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Breakage of Human Cell DNA After Exposure to 3-Methylcholanthrene-11,12-oxide[1]

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SUMMARY

Damage to and repair of DNA isolated from human neonatal and fetal skin cells were measured by alkaline sucrose gradient analysis. 3-Methylcholanthrene did not induce single-strand breaks in DNA of the cells in culture, whereas the 11,12-oxide of 3-methylcholanthrene was very effective in this regard. The *cis*-1,2-dihydroxy, *trans*-11,12-dihydroxy, and *cis*-11,12-dihydroxy derivatives of 3-methylcholanthrene exerted little effect. The breaks in DNA caused by 3-methylcholanthrene oxide occurred during a 60-min incubation period and were repaired during the following 60 min. Methylmethane sulfonate also induced breaks in the DNA within 60 min.

INTRODUCTION

Exposure to certain environmental chemicals, *e.g.*, 3MC,[3] can lead to the development of neoplasia as first noted in humans over 200 years ago by Pott. Since then, numerous reports have appeared establishing the carcinogenic potential of certain polycyclic hydrocarbons in a variety of animal systems. The results of more recent investigations have suggested that carcinogenicity, toxicity, and mutagenicity of this class of compounds require prior metabolic activation catalyzed by the microsomal mixed-function oxidase, aryl hydrocarbon hydroxylase. Furthermore, it is now generally accepted that the initial step in the metabolism of these compounds is the oxidation of aromatic double bonds to form an arene oxide (13, 14). Indeed, in several recent reviews (5, 12), evidence was presented in which the arene oxide was depicted as the causative agent not only in carcinogenicity, but also in toxicity and mutagenicity. In this regard, an increased rate of transformation of mammalian cells by K-region oxides of polycyclic hydrocarbons is observed when compared to the parent compounds *per se* (10, 11, 21).

The toxicity, carcinogenicity, and mutagenicity of the arene oxides in mammalian systems appear to correlate well with the extent of their binding to macromolecules within the susceptible cell (reviewed in Ref. 12). In the present report, we have examined the relative ability of 3MC and its K-region oxide, 3MC-11,12-oxide, to induce single-strand breakage in DNA of human fetal and neonatal skin cells; we have also examined DNA repair in the latter system. Toward this end, we have utilized the alkaline sucrose gradient technique as the tool for demonstrating single-strand breaks. The results of this study indicate that in the presence of the K-region oxide the DNA of these human cells does undergo single-strand breakage and, furthermore, these breaks are readily repaired.

MATERIALS AND METHODS

Cell Culture. Diploid human fibroblast cell cultures were initiated with skin biopsy samples obtained from a 16-week-old fetus and from neonatal foreskin, respectively. The fetal skin and the foreskin were obtained through the courtesy of Dr. Paul McDonough and Dr. William Scoggin, respectively, of the Department of Obstetrics and Gynecology, Medical College of Georgia, Augusta, Ga.

With the exceptions noted, all media and additives were obtained from the Grand Island Biological Co., Grand Island, N. Y. The fetal skin was minced with scalpel blades in McCoy's medium (Hsu's modification) which contained neomycin, 0.1 mg/ml, Fungizone, 0.2 μg/ml, and 20% fetal bovine serum (v/v). Several dozen small tissue fragments were placed on the growing surface of plastic, 30-ml T-30 cell culture flasks (Bioquest, Baltimore, Md.). After 30 min at room temperature, during which time the tissue attached to the plastic substrate, a small amount of 20% serum-supplemented McCoy's medium, which just covered the floor of the T-30 flask, was added. The preparation was incubated at 37° in an atmosphere of 5% CO_2:95% air, and the medium was replaced every 48 hr. Fibroblasts grew from the tissue fragments as a monolayer.

The foreskin tissue was minced and then digested overnight at 37° in McCoy's medium that contained 20% fetal bovine serum (v/v) and collagenase, 1 mg/ml (type 1 *Clostridium histolyticum;* Sigma Chemical Co., St. Louis, Mo.). Following digestion, the tissue was dispersed by gentle pipetting with a wide-mouthed 10-ml pipet. The suspension was filtered through sterile gauze and centrifuged at 60 × *g* for 3 min. The cell pellet was washed twice with 20% serum-supplemented McCoy's medium, diluted to approximately 10^7 cells/ml, and placed in plastic T-30 culture flasks. The latter were incubated at 37° in 5% CO_2:95% air for 24 hr. At this time, cells which had not attached to the growing surface were discarded. The attached cells grew as a monolayer of fibroblasts. The medium was changed every 48 hr.

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[3] The abbreviations used are: 3MC, 3-methylcholanthrene; MMS, methylmethane sulfonate; TCA, trichloroacetic acid.

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Both the fetal skin and foreskin cultures produced confluent primary monolayers. For subculture, the monolayers were rinsed once (1 min) with citrate solution (0.018 M sodium citrate, pH 7.4:0.01 M KCl-0.001% phenol red (w/v) and then rinsed again (1 min) with citrate solution which contained 0.125% trypsin (w/v). After 5 min at 37°, the cells detached from the surface of the culture vessel. These were resuspended in fresh culture medium and transferred to new culture flasks. In this manner, several primary cell lines from human fetal skin and foreskin were obtained. After the 1st passage, all strains were grown in McCoy's medium supplemented with 10% fetal bovine serum (v/v); medium changes were made at 3-day intervals. For the production of large numbers of cells, cells were grown as flat cultures in 30-oz glass prescription bottles. The fetal skin line FS-1 was used in these experiments in passages 15 to 25; the foreskin line Ib was used in passages 3 to 12.

Labeling of DNA and Analysis of DNA Repair in Tissue Culture. The medium from cultures of nonconfluent, growing human fetal skin or foreskin cells (approximately 10^6 cells) was replaced by 15 ml of fresh medium containing 15 μCi of [*methyl*-^{3}H]thymidine (Schwarz/Mann, Orangeburg, N. Y.; specific activity 0.36 Ci/mole). Incorporation of the DNA precursor was allowed to proceed for 24 hr. The specific activity of DNA from control cultures under these conditions was 5,000 to 10,000 dpm/μg.

Following the labeling period, the medium was discarded and fresh medium was added which included either 3MC, 3MC-11,12-oxide, or MMS for the experimental systems or the solvent alone for control cells, *i.e.,* dimethyl sulfoxide. After this treatment period, the cells were rinsed 4 times with 30 ml cold 0.024 M EDTA:0.075 M 0.9% NaCl solution, pH 7.5, harvested by scraping with a rubber policeman into 5 ml of cold EDTA:0.9% NaCl solution and centrifuged for 2 min at 600 to 800 rpm in an International PR-2 centrifuge. The cells were resuspended in 1 ml of rinsing solution and 0.15 ml of the cell suspension was added to 0.3 ml of a lysing solution (0.3 M NaCl:0.03 M EDTA:0.5% sodium dodecyl sulfate:0.1 Tris, pH 10) which was layered on top of 5 to 20% alkaline sucrose gradient (containing also 0.9 M NaCl and 0.3 M NaOH). The cell suspension added to the gradients contained 1 to 5 μg DNA. Alkaline sucrose gradients prepared in this manner allow for the rapid sedimentation of [^{3}H]thymidine-labeled DNA. RNA and protein remain near the top of the gradients during this brief centrifugation (4). The gradient had been formed on 0.5 ml of a 2.3 M sucrose cushion in a 5-ml cellulose nitrate tube. The gradients were allowed to stand at room temperature for 10 min (the lysing period) and then were centrifuged for 30 min at 25,000 rpm in a SW 50.1 rotor in an L3-50 ultracentrifuge maintained at 20°. This procedure represents a modification of that used by Laishes and Stich (17). Under these conditions, unbroken, single-stranded DNA sedimented between Fractions 3 and 6. Long pieces of DNA will accumulate in 1 region of the gradient. This method does not yield quantitative estimates of the size of DNA but only informs us that repair is taking place.

Determination of Radioactivity. Approximately 20 sequential 16-drop fractions were collected from the bottom of the gradient tubes and 1 ml of cold 5% TCA was added. The TCA-precipitable material was collected on Millipore nitrocellulose filters, and the filters were rinsed 5 times with 2 ml cold 5% TCA. The washed filters were then transferred to scintillation vials. NCS solubilizer, 0.5 ml, was added and the vials were allowed to stand at room temperature for 15 min. A toluene-based scintillation fluid, 10 ml (0.5% PPO:0.03% POPOP), was added, and the radioactivity was determined in a Beckman Model LS-150 liquid scintillation spectrometer.

RESULTS

Effect of 3MC, 3MC-11,12-oxide, and MMS on Human Cell DNA. Following the 24-hr labeling period of the human foreskin cells, the medium was changed; 3MC, 3MC-11,12-oxide, or MMS was added to experimental cultures. The results presented in Chart 1 show that 3MC did not induce single-strand breaks in DNA as measured by the alkaline sucrose technique, since the radioactive DNA sedimented to the bottom of the gradient as in the untreated control culture (Chart 1*A*). As a positive control in these experiments we used MMS, a known inducer of single-strand breaks. As is evident from Chart 1*D*, MMS treatment effected a marked shift in the migration of foreskin cell DNA. Under similar conditions, 3MC-11,12-oxide also induced single-strand breaks in the DNA, as indicated by the appearance of radioactivity peaks toward the top of the gradients (Chart 1*C*). Similar results were noted when fetal skin was used as the source of cells instead of neonatal foreskin.

The sharp peaks occurring near the bottom of the control gradients are similar to those obtained in other studies using this technique and they represent a range of high-molecular-weight DNA single strands (4, 17). DNA from untreated cells is left relatively intact in this procedure. More conventional methods of preparing DNA can result in shearing, which makes the comparison of gradient profiles

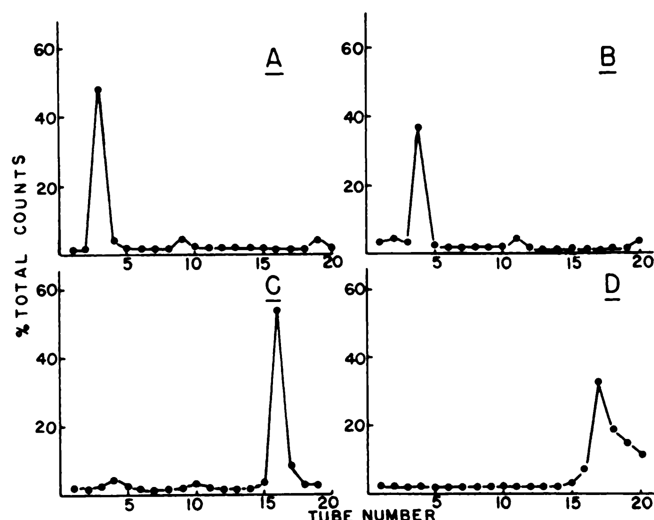

Chart 1. Alkaline sucrose gradient analysis of foreskin cell DNA. Neonatal foreskin cells were treated for 60 min with 15 μl of dimethyl sulfoxide alone (*A*); 10^{-5} M 3MC in dimethyl sulfoxide (*B*); 10^{-5} M 3MC-11,12-oxide in dimethyl sulfoxide (*C*); and 1 mM MMS in dimethyl sulfoxide (*D*). The cells were washed, placed into the lysing solution on top of alkaline sucrose gradients, and analyzed as described in the text. The top of the gradient is at the right. Control DNA sedimented to the bottom as indicated at Fraction 3-4 in at least 4 different experiments. Data represent 4 different experiments.

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more difficult. Therefore, application of whole cells to alkaline sucrose gradients allows for much greater sensitivity in detection of single-strand damage to DNA.

Titration of the 3MC oxide effect upon human cell DNA revealed that the greatest damage was achieved at a final concentration of 10^{-5} M, while 10^{-7} M appeared to exert little effect; at 10^{-6} M, some breakage was observed. In experiments not shown here, 3MC-oxide was found to exert little effect on foreskin cell DNA when the incubation time in the presence of the polycyclic hydrocarbon was 30 min or less. Nor was any effect seen with the parent compound, 3MC, when the incubation time was prolonged to 90 min.

Other metabolites of 3MC were tested for their ability to cause DNA breakage in human foreskin cells as ascertained by the alkaline sucrose gradient technique. At 10^{-5} M, neither *cis*-1,2-dihydroxy-, *trans*-11,12-dihydroxy-, nor *cis*-11,12-dihydroxy-3MC derivatives were able to cause damage to DNA which was detectable by this technique.

Kinetics of Repair. Experiments were performed to determine the time course of repair of foreskin cell DNA following treatment with 3MC-oxide. After a 60-min treatment period with 10^{-5} M of the latter, the culture medium was changed and the cells were incubated for additional periods of time in the absence of the polycyclic hydrocarbon. The results shown in Chart 2 indicate that the DNA is repaired within 1 hr.

DISCUSSION

The binding of K-region arene oxides to macromolecules has been studied extensively *in vitro* and in cultured cell lines (1, 7–9, 15, 16, 29). In this regard, Gelboin (6) has shown that the *in vitro* binding of benzo(*a*)pyrene to DNA requires metabolic activation that depends upon a NADPH-requiring microsomal reaction. Furthermore, we have reported recently (2) that the NADPH-requiring microsome-catalyzed *in vitro* binding of benzo(*a*)pyrene and 3MC to DNA is markedly enhanced by addition to the incubation medium of a known inhibitor of epoxide hydrase, 1,1,1-trichloropropene oxide (23, 27). We have also shown an increased number of skin tumors appearing in shorter time in mice that had received topically both 3MC and 1,1,1-trichloropropene oxide (2, 3). Apparently, the intracellular concentration of the K-region oxide, as determined in part by the epoxidase component of aryl hydrocarbon hydroxylase (*i.e.*, the enzyme catalyzing the formation of oxide) and the epoxide hydrase (*i.e.*, the enzyme catalyzing the detoxification of oxide as well as glutathione-*S*-epoxide transferase), is most important in any consideration of polycyclic hydrocarbon-induced carcinogenesis. Unfortunately, attempts to demonstrate by percutaneous or s.c. administration the increased carcinogenesis potency of the oxides over the parent compound have not been successful (3, 22, 24, 28), suggesting that its *in situ* formation is a prerequisite.

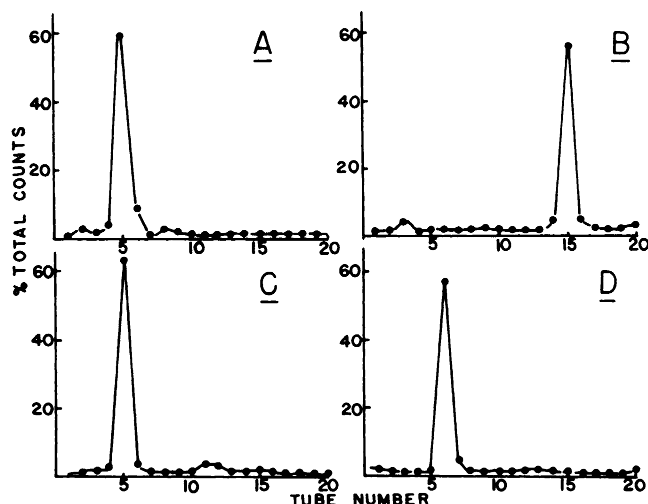

Chart 2. Kinetics of repair after 3MC-11,12-oxide treatment (*A*). Neonatal foreskin cells were treated with dimethyl sulfoxide or 10^{-5} M 3MC-11,12-oxide for 60 min (*B*) and then fresh medium was added to the cultures. Repair was allowed to proceed in the absence of polycyclic hydrocarbon for 60 (*C*) or 120 min (*D*). See the text and Chart 1 for details. These experiments have been repeated from 2 to 4 times with identical results.

In this study, we have shown that 3MC-11,12-oxide, but not the parent compound or several other of its metabolites, will cause breakage of DNA presumably after "arylation" of one or more of its components. Of even greater significance is finding that this breakage is rapidly repaired by these cells. Rapid repair of DNA lesions in human cells has also been observed in human lymphocytes exposed to alkylating agents (18, 20), or to a variety of carcinogens including 7-bromomethylbenz(a)anthracene (25) to procarcinogens such as dimethylnitrosamine plus a microsomal activating system (12), or to activated polycyclic hydrocarbons (19, 26). The latter study (26) is particularly interesting since these investigators have demonstrated the DNA repair-inducing capacity of the K-region oxide of benz(a)anthracene and the lack of such ability of the parent compound as well as the transdihydrodiol.

In summary, the present study demonstrates the existence of a repair process in cells prepared from prepuce material. It would be of interest to ascertain the extent of such capability periodically throughout the life-span of the individual by techniques for preparing fibroblasts from skin as utilized by many laboratories. Such studies should provide information as to the quantitative ability of human populations to repair lesions of their DNA induced by environmental pollutants such as polycyclic hydrocarbons, but unfortunately these studies will not tell us about the qualitative aspects of the repair, *i.e.*, the fidelity of repair.

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