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Induction of in vivo DNA adducts by 4 industrial by-products in the rat-lung-cell system

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Abstract

Benz[*a*]anthracene (BA), dibenz[*a*,*h*]anthracene (DBA), dibenzo[*a*,*i*]pyrene (DBP), and dibenz[*a*,*h*]acridine (DBAC) are by-products found in many industrial wastes and emissions. Workers in the related occupational settings are potentially exposed to these substances through inhalation. In the present study, induction of DNA adducts in vivo by these chemicals was investigated using ^{32}P-postlabeling analysis in the rat-lung-cell system. The potency of DNA-adduct inducing activity was also compared to that of two cytogenetic endpoints i.e., sister-chromatid exchange (SCE) and micronucleus formation. Via intratracheal instillation, male CD rats (6/group) were dosed 3 times with BA, DBA, DBP or DBAC in a 24-h interval. Lung cells were enzymatically separated and used to determine the frequency of DNA adducts, SCE and micronuclei. Results show that all 4 test compounds induced DNA adducts, SCEs, and micronuclei in the rat-lung cell in vivo and that the postlabeling DNA adduct assay detected genotoxic activity at lower dose levels than the two cytogenetic assays. These findings suggest that BA, DBA, DBP or DBAC are rat pulmonary genotoxicants and the DNA-adduct assay is more sensitive than SCE or micronucleus assays for detecting the pulmonary genotoxicity of these industrial PAHs in the in vivo rat-lung-cell system.

Key words: PAH; DNA adduct; SCE; Micronucleus; Rat-lung cell

1. Introduction

Benz[*a*]anthracene (BA), dibenz[*a*,*h*]anthracene (DBA), dibenzo[*a*,*i*]pyrene (DBP), and dibenz[*a*,*h*]acridine (DBAC) are toxic constituents of coke-oven emission, asphalt vapor, and/or coal tar, resulting from incomplete combustion of organic compounds in coal (NTP, 1989). The first three of these compounds are also by-products in gasoline or diesel-motor vehicle exhaust. Inhalation and/or dermal contact are the primary routes of potential human exposure to these toxic polycyclic aromatic hydrocarbons (PAHs). Potential occupational exposure to these agents exist at coke-oven plants, coal gasification and liquefaction manufacturing sites, foundries, asphalt roofing and paving, and coal-tar production plants (NTP, 1989). It has been estimated that more than 200 thousand workers were potentially exposed to DBP via inhalation of coke-oven emissions and the volatiles of asphalt

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and coal-tar pitch from 1972 to 1974 (NIOSH, 1976).

All of these four PAHs have been shown to be carcinogenic in experimental animals (e.g., mice, rats and/or hamsters), and the carcinogenic effects are specific as a function of administration (IARC, 1983, 1984, 1985; NTP, 1989). Tumors induced by these PAHs have been found in the skin (by topical administration), respiratory tract, and lung (by inhalation and intratracheal instillation) (IARC, 1983, 1985; NTP, 1989). Epidemiologic studies have demonstrated a higher incidence of lung cancer mortality among coke-oven workers (IARC, 1984; Lloyd, 1971; Redmond et al., 1972). An increased incidence of lung-cancer in workers exposed to coal tar and coal-tar pitch has also been reported (IARC, 1985).

It has been shown that these aromatic by-products induced gene mutations in *Salmonella typhimurium* (IARC, 1983; Phillipson and Ioannides, 1989). However, the information regarding their genotoxic potential in the lung-cell system is limited. It is important to build up the data base on these and other compounds for the DNA-adduct/lung-cell system versus other assays to further validate that system. In the present study, a comparative genotoxicity assay was conducted for BA, DBA, DBP, and DBAC in the rat-lung-cell system using ^{32}P-postlabeling DNA-adduct analysis. The formation of DNA adducts by these agents was also compared to two cytogenetic endpoints, i.e., sister-chromatid exchange (SCE) and micronuclei.

2. Materials and methods

Test chemicals. DBA, DBP, BA and DBA were purchased from Sigma Chemical (St. Louis, MO). All test chemicals were prepared in dimethyl sulfoxide (DMSO) just prior to use.

Reagents and enzymes. T4 polynucleotide kinase, nuclease P_1, micrococcal nuclease, proteinase K, RNAase A and T_1, and spleen phosphodiesterase were obtained from Sigma Chemical; [γ-^{32}P]ATP (3000 Ci/mmole, 10 mCi/ml) from DuPont/NEN (Boston, MA) polyethyleneimine (PEI) cellulose plates from Alltech (Deerfield, IL); and 1-butanol from Mallinckrodt, (Paris, KY).

Treatment. 6 male Sprague–Dawley rats (4–6 weeks old) per group were dosed 3 times during a 24-h interval with test chemicals (3 doses/chemical) via intratracheal instillation (Whong et al., 1992). 6 h after the third administration, animals were anesthetized by pentobarbital ($\sim$ 26 μg/rat) and lungs were harvested. In order to separate individual lung cells, each lung was infused with 8 ml of protease 14 solution (2 μg/ml) in Eagle's minimum essential medium (MEM) and incubated at 4°C for 16 h. The lungs were then cut into small pieces ($\sim$ 1–2 mm^3) with scissors and further incubated at 37°C for 30 min. At the end of incubation, 4 ml MEM and 1 ml each of fetal bovine serum (FBS) and DNAase (0.6 μg/μl) solution were added to each digested cell suspension, which was then filtered through 2 layers of screen (nylon blotting and silk cloths; 100 and 305 meshes), respectively. The separated lung cells were used for DNA adduct, SCE and micronucleus determinations.

DNA adduct assay. ^{32}P-Postlabeling analysis was used as the assay system (Randerath et al., 1981; Whong et al., 1992). DNA was extracted from enzyme-separated lung cells using the standard phenol/ethanol method and purified with RNAase digestion (Sambrook et al., 1989). Each DNA sample (12.5 μg) was enzymatically digested to 3′-monophosphate nucleotides by incubating with micrococcal nuclease and spleen phosphodiesterase at 37°C for 4 h. The enzyme-digested samples were then subjected to adduct-detection enhancements with both the butanol extraction (Gallagher et al., 1988; Gupta, 1985) and nuclease P_1 (Reddy and Randerath, 1986) methods. For the butanol enhancement, 5 μg of DNA digested in 24 μl total volume was mixed with 15 μl each of 10 mM transferring agent (tetrabutylaminonium chloride) and 100 mM ammonium formate (pH 3.5) and 70 μl distilled water (dH_2O). The mixture was extracted twice with 100 μl 1-butanol and then back-extracted two times with 500 μl water to remove normal nucleotides from butanol fractions. In the nuclease

P_1 enhancement, an equal amount of DNA digest (5 μg/24 μl) was incubated with 2.4 μl nuclease P_1 (5 μg/μl) and 9.6 μl reaction buffer (112 μM zinc chloride and 156 μM sodium acetate, pH 5.0) at 37°C for 1 h. The enhancement samples were dried and redissolved in 7.5 μl dH_2O. For the ^{32}P-labeling, a 7.5 μl radioactive mixture containing 5 μl ^{32}P-ATP, 20% kinase buffer (100 mM dithiothreitol, 10 mM spermidine, and 300 mM tris–base), and 2.5 units of T4 polynucleotide kinase was added to each sample and incubated at 37°C for 1 h. At the end of the labeling process, 14 μl of labeled sample was spotted onto a PEI–cellulose plate. DNA adducts were developed using the multi-directional TLC method (Gallagher et al., 1988; Gupta, 1985; Whong et al., 1992): (1) removal of unused ^{32}P-ATP and normal nucleotides with D1 (1 M sodium phosphate, pH 6.8); (2) first dimensional separation of adducts with D2 (25 M ammonium formate, pH 3.5) and D3 (4 M lithium formate and 7 M urea, pH 3.45); (3) second dimensional separation of adducts with pre-D4 (0.5 M tris–HCl, pH 8.0) and D4 (0.8 M lithium chloride, 0.5 M tris · HCl and 7 M urea, pH 8); and (4) background cleanup with D5 (0.35 M magnesium chloride). To determine the total amount of nucleotides, 4 μl of diluted DNA digest, which contained 4.16 ng nucleotides, was labeled with 2 μl of radioactive mixture, as described above, at 37°C for 1 h and treated with 4 μl apyrase and bicine for another 30 min. After adding 615 μl termination solution (15 mM EDTA and 10 mM tris–HCl, pH 9.5), 5 μl of reaction mixture was spotted onto a PEI–cellulose plate and developed with 40 mM ammonium sulfate (Kato et al., 1988). Adduct spots on PEI–TLC plates were visualized by autoradiography on Kodak XAR-5 film at −70°C for 24–48 h using intensifying screens. DNA adducts were quantified by scintillation counting and adduct levels were calculated using the following formula:

$$\text{Adducts}/10^8 \text{ nucleotides} = \frac{\text{CPM in adducted nucleotides}}{\text{CPM in total nucleotides}} \times 10^8$$

Sister-chromatid exchange assay. Enzyme-separated lung cells (~ 10^6 cells) were inoculated into a 75-cm^2 flask containing 15 ml of Ham's F-12 medium supplemented with 15% fetal bovine serum (FBS) and antibiotics (100-U penicillin/ml, 100 μg streptomycin/ml, and 50 μg gentamicin/ml). The cells were incubated for 1 day and then the medium in each flask was changed. BrdU (12.5 μM) was added to each culture and the cells were incubated at 37°C for 54 h. 6 h before the end of the incubation, colcemid (0.1 μg/ml) was added to each flask in order to arrest dividing cells at metaphase. Cells in each flask were then harvested by trypsinization and washed twice with PBS by centrifugation (500 × *g*). The washed cells were incubated at 37°C for 25 min in a hypotonic solution (0.075 M KCl) and fixed twice with a mixture of methanol and acetic acid (3 : 1). The fixed cells were then dropped on cold, wet slides and were stained following the method of Perry and Wolff (1974). 25 well-differentiated M2 cells (each containing 42 ± 4 chromosomes) were scored and the replicative index was calculated as below:

$$\text{Replicative index} = \frac{1 \times M1 + 2 \times M2 + 3 \times M3}{100}$$

Statistical significance was analyzed using the *t* test ($\alpha < 0.05$).

Micronucleus assay. Binucleated primary lung cells induced by cytochalasin B (CYB) were used for measuring the induction of micronuclei by test chemicals (Fenech and Morley, 1986; Whong et al., 1990). This procedure provided a more precise scoring method for micronuclei than the conventional method, because the enumeration of micronuclei was restricted to the dividing cells, which were required for the expression of micronuclei. In this assay, enzyme-separated primary lung cells (6×10^6) were seeded in a 75-cm^2 flask containing 15 ml of F-12 medium as described in the SCE assay. The medium was changed after incubation at 37°C for 1 day and the lung-cell cultures were incubated for 2 more days in the presence of 2 μg CYB/ml. At the end of incubation, cells were harvested by trypsinization and centrifugation and then treated with 0.075 M KCl at 37°C for 10 min. After being

centrifuged and resuspended, the lung cells were smeared on slides and fixed with methanol for 5–10 min. The slides were stained with Diff–Quik stain for 1 min with stain solution 1 and 0.5 min with stain solution 2 (Baxter Scientific Products, Obetz, OH). The number of micronucleated cells among 1000 binucleated cells was enumerated. The criteria for a positive response were: (1) at least two times the control at one dose level and (2) statistically significant based on the trend test.

3. Results

DNA adducts, induced by BA, DBA, DBP and DBAC, detected with both the butanol and nuclease P_1 enhancements are given in Table 1 and Fig 1. The doses used in the experiments were the highest concentrations possible without causing a lethal effect in rats. All of the 4 coke-oven and/or petroleum-related chemicals showed positive activities for in vivo DNA-adduct formation at all dose levels tested in the rat-lung-cell system. Under the conditions tested, both DBP and DBAC caused one type of DNA adduct, whereas BA and DBA induced 3 and 2 adduct spots, respectively. With respect to enhancements of adduct detection, a higher DNA adduct was detected with the butanol than with the nuclease P_1 enhancement method. With the nuclease P_1 enhancement, a lower amount of adduct activity (or a missing spot) was found for all 4 chemicals tested. Based on the mass of the chemicals tested, the potency of adduct induction among the 4 chemicals studied ranked in the order of DBP > DBA > BA > DBAC.

The 4 industrial by-products were also studied in regard to their relationship between DNA-adduct induction and 2 cytogenetic endpoints (i.e., SCE and micronuclei). As shown in Table 2, all 4

Table 1
DNA adducts induced by industrial chemicals related to coke-oven and petroleum industries in rat-lung cells

Test chemical	Chemical dose (mg/kg b.w.)	Adduct spot	Adducts/10^8 nucleotides [a]	
			Butanol	Nuclease P1
BA	25	1	1.6 [c]	0.6 [c]
		2	0.4	– [b]
		3	0.1	–
	50	1	4.1 [c]	2.6 [c]
		2	0.6 [c]	–
		3	0.4	–
	100	1	6.7 [c]	4.5 [c]
		2	0.7 [c]	0.4
		3	0.6 [c]	–
DBA	8.52	1	0.9 [c]	0.3
		2	0.2	–
	17.05	1	1.1 [c]	0.5 [c]
		2	0.2	–
	34.10	1	3.8 [c]	1.8 [c]
		2	0.6 [c]	–
DBP	2.5	1	0.7 [c]	0.2
	5.0	1	0.9 [c]	0.4
	10.0	1	2.1 [c]	0.9 [c]
DBAC	25	1	0.6 [c]	0.4
	50	1	1.4 [c]	0.6 [c]
	100	1	1.9 [c]	0.7 [c]

[a] Background adduct level was between 0.13 and 0.04 adduct/10^8 nucleotides.
[b] No corresponding spot.
[c] Post response (at least 3 × higher than background level).

test agents induced SCE in the rat-lung cells in vivo. However, except for DBP, induction of SCE was not found at the lowest dose tested for BA, DBA and DBAC. The range of positive activity was between 10.4 and 17.0 SCE/cell, of which DBP showed the highest and DBAC the lowest activities, respectively. In examining micronucleus formation, DBP and DBA showed positive activity at the 2 high doses tested, while BA and DBAC displayed activity only at the highest dose tested (Table 3). Moreover, the sensitivity of this cytogenetic assay for detecting the genotoxicity of these industrial-related chemicals was generally lower than the SCE assay, because higher doses were needed for the micronucleus assay to exert a positive activity in this study.

4. Discussion

It has been evident that covalent binding of chemicals to DNA resulting in DNA adducts is a common property of many carcinogens and genotoxicants (Lutz, 1979). The formation of certain types of DNA adducts is a crucial step in the initiation of cancer and the first step of mutagenesis. The requirement of additional DNA-adduct events in the later stages of carcinogenesis has also been demonstrated (Hennings et al., 1983). Formation of covalent DNA adducts has been considered to be a useful dosimeter for exposure. Therefore, it has been used as a biomarker for determining the internal dose of exposure to genotoxicants. In the present study, the 4 indus-

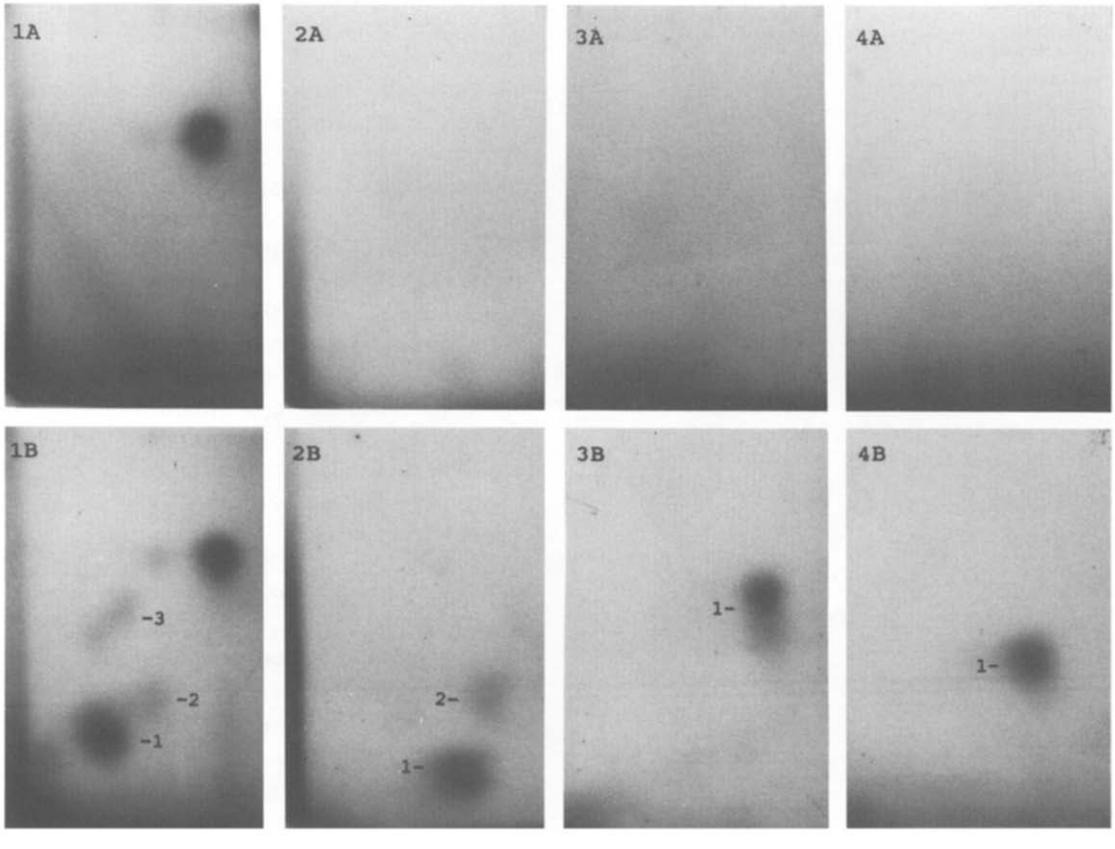

Fig. 1. Autoradiograms of DNA adducts induced by BA (1), DBA (2), DBP (3) and DBAC (4) in rat-lung cells. (A) control; (B) treatment.

Table 2
Sister-chromatid exchange induced by industrial pulmonary carcinogens with the in vivo/in vitro lung-cell assay

Test chemical	Chemical dose (mg/kg b.w.)	SCE/cell (mean ± SE)	Replicative index
BA	0	9.4 ± 2.4	1.84
	25	10.2 ± 2.1	1.76
	50	12.7 ± 3.4 *	1.78
	100	15.3 ± 2.8 **	1.63
DBA	0.00	8.8 ± 1.7	2.11
	8.52	9.2 ± 2.2	2.32
	17.05	10.9 ± 1.9 *	1.74
	34.10	10.4 ± 0.7 *	1.71
DBP	0.0	7.9 ± 2.1	1.92
	2.5	10.6 ± 2.8 *	1.84
	5.0	12.4 ± 3.2 **	1.65
	10.0	17.0 ± 4.3 **	1.50
DBAC	0	7.4 ± 0.8	2.04
	25	8.1 ± 2.4	1.86
	50	10.6 ± 3.5 *	1.90
	100	11.2 ± 3.8 *	1.54

* Significant ($p < 0.05$, t test).
** Significant ($p < 0.01$, t test).

Table 3
Micronuclei induced by industrial pulmonary carcinogens in rat-lung cells

Test chemical	Chemical dose (mg/kg b.w.)	Micronucleated cells/10^3 binucleated cells (mean ± SE) [a]	Binucleated cells (%)
BA	0	18.4 ± 2.6	12.8
	25	22.7 ± 3.5	14.6
	50	29.6 ± 5.3	14.1
	100	54.7 ± 4.2 [b]	10.5
DBA	0.00	20.5 ± 3.2	8.7
	8.52	29.4 ± 4.7	7.3
	17.05	43.6 ± 6.8 [b]	6.5
	34.10	74.7 ± 11.5 [b]	7.2
DBP	0.0	15.8 ± 4.2	11.4
	2.5	25.2 ± 7.3	9.2
	5.0	45.3 ± 6.1 [b]	10.3
	10.0	67.5 ± 5.7 [b]	7.5
DBAC	0	17.5 ± 4.6	13.8
	25	25.4 ± 3.8	11.7
	50	29.1 ± 6.6	14.5
	100	40.6 ± 8.3 [b]	10.1

[a] Significant for all the test chemicals (by the trend test).
[b] Positive activity (2× equal to or more than the control).

trial by-products (BA, DBA, DBP and DBAC) were investigated for DNA adduct formation in rat-lung cells in vivo using ^{32}P-postlabeling analysis. The study showed that all 4 agents induced DNA adducts in this lung-cell system. The findings are comparable to previous results in other cell systems or in other species. For instance, it has been reported that DBP induced DNA adducts in the lung and skin of mice, and the DNA-adduct formation was consistent with its carcinogenicity in mouse skin (Hughes and Philips, 1990). In vitro DNA adducts induced by BA have been demonstrated in human lymphocytes (Gupta et al., 1988) and calf-thymus DNA (Bryla and Weyand, 1992). Lecoq et al., (1991a,b) has revealed that DBA induces adducts in DNA after being activated by rat-liver microsomes and mouse skins, in which the major adduct was derived from the activation. Since PAHs require metabolic activation for their genotoxic and carcinogenic actions, the positive results of DNA-adduct formation from our study imply that the rat-lung-cell system possesses the necessary metabolic activity for the toxic action, and that this cell system is promising for the study of the genotoxicity of industrial PAHs in this animal model.

BA, DBA, DBP and DBAC are common industrial by-products found in exhausts from coke-oven and iron-foundry plants and in vapors from roofing and paving. Using ^{32}P-postlabeling analysis, it has been reported that workers from these industries have shown an elevated DNA-adduct level in white-blood-cell samples. Two studies on foundry workers revealed that there is a good correlation between DNA-adduct formation and the intensity of exposure (Reddy et al., 1981; Savela et al., 1989). It has also been shown that much higher DNA adducts were found in coke-oven workers than in the rural control (Hemminki et al., 1990). In the roofing setting, a significant increase in DNA adducts was observed in workers compared to nonworkers (Herbert et al., 1990). The positive results in our study suggest that the elevated DNA adducts in these workers may, at least in part, be attributed to the exposure of BA, DBA, DBP and/or DBAC in the workplace.

All 4 PAHs tested showed a higher DNA-adduct detection with the butanol than with the nuclease P_1 enhancement. The similar finding has also been reported for other aromatic compounds by several independent studies (Gallagher et al., 1988; Whong et al., 1992). The nature of a higher sensitivity with the butanol enhancement for these aromatics is not known. However, it is possible that the induced adducts by these tested agents are considerably non-polar, which are more readily extracted by butanol, and/or that the adducts are vulnerable to the cleavage action of nuclease P_1. Furthermore, the site and conformation of adducts induced by these PAHs may also be contributed to the different sensitivity of the two enhancements (Gallagher et al., 1988; Gupta and Farley, 1988; Reddy and Randerath, 1986).

In the correlation study between DNA-adduct induction and other genotoxic endpoints (SCE and micronuclei), all 4 aromatic compounds tested not only induced DNA adducts, but also induced SCEs and micronuclei in the same lung-cell system, suggesting a good correlation among those 3 genetic effects. However, at the lowest dose tested, these substances displayed positive activities with the postlabeling/DNA-adduct assay (when the butanol enhancement was used), but not all with SCE and/or micronucleus test systems. These observations indicate that with the butanol enhancement, the postlabeling DNA-adduct analysis is a more sensitive assay than the two cytogenetic assay systems (i.e., SCE and micronuclei) for detecting the pulmonary genotoxicity and potential carcinogenicity of these industrial by-products.

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