Formation and Removal of Benzo(a)pyrene Adducts of DNA in Hamster Tracheal Epithelial Cells

Alan Eastman, Brooke T. Mossman, and Edward Bresnick

ABSTRACT

A cloned cell line derived from normal hamster tracheal epithelium has been characterized with respect to its response to the environmental pollutant and carcinogen benzo(a)pyrene \([B(a)P]\). These cells metabolize \(B(a)P\) to ultimate reactive forms as assayed by alkylation of DNA. Alkylation with radiotracer amounts of \(B(a)P\) was maximum at 8 hr, at which time 70\% of the applied hydrocarbon had been converted to water-soluble forms. At longer incubation times, the rate of removal of adducts exceeded the rate of formation. When \(B(a)P\)-containing medium was replaced with fresh medium at two or four hr, a subsequent biphasic removal of adducts occurred, a rapid removal for the first four hr postincubation and then a slower repair. About 50\% of the DNA-bound hydrocarbon remained in DNA after 48 hr. Cells were able to divide in the presence of these lesions, undergoing five doublings (five days), while only 60\% of the adducts were removed from the DNA.

Integrity of DNA during this period was monitored by the alkaline elution technique. A toxic dose of \(B(a)P\) was required to cause any increase in the rate of elution. Minimal single-strand breakage was observed from two to eight hr of \(B(a)P\) treatment, but at 15 hr DNA appeared normal. Comparison was made with a nontoxic dose of methyl methanesulfonate which caused very rapid elution of DNA after only one hr treatment.

At least 15 deoxyribonucleoside-bound \(B(a)P\) adducts were separated by high-pressure liquid chromatography. Four adducts, probably deoxyadenosine-\(B(a)P\), were removed almost completely in 24 hr, while the others appeared to be poorly removed. The possible significance to neoplasia of persistent and repairable lesions is discussed.

INTRODUCTION

A large variety of carcinogens cause damage to DNA, a reaction which can potentially result in mutation and in transformation. Cells, however, contain various processes for excising or circumventing these lesions. Evidence from a number of pathological conditions, such as xeroderma pigmentosum (5, 29), shows that deficient repair processes predispose to neoplasia. Therefore, it has been proposed that persistence of \(O^6\)-ethylguanine in rat nervous tissue (16) and DNA-bound polycyclic hydrocarbons in mouse lung (10) both correlate with susceptibility of these tissues to neoplasia.

The reported rate of repair after exposure of cells to polycyclic hydrocarbons depends upon the method of analysis. Indirect methods such as unscheduled DNA synthesis (23, 28) or DNA strand breakage (25, 31) have been used to measure repair up to 20 hr, with apparently significant repair occurring within 4 hr. Direct measurement of adducts in DNA has generally involved 24 to 48-hr incubation periods with the hydrocarbon, which is too long a time to allow observation of such rapid processes. After these longer incubations, mouse embryo cell cultures excised greater than 50\% of either 3-methylcholanthrene or 7-bromomethylbenz(a)anthracene adducts in the following 48 hr (7). Epitheloid human lung cells excised about 50\% of \(B(a)P^2\) adducts in 72 hr (14), while mouse embryo fibroblasts removed 50 to 70\% in 68 hr (3). After a relatively short incubation of 2.5 hr with 7-bromomethylbenz(a)-anthracene, approximately 50\% of the hydrocarbon adducts were removed during the following 30 hr with a preferential excision of modified adenine moieties (8). In only one communication have results been presented on both adduct disappearance and DNA strand breakage after shorter periods of incubation with polycyclic hydrocarbons (4). After incubation of \(B(a)P\)-diol-epoxide with a human alveolar tumor cell line for 15 min, DNA strand breakage was maximum after 3 hr although 30 to 50\% of the adducts still remained by 30 hr.

In our previous studies, we detected hydrocarbon adducts under in vivo conditions in both mouse lung and liver (10-12). We have chosen to analyze the hydrocarbon adducts in a tissue of a species which responds similarly to that of the human, i.e., hamster tracheal epithelium. Unfortunately, because of the extremely small yields of DNA, we could not perform these experiments under in vivo conditions. We have therefore used a cloned cell line derived from a normal hamster tracheal epithelium for these studies (13, 20, 24). In this paper, we present an analysis of the rates of formation and removal of \(B(a)P\)-DNA adducts in these hamster tracheal cells. These have been analyzed by HPLC, a method which resolves considerably more adducts than does Sephadex LH-20 chromatography. In addition, the presence of adducts was compared to the integrity of the cell DNA using the method of alkaline elution for the detection of single-strand breaks.

MATERIALS AND METHODS

Cell Culture. HTE-B cells were grown as monolayers in Ham's Medium F-12 supplemented with Mycostatin (25 units/ml), 10\% calf serum (Grand Island Biological Co., Grand Island, N. Y.) and gentamicin (100 \(\mu\)g/ml) (Microbiological Associates, Walkersville, Md.). Cells were subcultured (a 1:4 split) twice weekly by gentle trypsinization 0.25\% trypsin. Plates were incubated at 37° at 100\% relative humidity under an atmosphere of 5\% \(CO_2\) in air. Passages 40 to 50 were used in the present study.

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2 The abbreviations used are: \(B(a)P\), benzo(a)pyrene; HPLC, high-pressure liquid chromatography; HTE-B, hamster tracheal epithelial cells, clone B. DMSO, dimethyl sulfoxide; ODS, octadecysilane.

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For the experiments reported here, confluent monolayers were harvested and plated at approximately 2 x 10^6 cells/ Falcon T-150 plastic flask containing 25 ml medium. After 24 hr incubation [2-^14C]thymidine (0.01 µCi/ml; (56mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was added for 65 hr to radiolabel the DNA. Cell replication could be monitored throughout the experiments by a progressive reduction in the ^14C specific activity of purified DNA. Confluent radiolabeled cultures were trypsinized and plated at approximately 1 x 10^6 cells in replicate T-150 flasks before the addition of B(a)P.

Alkylation of DNA. [G-^3H]B(a)P (50 to 250 µCi; 37 Ci/mmol; Amersham) or unlabeled B(a)P was added to cultures in 10 µl DMSO per flask as required. After various incubation periods, cultures were washed 3 times with Hanks' balanced salt solution and postincubated in 25 ml of fresh medium with serum. Cells were harvested with a rubber policeman into 75 mm NaCl-24 mm EDTA, pH 7, and centrifuged at 900 x g for 5 min. The pellet was resuspended in 15 mm NaCl-1.5 mm sodium citrate-1% sodium dodecyl sulfate, the DNA was purified, and specific activities were obtained as detailed previously (12).

The purified DNA was redissolved in 1 ml of 20 mm sodium acetate-50 mm NaCl-1 mm ZnSO_4-10 mm MgCl_2, pH 4.6, and digested to deoxyribonucleosides sequentially for 8 hr with 200 Kunitz units of DNase I (bovine pancreas; Sigma Chemical Co., St. Louis, Mo.), for 16 hr with 1000 units of S_nuclease (Bethesda Research Laboratory, Rockville, Md.), and for 8 hr with 0.2 unit of acid phosphatase (wheat germ; Sigma). The digest was applied to a 2- x 0.5-cm Sephadex LH-20 column equilibrated with water. Unmodified nucleosides were eluted with 5 ml water, and B(a)P-modified nucleosides were eluted with 5 ml methanol. The latter fraction was evaporated to 100 µl and injected onto an Altex Ultrasound ODS column (250 x 4.6 mm) attached to a Varian Model 5000 high-pressure liquid chromatograph. A precolumn of Waters Associates C_18-Corasil was used. The deoxyribonucleoside-bound adducts were eluted at a flow rate of 1 ml/min with 45% methanol in water for 45 min followed by a linear gradient to 60% methanol at 105 min. Fractions (0.5 ml) were collected, and the radioactivity was determined by scintillation counting.

Reference adducts were synthesized using the DNA homopolymers of dAMP, dCMP, and dGMP (P-L Biochemicals Inc., Milwaukee, Wisc.). The ^3H-syn and ^14C-anti forms of (±)-trans-7,8-diol-9,10-epoxy-7,8,9,10-tetrahydro-B(a)P (kindly procured from the National Cancer Institute Chemical Carcinogen Reference Repository by Dr. T. Slaga) were synthesized according to previously published techniques (18).

Alkaline Elution Analysis. HTE-B cells plated at 5 x 10^5 cells/dish were grown in 60-mm Falcon plastic Petri dishes containing 5 ml of fresh medium. [methyl-^3H]thymidine (43 Ci/mmoll; Amersham) was added to give 0.1 µCi/ml. After a 48-hr incubation, the medium was replaced so that the radiolabel would not be present in the short newly synthesized DNA during analysis. B(a)P at various concentrations was added in DMSO (final concentration, 0.33%) at various times during a subsequent 24 hr of incubation, while controls received DMSO only. Cells were harvested with a rubber policeman after 72 hr, and aliquots were subjected to alkaline elution analysis as reported previously (9). Resolution between treated and control cells was enhanced by increasing the flow rate in tetrapropylammonium hydroxide, pH 12.1, to 3 ml/hr and collecting 3-ml fractions for 24 hr. The radioactivity of these fractions was then determined. Positive elution controls were obtained after 1 hr treatment of cells with methyl methanesulfonate (20 µg/ml).

RESULTS

Toxicity of B(a)P. HTE-B cells that had been prelabeled with [^14C]thymidine were trypsinized and diluted. After 48 hr incubation, unlabeled B(a)P was added at various concentrations. Cells were harvested at this time or after 24 and 48 hr. DNA was purified, and growth was calculated from the decrease in specific radioactivity. Chart 1 shows that 10^-6 M B(a)P was the lowest dose tested that caused some inhibition of growth.

Alkaline Elution Analysis. When cells treated only with DMSO were subjected to alkaline elution, 80 to 90% of the DNA was routinely retained on the filter after 24 hr (Chart 2). Under identical conditions, DNA from methyl methanesulfonate-treated cells eluted rapidly, with less than 10% retained at this time. This dose did not result in growth inhibition. HTE-B cells required a 100-µg/ml dose of methanesulfonate before growth was slightly inhibited (data not shown).

With 10^-5 M B(a)P, a slight but consistent increase in elution over control was obtained. The extent of increase was 5 to 10% after 24 hr elution. This was the minimum increase reliably detectable; in order to resolve this slight change, an increased elution time and volume were required over our previously published study (9). This increase in elution, which was observed between 2 and 8 hr, was no longer detectable by 15 hr. No increase in elution was observed when 10^-6 M B(a)P was used.

Analysis of Adduct Formation. Analysis of hydrocarbon-DNA adducts has been performed with nontoxic radiotracer amounts of B(a)P. Cells prelabeled with [^14C]thymidine were grown to confluent monolayers and incubated with [^3H]B(a)P, and DNA was purified from 2 to 48 hr later. The specific activity of...
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Chart 2. Alkaline elution of DNA from HTE-B cells treated with B(a)P for various time periods. The positive elution control was obtained by 1-hr treatment of cells with methyl methanesulfonate (20 μg/ml) (△). Although the results of a single experiment are presented, the rate of elution in 4 other studies was similar. The duration of treatment of the cells with 10^{-6} M B(a)P varied from 0 to 2 to 8, to 15 hr.

[^14C]thymidine in DNA indicated less than one doubling during this period. Specific activities of B(a)P in DNA have been corrected for the dilution due to DNA replication. Alkylation of the DNA of these cells was directly proportional to the amount of radioactive B(a)P proffered from 50 to 250 μCi/dish. B(a)P alkylation of DNA was maximum at 8 hr with about 20% reduction by 48 hr (Chart 3). In the same experiment, B(a)P was washed off some dishes at 2 or 4 hr, and the cells were postincubated in fresh medium. Between 2 and 4 hr, alkylation usually increased even in the absence of B(a)P in the medium. The cells presumably absorbed the hydrocarbon but did not metabolize it to its ultimate reactive form. Between 4 and 8 hr, a rapid removal of adducts was always seen after which the rate of removal slowed markedly. By 48 hr, approximately 50% of the adducts remained in the cells compared to the maximum observed at 4 hr. Both rapidly dividing cells and confluent cultures (relatively static) were compared, but no significant differences were observed in either the rates of formation or the removal of adducts.

The repair process was followed for 5 days in actively dividing cells. Cells were prelabeled with [^14C]thymidine, grown to confluency, and treated with 250 μCi[^3H]B(a)P for 4 hr. Cells were then trypsinized, reseeded at lower density, and grown for 5 days. During this period, the cells underwent 5 doublings, while only 60% of the adducts were removed from the DNA (Chart 4).

Analysis of individual lesions has been followed by HPLC using predominantly confluent cell cultures. The cells from 3 T-150 flasks were treated with 250 μCi[^3H]B(a)P per flask for 4 hr and harvested then or after various post-incubations in fresh medium. When the purified DNA was chromatographed on a small Sephadex LH-20 column, approximately 25% of the radioactivity was eluted in the aqueous fraction. The amount of this radioactivity did not change significantly with the various incubation times investigated. The possible identity of this radioactivity has been discussed recently (10, 27) but still remains obscure. It seems unlikely that the radioactivity arises as a result of poor enzyme digestion as the B(a)P-diol-epoxide-
alkylated homopolymers gave only about 2% in the aqueous fraction. The methanol eluate from Sephadex LH-20 was subjected to HPLC (Chart 5). At least 15 radioactive peaks were detected excluding the residual early peak that was incompletely removed by Sephadex LH-20. In some chromatograms, Peaks 6 and 14 appeared to contain more than one constituent. The most striking difference between the adduct profiles from cells incubated with B(a)P for 4 hr (Chart 5A) and those postincubated for 20 hr (Chart 5B) is the marked reduction in Peaks 12 to 15.

In an attempt to identify these peaks, DNA homopolymers which were reacted with B(a)P-diol-epoxides were used. The HPLC profiles obtained are shown in Chart 6. The considerable overlap of deoxycytidine and deoxyguanosine adducts did not facilitate identification of the majority of the adducts obtained from the cells. However, deoxyadenosine adducts all eluted late in the chromatograms, which suggested that adduct Peaks 12 to 15 (Chart 5) probably derive from this deoxyribonucleoside.

**DISCUSSION**

It is evident that in the tracheal epithelial cells a variety of processes occur that facilitate the analysis of DNA repair subsequent to polycyclic hydrocarbon alkylation. These cells obviously metabolize B(a)P to ultimate forms that can give rise to DNA adducts, thus avoiding complications that can arise from the use of microsomal activating systems. Furthermore, it would seem that the generation of active intermediates of B(a)P within the cell and the subsequent alkylation reaction can provide data which more closely approximate the situation during human exposure. The formation of DNA adducts in our HTE-B cells occurs rapidly and reaches a maximum by 8 hr. This correlates with the availability of hydrocarbon. At 4 hr, approximately 45% of the applied radioactive hydrocarbon can no longer be extracted from the medium into ethyl acetate. At 8 hr, 70% remains in the aqueous phase; at 24 hr, this value increases to about 80%. Presumably, in cell lines used previously, metabolism occurred slower; hence, alkylation increased over longer periods of time.

The DNA-bound adducts are removed in a biphasic manner with a rapid excision for about 4 hr, which is followed by a much slower process, with about 50% remaining after 48 hr. This initial rapid repair could not be explained by the removal of any particular adduct. It might be due to the repair of a specific fraction of DNA such as actively transcribing chromatin. Dividing cells were able to remove 60% of the adducts during 5 doublings (5 days) and must therefore be capable of synthesizing DNA on a damaged template, presumably by some "postreplication repair mechanism."

Analysis of adducts derived from homopolymers and B(a)P-diol-epoxides showed profiles (Chart 6) that were similar to those published previously (18). In our work, we have used an Altex Ultrasphere ODS HPLC column which was recently shown to exhibit the best resolution of the analogous ribonucleoside-B(a)P-diol-epoxide adducts (26). This explains the greater number of peaks than was previously reported (3, 17) that were obtained in the present study on incubating cells with B(a)P. It is generally believed that the majority of these adducts are derived from the reactive diol-epoxide derivatives. However, the use of B(a)P rather than the diol-epoxides (21, 22) may result in the production of more adducts. Further metabolism of B(a)P phenols, quinones, and the 4,5-oxide reportedly can also give rise to DNA-bound adducts (27). Our adduct Peaks 1 and 2 eluted from HPLC before any of the synthesized standards. Peaks 3 to 11 elute in the region where considerable overlap occurs with the deoxycytidine- and deoxyguanosine-B(a)P-diol-epoxide standards and therefore does not facilitate their characterization. The tetralol derivatives of the diol-epoxides also chromatograph in this region. However, Peaks 12 to 15 all cochromatographed with deoxyadenosine standards. These 4 peaks also appear to be removed during postincubation of the cells. It has been reported previously that deoxyadenosine-7-bromethylbenz(a)anthracene adducts were excised preferentially (8), which is in concert with the characterization of these peaks as representing deoxyadenosine derivatives.

Several additional points are worth noting which relate to our observations. A 4-hr incubation time with B(a)P shows a greater contribution of deoxyadenosine adducts than was observed by other investigators who measured removal after 24 hr incubation with hydrocarbon (3, 7, 14). During these longer incubation times, the sum of both formation and removal of adducts will reduce the apparent contribution of the repairable lesions. Indeed, even after 4-hr incubations, the real amount of these lesions that form may be underestimated. These HTE-B cells may provide a good system for analyzing the significance of short-lived lesions because the rapid rate of metabolism in these cells facilitates their detection.

A different protocol for enzyme digestion has been used in which S, nuclease and acid phosphatase are used rather than phosphodiesterase and alkaline phosphatase (1). We have compared the 2 digestion systems with a single DNA preparation and found that our new conditions give rise to a greater proportion of deoxyadenosine adducts. It has been suggested
that alkaline phosphatase contains contaminating adenosine deaminase that could degrade modified adenosines, at least with methylated derivatives (19). We have not noted similar deaminase that could degrade modified adenosines, at least under our conditions.

An important question remains as to which adducts are most likely to contribute to the development of neoplasia or whether all are equally important. Many of these adducts appear to be poorly repaired and could be of significance to the transformation process since persistence of lesions in DNA appears to correlate with susceptibility to neoplasia (5, 10, 16). However, the fact that a mechanism exists for more rapid removal of certain other lesions, presumably deoxyadenosine adducts, suggests that these lesions might be more critical to the perpetuation of living systems. This suggestion is supported by a reported correlation between deoxyadenosine adducts and tumorigenesis in mouse skin (6). An analogous example is provided by the lack of miscoding during replication of the poorly repaired, alkylated base, N'-methylguanine, while O6-methylguanine represents a critical lesion for which a repair process normally exists. Deficiency in the latter repair process appears to result in transformation (15). It would therefore seem important to seek conditions under which the repair of deoxyadenosine adducts is inhibited in order to determine their significance to cell transformation.

Alkaline elution analysis of B(a)P-treated cells showed only a minimal increase in DNA strand breakage at toxic doses, while methyl methanesulfonate caused extensive strand breakage without exhibiting toxicity. This demonstrated that B(a)P did not cause significant alkali-sensitive lesions in the DNA of HTE-B cells. Other investigators have reported small increases in strand breakage by the alkaline elution technique only either after microsomal activation of B(a)P (30) or after treatment with B(a)P-diol-epoxide (4). The observation that many chemical carcinogens cause DNA strand breakage and repair has been considered as a possible tool in predicting carcinogenic potential (2, 30). However, our experiments demonstrate that the potent carcinogen B(a)P caused only minimal breakage and would probably not be detected in such a screen. Alkaline elution analysis depends on either the ability of a chemical to cause alkali-sensitive lesions or DNA strand breakage per se and/or the ability of an endonuclease to recognize and cleave at the site as in excision repair. For the most part, such repair probably has high fidelity. Many carcinogens do cause strand breakage. However, more importance should be placed on the lesions that remain in the DNA as these are more likely to have subsequent mutagenic effects on the cells. It is the absence of a repair process in pathological conditions such as xeroderma pigmentosum that increases transformability of these cells. It is probably, therefore, the persistence of a lesion, not its repair, which may correlate with transformation. Considerable experimental work must be undertaken to identify the critical lesions and the mechanism by which they elicit neoplastic transformation.

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