ABSTRACT

The persistence of benzo(a)pyrene (BP) metabolite:DNA adducts has been studied in lung and liver of A/HeJ and C57BL/6J mice after a dose of BP (6 mg/mouse) which induces pulmonary adenomas in A/HeJ mice but not in C57BL/6J mice. BP is not a hepatic carcinogen in either strain. Following p.o. administration of [3H]BP, animals were killed at times ranging from 10 hr to 28 days, and BP metabolite:DNA adducts were analyzed by high-pressure liquid chromatography. The major adduct identified in each tissue was the (+)-7,8,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene: deoxyguanosine adduct. A 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene: deoxyguanosine adduct, a (-)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-BP adduct in A/HeJ mice followed first-order kinetics over the time period examined, with a half-life of 18 and 9 days in lung and liver, respectively. The decay of this adduct in C57BL/6J mice was biphasic in both tissues. Our data on cell turnover suggest that there is active removal of adducts in liver, but that normal DNA turnover can account for the partial or possibly total observed disappearance of adducts in lung. These results suggest that the tissue specificity for BP-induced neoplasia in A/HeJ mice may be related to the relative persistence of adducts and high cell turnover rates in lung. In contrast, the results on formation and persistence of adducts and cell turnover do not provide an explanation for the strain difference in susceptibility to BP-induced pulmonary adenomas. It was also shown that the rates of removal of BP metabolite:DNA adducts in A/HeJ mice are not significantly different at a 500-fold lower BP dose.

INTRODUCTION

Mutation and malignant transformation of cells by chemicals may be a consequence of DNA synthesis on parent-strand templates containing unexcised chemically induced lesions. The ability of a cell to repair the damaged DNA by an error-free pathway prior to cell replication could constitute a critical protective mechanism against mutagenesis and carcinogenesis (5, 11, 14). Yang et al. (36, 37) demonstrated a linear relationship in normal human fibroblasts between the number of BPDEI2:DNA adducts present at the time of DNA replication and BPDEI-induced mutation frequency. If the cells were allowed to remain in confluenence until the BPDEI adducts had been removed by excision repair, then no mutations were detected. In contrast, the length of time that repair-deficient xeroderma pigmentosum cells remained in confluence had no effect on the frequency of BPDEI-induced mutations. In vivo evidence suggests that the deficient DNA repair processes found in individuals with xeroderma pigmentosum (13, 30) and ataxia telangiectasia (26) predispose them to neoplasia. Also, some investigators have suggested that the persistence of specific carcinogen:DNA adducts in the target tissue correlates with the susceptibility of the tissue to neoplasia. For example, the persistence of O6-ethylguanine adduct appears to correlate with the susceptibility of rat brain to neoplasia induced by ethyl-nitrosourea (20). Eastman and Bresnick (15) claim that 3-MC metabolite:DNA adducts are more persistent in lungs of mice strains susceptible to PAH-induced pulmonary neoplasia than in resistant strains, and Abbott and Crew (1) argue that the persistence of DNA adducts formed from metabolites of 15,16-dihydro-11-methylcyclopenta(a)phenanthrene-17-one, together with the relatively high rate of cell division, may be related to the tissue-specific carcinogenesis of this polycyclic ketone in TO mice.

PAHs such as BP are a major class of ubiquitous environmental carcinogens. Recent studies have characterized the in vivo formation of PAH metabolite:DNA adducts in a variety of tissues (1, 4, 6, 9, 15, 17, 22, 29). However, there have been few studies on the persistence of these adducts (1, 15, 29). The persistence of BP metabolite:DNA adducts in vivo in mouse skin after topical application of BP has been examined (29), but no correlation was observed between persistence of adducts and susceptibility of various strains of mice to PAH-induced neoplasia, a result that contrasts with that of Eastman and Bresnick (15). We examined the persistence of BP metabolite:DNA adducts in lung and liver of A/HeJ mice. The lungs of C57BL/6J mice and the livers of both strains are resistant to BP-induced neoplasia. The disappearance of adducts in lung and liver of A/HeJ mice was also examined at a much lower dose of BP in order to assess the effect of initial BP metabolite:DNA adduct levels on persistence of the adducts. To determine the contribution of normal DNA turnover to the observed in vivo rates of disappearance of DNA adducts, we also measured the rates of DNA turnover in the various tissues.

MATERIALS AND METHODS

Chemicals. [G-3H]BP (specific activity, 56 Ci/mmol) and unlabeled BP were obtained from Amersham Corp. (Arlington Heights, III.) and Sigma Chemical Co. (St. Louis, Mo.,) respectively. The fluorescent dye H33258 was purchased from Cabibochem-Behring Corp. (La Jolla, Calif.). Hydroxylapatite (DNA grade; Bio-Gel HTP) was obtained from Bio-Rad Laboratories (Richmond, Calif.). Call thymus DNA, alkaline phosphatase type III.
RESULTS

Disappearance of BP Metabolism:DNA Adducts in Mouse Lung and Liver. The rate of disappearance of BP metabolite-DNA adducts in lung liver of A/HeJ and C57BL/6J mice was studied after a single p.o. dose of [3H]BP (6 mg/mouse). This dose, administered twice, 2 weeks (3), or 6 weeks apart (33), induced 21 (3) or 17 (33) pulmonary adenomas/mouse in A/HeJ mice. Animals were sacrificed at times ranging from 10 hr to 28 days following p.o. administration of BP. The specific activities of BP metabolite:deoxyribonucleoside adducts were determined by HPLC analysis. A typical HPLC analysis is shown in Chart 1. Four peaks, labeled I, II, III, and IV, were observed in each tissue and in each mouse strain. Peaks II and III have been identified previously as (−)-BPDEI:dGuo and (+)-BPDEI:dGuo, respectively. Peak IV was identified as a BPDEII:dGuo adduct. Peak I could be either a benzo(a)pyrene-phenol-oxide:DNA adduct or a BPDEI:deoxyxytidine adduct. In addition to Peaks I to IV, uncharacterized radioactivity eluted in the WF and early in the methanol:water gradient (4, 15).

The time-dependent decrease in (+)-BPDEI:dGuo adduct levels (Chart 1, Peak III) in lung and liver of A/HeJ and C57BL/6J mice after a p.o. dose of BP is shown in Chart 2. In A/HeJ mice, this BPDEI adduct decayed exponentially in both lung and liver, the half-life being 18 and 9 days, respectively (Chart 2). In contrast, the BPDEI adduct levels in C57BL/6J mice did not

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(CE 3.1.3.1), and phosphodiesterase I (CE 3.1.4.1) were purchased from Sigma. DNase I was purchased from Worthington Biochemical Corp. (Freehold, N. J.) [3H]Thymidine (specific activity, 60 to 80 Ci/mmol) was obtained from New England Nuclear. All other chemicals were reagent grade or better.

Animals. Seven- to 9-week-old female A/HeJ and C57BL/6J mice (The Jackson Laboratory Bar Harbor, Maine) were used in all experiments. The mice were randomly selected by age before allocation to treatment groups. They were fed NIH-31 rat and mouse ration (Zeiger Brothers, Inc., Gardner, Pa.) and given water ad libitum for the duration of each study.

Treatment of Animals for Adduct Disappearance Studies. The BP dosing regimen is based on a previous study of Wattenberg (33) for the induction of pulmonary adenomas in A/HeJ mice. A/HeJ and C57BL/6J mice received a total of 6 mg of BP administered as 2 equal p.o. doses (2 x 3 mg) 2 hr apart. BP dose consisted of 3 mg of unlabeled BP and 1 mCi of [3H]BP dissolved in 0.25 ml corn oil. Animals were killed by cervical dislocation at times ranging from 10 hr to 28 days following treatment with BP (15 animals for each time point). The lungs and livers were removed and frozen in liquid nitrogen. In another experiment with A/HeJ mice, a much lower BP dose (0.012 mg/mouse) was used. Animals received a total of 2.6 mCi of [3H]benzo(a)pyrene, administered as 2 equal p.o. doses 2 hr apart.

Isolation and Analysis of BP Metabolites Bound to DNA. DNA was isolated from each pooled tissue sample by the hydroxylapatite procedure described previously (2). Samples of the purified DNA were assessed for DNA content by using the fluorescent dye (H33258) adduct method of Cesarone et al. (12). Calf thymus DNA was used as a reference standard. UV absorbance measurements were also made using the relationship 1 mg DNA = 20 A260 units.

DNA was digested enzymatically to deoxyribonucleosides and their BP metabolite adducts according to the method of Baird and Brooks (7). These adducts were analyzed by a modified version of the HPLC and liquid scintillation counting procedure described previously by Anderson et al (4). The HPLC system (Waters Associates, Milford, Mass.) consisted of a dry-packed precolumn containing the same material as that used in a Waters 10 μm C-18 Radial Pak analytical column. The precolumn was freshly packed and washed with methanol before each sample run. The elution program was carried out at a flow rate of 0.8 ml/min. The sample and internal standards (acetophenone, butyrophenone, and BP-9,10-diol) were loaded on to the precolumn and washed with 100% water for 10 min followed by methanol:water containing 0.1% triethylammonium acetate, pH 7, (40:60) for 5 min. Fifty drops were collected per vial. These samples were called the water fractions. The precolumn, containing retained adducts, was then connected to the analytical column, and the gradient fraction eluted with a linear methanol:water:triethylammonium acetate gradient (40 to 70% in 40 min). Fifty drops/vial were still collected until after the first internal standard peak (acetophenone) was seen. The drop number was then changed to 15 drops/vial throughout the remainder of the run.

Reference standards of BP metabolite:deoxyribonucleoside adducts were prepared by incubation of [G-3H]BP with 3-MC-induced liver microsomes in the presence of DNA as described by Pelkonen et al. (27). Other standards were also prepared by reacting [G-3H]±7-bromo-8,8-dinhydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene and BPDEI with DNA as described by Yamasaki et al. (35). Chromatograms of the reference standards are given by Anderson et al. (4).

Rate of DNA Turnover in Lung and Liver. Mice received [3H]thymidine (15 μCi/mouse i.p.) on 2 consecutive days (1). After 3 days, one group of animals was killed (zero time point), and the remaining animals received unlabeled BP (6 mg/mouse) or vehicle. Mice were killed at times ranging from 1 to 21 days following BP administration (5 animals/time point). DNA was isolated from lung and liver and quantitated as described above. Aliquots of isolated DNA samples were measured for radioactivity. Labeled DNA (dpm/mg DNA) at various time points was compared to the amount of DNA labeled at zero time point. Relative percentage of loss of labeled DNA was used as a measure of DNA turnover.

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Persistence of BP:DNA Adducts in Lung and Liver

Chart 2. Disappearance over time of BPDEI:DNA adduct from lung and liver of A/HeJ and C57BL/6J mice. Mice were sacrificed at times ranging from 10 hr to 28 days following p.o. administration of [3H]BP (6 mg/mouse). DNA isolated from lung or liver was enzymatically digested, and the deoxyribonucleosides were chromatographed on HPLC. The specific activity (pmol/mg DNA) of the BPDEI:DNA adduct was calculated from the area under Peak III in a chromatogram similar to those presented in Chart 1. Points, average of 2 HPLC determinations.

decay in a monophasic manner (Chart 2). The same general kinetics were observed in a repeat experiment in which adduct levels were only determined at 1, 7, 14, and 28 days following BP dose (data not shown). In the repeat experiment, BPDEI adduct levels decayed exponentially in lung and liver of A/HeJ mice with a half-life of 17 and 13 days, respectively, and the decay of this adduct in C57BL/6J mice was non-monophasic. In lung of A/HeJ mice, the disappearance of BPDEII (Peak IV) and Peak I adduct levels paralleled the decrease in BPDEI adduct levels. The half-lives for removal of BPDEII and Peak I adducts in lung of A/HeJ mice were 14 and 17 days, respectively. These adducts could not be quantitated in liver over the entire time span. (We did not attempt to determine the specific activity of a peak unless the counts in the peak were at least 100 dpm above background.)

The disappearance of BP metabolite:DNA adducts in lung and liver of A/HeJ mice was also examined at a much lower dose of BP (0.012 mg/mouse) to determine if the rates of disappearance for the adducts depended on the initial levels of the adduct. As with the higher dose of BP, the BPDEI adduct levels decayed exponentially (Chart 3). The half-lives for loss of the adduct were 17 and 13 days in lung and liver, respectively. Although the initial BPDEI adduct levels in lung and liver at the higher BP dose were approximately 1700- and 2700-fold larger, respectively, than at the lower dose, the removal rates of the adduct were similar at the 2 dose.

DNA Turnover Rates in Lung and Liver of Mice. The rate of DNA turnover in lung and liver of BP-treated and control mice was measured by prelabeling the DNA with [3H]thymidine. No difference in DNA turnover rates was observed in BP- and vehicle-treated mice. After BP treatment, there was an initial rapid loss of [3H]thymidine from lung in both A/HeJ and C57BL/6J mice (Chart 4). Approximately 7 days after BP treatment, the amount of [3H]thymidine in lung appeared to either approach a constant level, being approximately 20 to 30% of the level at time of BP treatment, or to decrease at a much slower rate (Chart 4). [3H]Thymidine levels in liver of C57BL/6J mice also decreased after BP dose and then approached a constant level of 60% of the initial level. In contrast, [3H]thymidine levels in liver of A/HeJ mice did not decrease significantly for the first 7 days after BP dose. The levels then decreased to 50% of initial level. Each time course for [3H]thymidine disappearance was done in

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Chart 3. Disappearance of BPDEI:DNA adduct from lung and liver of A/HeJ mice. Mice were sacrificed at times ranging from 10 hr to 28 days following p.o. administration of [3H]BP (0.012 mg/mouse). DNA isolated from lung and liver was enzymatically digested, and the deoxyribonucleosides were chromatographed on HPLC. The specific activity (pmol/mg DNA) of the BPDEI:DNA adduct was calculated from the area under Peak III in a chromatogram similar to those presented in Chart 1. Points, average of 2 HPLC determinations.

Chart 4. Loss of [3H]thymidine-labeled DNA from lung and liver of A/HeJ and C57BL/6J mice over 21 days following treatment with BP (6 mg/mouse). DNA was prelabeled with [3H]thymidine as described in "Materials and Methods." After administration of unlabeled BP (6 mg/mouse), animals were sacrificed at times ranging from 1 to 21 days. DNA was isolated from lung and liver, and specific activity (dpm/mg DNA) was determined. Ordinate, amount of DNA labeling is percentage of label at time of BP administration.
duplicate. The values at each time point are averages from the 2 separate experiments. The shapes of the time curves for \[^3H\]thymidine disappearance in the 2 experiments were very similar.

DISCUSSION

We examined the formation and disappearance of BP metabolite:DNA adducts in lung and liver of A/HeJ and C57BL/6J mice. We also compared the rate of cell turnover in these tissues and strains of mice. Although adducts disappeared at detectable rates in both the tissues, it is not possible at present to conclude that the removal in lung is due to DNA repair processes. The normal rate of DNA turnover could account for the partial or possibly total observed disappearance of adducts in lung of both strains. The relative rate of loss of \[^3H\]thymidine-labeled DNA in lung of both strains was greater than the rate of decay of the adducts as shown in Charts 2 and 4. Since there is very little DNA turnover in liver of A/HeJ mice for the first 7 days following BP dose, the removal of adducts in this tissue may be due to DNA repair. Although there is some DNA turnover for the first 4 days in liver of C57BL/6J mice, the initial loss of adducts is more rapid as well as sustained for the entire period of study. Abbott and Crew (1) examined the removal of DNA adducts formed from metabolites of 15,16-dihydro-11-methyl-cyclopenta(a)-phenanthren-17-one and DNA turnover rates in lung, skin, and liver of TO mice. Their data also suggest that there is a active removal of adducts in liver, whereas normal DNA turnover could account for the removal of adducts in lung and skin. If adducts do persist in some specific cell types in lung (skin) but are removed by enzymatic repair in the liver before cell division, then this, in part, may account for the susceptibility of lung (skin) and resistance of the liver to PAH-induced neoplasia in A/HeJ and TO mice. Several investigators (21, 23, 24) have induced liver hepatomas and enzyme-altered foci in rats when the PAH is administered after partial hepatectomy. Thus, PAH can be carcinogenic in the liver under altered conditions of cell replication.

On the contrary, our data on formation and persistence of BP metabolite-DNA adducts and on cell turnover rates offer no explanation for the strain difference in susceptibility to BP-induced pulmonary adenomas. Although the disappearance curves for adducts in lung are distinctly different in A/HeJ and C57BL/6J mice, one being a first-order decay and the other a biphasic decay, the levels of adducts are similar initially as well as at 28 days following the BP dose. Conclusions similar to ours were reached by several other workers. Phillips et al. (29) studied the formation and disappearance of DMBA metabolite-DNA adducts in skin of several mice strains. Their data could not explain the strain difference in susceptibility to DMBA-induced neoplasia in skin of mice. Similar conclusions were reached with BP and 3-MC, although adduct levels were examined at only 2 time points (29). Pelkonen et al. (27) studied the disappearance of BP adducts in skin and s.c. tissue of C3H and C57BL/6J mice. The rate of disappearance of the adducts did not differentiate between the C57BL/6J resistance and the C3H susceptibility to BP-induced s.c. fibrosarcomas. In contrast, Eastman and Bresnick (15) reported that the persistence of 3-MC metabolite-DNA adducts in mouse lung correlated with susceptibility of the various mice strains to 3-MC-induced pulmonary adenomas. The reasons for the discrepancy between the results of Eastman and Bresnick (15) and the other studies on correlation between persistence of PAH metabolite-DNA adducts and strain susceptibility to PAH-induced neoplasia is unclear.

It should be emphasized that the specific activities of the PAH metabolite-DNA adducts reported in this and the above-mentioned studies are calculated on the basis of the total DNA in the organ. It is possible that the amounts of adducts formed as well as their repair rates in different cell types of the target organ may vary considerably. Several investigations have demonstrated cell specificity in the in vivo formation and repair of carcinogen:DNA adducts in liver for 1,2-dimethylhydrazine and 2-acetylaminofluorene (8, 32, 34). Correlations were observed between the persistence of adducts and cell specificity for liver carcinogenesis (8, 32). Examination of the formation and persistence of PAH metabolite:DNA adducts in individual cell types of the target tissue might allow differentiation of tissues with respect to susceptibility and resistance to PAH-induced neoplasia.

The persistence of BP metabolite:DNA adducts in lung and liver of A/HeJ mice was examined at both a carcinogenic dose level and at a 500-fold lower dose. No significant difference in the rates of disappearance was observed, even though there was a 700-fold difference in the initial levels of the adducts. Consideration of the dose dependency of adduct formation and persistence is important in the low-dose extrapolation of carcinogenic data (5, 15, 28), and we know of no previous study on the dose dependency of the persistence of PAH metabolite:DNA adducts in vivo.

The excisability and persistence of BPDE:DNA adducts have been studied in several cell culture systems (10, 11, 16, 18, 19, 31, 36, 37). In these studies, DNA turnover was accounted for by keeping the cells in confluence (36, 37), or by prelabeling the cellular DNA prior to BPDE treatment (11, 16, 18, 19, 31) and, thus, the removal of adducts can probably be equated with excision repair. Feldman et al. (18) found the excisability of BPDE:DNA adducts in a human lung tumor cell line to be poor, and 50% persisted for several generations. They speculated that the persistent adducts could have resulted from the loss of excision repair capacity during prolonged incubation or from the location of the adducts on a portion of the DNA that cannot be repaired by the excision pathway. Eastman et al. (16) made similar observations in hamster tracheal epithelial cells.

In conclusion, although the persistence of carcinogen metabolite-DNA adducts in tissues with high DNA turnover is conducive for the initiation of neoplasia, the persistence of the adducts per se is apparently not sufficient for the induction of neoplasia. Elucidation of the protective role of excision repair in PAH-induced neoplasia requires further examination in target and nontarget tissues.

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Persistence of Benzo(a)pyrene Metabolite:DNA Adducts in Lung and Liver of Mice

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