions to reach 100% survival when allowed to form colonies.

Correlation of Kinetics of Excision of Covalently Bound Bz*a*P-Diol Epoxide Residues from DNA with Rates of Recovery from Potentially Cytotoxic and Mutagenic Lesions. To determine the rate of removal of Bz*a*P-diol epoxide residues from cellular DNA and compare this with the rate of recovery from potentially cytotoxic or mutagenic lesions, we exposed large populations of normal or XP12BE cells (≈100×10^6 cells per determination) to tritiated Bz*a*P-diol epoxide at concentrations that would yield 12–27% survival. The concentration resulting in ≈25% survival in the excision repair-deficient XP12BE cells (0.025 μM) was much lower than for normal fibroblasts (0.2 μM). One set of cultures was immediately harvested and assayed for the number of Bz*a*P-diol epoxide residues bound to DNA, for percent survival, and for the frequency of mutations induced.

The remaining cultures were prevented from replicating but allowed to carry out excision repair. After various lengths of time, additional sets of cultures were similarly released and assayed. The results are shown in Fig. 1. The data in Fig. 1*B* show that normal fibroblasts held in confluence gradually removed the Bz*a*P-diol epoxide residues from their DNA whereas the XP12BE cells did not. The corresponding recovery data in Fig. 1*A* (i.e., the data showing 27% and 12% survival on day 0) indicate that the rate of removal of bound residues over a period of 8 days was closely correlated with the rate of recovery of normal fibroblasts from potentially cytotoxic lesions. No increase in survival could be detected with the XP12BE cells over a 6-day period. In the normal fibroblasts treated with 0.2 μM Bz*a*P-diol epoxide, the average number of residues per 10^6 DNA nucleotides was reduced from 10.2 to 3 in ≈5 days, but then remained virtually constant (Fig. 1*B*). Similarly, these fibroblasts exhibited 80% survival if released from confluence and plated on day 5, 6, 7, or 8 (Fig. 1*A*). This high survival for normal fibroblasts contrasted with the 27% survival exhibited by XP12BE cells containing the same number of residues per 10^6 nucleotides. This is to be expected if normal fibroblasts, but not XP12BE cells, are capable of removing additional residues from their DNA during the period between plating and the beginning of colony formation. We have recently shown that this is indeed the case (unpublished observations).

The sets of normal fibroblast and XP confluent cultures treated with concentrations of tritiated Bz*a*P-diol epoxide that resulted in ≈27% survival (0.2 and 0.025 μM, respectively) were

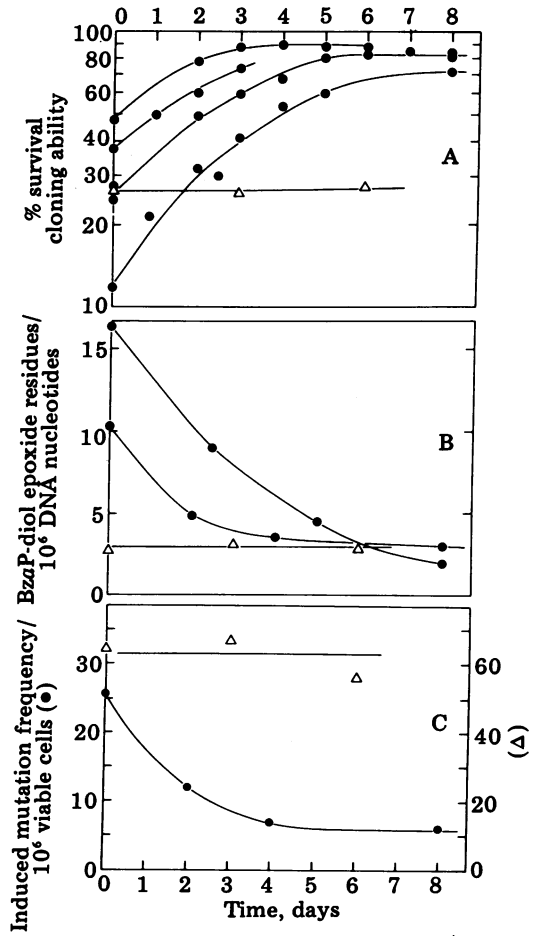

FIG. 1. Kinetics of removal of covalently bound adducts (*B*) and recovery of human cells from the potentially cytotoxic (*A*) and mutagenic (*C*) effects of BzaP-diol epoxide. ●, Normal fibroblasts; △, XP12BE cells. For XP cells: 0.025 μM BzaP-diol epoxide was administered (*A–C*). For normal fibroblasts: (*A*) identified by the percent survival on day 0, 0.1 μM at 49%, 0.15 μM at 39%, 0.2 μM at 27%, and 0.3 μM at 12%; (*B*) 0.2 μM (lower curve) and 0.3 μM (upper curve); and (*C*) 0.2 μM. The survival data were averaged from 7–14 duplicate dishes per point. The number of carcinogen residues bound was averaged from two to six determinations per point, each taken from 35–80 × 10^6 cells. The induced mutation frequencies were calculated, by the *P*(0) method (9), from 80–96 100-mm-diameter dishes (>2.2 × 10^6 cells) per point.

also assayed for the number of potentially mutagenic lesions remaining with time held in confluence (Fig. 1*C*). Each experimental point represents ≳2.5 × 10^6 cells assayed for thioguanine resistance. The data showed that the rate of decrease in potentially mutagenic lesions in normal fibroblasts was closely correlated with the rate of loss of residues from DNA (Fig. 1*B*) and rate of recovery from the potentially cytotoxic effects (Fig. 1*A*). Again, no significant decrease in the frequency of induced mutations with time at confluence was detected in the XP12BE cells. Note that normal fibroblasts containing three residues per 10^6 nucleotides at the beginning of expression exhibited a significantly lower frequency of induced mutations than did XP12BE cells containing that number of residues. This is to be expected because we found that normal fibroblasts that have ceased removing residues in the confluent state are capable of removing additional adducts after release from confluence (see above).

Identification of Cytotoxic and Mutagenic BzaP-Diol Epoxide–DNA Adduct. To determine the nature of the BzaP-diol epoxide residues bound to DNA, we enzymatically digested a portion of the radioactively labeled DNA corresponding to the data in Fig. 1 (i.e., the samples that initially contained 10.2 residues per 10^6 nucleotides) and analyzed the adducts by Sephadex LH-20 column chromatography followed by high-pressure liquid chromatography. The high-pressure liquid chromatography profiles for DNA samples derived from confluent cultures of normal fibroblasts 0, 2, 4, or 8 days after treatment are shown in Fig. 2.

Identification of the peak was made by comparison of the retention time (elution volumes) with those of markers synthesized by treating deoxyguanosine, deoxyadenosine, and deoxycytidine with BzaP-diol epoxide (7). The only detectable component, which eluted at 35–40 min, cochromatographed with the BzaP-diol epoxide–dG marker. The amount of this N^2-deoxyguanosine–BzaP-diol epoxide adduct (10) per amount of DNA analyzed on the column for each day assayed was quantitated from the specific radioactivity of fractions collected. Table 1 summarizes the amount of N^2-deoxyguanosine adduct remaining unexcised from the DNA as a function of the time the cells were held confluent and also cites the loss of total radioactivity from the purified DNA samples. The correspondence between the two rates is obvious.

Relationship Between Number of N^2-Deoxyguanosine BzaP-Diol Epoxide Adducts and Biological Consequences of Exposure to BzaP-Diol Epoxide. A series of normal fibroblast cultures initially exposed to tritiated BzaP-diol epoxide

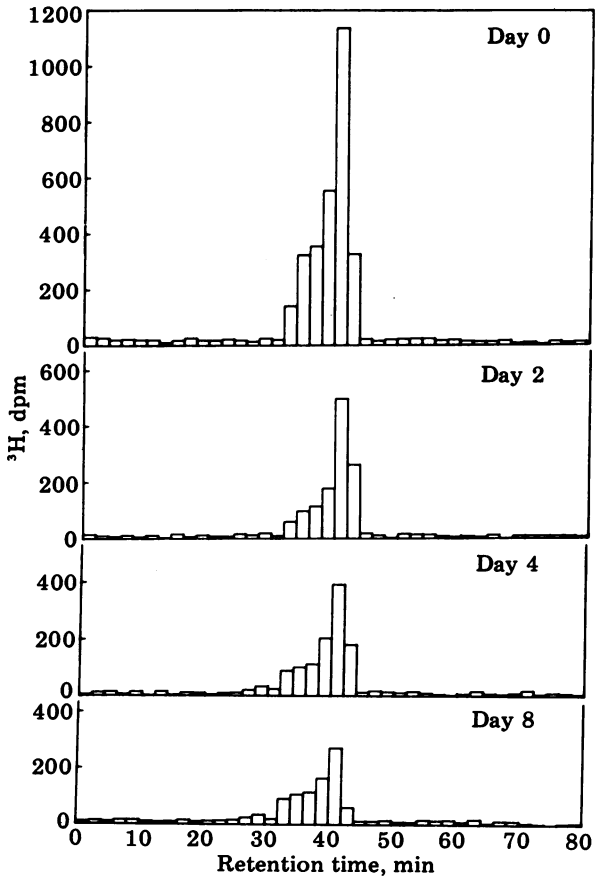

FIG. 2. High-pressure liquid chromatography profile of enzymatically hydrolyzed DNA adducts remaining in normal fibroblasts treated with tritiated BzaP-diol epoxide and held in confluence for 0, 2, 4, or 8 days. The DNA from each culture was extracted and 120 μg was hydrolyzed to nucleosides. Nucleoside adducts were further purified by Sephadex LH-20 column chromatography and then analyzed by high-pressure liquid chromatography on a C^{18} reverse-phase column.

Table 1. Comparison of kinetics of loss of radioactively labeled BzaP-diol epoxide residues from DNA and removal of the specific N^2-deoxyguanosine adduct

Time after treatment, days	BzaP-diol epoxide, residues/ 10^6 DNA nucleotides*	% of bound residues removed†	Total radio-activity in N^2-dG peak,‡ cpm	% N^2-dG–BzaP-diol epoxide residues removed§
0	10.2	0	2810	0
2	4.9	52	1230	56
4	3.7	64	1090	62
8	3.1	71	870	70

* Calculated from the radioactivity of the treated DNA compared to the specific activity of the BzaP-diol epoxide (400 Ci/mol).

† Calculated from the number of BzaP-diol epoxide residues remaining per μg of DNA nucleotides at the designated time divided by the number present immediately after treatment.

‡ N^2-Deoxyguanosine peak upon high-pressure liquid chromatography; cpm per 120 μg of DNA.

§ Calculated from the radioactivity remaining in the high-pressure liquid chromatography peak (Fig. 2) at the designated time divided by the radioactivity in the peak derived from DNA harvested immediately after treatment.

at 0.15, 0.2, 0.25, or 0.3 μM was assayed immediately (i.e., on day 0) for percent survival and number of adducts per 10^6 DNA nucleotides. The results, identified with the numeral 0, are shown in Fig. 3. Initial binding levels of 6.5, 10.2, 14.7, and 16.3 residues per 10^6 nucleotides resulted in initial survival levels of 40%, 27%, 16%, and 12%, respectively. More significantly, Fig. 3 shows that when the data from Fig. 1, derived from cells treated and then held in confluence, were analyzed in the same manner and replotted with these data, the points fell on the identical line. Cells initially exposed to 0.3 μM tritiated BzaP-diol epoxide bound 16.3 residues per 10^6 DNA nucleotides and exhibited an initial survival of 12%. After 2.5 days in the nonreplicating state, when they had reduced the number of such residues per 10^6 nucleotides from 16 to ≈10, the survival they exhibited when released from confluence was the same as that obtained with cells exposed *initially* to a concentration giving 10 residues per 10^6 nucleotides and assayed immediately. Thus, at these low doses, the cytotoxic effect of BzaP-diol epoxide treatment while cells are held in confluence is negligible. The cell killing and mutation induction seen when cells are plated at lower densities reflect DNA lesions remaining unexcised in the cells at the time of some critical event in the replicating state. This conclusion is supported by the lack of alteration in cytotoxicity or mutagenicity in the XP cells, which are unable to remove these DNA adducts.

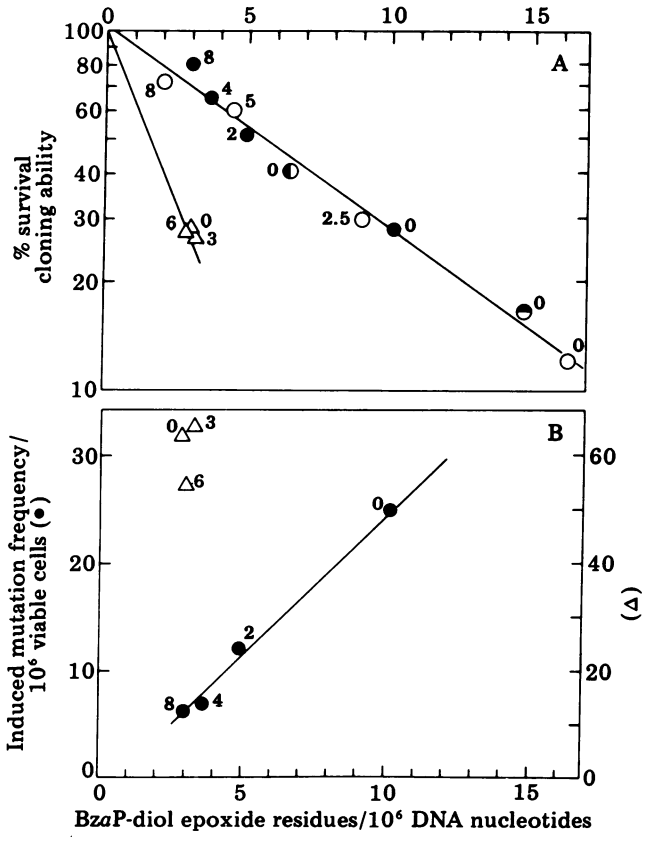

FIG. 3. Cytotoxicity (*A*) and induced mutation frequency (*B*) as a function of the number of BzaP-diol epoxide-induced adducts present in DNA of normal fibroblasts (circles) and XP12BE cells (triangles) at the time they were released from confluence and plated for survival or for the beginning of expression of 6-thioguanine resistance. The number of days cells were held in confluence before being analyzed is indicated next to each data point. The data represented by ○, ●, and △ have been replotted from the data given in Fig. 1. Concentrations (μM) of BzaP-diol epoxide administered on day 0 were: △, 0.025; ◐, 0.15; ●, 0.2; ◓, 0.25; and ○, 0.3.

DISCUSSION

The ability of cells to remove potentially lethal damage induced by x-rays in mammalian cells (11, 12) and UV radiation in normal human fibroblasts (6, 8, 13, 14) while being held in a density-inhibited, nonreplicating state has been demonstrated by several laboratories. The results with UV radiation suggest that this cellular recovery phenomenon reflects DNA excision repair (6, 13, 14) because excision repair-deficient XP cells are incapable of such recovery. Furthermore, the data suggest that excision repair in these confluent normal fibroblast cultures can also eliminate potentially mutagenic lesions induced by UV radiation—i.e., is virtually error free (6). Heflich *et al.* (3) reported similar recovery of normal human fibroblasts from the potentially cytotoxic effects of reactive derivatives of several aromatic amide carcinogens and showed that the rate of recovery is comparable to the rate of removal of DNA bound residues and to the rate of carcinogen-induced repair synthesis.

The data presented in this paper indicate that normal human fibroblasts can also excise residues of BzaP-diol epoxide from DNA and that this excision process results in a gradual decrease in the potentially cytotoxic and mutagenic effects of this carcinogen. The fact that XP12BE cells showed no increase in survival and no loss of bound residues even when held in confluence for 6 days indicates that the increase observed in normal fibroblasts reflects excision repair and not gradual cell turnover. [Autoradiography studies show that less than 1% of the cells replicate during 24 hr in confluence in spent medium (6).] The fact that XP12BE cells are incapable of removing the DNA adduct formed by BzaP-diol epoxide explains why they are so much more sensitive than normal fibroblasts to the potentially lethal and mutagenic effects of this carcinogen. The correlation between the rate of removal of bound residues and the rate of decrease of induced mutation frequency suggests that the excision repair process that removes the BzaP-diol epoxide–DNA adduct is essentially error free. Mutation induction by BzaP-diol epoxide, therefore, must be attributed to processes by which the cells attempt to deal with the lesions remaining unexcised at the time of some critical event, e.g., DNA replication.

The data in Table 1 showing that the normal fibroblasts failed to remove all of the DNA-bound carcinogen residues during the period they were held in confluence are similar to that re-

ported by Feldman *et al.* (15) for human lung carcinoma cells. These investigators suggested that a certain fraction of the original lesions are somehow inaccessible and, therefore, persist. Our data in Fig. 1*A* suggest that at each concentration a certain proportion of the initial lesions induced in the confluent cultures are or become inaccessible to excision repair processes operating in these resting cells. It is of interest to determine if, as suggested by Feldman *et al.* (15), these consist of adducts located predominantly in the core region of the DNA. Whatever their distribution, we have found that resting cells that have not removed additional lesions for several days are quite capable of removing them when released from confluence. The failure to continue excision by cells in the confluent state after a few days cannot be the result of the death of a certain fraction of the population accompanied by continued excision in the remaining fraction of viable cells. This is because only the viable fraction contributes to the frequency of induced mutations, and the mutation frequency does not continue to decrease but also levels off with time (Fig. 1*C*). This is additional evidence in favor of the explanation of inaccessible lesions in confluent cells.

It has been shown that Bz*a*P-diol epoxide can react with DNA *in vitro* to form adducts with deoxyguanosine, deoxycytidine, and deoxyadenosine (7, 10, 15–17). Studies with animal cells in culture also indicate that more than one adduct can be formed. For example, in the 10T½ mouse embryo cell line both deoxyguanosine and deoxycytidine adducts are present, but no deoxyadenosine adduct is seen (18), whereas in hamster embryo cells all three adducts are formed (19). In each instance cited, the N^2-deoxyguanosine adduct predominates. The only detectable DNA adduct we observed in diploid human skin fibroblasts cochromatographed with the Bz*a*P-diol epoxide-dG marker, which has been identified as the N^2-deoxyguanosine adduct (10, 16). Of course, it is always possible that very minor amounts of deoxyadenosine or deoxycytidine adducts may have been formed but that the specific activity of the Bz*a*P-diol epoxide was too low for them to be detected. There is also the possibility that some unstable adduct, such as the N^7-deoxyguanyl derivative (20), is formed in the cells but lost during DNA extraction. However, if any of these latter possible adducts are responsible for the cytotoxic or mutagenic effect of Bz*a*P-diol epoxide in these cells, it is necessary that they be excised with *exactly* the same kinetics as the detectable N^2-deoxyguanosine adduct in order to account for the data in Table 1 and Fig. 1. Therefore, we conclude that the N^2-deoxyguanosine adduct is most likely the potentially cytotoxic and mutagenic adduct responsible for the observed biological effects and that excision repair of this lesion is error free.

This research was supported in part by Contract 78-4659 from the Department of Energy, and by grants from the Elsa U. Pardee Foundation and the College of Osteopathic Medicine, Michigan State University.

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