Appendix 1

B.Binkova, J.Topinka, R.J.Sram, O.Sevastyanova, Z.Novakova, J.Schmuczerova, I.Kalina, T.Popov, P.B.Farmer

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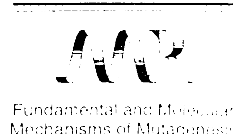

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In vitro genotoxicity of PAH mixtures and organic extract from urban air particles Part I: Acellular assay

B. Binkova [a], J. Topinka [a,*], R.J. Sram [a], O. Sevastyanova [a], Z. Novakova [a], J. Schmuczerova [b], I. Kalina [b], T. Popov [c], P.B. Farmer [d]

[a] *Laboratory of Genetic Ecotoxicology, Institute of Experimental Medicine, AS CR and Health Institute of Central Bohemia, Videňská 1083, 142 20 Prague 4, Czech Republic*

[b] *Department of Medical Biology, Medical Faculty of P.J. Safarik University, Tr. SNP 1, 040 60 Košice, Slovak Republic*

[c] *Department of Toxicology, National Center of Public Health Protection, Sofia, Bulgaria*

[d] *Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, Leicester LE1 7RH, UK*

Abstract

Acellular assay of calf thymus DNA ± rat liver microsomal S9 fraction coupled with ^{32}P-postlabelling was used to study the genotoxic potential of organic compounds bound onto PM10 particles collected in three European cities—Prague (CZ), Kosice (SK) and Sofia (BG) during summer and winter periods. B[a]P alone induced DNA adduct levels ranging from 4.8 to 768 adducts/10^8 nucleotides in the concentration dependent manner. However, a mixture of 8 c-PAHs with equimolar doses of B[a]P induced 3.7–757 adducts/10^8 nucleotides, thus suggesting the inhibition of DNA adduct forming activity by interaction among various PAH. Comparison of DNA adduct levels induced by various EOMs indicates higher variability among seasons than among localities. DNA adduct levels for Prague collection site varied from 19 to 166 adducts/10^8 nucleotides, for Kosice from 22 to 85 and for Sofia from 6 to 144 adducts/10^8 nucleotides. Bioactivation with S9 microsomal fraction caused 2- to 7-fold increase in DNA adduct levels compared to −S9 samples, suggesting a crucial role of indirectly acting genotoxic EOM components, such as PAHs. We have demonstrated for the first time a significant positive correlation between B[a]P content in EOMs and total DNA adduct levels detected in the EOM treated samples ($R = 0.83$; $p = 0.04$). These results suggest that B[a]P content in EOM is an important factor for the total genotoxic potential of EOM and/or B[a]P is a good indicator of the presence of other genotoxic compounds causing DNA adducts. Even stronger correlation between the content of genotoxic compounds in EOMs and total DNA adduct levels detected ($R = 0.91$; $p = 0.005$) was found when eight c-PAHs were taken into consideration. Our findings support a hypothesis that a relatively limited number of EOM components is responsible for a major part of its genotoxicity detectable as DNA adducts by ^{32}P-postlabelling.
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Keywords: Acellular assay; DNA adducts; ^{32}P-postlabelling; PAHs; Complex mixtures

Abbreviations: B[a]P, benzo[a]pyrene; B[b]F, benzo[b]fluoranthene; B[k]F, benzo[k]fluoranthene; B[a]A, benz[a]anthracene; B[ghi]P, benzo[g,h,i]perylene; BPDE, benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±]; CHRY, chrysene; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; DB[al]P, dibenzo[a,l]pyrene; DB[ah]A, dibenz[a,h]anthracene; 7,12-DMBA, 7,12-dimethylbenz[a]anthracene; DMSO, dimethyl sulphoxide; I[cd]P, indeno[c,d]pyrene; EOM, extractable organic matter adsorbed to respirable air particles; PAHs, polycyclic aromatic hydrocarbons; RAL, relative adduct labelling; TLC, thin layer chromatography

* Corresponding author. Tel.: +420 2 4106 2675; fax: +420 2 4106 2785.
E-mail address: jtopinka@biomed.cas.cz (J. Topinka).

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1. Introduction

Investigation of the biological effects of ambient air particulate matter has involved a number of different approaches, including the studies of particle induced genotoxicity. The latter was shown to be related to chemical compounds bound onto the particles or to particles themselves [1]. Some studies suggest that genotoxic effect of the particulate matter is due to polycyclic aromatic hydrocarbons (PAHs) and their derivatives present in the organic fraction of PM [2–4] while other studies indicate that some metals, forming PM can catalyze reactions resulting in oxidative stress and DNA damage [5–7]. A wide variety of *in vitro* systems was developed in order to study the genotoxicity of chemicals and their mixtures, including complex mixtures of environmental pollutants adsorbed onto respirable air particles (<2.5 μm). Complex mixtures of organic compounds to which humans are exposed through air pollution are only partially characterized with respect to their chemical composition due to difficulties with chemical analysis of the individual components. Therefore, alternative assays based on biological effects of complex mixture components may be a suitable alternative to a circumstantial chemical analysis.

PAHs constitute a major class of environmental pollutants generated by combustion of organic matter in mobile sources, such as motor vehicles as well as in stationary sources, such as power plants, residential heating, etc. Many PAHs, particularly the larger five- and six-ring compounds that can be metabolized to diol epoxides are mutagens and carcinogens [8–10]. It has been repeatedly shown that PAHs formed DNA adducts after metabolic activation by P450 enzymes to diol epoxides [11–13]. Rat liver microsomal fraction (S9) has been frequently used for metabolic activation of compounds like PAHs contained in the complex mixtures to test their mutagenic effect in bacteria [14,15]. The same activation system was also used in an acellular assay coupled with ^{32}P-postlabelling [16–20] to assess genotoxic potential of complex environmental mixtures via the analysis of DNA adduct forming activity of the mixtures in native DNA.

The extraction of particles collected from ambient air by various organic solvents was repeatedly used to characterize the chemical composition and biological effects of extractable organic matter (EOM) [2,21–23]. Since the EOM mixture is extremely complex, it is challenging to determine which components or groups of components are responsible for the observed genotoxic effect.

This study used the acellular model consisting of calf thymus DNA ± S9 (rat liver microsomal fraction) to assess the genotoxic potential of complex mixtures of organic air pollutants adsorbed onto PM10 particles in the air by means of DNA adduct analysis.

2. Materials and methods

2.1. Chemicals and biochemicals

Spleen phosphodiesterase was purchased from ICN Biomedicals, Inc.; micrococcal nuclease, nuclease P1 from Sigma (Deisenhofen, Germany); polyethylene imine cellulose TLC plates (0.1 mm) from Macherey-Nagel (Düren, Germany); c-PAHs (99% pure) from Supelco, Inc.; T4 polynucleotide kinase (USB); γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Amersham Biosciences. All other chemicals and solvents were of HPLC or analytical grade.

2.2. Air sampling, EOM extraction and chemical analysis

Particulate matter <10 μm (PM10) was collected in cities of Prague (Czech Republic), Kosice (Slovak Republic) and Sofia (Bulgaria) by HiVol air samplers (Anderson) 24 h daily during 3 months in winter and summer seasons. Both the dichloromethane extractions of organic complex mixture (EOM) from the Pallflex filters 20 cm × 20 cm (T60A20) with PM10 samples as well as the chemical analyses of PAHs were performed uniformly in the laboratories of the certified company Ecochem a.s. Prague (EN ISO CSN IEC 17025). Details of these procedures have been already described elsewhere [22,23]. The concentrations of eight polycyclic aromatic hydrocarbons (PAHs) regarded as carcinogenic PAHs (c-PAHs) according to IARC [8], namely, benz[a]anthracene (B[a]A), chrysene (CHRY), benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P), dibenzo[a,h]anthracene (DB[ah]A), benzo[g,h,i]perylene (B[ghi]P), and indeno[1,2,3-c,d]pyrene (I[cd]P) were analyzed in each EOM sample as described earlier [22]. For the *in vitro* experiments, EOM samples were evaporated to dryness under a stream of nitrogen and the residue re-dissolved in dimethylsulfoxide (DMSO). The stock solution of each EOM sample contained 50 mg EOM/ml DMSO.

2.3. *In vitro* acellular assay with DNA adduct analysis

The assay was performed as previously described [18,21]. Calf thymus DNA (1 mg/ml) was incubated with B[a]P (0.1–100 μM), synthetic mixture of 8 c-PAHs (0.1–100 μM), or various EOM samples (100 μg EOM/ml) for 24 h at 37 °C with and without metabolic activation using the S9 fraction (0.5 mg protein/ml). Rat liver S9 fraction, induced by Aroclor 1254 was purchased from Organon Teknika Corp (West Chester, PA). DMSO treated calf thymus DNA samples were used as a negative control. DNA was isolated by phenol/chloroform/isoamylalcohol extraction and ethanol

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precipitation [25], and the samples were kept at −80 °C until analysis.

^{32}P-postlabelling analysis was performed as previously described [26,27]. Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37 °C. Nuclease P1 was used for adduct enrichment. The labelled DNA adducts were resolved by multidirectional thin layer chromatography on 10 cm × 10 cm PEI-cellulose plates. Solvent systems used for TLC were the following: D-1: 1 M sodium phosphate, pH 6.8; D-2: 3.8 M lithium formate, 8.5 M urea, pH 3.5; D-3: 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. Autoradiography was carried out at −80 °C for 1 to 24 h. The radioactivity of distinct adduct spots was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (0.5 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA as well. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A BPDE-DNA adduct standard was run in triplicate in each postlabelling experiment in order to control for interassay variability and to normalize the calculated DNA adduct levels.

2.4. *Statistical analysis*

Pearson's correlation coefficient was used to analyze the correlation of DNA adduct levels with B[a]P and c-PAH content in EOMs from various localities and sampling period.

3. Results

3.1. *Air sampling, EOM extraction, and chemical analysis*

Detailed PM10 and EOM data were reported elsewhere [24]. Some details of HiVol samplings are shown in Table 1. In summer period, the highest concentrations of PM10 in the air were detected in Prague, followed by Sofia and Kosice, while in winter period, PM10 was most abundant in Sofia, followed by Prague and Kosice. Higher content of extractable organic compounds (EOM) was observed in summer in Prague (4.96 μg/m^3) and Sofia (3.95 μg/m^3) as compared to Kosice (1.67 μg/m^3). Similarly to PM10, the highest content of EOM extracted from samples collected in winter period was found in Sofia (24.6 μg/m^3) as compared to Prague (14.9 μg/m^3) and Kosice (15.3 μg/m^3). The data suggest stronger seasonal differences in PM10 concentrations and EOM contents rather than differences between localities. Different proportions of individual c-PAHs were seen in summer samples as compared to winter samples (Table 2). B[ghi]P and I[cd]P were relatively more apparent in summer samples at all three localities, whereas B[a]A and CHRY were more abundant in winter samples. Higher proportion of B[ghi]P indicates motor vehicles as a main emission source of c-PAHs during the summer season [28]. More abundant B[a]A and CHRY indicate residential heating as a main emission source during the winter period. Interestingly, relative seasonal differences in the total content of c-PAHs and the level of B[a]P are almost equal, thus suggesting that the level of B[a]P is a good marker of total c-PAH concentration in EOM. Despite the fact that all three monitoring sites are classified according to the European Environmental Register of monitoring stations as commercial, residential, traffic, and urban [23], substantially different c-PAH levels were found between cities of Prague and Sofia on one hand and city of Kosice on the other hand (Table 2). While in Kosice summer and winter c-PAH levels in EOM are almost equal, in Prague and Sofia c-PAH concentrations in EOMs are 4-fold and 2.5-fold higher in winter, respectively.

3.2. *DNA adducts induced by B[a]P and c-PAH artificial mixture*

Benzo[a]pyrene incubated for 24 h with calf thymus DNA + S9 fraction induced at concentration range of 0.1–100 μM DNA adduct levels ranging from 4.8 to 768

Table 1
Characteristics of HiVol samples of PM10 collected during winter and summer seasons in three cities: Prague (Czech Republic), Kosice (Slovak Republic) and Sofia (Bulgaria)

Season	Monitoring site	PM 10 (μg/m^3)	EOM (μg/m^3)	B[a]P (ng/m^3)	c-PAHs[a] (ng/m^3)
Winter	Prague	62.59	14.93	3.50	24.69
	Kosice	57.99	15.30	1.37	11.87
	Sofia	89.88	24.60	4.84	36.44
Summer	Prague	36.91	4.96	0.28	2.29
	Kosice	24.30	1.67	0.15	1.20
	Sofia	29.72	3.95	0.36	2.43

[a] Sum of carcinogenic PAHs.

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Table 2
Carcinogenic PAHs in the organic matters (EOMs) extracted from PM10 particles collected in various localities during winter and summer season[a]

PAHs in EOM	Prague			Kosice			Sofia		
	Winter [ng/mg][c]	Summer [ng/mg][c]	W/S[b]	Winter [ng/mg]	Summer [ng/mg][c]	W/S	Winter [ng/mg]	Summer [ng/mg]	W/S
Benzo[a]pyrene	234	50	4.7	90	90	1.0	197	92	2.1
Benz[a]anthracene	348	34	**10.2**	182	71	**2.6**	249	51	4.9
Benzo[b]fluoranthene	308	74	4.2	165	159	1.0	213	122	1.7
Benzo[k]fluoranthene	134	36	3.7	58	66	0.9	121	55	2.2
Benzo[g,h,i]perylene	127	132	**0.96**	48	149	**0.3**	129	134	1.0
Chrysene	315	39	**8.1**	176	18	**9.8**	376	65	5.8
Dibenz[a,h]anthracene	19	10	1.9	6.4	15	0.4	35	10	3.5
Indeno[c,d]pyrene	167	87	**1.9**	50	149	**0.3**	162	85	1.9
Total c-PAHs	1652	462	3.6	775	717	1.1	1482	614	2.4

[a] Reliability of PAHs analysis expressed as percent relative standard deviation (% R.S.D.) was less than 20% with exception of dibenz[a,h]anthracene (±26%).
[b] $W/S = c_{PAH\ winter}/c_{PAH\ summer}$.
[c] Refers to the ng/mg of total EOM.

adducts/10^8 nucleotides (Table 3). To assess the interaction among various carcinogenic PAHs forming complex environmental mixtures, an artificial mixture of eight c-PAHs was prepared (B[a]P 14.3%; B[a]A 21%; B[b]F 18.6%; B[k]F 8%; B[ghi]P 7.7%; CHRY 19%; D[ah]A 1%; I[cd]P 10%) based on chemical analysis of c-PAHs in EOM extracted from particles collected in Prague in the winter period (Table 2). The corresponding amounts of c-PAHs were calculated according to the B[a]P in order to keep relative abundance of all components as detected in real EOM. This c-PAH mixture, with concentration range of B[a]P 0.1–100 μM, induced 3.7–757 adducts/10^8 nucleotides (Table 3) corresponding to the inhibition of DNA adduct forming activity by 23–78%; no effect at 100 μM B[a]P was observed. The inhibitory effect of some c-PAHs on major B[a]P-induced adduct spot is clearly seen by comparing the adduct pattern in Fig. 1A and B. In fact, this effect is even stronger than shown in Fig. 1 because, as observed earlier [2,3], other c-PAHs than B[a]P should also contribute to the total adduct levels by their own genotoxicity.

3.3. *DNA adducts induced by EOMs*

Total DNA adduct levels induced by EOMs extracted from PM10 particles were quantified by ^{32}P-radioactivity detected in diagonal radioactive zone (DRZ) of TLC chromatograms (Fig. 2). Qualitatively similar DNA adduct pattern was observed for EOMs from various localities. DNA adduct levels for Prague collection site varied from 19 to 166 adducts/10^8 nucleotides, for Kosice from 22 to 85, and for Sofia from 6 to 144 adducts/10^8 nucleotides (Table 4). Bioactivation by rat liver microsomal fraction caused 2- to 7-fold increase of DNA adduct induction (Table 4) thus suggesting a crucial role of indirectly acting genotoxic

Table 3
Total DNA adduct levels induced in acellular system (calf thymus DNA + S9) incubated with B[a]P and c-PAHs[a] at equimolar concentration of B[a]P

B[a]P concentration[a] (μM)	Total DNA adducts		Amplification/inhibition (%)[b]
	B[a]P[c]	c-PAHs[c]	
0.1	4.8 (1.0)	3.7 (1.2)	23
1	38.5 (5.8)	8.4 (2.6)	78
10	375 (28.6)	209 (14.2)	−44
100	768 (201)	757 (156)	−1

Data from at least three independent incubations are shown.
[a] c-PAH mixture was prepared on the basis of chemical analysis of PAHs in EOM extracted from PM10 particles collected in Prague in the winter period (see Table 1). The abundance of each individual c-PAH was calculated in relation to B[a]P.
[b] Amplification (+) or inhibition (−) of total DNA adduct levels expressed in (%) were calculated as follows: (RAL[illegible], RAL[illegible]) [illegible] where RAL × 10^8 (relative adduct labelling) = adducts per 10^8 nucleotides.
[c] RAL × 10^8 (±S.D.).

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Fig. 1. Autoradiographs of TLC maps of ^{32}P-labelled DNA adducts derived from B[a]P (100 μM) (A) and c-PAH mixture (100 μM) (B) (see list in Table 1) after incubation of calf thymus DNA + microsomal S9 fraction. DNA (5 μg) was analyzed using the nuclease P1 method of sensitivity enhancement. Screen-enhanced autoradiography was performed at −70 °C for 1 h.

EOM components, such as c-PAHs. High relative contribution (~25–37%) of directly acting genotoxicants was detected in EOM from Kosice for both summer and winter sampling period, while in Sofia a strong induction of DNA adducts by EOM components requiring metabolic activation was observed (80–90%). In Prague, the contribution of directly acting genotoxicants varied substantially between summer (28%) and winter (14%) sampling periods. Samples with metabolic activation (+S9) exhibited more than 3-fold increase of total adduct levels (Prague and Sofia EOM samples) collected in winter period compared to summer period. However, for Kosice samples almost no seasonal variation was observed. Similar seasonal variability was found in samples without external metabolic activation (−S9) indicating higher levels of directly acting genotoxic compounds during winter period, such as nitro-PAHs. With exception of Kosice in winter, the differences among all three localities in DNA adduct forming activity of EOM samples are substantially lower (±20%) for samples with metabolic activation (+S9) than for samples without metabolic activation (−S9).

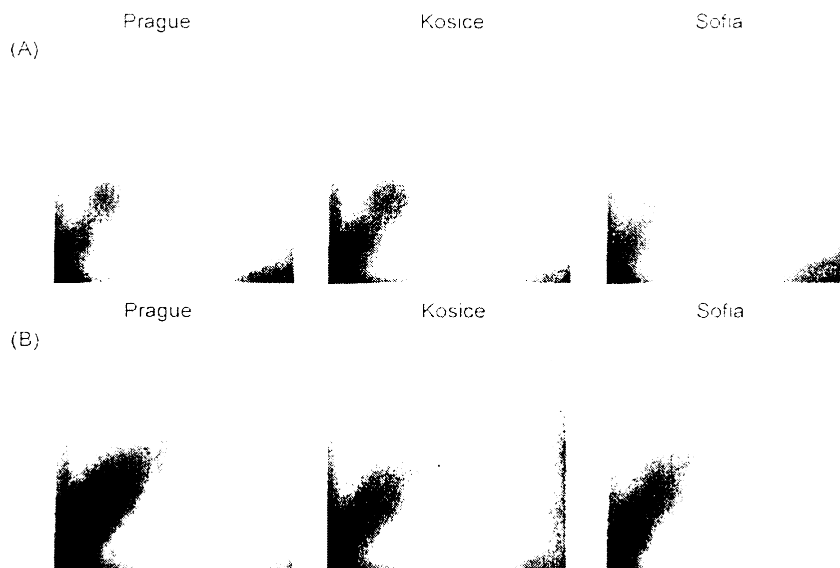

Fig. 2. Autoradiographs of TLC maps of ^{32}P-labelled DNA digests after incubation of calf thymus DNA (±S9 fraction) with 100 μg/ml extractable organic matter (EOM) from PM10 particles collected during summer and winter sampling period in Prague (CZ), Kosice (SK), and Sofia (BG). The panels depict analyses of calf thymus DNA treated with: EOMs + S9 fraction during summer sampling period (A); EOMs + S9 fraction during winter sampling period (B). DNA (5 μg) was analyzed using the nuclease P1 method of sensitivity enhancement. Screen-enhanced autoradiography was performed at −70 °C for 6 h.

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Table 4
Total DNA adduct levels induced in acellular system (calf thymus DNA ± S9) incubated with EOMs[a] collected in Prague, Kosice and Sofia during winter and summer period

Locality	Total DNA adducts					
	Summer			Winter		
	+S9[b]	−S9[b]	+S9/−S9	+S9[b]	−S9[b]	+S9/−S9
Prague	49(6.1)	19 (4.6)	2.6	166 (15.5)	28 (6.4)	6.0
Kosice	62(5.2)	22 (5.2)	2.9	85 (12.5)	50 (7.1)	1.7
Sofia	45(7.2)	6 (1.9)	7.1	144 (13.8)	33 (4.8)	4.3

Data from at least three independent incubations are shown. RAL × 10^8 (relative adduct labelling) = adducts per 10^8 nucleotides.
[a] Samples were incubated with 100 μg EOM/ml for 24 h. DNA was isolated and ^{32}P-postlabelling was performed as described in Section 2.
[b] RAL × 10^8 (± S.D.).

4. Discussion

Our model study is based on the assumption that the genotoxic components adsorbed on surface of PM10 are released from particles in the lung. One of the most detailed studies [29] demonstrated that highly lipophilic carcinogens, such as B[a]P have a dual dosimetry in the lungs, with 80% of B[a]P deposited in the alveolar region and rapidly passed into the blood without much metabolism while 20% is deposited on the conducting airways and is slowly absorbed under intense metabolism. The extraction of PM10 particles by dichlormethane (DCM) might overestimate real risk (the extraction of PAHs from particles by DCM is quantitative), but as indicated above, there is no question that most of c-PAHs is desorbed very quickly after deposition of respirable particles in the lung.

The question has been addressed whether an acellular assay, based on the DNA adduct analysis in calf thymus DNA in the presence or absence of the rat liver microsomal fraction, could be used to evaluate the genotoxic potential of complex mixtures of organic air pollutants (EOMs) adsorbed onto PM10 particles collected in three European cities—Prague (CZ), Kosice (SK) and Sofia (BG) during summer and winter period. The results support the conclusion that this system represents a suitable, simple and reliable method to detect the genotoxic potential of EOM components, particularly PAHs, as well as to distinguish among direct and indirect genotoxicants in the mixture.

4.1. *DNA adducts induced by B[a]P and c-PAH artificial mixture*

Although humans are never exposed to single substances or even well defined mixtures, we have for the first time confirmed that acellular system consisting of calf thymus DNA ± rat liver microsomal S9 fraction is sensitive enough to detect DNA adducts induced by B[a]P as the most thoroughly studied PAH found in the environment [30]. B[a]P was detected in significant amounts in all EOM samples within this study. Chemical analysis of EOMs proved also the presence of other carcinogenic PAHs (c-PAHs) listed in Table 2. The strong correlation between B[a]P and other c-PAH content in all EOMs tested in this study ($R = 0.98$; $p = p < 0.001$) suggests that B[a]P may be used as an indicator of the presence of other c-PAHs in mixtures. The crucial role of B[a]P in the genotoxicity of complex PAH mixtures was confirmed earlier by Randerath et al. [31]. The question concerning the interaction of c-PAHs in the process of bioactivation (competition for activating enzymes in S9 fraction) as well as in the process of DNA adduct formation, was addressed. We have demonstrated earlier [23] that all components of c-PAH mixture used in this study induced DNA adduct levels in the acellular test with calf thymus DNA (+S9) comparable with those induced by B[a]P. Thus, in case of the additivity of the effects, one should expect that total DNA adduct levels induced by c-PAH mixture (including B[a]P) should be substantially higher than adduct levels induced by B[a]P alone. In most cases, however, we observed lower DNA adduct levels in c-PAH mixture treated samples, suggesting a strong inhibitory effect, also observed by others [10]. Even stronger inhibition of DNA adduct formation was observed in human derived cell lines (HepG2, HEL, THP-1) treated with c-PAH mixtures under comparable conditions [32]. Therefore, it seems likely that the acellular system used in this study is less sensitive to the general toxic effects of c-PAHs than living cells and that the inhibition of adduct forming activity is predominantly caused by competition of c-PAHs for activating enzymes in S9 fraction.

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4.2. DNA adducts induced by EOMs

Several studies have demonstrated that EOMs extracted from ambient air particles consist of many diverse classes of compounds, such as PAHs, nitro- and alkyl-substituted PAHs, and heterocyclic compounds, that have both mutagenic and carcinogenic activity [33,34]. The question is whether the majority of these EOM components induce DNA adduct formation. DNA adduct levels detected in this study for EOMs from various localities suggest that the adduct levels are mostly related to c-PAH content in the EOMs. We found a positive correlation (Fig. 3A) between B[a]P content in EOMs from various localities and sampling periods and total DNA adduct levels detected in the EOM treated samples ($R = 0.83$; $p = 0.04$). This finding suggests that B[a]P content in EOM is the most important factor in its genotoxic potential and/or B[a]P is a good indicator of the presence of other genotoxic compounds causing DNA adducts. Even stronger correlation between the content of genotoxic compounds in EOMs and total DNA adduct levels detected ($R = 0.94$; $p = 0.005$) was found when not only B[a]P, but eight c-PAHs listed in Table 2, were taken into consideration (Fig. 3B). This correlation supports our earlier hypothesis [2] that a relatively limited number of EOM components is responsible for a major part of EOM genotoxicity detectable as DNA adducts by ^{32}P-postlabelling. As observed earlier using a similar model system [4], the genotoxic compounds derived from different test areas and sampling periods are qualitatively similar as shown by the finding that the pattern of DNA adducts resemble each other. Quantitatively, the differences in EOM genotoxicity among all three cities are weak compared to the differences between total adduct levels detected in samples treated with EOMs derived from particles collected in summer and winter. Similar observations were reported earlier [2,21,35].

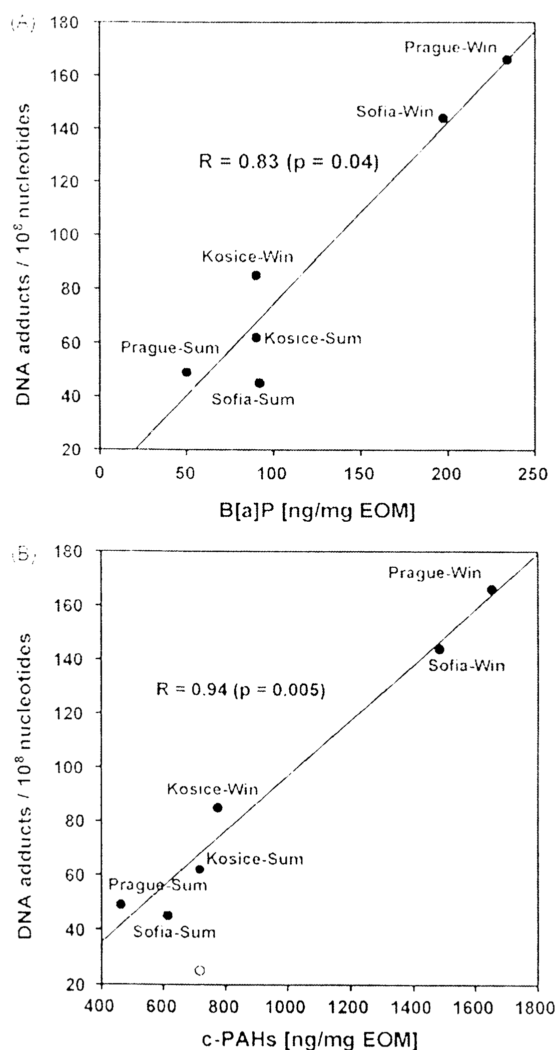

Fig. 3. Relation of B[a]P (A) and c-PAH (B) content in EOMs from various localities and seasons to DNA adduct levels induced by EOMs in acellular system (calf thymus DNA + S9 fraction).

In order to get more insight into the real human exposures resulting from the air pollution in all three localities and both sampling periods, the results of total DNA adduct levels were normalized to the amounts of EOMs per meter cube. Such a comparison underlines the difference between summer and winter sampling period indicating that organic compounds bound onto PM10 particles collected from 1 m^3 of ambient air in winter represents, depending on locality, 15–20-fold higher genotoxic potential than particles collected in summer (Fig. 4). This might be explained by increased levels of particle-associated genotoxic compounds resulting from local heating and other combustion emissions, frequent atmospheric inversions and increased deposition of vapor-phase mutagens onto particles during low temperature periods.

The relative comparison of DNA adducts forming activity normalized, to the amounts of EOMs per meter cube, also suggests that in the winter period the highest human exposure to genotoxic compounds resulted from the air pollution in Sofia, followed by Prague and Kosice. This conclusion corresponds to our finding in HepG2 cells reported in [32] suggesting that both models—calf thymus DNA + rat liver S9 fraction used in this study and human hepatoma cell line HepG2 used in [32] are capable of activating similar groups of genotoxic compounds as might be expected by liver origin of both systems.

Finally, the study has shown that a cell-free system in conjunction with the sensitive ^{32}P-postlabelling is a

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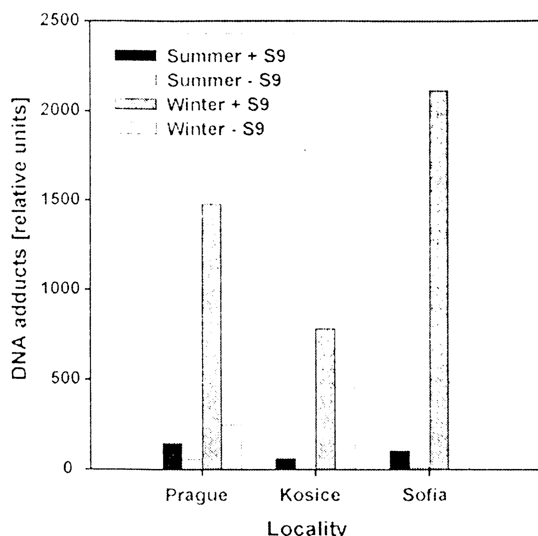

Fig. 4. Relative comparison of DNA adduct forming activity in acellular system (calf thymus DNA ± S9 fraction) detected for various EOMs of the air particulate matter sampled in Prague (CZ), Kosice (SK), and Sofia (BG) in summer and winter sampling periods. Samples were incubated with 100 μg EOM/ml for 24 h. Differences in EOM concentrations between localities and sampling periods were taken into the consideration—adducts/10^8 nucleotides were multiplied by a factor correcting for differences in EOM/m^3 of air. DNA adduct levels are expressed in relative units.

suitable model to detect genotoxic potential of EOMs, particularly those containing c-PAHs, as well as to distinguish between direct and indirect genotoxicants in the complex mixtures of environmental pollutants. Our results indicate that c-PAHs contribute predominantly to the total genotoxicity of various EOMs. We have demonstrated for the first time a significant positive correlations between B[a]P and/or c-PAH contents in EOMs from various localities and sampling periods and total DNA adduct levels detected in the EOM treated DNA samples. Taking into account the amount of c-PAHs in EOMs, it seems that a relatively limited number of EOM components is responsible for a major part of EOM genotoxicity detectable as DNA adducts by ^{32}P-postlabelling.

Since the interaction of EOM components with living cells may substantially differ from the interaction with native DNA, the Part II of this study [32] employs three human cell lines: the human hepatoma cell line (HepG2), human diploid lung fibroblasts (HEL), and human acute monocytic leukemia cell line (THP-1) to assess genotoxic potential of various EOMs.

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Appendix 2

O.Sevastyanova, B.Binkova, J.Topinka, R.J.Sram, I.Kalina,
T.Popov, Z.Novakova, P.B.Farmer

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In vitro genotoxicity of PAH mixtures and organic extract from urban air particles Part II: Human cell lines

O. Sevastyanova[a], B. Binkova[a], J. Topinka[a,*], R.J. Sram[a], I. Kalina[b], T. Popov[c], Z. Novakova[a], P.B. Farmer[d]

[a] *Laboratory of Genetic Ecotoxicology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic*
[b] *Department of Medical Biology, Medical Faculty of University P.J. Šafárik, Tr. SNP 1, 040 60 Košice, Slovak Republic*
[c] *Department of Toxicology, National Center of Public Health Protection, Sofia, Bulgaria*
[d] *Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, Leicester LE1 7RH, UK*

Abstract

Principal aims of this study were at first, to find a relevant human derived cell line to investigate the genotoxic potential of PAH-containing complex mixtures and second, to use this cell system for the analysis of DNA adductforming activity of organic compounds bound onto PM10 particles. Particles were collected by high volume air samplers during summer and winter periods in three European cities (Prague, Kosice, and Sofia), representing different levels of air pollution. The genotoxic potential of extractable organic matter (EOM) was compared with the genotoxic potential of individual carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) as well as their artificial mixtures metabolically competent human hepatoma HepG2 cells, confluent cultures of human diploid lung fibroblasts (HEL), and the human monocytic leukemia cell line THP-1 were used as models. DNA adducts were analyzed by ^{32}P-postlabeling. The total DNA adduct levels induced in HepG2 cells after exposure to EOMs were higher than in HEL cells treated under the same conditions (15–190 versus 2–15 adducts/10^8 nucleotides, in HepG2 and HEL cells, respectively). THP-1 cells exhibited the lowest DNA adduct forming activity induced by EOMs (1.5–3.7 adducts/10^8 nucleotides). A direct correlation between total DNA adduct levels and c-PAH content in EOM was found for all EOMs in HepG2 cells incubated with 50 μg EOM/ml ($R = 0.88$; $p = 0.0192$). This correlation was even slightly stronger when B[*a*]P content in EOMs and B[*a*]P-like adduct spots were analyzed ($R = 0.90$; $p = 0.016$). As THP-1 cells possess a limited metabolic capacity for most c-PAHs to form DNA reactive intermediates and are also more susceptible to toxic effects of PAHs and various EOM components, this cell line seemed to be an inappropriate system for genotoxicity studies of PAH-containing complex mixtures. The seasonal variability of genotoxic potential of extracts was stronger than variability among the three localities studied. In HepG2 cells, the highest DNA adduct levels were induced by EOM collected in Prague in the winter period, followed by Sofia and Kosice. However, in the summer sampling period, the order was quite opposite: Kosice > Sofia > Prague. When the EOM content per m^3 of air was taken into consideration in order to compare real exposures of humans to genotoxic compounds in all three localities, extracts from respirable dust particles collected in Sofia

Abbreviations: B[*a*]P, benzo[*a*]pyrene; B[*b*]F, benzo[*b*]fluoranthene; B[*k*]F, benzo[*k*]fluoranthene; B[*a*]A, benz[*a*]anthracene; B[*ghi*]P, benzo[*ghi*]perylene; BPDE, benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±]; CHRY, chrysene; DB[*al*]P, dibenzo[*al*]pyrene; DB[*ah*]A, dibenz[*ah*]anthracene; 7,12-DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethylsulphoxide; I[*cd*]P, indeno[*cd*]pyrene; EOM, extractable organic matter adsorbed to respirable air particles; HEL, human embryonic lung fibroblasts; RAL, relative adduct labelling; TLC, thin layer chromatography; PM10, air particles <10 μm; PAH, polycyclic aromatic hydrocarbons; c-PAH, carcinogenic polycyclic aromatic hydrocarbons; DRZ, diagonal radioactive zone; HiVol, high volume

* Corresponding author. Tel.: +420 2 4106 2675; fax: +420 2 4106 2785.
E-mail address: jtopinka@biomed.cas.cz (J. Topinka).

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exhibited the highest genotoxicity regardless of the sampling period. The results indicate that most of DNA adducts detected in cells incubated with EOMs have their origin in low concentrations of c-PAHs representing 0.03–0.17% of EOM total mass. Finally, our results suggest that HepG2 cells have a metabolic capacity for PAHs similar to human hepatocytes and represent therefore the best *in vitro* model for investigating the genotoxic potential of complex mixtures containing PAHs among the three cell lines tested in this study.
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Keywords: DNA adducts; ^{32}P-postlabeling; PAHs; Complex mixtures; Genotoxicity

1. Introduction

Several thousands of chemicals, including polycyclic aromatic hydrocarbons (PAHs), halogenated and nitrated organic compounds, sulphur compounds, organic acid derivatives, and organic metal compounds have been identified in polluted ambient air [1]. Many of these compounds adsorb onto the surface of respirable dust particles. PAHs and their derivatives are of primary concern, since some of them are known or suspected human carcinogens [2,3]. Cell culture studies have shown that PAHs are bound to DNA after being activated by cytochrome P450 enzymes to electrophilic metabolites [4–6]. Concentrations of many biologically active components in ambient air are very low and their detection represents a difficult analytical task [4]. Moreover, the knowledge of chemical composition of complex mixtures does not take into account interactions of various components which may produce synergistic, antagonistic, or additive effects [5]. Although the biological properties of many pure PAHs have been investigated, less is known about their effects when present as components of mixtures. The assumption that the total genotoxicity of a complex mixture is the weighted sum of the individual contributions of the mixture components, is not satisfactory [6].

Our previous studies [7,8] suggest that instead of a comprehensive chemical analysis, a "biological approach" based on the analysis of DNA adducts in cultured mammalian cells different in the expression of xenobiotic metabolizing enzymes could be used to determine the genotoxicity of complex mixtures such as EOM from coke oven emissions or extracts of ambient air particles.

In order to find an appropriate human cell line to test the genotoxicity of complex environmental mixtures *in vitro*, we used in this study several human-derived cell lines to assess the genotoxicity of individual PAHs, their binary and more complex artificial mixtures containing eight carcinogenic PAHs (B[*a*]P, B[*b*]F, B[*k*]F, B[*a*]A, B[*ghi*]P, CHRY, DB[*ah*]A, and I[*cd*]P), and, finally, the genotoxic potential of real EOMs extracted from PM10 particles collected in three European cities representing various levels of air pollution by genotoxic compounds—Prague (Czech Republic), Kosice (Slovak Republic), and Sofia (Bulgaria). In contrast to the acellular assay attempting to assess the genotoxic potential of the same complex mixtures using the system of calf-thymus DNA ± S9 liver microsomal fraction reported in Part I [9], three cell types were employed in this study: the human hepatoma cell line (HepG2), human diploid lung fibroblasts (HEL), and human acute monocytic leukemia cells (THP-1). The human hepatoma cell line HepG2 retains many characteristic enzyme pathways of human hepatocytes [10], being regarded as a "gold standard" in toxicology. Similar to hepatocytes, HepG2 cells contain PAH inducible CYP1A1 [11,12] and their overall xenobiotic metabolizing capacity is comparable to that observed in human hepatocytes in primary culture [13]. HepG2 cells are able to activate PAHs including B[*a*]P [14,15], 7, 12—DMBA [16], and DB[*ah*]A as well as many others to DNA reactive metabolites [17]. However, toxicogenomic studies [18] indicated that although HepG2 cells express genes similar to human liver and primary cultured human hepatocytes, they also express a significant number of other genes, not found in human hepatocytes and probably related to a transformed phenotype. In contrast to HepG2 cells, human diploid lung fibroblasts (HEL) at confluency are closer to *in vivo* conditions, since tumor cell lines are likely to contain multiple mutations that may affect the DNA damage response [19,20]. Finally, THP-1 cells were tested as a possible surrogate for lymphocytes, since they are known to keep many characteristics of monocytes for many months [21]—these being the most frequently used cell type in human biomarker studies [22–24].

2. Materials and methods

2.1. Chemicals and biochemicals

Spleen phosphodiesterase was purchased from ICN Biomedicals, Inc.; ribonuclease A and T1, proteinase K, micrococcal nuclease, nuclease P1, and protein assay kit (No. 5656) from Sigma (Deisenhofen, Germany); polyethyleneimine cellulose TLC plates (0.1 mm) from Macherey–Nagel

(Düren, Germany); DB[al]P from Midwest Research Institute (Kansas City, MO); all other c-PAHs (99% pure) from Supelco, Inc; T4 polynucleotide kinase from USB; and γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Amersham Biosciences. All other chemicals and solvents were of HPLC or analytical grade.

2.2. Air sampling, EOM extraction, and chemical analysis

Particulate matter <10 μm (PM10) was collected in cities of Prague (Czech Republic), Kosice (Slovak Republic), and Sofia (Bulgaria) by high volume (HiVol) air samplers (Anderson) 24 h daily within 3 months in summer and winter seasons. Details are described by Binkova et al. [9].

2.3. Cell culture conditions and treatment

2.3.1. HepG2 cells

The human HepG2 cell line was originally established from a human liver biopsy [25] and was obtained from A. Gabelova (Institute of Cancer Research, Bratislava, Slovakia). Cells (2–4 × 10^6) were cultured in 75 cm^2 flasks. The cells were grown in William's medium (Gibco) containing 10% FBS (Gibco), 2 mM glutamine, and supplemented with penicillin (50 U/ml) for 3–4 days until confluency, prior to treatment with PAHs, their mixtures or EOMs. The stock solutions of individual PAHs, artificial mixtures, and EOMs were diluted in DMSO immediately before use and added to serum-free medium at required concentrations. The concentration of DMSO never exceeded 0.5% (v/v). After 24-h exposure, the medium was aspirated and cells were washed twice with physiological saline. The cells were harvested into 15 ml centrifuge tubes with 4 ml physiological saline and centrifuged for 5 min. Cell pellets were washed twice with 13 ml physiological saline and resuspended in 3 ml physiological saline. Finally, 2 ml aliquots of all suspensions were stored in liquid nitrogen for the analysis of DNA adducts.

2.3.2. HEL cells

Human embryonic lung diploid fibroblasts (HEL, Sevapharma, Prague) were cultured and treated with individual PAHs, PAH artificial mixtures or EOMs as described by Binkova and Sram [26].

2.3.3. THP-1 cells

Human acute monocytic leukemia cells (THP-1, European Collection of Cell Cultures, ECACC, No. 88081201, UK). were cultivated at 37°C and 5% CO_2 in RPMI 1640 media (Sevapharma, Czech Republic) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM glutamine, and 100 U/ml penicillin. Cells were incubated in plastic bottles (25 cm^2, Costar) at the density of 5 × 10^5 cells/ml medium. Before cell treatment, the medium was replaced with fresh medium supplemented with 1% bovine serum. For cell treatment, the density of 8 × 10^5 cells/ml medium was employed. PAHs, their mixtures, and EOMs were dissolved in DMSO. The final concentration of DMSO did not exceed 0.1% of total incubation volume. Control samples included in each incubation set were treated with DMSO alone. Cells were incubated for 24 h. The harvested cells were washed three times in 50 ml physiological saline. Selected cultures were tested for viability by trypan blue test. Washed cells were resuspended in 2 ml physiological saline and stored in liquid nitrogen for the analysis of DNA adducts.

2.4. DNA isolation

The cell pellets were homogenised in a solution of 10 mM Tris–HCl, 100 mM EDTA, and 0.5% SDS, pH 8.0. DNA was isolated using RNAses A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol as previously described [27]. DNA concentration was estimated spectrophotometrically by measuring the UV absorbance at 260 nm. DNA samples were kept at −80 °C until analysis.

2.5. DNA adduct analysis

^{32}P-postlabeling analysis was performed as previously described [28,29]. Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37 °C. Nuclease P1 was used for adduct enrichment. This method is suitable to detect lipophilic DNA adducts with exception of some amino- and nitro-PAHs. The levels of these PAHs derivatives in EOMs from ambient air particulate matter are according to our previous experience almost negligible. The labeled DNA adducts were resolved by two-directional thin layer chromatography on 10 cm × 10 cm PEI-cellulose plates. Solvent systems used for TLC were the following: D-1: 1 M sodium phosphate, pH 6.8; D-2: 3.8 M lithium formate, 8.5 M urea, pH 3.5; D-3: 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. Autoradiography was carried out at −80 °C for 1–24 h. The radioactivity of distinct adduct spots was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of DNA enzymatic digest (0.5 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A BPDE-DNA adduct standard was run in triplicate in each postlabeling experiment to control for interassay variability and to normalize the calculated DNA adduct levels.

2.6. Statistical analysis

Linear regression was used to analyze correlation between c-PAH and B[a]P levels in EOMs and DNA adduct levels induced in HepG2, HEL, and THP-1 cells.

3. Results

3.1. DNA adducts induced by individual PAHs

To test for the ability of HepG2, HEL, and THP-1 cells to activate c-PAHs (as compounds implicated in the

Table 1
DNA adduct levels induced after 24 h exposure of HepG2, HEL, and THP-1 cells to single c-PAH compounds

Compound	Total DNA adducts					
	HepG2		HEL		THP-1	
	Concentration (μM)	RAL × 10^8 (±S.D.)	Concentration (μM)	RAL × 10^8 (±S.D.)	Concentration (μM)	RAL × 10^8 (±S.D.)
Dibenzo[*al*]pyrene	1	1293 (*616*)	0.1	2256 (*538*)	0.1	234 (*45*)
Benzo[*a*]pyrene	10	2865 (*1180*)	10	216 (*62*)	0.1	104 (*73*)
Benz[*a*]anthracene	10	216 (*26*)	10	3.5 (*0.4*)	10	0.6*
Benzo[*b*]fluoranthene	10	972 (*724*)	10	8.1 (2.5)	10	1.9*
Benzo[*k*]fluoranthene	10	74 (3.2)	10	4.0 (*1.5*)	0.1–10	n.d.
Benzo[*ghi*]perylene	10	1.3*	10	0.6 (*0.2*)	0.1–10	n.d.
Chrysene	10	294*	10	5.7 (*2.1*)	0.1–10	n.d.
Dibenz[*ah*]anthracene	10	233 (*126*)	10	2.8 (*0.5*)	0.1–10	n.d.
Indeno[*cd*]pyrene	10	25 (18)	10	2.2 (*0.8*)	0.1–10	n.d.

Data from at least three independent incubations are shown with exception of (*) labelled data representing mean value from two incubations only.
n.d., not detectable

carcinogenicity of EOM components), to DNA adduct-forming metabolites, the cell cultures were incubated with selected PAHs for 24 h at concentrations indicated in Table 1 and Fig. 1. Multiple adduct spots were detected in HepG2, HEL, and THP-1 cells treated with B[*a*]P and DB[*al*]P (Fig. 2). B[*a*]P induced a major BPDE-derived adduct spot in all three cell types. With the exception of DB[*al*]P, the most genotoxic compound in HEL cells, all other c-PAHs were by far more active in human hepatoma HepG2 cells, yielding 10–100-fold higher total DNA adduct levels (the sum of all adduct spots identified) than in HEL cells at 10 μM concentration. THP-1 cells seemed to be especially sensitive to B[*a*]P and DB[*al*]P at low concentrations. Although high total DNA adduct levels were observed at 0.1 μM B[*a*]P or DB[*al*]P (Fig. 1), adduct levels decreased rapidly with further increase in B[*a*]P or DB[*al*]P concentrations (Fig. 1C). As no DNA adducts were induced in THP-1 by c-PAHs other than B[*a*]P and DB[*al*]P (Table 1), these cells seem inappropriate for the study of adduct induction by PAH mixtures.

3.2. *DNA adducts induced by binary and c-PAH artificial mixtures*

The interactions of c-PAHs in complex environmental mixtures were modeled first on the binary mixtures of B[*a*]P with other c-PAHs at equimolar concentrations

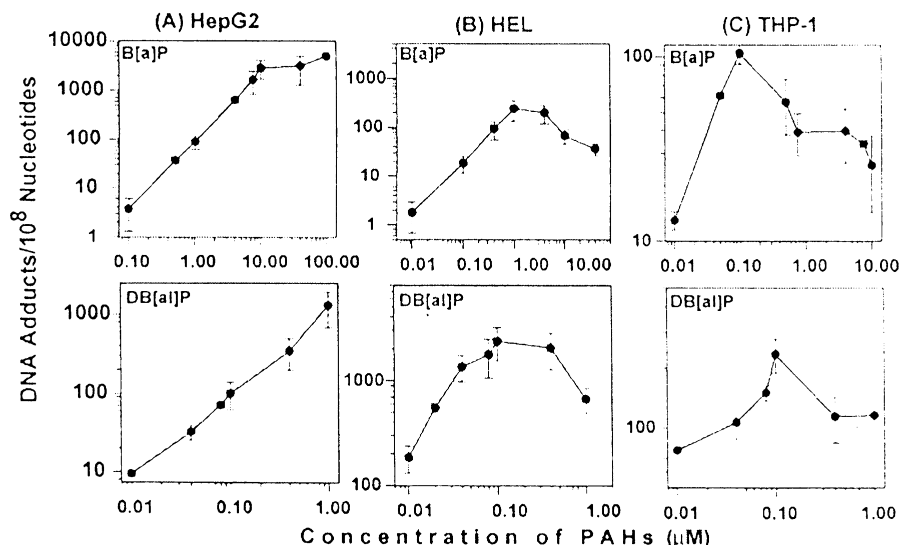

Fig. 1. Dose–response relationships of the total DNA adduct levels induced by benzo[*a*]pyrene and dibenzo[*al*]pyrene in HepG2 cells (A), HEL cells (B) and THP-1 cells (C). Cells were incubated for 24 h.

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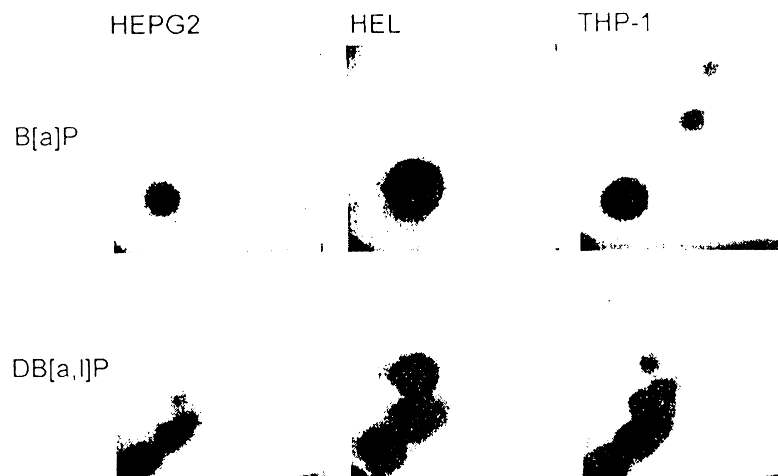

Fig. 2. Autoradiographs of TLC maps of ^{32}P-labelled DNA digests after incubation of HepG2, HEL and THP-1 cells with B[a]P (0.1 μmol/l) and DB[al]P (0.1 μmol/l) for 24 h. Screen-enhanced autoradiography was performed at −70 °C for 1–6 h.

of 1 μM (Table 2). HepG2 and HEL cells exhibiting higher levels of DNA adduct with individual PAHs were employed because THP-1 cells yielded no detectable adduct levels with most of individual c-PAHs (Table 1). Moreover, the B[a]P concentration of 1 μM was toxic for THP-1 cells already. The levels of the major B[a]P induced adduct (BPDE-derived) were analyzed in cells treated with B[a]P alone as well as with B[a]P in the mixtures with other c-PAHs. In cells treated with binary mixtures, HepG2 cells exhibited a synergistic effect amounting to ~20–140% enhancement of BPDE-DNA adduct almost exclusively, while a significant inhibition of DNA adduct forming activity was observed in HEL cells. The overlap of the second PAH adduct spot with the BPDE adduct spot was taken in the consideration. B[k]F and DB[ah]A blocked the formation of the major B[a]P induced DNA adduct in HEL cells almost completely. Interestingly, B[k]F was also the only c-PAH exhibiting inhibition of adduct formation in HepG2 cells.

To better mimic real exposure to many compounds in EOMs, a c-PAH mixture was prepared on the basis of chemical analysis of PAHs in EOM extracted from PM10 particles collected in Prague in the winter period [9]. The abundance of each individual PAH was calculated in relation to B[a]P. This relation was taken into account when preparing the artificial c-PAH mixture tested in all three cell types. The mixture was prepared at various PAH concentrations in the range of 0.01–1 μM calculated on B[a]P basis. Since the BPDE induced DNA adducts could be, in the case of incu-

Table 2
The effects of c-PAHs on BPDE-DNA adduct levels induced in HepG2 and HEL cells treated with binary mixtures of B[a]P and c-PAHs at equimolar concentrations (1 μM)

PAHs (1 μM)	BPDE adduct			
	HepG2		HEL	
	RAL × 10^8 (±S.D.)	Amplification/inhibition[a] (%)	RAL × 10^8 (±S.D.)	Amplification/inhibition (%)
B[a]P alone	81 (20)	–	228 (52)	–
B[a]P + B[a]A	134*	+22	149 (16)	−35
B[a]P + B[b]F	374 (4)	+75	118 (26)	−48
B[a]P + B[k]F	50 (10)	−45	7.1 (2.5)	−97
B[a]P + B[ghi]P	152*	+87	164 (33)	28
B[a]P + CHRY	172*	+92	60 (23)	−74
B[a]P + DB[ah]A	185*	+93	1.8 (0.5)	−99
B[a]P + I[cd]P	198*	+142	55 (19)	−76

Data from at least three independent incubations are shown with exception of (*) labelled data representing mean value from two incubations only.
[a] Amplification (+) or inhibition (−) of BPDE-derived DNA adduct levels expressed in (%) were calculated as follows: [(RAL$_{mixture}$/RAL$_{B[a]P}$ + RAL$_{second\ cPAH}$) − 1] × 100; where RAL × 10^8 (relative adduct labelling) = adducts per 10^8 nucleotides.

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Table 3
B[*a*]P-like[a] and total DNA adduct levels induced after 24 h exposure of HepG2, HEL, and THP-1 cells to artificial c-PAH mixtures[b]

B[*a*]P concentration in mixture [μM]	DNA adduct levels [RAL × 10^8] (±S.D.)					
	HepG2		HEL		THP-1	
	B[*a*]P-like spot	Total adducts	B[*a*]P-like spot	Total adducts	B[*a*]P-like spot	Total adducts
0.01	n.d.[c]	n.d.	0.3 (0.1)	0.6 (0.2)	n.d.	n.d.
0.05	n.d.	n.d.	1.8 (0.4)	2.9 (0.5)	n.d.	n.d.
0.1	1.2*	3.5*	3.5 (0.5)	4.5 (1.3)	3.6 (3.1)	4.0 (3.7)
0.5	9.8 (3.8)	16.5 (4.3)	15.4 (5.0)	20.2 (8.0)	3.5 (2.3)	3.9 (2.8)
1	20.0 (16)	28.7 (21.2)	14.4 (6.2)	16.9 (10.1)	2.7 (0.8)	2.9 (0.6)

n.d., not detectable; detection limit, 0.05 adducts/10^8 nucleotides. Data from at least three independent incubations are shown with exception of (*) labelled data representing mean value from two incubations only.

[a] B[*a*]P-like adduct levels were calculated from the radioactivity detected on TLC chromatogram in the same position as major B[*a*]P-derived DNA adduct.

[b] Artificial c-PAH mixtures were prepared on the basis of B[*a*]P concentrations indicated in the table. The concentrations of other c-PAHs were calculated on the basis of c-PAH concentrations in real EOM (Prague, winter sampling period, see Table 1).

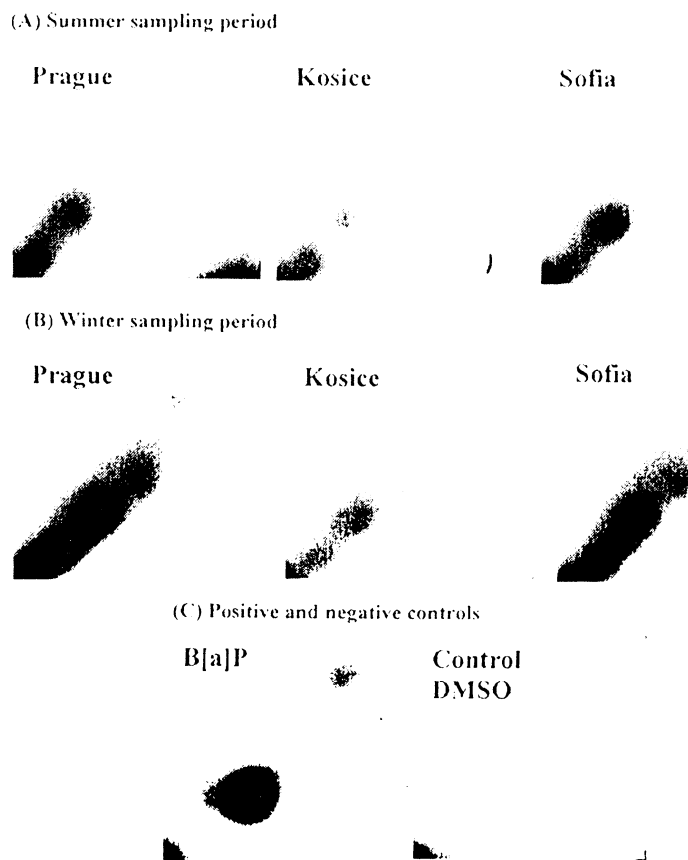

Fig. 3. Autoradiographs of TLC maps of ^{32}P-labelled DNA digests after incubation of Hep G2 cells with extractable organic matter (EOM) from Prague (CZ), Kosice (SK), and Sofia (BG). The panels depict analyses of DNA isolated from HepG2 cells treated with: 50 μg/ml EOM collected during summer period (A), 50 μg/ml EOM collected during winter period (B), positive (B[*a*]P, 100 μmol/l) and negative (DMSO) controls (C). DNA (5 μg) was analyzed using the nuclease P1 method of sensitivity enhancement. Screen-enhanced autoradiography was performed at −70 °C for 24 h.

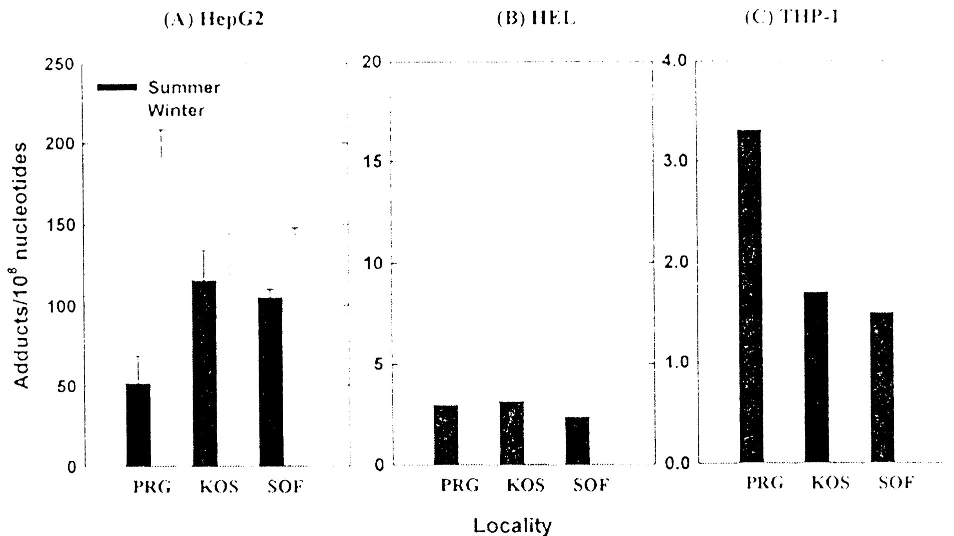

Fig. 4. Total DNA adduct levels induced by EOMs (50 μg/ml medium) extracted from PM_{10} particles from various localities in: human hepatoma (HepG2) cells (A), human diploid lung fibroblasts (HEL) cells (B) and human monocytic acute leukemia (THP-1) cells (C). Cells were incubated with EOMs for 24 h, DNA was isolated and DNA adducts were analyzed by ^{32}P-postlabelling using the nuclease P1 method of sensitivity enhancement.

bation with the c-PAH mixture, affected substantially by the adducts induced by other PAHs, the adduct having the same chromatographic mobility as BPDE-DNA is further referred to as B[*a*]P-like adduct. A dose-dependent increase of B[*a*]P-like and total DNA adduct levels was observed in HepG2 and HEL cells (Table 3). HepG2 cells did not exhibit any saturation effect in the whole range of PAH concentrations tested (0.01–1 μM). In contrast, B[*a*]P-like and total DNA adduct levels in HEL cells declined from an almost linear dose–response curve at the highest B[*a*]P concentration (1 μM). In THP-1 cells, very low DNA adduct levels were detected and no dose–response relationship was observed.

3.3. DNA adducts induced by EOMs

The DNA adduct patterns observed in HepG2 cells incubated with EOMs from all three localities and both summer and winter sampling periods exhibited diagonal radioactive zones (DRZ) of various intensities (Fig. 3), which are typical adduct patterns for the exposure to complex mixtures of genotoxic compounds in various experimental systems. The B[*a*]P-like spot was detectable in chromatograms derived from EOM samples in HepG2 (Fig. 3) and HEL cells (pictures not shown). At the dose of 50 μg EOM/ml, DNA adduct levels induced in HepG2 cells were ∼10–50-fold higher than those detected in HEL cells. At the same dose, very low total DNA adduct levels (∼1–3 adducts/10^8 nucleotides) were observed in all EOM samples incubated with THP-1 cells without any visible B[*a*]P-like spot.

In HepG2 cells (Fig. 4A), the highest DNA adduct levels were induced by EOM collected in Prague in the winter period followed by Sofia and Kosice. However, the order was opposite for the summer sampling period: Kosice > Sofia > Prague. In HEL cells (Fig. 4B), the seasonal differences of EOM adduct forming activities were much more significant than differences among localities: total DNA adduct levels were 3–6-fold higher in winter than in summer EOM-treated HEL cells. EOM samples obtained in all three localities during the summer sampling period gave almost equal and very low adduct levels in HEL cells (2–3 adducts/10^8 nucleotides), while winter samples varied between 9 and 15 adducts/10^8 nucleotides, being highest for Sofia. Results of DNA adduct analysis in THP-1 cells (Fig. 4C) suggest that adduct levels induced by EOMs are too low to draw any conclusion.

The dose–response relationship for total and B[*a*]P-like DNA adducts induced by various EOMs was studied in HepG2 cells (Fig. 5) as the most sensitive cell line in this study to detect adducts formed by EOMs. With the exception of EOM collected in Prague in the summer period, which showed a linear dose–response relationship in the whole range of 10–100 μg/ml, all other extracts exhibited an increase of adduct forming activity only for EOM doses from 10 to 50 μg/ml. Saturation or even a decrease of adduct levels was observed for the highest dose of 100 μg EOM/ml. Interestingly, the curves for B[*a*]P-like DNA adduct levels almost quan-

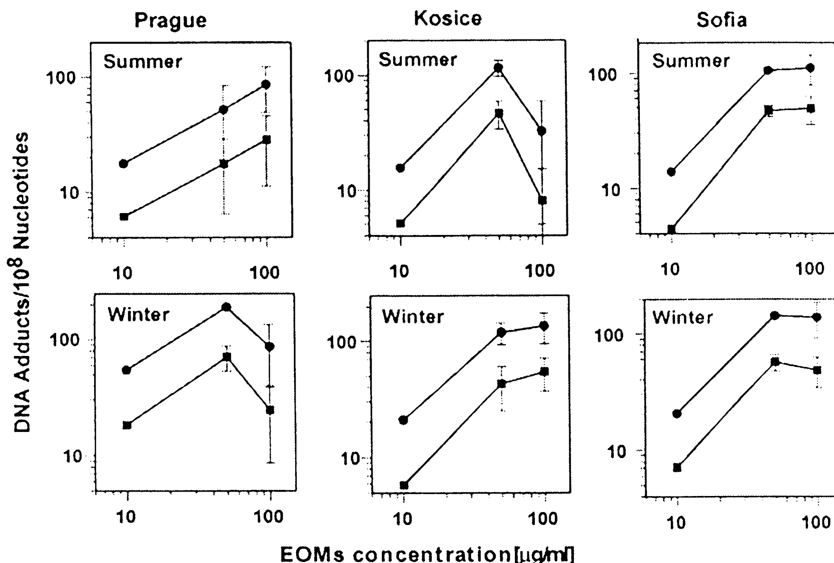

Fig. 5. Dose–response relationship of the DNA adduct forming activity of EOMs extracted from PM10 particulate matter collected in cities of Prague (Czech Republic), Kosice (Slovak Republic) and Sofia (Bulgaria) during summer and winter sampling period. Human hepatoma HepG2 cells were incubated with 10–100 µg of EOM/ml medium for 24 h and total (circles) and B[*a*]P-like (squares) DNA adduct levels were determined (see Section 2 for details).

titatively followed the dose–response curves for total DNA adduct levels, indicating a constant factor for the difference between these levels (Fig. 5).

4. Discussion

The two major aims of the present study were, firstly, to find an appropriate human derived cell line to determine *in vitro* the genotoxic potential of organic material bound onto particulate matter <10 µm (PM10) collected from ambient air, and secondly, to use this cell line to compare this potential in EOM samples collected in three European cities during both summer and winter sampling periods. In order to find a suitable cell line to test genotoxicity of complex mixtures *in vitro*, one should define the basic properties of such a model system: (1) It should be sensitive enough to detect low levels of genotoxic mixture components; (2) it should be metabolically competent to cover the wide spectrum of enzyme activities needed for bioactivation of indirect genotoxins such as PAHs; (3) it should mimic as well as possible the *in vivo* situation (for example confluent cultures of cells derived from target tissues for mutagenesis and carcinogenesis; (4) it should exhibit a dose–response relationship over wide concentration ranges without saturation of activating enzymes or toxic effects causing a plateau and a decrease of the dose–response curve. It is clear that a cell culture satisfying fully all four basic criteria does not exist. However, an attempt has been made to compare the competence of three cell lines in this study keeping in mind the above-mentioned properties.

4.1. DNA adducts by individual PAHs and binary mixtures

It is well established that c-PAHs are probably the most important components of complex environmental mixtures [30]. In order to get more insight into the mechanism of the interaction of these EOM components, we studied first the genotoxic potential of individual c-PAHs in comparison with selected binary mixtures. Strong interactions of components were observed even on the level of binary PAH mixtures as indicated by changes in BPDE-derived adduct spots caused by the presence of the second PAH. Depending on the cell type, we found either a synergistic interaction of B[*a*]P with the second c-PAH in HepG2 cells leading to the enhancement of BPDE-DNA adduct formation, or a strong inhibitory effect in HEL cells. Although such results seem to be contradictory, several HepG2 and HEL cells properties affecting the interaction of mixture components with DNA in cells should be taken into consideration. First, it is clear from DNA adduct analysis by individual PAHs that HepG2 cells are less susceptible to saturation of PAH activating enzymes and to toxic effects leading to a decrease of DNA adduct levels at high doses of PAHs and their mixtures than HEL cells. Similar to hepatocytes [7], HepG2 cells contain inducible CYP 1A1 [11,31]

which is not, under the experimental conditions used in this study, saturated by treatment with artificial c-PAH mixtures (as indicated by a linear dose–response relationship of DNA adduct levels) as is the case for HEL cells [26]. Depending on the system used, synergistic and inhibitory effects of simultaneous PAH treatment were also observed by other workers using different experimental systems, suggesting that B[*k*]F and DB[*ah*]A are the most potent inhibitors [32,33], which was confirmed also in this study.

4.2. DNA adduct formation by artificial c-PAH mixtures

Approaching further the situation of exposure to real EOMs, we analyzed the DNA adduct forming activity of an artificial c-PAH mixture containing eight carcinogenic PAHs. The mixture was prepared according to the chemical analysis of c-PAH in EOM obtained in Prague during the winter sampling period. The concentration of B[*a*]P was taken as a basis (0.01–1 μM) and the concentrations of other components were calculated accordingly to the relative abundance of other c-PAHs as detected in real EOM. DNA adduct analysis at various doses of this c-PAH mixture confirmed our previous results with individual PAHs and their binary mixtures, showing that HEL cells were more sensitive to detect adducts at lower c-PAH concentrations (0.01–0.5 μM), while HepG2 cells yielded an almost linear dose–response curve at higher doses (0.1–1 μM). Very low adduct levels and the absence of any dose–response relationship in THP-1 cells suggested that this cell line was strongly affected by toxic effects and/or that its capacity to metabolize c-PAHs was exhausted at all of the tested concentrations. Interestingly, the B[*a*]P-like DNA adduct spot, representing 50–80% of all DNA adducts detected in HepG2 and HEL cells, exhibited qualitatively an almost identical dose–response relationship when compared with total DNA adduct levels. This finding supports the conclusion made by others that B[*a*]P is of outstanding importance as a reference PAH for many complex mixtures in mutagenicity and carcinogenicity studies [30,34,35] and may be therefore used as the indicator of c-PAH concentrations and biological activity.

4.3. DNA adducts induced by EOMs

Linking the results of the DNA adduct analysis induced by various EOMs in this study and chemical analyses of c-PAHs reported earlier [36,37], we found a good correlation between these two parameters: the ranking of DNA adduct forming activity by EOMs from all localities in HepG2 cells is the same as the ranking of the content of c-PAHs (winter: Prague > Sofia > Kosice; summer: Kosice > Sofia > Prague). This finding suggests that the content of c-PAHs in EOM is crucial for the total genotoxic potential of the whole extract as detected by DNA adduct analysis. Taking into account that c-PAHs, depending on the locality and sampling period, formed 0.04–0.17% of the total mass of EOM, they form a very small portion of the material extracted from PM10, but they account for most of its genotoxicity. A direct correlation was found between the B[*a*]P content in EOMs and B[*a*]P-like adduct spots ($R = 0.90$; $p = 0.016$; Fig. 6A). A similar direct correlation exists between c-PAH content and the total DNA adduct levels in HepG2 cells incubated with 50 μg EOM/ml ($R = 0.88$; $p = 0.0192$; Fig. 6B) for all EOMs in this study. The crucial role of c-PAHs was also observed in our previous studies and other studies when fractionated crude EOMs were tested for genotoxicity in primary rat hepatocytes and V79NH cells [8]

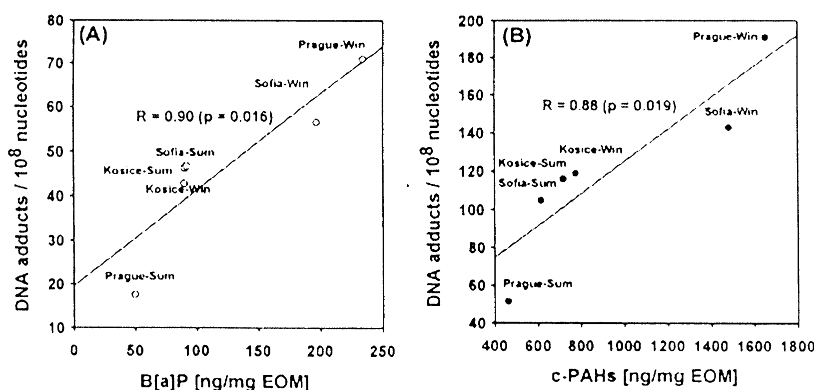

Fig. 6. Relation of c-PAH content in EOMs (A) to the total DNA adduct levels and B[*a*]P content in EOMs (B) to the B[*a*]P-like adduct levels. HepG2 cells were incubated with EOMs from various localities and seasons (Sum, summer; Win, winter) at concentrations of 50 μg EOM/ml medium.

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and when mutagenicity of EOMs was analyzed [34]. Furthermore, the B[*a*]P-like adduct spot, observed in all EOM treated samples and derived from BPDE and possibly from some other c-PAH diol-epoxides, represents 50–80% of all DNA adducts detected in HepG2 by EOMs. As observed earlier [8,35], the genotoxic compounds derived from different localities and sampling periods are qualitatively similar, which is indicated by the finding that DNA adduct patterns in all these tests resemble each other. This represents further evidence for a hypothesis that the spectra of EOM components forming detectable DNA adduct levels is limited and that the c-PAH content in EOM is crucial for the intensity of multiple adduct spots or diagonal radioactive zones and thus for the total DNA adduct levels.

A weak direct correlation between the c-PAH content in EOMs and total DNA adduct levels ($R = 0.74$; $p = 0.095$) was observed in HEL cells treated with various EOMs. In THP-1 cells, there is no relation between c-PAH content in EOM and DNA adduct levels.

To compare the real human exposures to genotoxic components of EOMs from various localities and sampling periods, the amounts of EOM per m^3 should be taken into consideration. Under such conditions, the observed differences between summer and winter sampling periods for all three localities are even more pronounced as demonstrated for HepG2 cells (Fig. 7). Differences among localities are less pronounced (Sofia ∼ Prague > Kosice). Similar differences among localities were observed when a cell-free system of calf thymus DNA + rat liver S9 fraction was employed for bioactivation of EOM components [9].

In conclusion, we have demonstrated, using HepG2 cells, that qualitatively similar adduct patterns with a dominating B[*a*]P-like DNA adduct were detected in all three localities. The total adduct levels are strongly related to the content of c-PAHs, which was several fold higher for samples collected in the winter season than in the summer season. The differences in EOM adduct forming activity between localities are less pronounced. Seasonal differences are even more distinguishable when the EOM quantities per m^3 are taken into consideration. Furthermore, we have shown that out of three cell lines tested for the ability to detect genotoxicity of extracts from ambient air particles via induction of DNA adducts, HepG2 cells represent the best compromise: they are metabolically competent to activate c-PAHs (as the most important genotoxic EOM components) and yield a dose–response relationship of adduct forming activity over a wide range of EOM concentrations. Confluent cultures of HEL cells, representing a relevant *in vitro* model of lung tissue as a major target tissue for air pollutants, are sensitive enough to detect DNA adducts of individual PAHs; however, strong inhibition of adduct formation occurred when artificial PAH mixtures were employed, and even lower sensitivity was observed for real EOMs. Finally, as THP-1 cells are unable to detect most of the c-PAH induced adducts, this cell line thus seems to be an inappropriate *in vitro* model to detect genotoxicity of PAH containing complex mixtures.

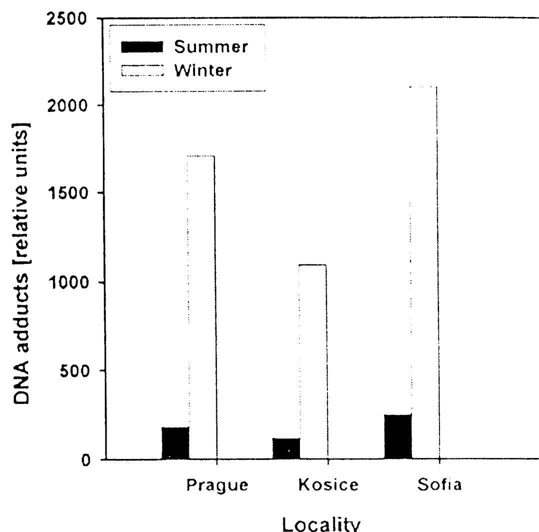

Fig. 7. Relative comparison of DNA adduct forming activity in HepG2 cells detected for various EOMs (50 μg EOM/ml) extracted from the air particulate matter (PM10) sampled in Prague, Kosice, and Sofia in summer and winter sampling periods. Differences in EOM concentrations per m^3 between localities and periods were taken into the consideration. DNA adduct levels are expressed in relative units. Adducts/10^8 nucleotides were multiplied by a factor correcting for differences in EOM/m^3 of air.

Our study suggests that a very small portion of the material extracted from PM10, in all likelihood c-PAHs, accounts for most of its genotoxicity.

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Appendix 3

A.Gabelova, Z.Valovicova, G.Bacova, J.Labaj, B.Binkova,
J.Topinka, O.Sevastyanova, R.J.Sram, I.Kalina, V.Habalova,
T.A.Popov, T.Panev, P.B.Farmer

Sensitivity of different endpoints for in vitro measurement of genotoxicity of extractable organic matter associated with ambient airborne particles (PM_{10})

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Sensitivity of different endpoints for in vitro measurement of genotoxicity of extractable organic matter associated with ambient airborne particles (PM_{10})

Alena Gábelová [a,*], Zuzana Valovičová [a], Gabriela Bačová [a], Juraj Lábaj [a], Blanka Binková [b], Jan Topinka [b], Oksana Sevastyanova [b], Radim J. Šrám [b], Ivan Kalina [c], Viera Habalová [c], Todor A. Popov [d], Teodor Panev [d], Peter B. Farmer [e]

[a] *Laboratory of Mutagenesis and Carcinogenesis, Cancer Research Institute SAS, Bratislava, Slovakia*
[b] *Laboratory of Genetic Ecotoxicology, Institute of Experimental Medicine AS CR and Health Institute of Central Bohemia, Prague, Czech Republic*
[c] *Department of Medical Biology, Medical Faculty University P.J. Šafárik, Košice, Slovakia*
[d] *Department of Toxicology, National Center of Public Health Protection, Sofia, Bulgaria*
[e] *Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, UK*

Abstract

Sensitivity and correlations among three endpoints were evaluated to assess the genotoxic potential of organic complex mixtures in vitro. This study was focused on DNA adduct formation, DNA single strand break induction and tumour suppressor p53 protein up-regulation produced by extractable organic matter (EOM) absorbed on respirable particulate matter PM_{10} (particulate matter < 10 μm) collected in three European cities (Prague, Sofia, Košice) during winter and summer period. To compare the sensitivity of particular endpoints for in vitro measurement of complex mixture genotoxicity, the metabolically competent human hepatoma cell line Hep G2 was treated with equivalent EOM concentration of 50 μg/ml. Cell exposure to EOMs resulted in significant DNA adduct formation and DNA strand break induction, however, a lack of protein p53 up-regulation over the steady-state level was found. While the maximum of DNA strand breaks was determined after 2 h cell exposure to EOMs, 24 h treatment interval was optimal for DNA adduct determination.

No substantial location- and season-related differences in EOM genotoxicity were detected using DNA strand break assessment. In agreement with these results no significant variation in DNA adduct levels were found in relation to the locality and season except for the monitoring site in Prague. The Prague EOM sample collected during summer period produced nearly three-fold lower DNA adduct level in comparison to the winter EOM sample.

Comparable results were obtained when the ambient air genotoxicity, based on the concentration of carcinogenic PAHs in cubic meter of air (ng c-PAHs/m^3), was elicited using either DNA adduct or strand break determination. In general, at least six-fold higher genotoxicity of the winter air in comparison to the summer air was estimated by each particular endpoint. Moreover, the genotoxic potential of winter air revealed by DNA adduct assessment and DNA strand break measurement increased in the same order: Košice ≪ Prague < Sofia.

Based on these data we suppose that two endpoints DNA breakage and DNA adduction are sensitive in vitro biomarkers for estimation of genotoxic activity of organic complex mixture associated with airborne particles. On the other hand, the measurement

* Corresponding author. Tel.: +421 259327512; fax: +421 259327506.
E-mail address: alena.gabelova@savba.sk (A. Gábelová).

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of protein p53 up-regulation manifested some limitations; therefore it cannot be used as a reliable endpoint for in vitro genotoxicity assessment.

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Keywords: Particulate matter; Carcinogenic polycyclic aromatic hydrocarbons; DNA adduct; Single strand break; In vitro genotoxicity

1. Introduction

The in vitro short-term genotoxicity assays form an important part of risk assessment of potential carcinogens. They are widely accepted around the world as useful tools to reduce the occurrence of hazardous compounds in the environment. Around 100 different types of short-term tests on prokaryotic and eukaryotic organisms have so far been developed to predict the potential health hazard of new chemicals. Various endpoints including gene mutations, clastogenic effects (e.g. chromosomal rearrangements, DNA breakage), DNA adducts or diverse cellular responses (e.g. DNA repair, apoptosis) are employed as biomarkers of genotoxicity. Among these endpoints, DNA adduct and DNA strand break formation have been proposed as standard biomarkers of DNA damage expression [1].

DNA adducts resulting from covalent binding of chemicals to DNA are a critical event in the initiation of cancer. Associations have been observed between DNA adduct formation, and mutagenesis [2], and tumorigenesis [3]. Various analytical methods have been developed to detect covalent DNA adducts including immunoassays, fluorescence and phosphorescence spectroscopy, mass spectrometry and ^{32}P-postlabelling [4]. The ^{32}P-postlabelling assay has been widely applied because of its high sensitivity and the requirement for only small amounts of DNA. This technique is able to detect a broad spectrum of hydrophobic compounds bound to DNA [5,6]; with some modifications, this method is additionally able to determine apurinic (AP) sites in DNA and oxidative DNA damage [7]. The ^{32}P postlabelling assay is especially useful for detection of adducts produced by single (individual) chemicals [8,9] but it is frequently employed also in human biomonitoring studies [10,11]. Compared with other methods for DNA adduct analyses the ^{32}P postlabelling assay has a unique potency for detection of DNA adducts from complex mixtures with unknown structures [12–14].

DNA strand breaks are readily detected as cell-response to exposure; breaks are induced directly by the agent or as a consequence of DNA damage removal in the process of DNA repair. A lot of biochemical techniques have been developed for DNA strand break estimation, based on different mechanisms and performed under different conditions [15]. Among them, the single cell gel electrophoresis (SCGE), known as the comet assay, is a sensitive, rapid and inexpensive fluorescence microscopic method measuring DNA damage at the level of a single cell [16]. Depending on the experimental conditions, this technique is able to detect a broad spectrum of DNA damage including single and double strand breaks, alkali labile sites, DNA–DNA/DNA–protein cross-linking and strand breaks associated with incomplete excision repair [17,18]. In addition, the application of repair specific DNA endonucleases enables detection of oxidative DNA damage and other base modifications induced by xenobiotics [19]. This technique has been successfully used to estimate the genotoxic potential of various chemical compounds [20–23] as well as organic complex mixtures [14,24–26].

The tumour suppressor protein p53 plays a key role in maintaining genomic stability and homeostasis. This protein regulates the expression of its downstream effector genes, whose products are implicated in critical cellular processes such as DNA repair [27], cell cycle control [28] and apoptosis [29]. Induction of this protein has been determined as the cellular response to genotoxic stress induced by multiple chemical DNA damaging agents, ionizing or UV radiation [30,31]. An up-regulation of p53 protein is triggered by damage accumulating in DNA due to exposure to genotoxins [32,33]. Based on this phenomenon, Yang and Duerksen-Hughes [34] proposed the measurement of p53 induction as a new promising in vitro approach for identifying the genotoxic carcinogens.

A valuable tool for detection of compounds which might pose a health hazard to man are human-derived metabolically competent cell lines such as the human hepatoma cell line Hep G2. This cell line has retained certain activities of various phase I and phase II enzymes [22,35] which play a key role in the activation/detoxification of various promutagens/procarcinogens. The capacity of Hep G2 cells to detect the genotoxicity of both direct and indirect carcinogens has already been well documented [21,23].

The goal of this work, which is part of an EC-funded study [36], was to evaluate the sensitivity and correlation among three in vitro endpoints, DNA adduct

formation, DNA strand break induction and p53 protein up-regulation, to assess the genotoxic potential of the complex mixture of organic compounds. EOMs were extracted uniformly from respirable particulate matter PM_{10} (Ø < 10 μm) collected in three European cities: Prague (Czech Republic), Košice (Slovak Republic) and Sofia (Bulgaria) during the winter and summer seasons. All monitoring sites are classified according to the European Environmental Register of monitoring stations as commercial, residential, traffic and urban [37].

2. Materials and methods

2.1. Cell line

The human hepatoma cell line Hep G2 was generously provided by Prof. Andrew R. Collins (University of Oslo, Norway). Hep G2 cells were maintained in William's modified medium supplemented with 10% fetal calf serum and antibiotics (penicillin 200 U/ml; streptomycin and kanamycin 100 μg/ml) in humidified 5% CO_2 atmosphere.

2.2. Chemicals

B[*a*]P (CAS No. 50-32-8) was purchased from Sigma. The stock solutions of B[*a*]P (2 mM in DMSO) and extractable organic mixture (EOM, 50 mg/ml in DMSO) from individual samplings were kept at −20 °C and diluted immediately before use. All chemicals used for cell cultivation (MEM, FCS, antibiotics) and agarose LMP (low melting point) were purchased from GIBCO BRL (Paisley, UK); agarose electrophoresis grade (NMP, normal melting point), spleen phosphodiesterase from ICN Biomedicals Inc., ethidium bromide, RNases A and T1, proteinase K, micrococcal nuclease and nuclease P1 from Sigma (Deisenhofen, Germany); T4 polynucleotide kinase from USB (Cleveland, OH, USA) 0.1 mm polyethylene-imine cellulose thin-layer chromatography (TLC) plates from Macherey-Nagel (Düren, Germany), primary antibodies Ab-6 (clone DO-1) and p53 Western blotting standard from Oncogene Research Products (Cambridge, MA, USA), secondary anti-mouse IgG (NA931), streptavidin–horseradish peroxidase conjugate (RPN1231), Hybond™ C-pure membranes, chemiluminiscence detection reagents and γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Amersham (Buckinghamshire, UK). All other chemicals and solvents were of HPLC or analytical grade.

2.3. Air samples collection, extraction of EOM and chemical analysis

Details about the air particles PM_{10} (particulate matter < 10 μm) collection, extractions of organic complex mixture (EOM) from the filters with PM_{10} samples and the chemical analyses of PAHs have been already described elsewhere [24,37]. The concentrations of selected PAHs, including carcinogenic PAHs (c-PAH) in each organic extract were determined by an HPLC method with fluorimetric detection.

2.4. Treatment of human cells

Human hepatoma cells Hep G2 were exposed to EOMs and positive control after reaching semi-confluency. The stock solutions of individual EOMs (50 mg/ml) were diluted in dimethylsulfoxide (DMSO) freshly before use, and added to serum free medium or medium with 1% FCS in dependence of the treatment interval, to reach the final concentration 50 μg/ml. The concentration of DMSO never exceeded 0.5%. Hep G2 cells were exposed to EOMs for 2 h (the comet assay) or for 24 h (DNA adduct detection and p53 induction). Control cells (negative control) were exposed to 0.5% DMSO. The treatment was finished by rinsing off the cells twice with PBS buffer. Then, the cells were harvested and used for SCGE (the comet assay) or cells were re-suspended in 3 ml PBS and divided into two aliquots (2 ml for DNA isolation; 1 ml for Western blotting). Benzo[*a*]pyrene (BaP, 7.5 μM) was used as a positive control.

2.5. DNA isolation and ^{32}P-postlabelling

The cell pellets were homogenised in a solution of 10 mM Tris–HCl, 100 mM EDTA and 0.5% SDS, pH 8.0. DNA was isolated using RNases A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation [6]. DNA concentrations were estimated spectrophotometrically by measuring of the UV absorbance at 260 nm. DNA samples were stored at −80 °C until analysis.

^{32}P-postlabelling analyses were performed as previously described [37]. Briefly, DNA samples (the exact amount of DNA was 6 μg) were digested by a mixture of micrococcal nuclease and spleen phosphodiesterase for 4 h at 37 °C. The nuclease P1 procedure was used instead of the butanol extraction method for adduct enrichment. According to our previous experience, no substantial contribution to the total DNA adduct levels might be expected from amino- and nitro-aromatics since the content of these PAH-derivatives in the extracts from ambient air particles is at least by three orders lower than that of PAHs. Adducted nucleotides were enzymatically labelled using γ-^{32}P-ATP and T4 polynucleotide kinase and separated by multidirectional polyethylenimine–cellulose TLC (10 cm × 10 cm). The solvents used were as follows: D1, 1 M sodium phosphate, pH 6.8; D2, 3.8 M lithium formate, 8.5 M urea, pH 3.5; D3, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0; D4 = D1, same direction as D3. After screen-enhanced autoradiography at −80 °C for 24 h, the distinct DNA adduct spots were cut out and evaluated by measuring ^{32}P-radioactivity using liquid scintillation spectroscopy. To determine the exact amount of DNA in each sample, aliquots of the enzymatic DNA digests (0.5 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed

as adducts per 10^8 nucleotides. A BPDE-derived DNA adduct standard was run in triplicate as part of each postlabelling experiment to control for inter-assay variability and to normalize the calculated DNA adduct levels.

2.6. Single cell gel electrophoresis

The procedure of Singh et al. [16], modified by Collins et al. [19] and Slamenova et al. [38] was followed. Hep G2 cells embedded in 0.75% LMP agarose and spread on a base layer of 1% NMP agarose in PBS buffer (Ca^{2+} and Mg^{2+} free) were placed in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10 and 1% Triton X-100) at 4 °C for 1 h. After lysis, slides were transferred to an electrophoretic box and immersed in an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13). After 40 min unwinding time, at 4 °C, a voltage of 25 V (300 mA) was applied for 30 min. Then the slides were neutralised with 3 × 5 min washes with Tris–HCl (0.4 M, pH 7.4), and stained with 20 μl of ethidium bromide (EtBr, 10 μg/ml). EtBr-stained nucleoides were examined with a Zeiss Jenalumar fluorescence microscope by image analysis using the software Komet 3.0 (Kinetic Imaging Ltd.). The percentage of DNA in the tail (%tail DNA) was used as a parameter for DNA damage measurement (DNA strand breaks). One hundred nucleoids were scored per each sample in one electrophoretic run.

2.7. Immunoblotting

The cell pellets were lysed in an appropriate volume of sample buffer (0.063 M Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol) containing protease inhibitors. The protein concentration was determined by the Peterson modification of the micro-Lowry methods using the Folin & Ciocalteau's phenol reagent (Sigma kit). The aliquots of these samples were stored at −80 °C until analysis.

Prior to Western blotting, the samples were diluted with sample buffer to contain 15 μg protein per 10 μl and heated to 100 °C for 5 min. The proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to Hybond™ C-pure membranes (Bio-Rad Mini-Protean and Trans-Blot systems). The membranes were blocked for 1.5 h at room temperature with TBS (10 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 5% non-fat dry milk powder. After repeated washing with TBS-T (TBS with 0.1% Tween-20) they were incubated overnight at 8 °C with primary antibodies for p53 (Ab-6, clone DO-1, 0.025 μg/ml). Subsequently, the membranes were repeatedly washed with TBS-T and incubated for 1 h at room temperature with the secondary antibody (anti-mouse IgG). The p53 protein was detected using an enhanced chemiluminescence technique (Amersham). P53 Western blotting standard (Oncogene) was used as a positive control for p53 protein detection. The intensity of bands was quantified with an image acquisition and analysis system (GDS-8000 Chemi System, UVP Inc., CA, USA).

2.8. Statistics

The data from all independent experiments (^{32}P-postlabelling, two experiments; SCGE, four experiments) were pooled together and evaluated statistically by the Student's *t*-test; significantly different at $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Characteristics of HiVol samples

The average seasonal concentrations of PM_{10}, EOM, benzo[*a*]pyrene (B[*a*]P) and c-PAHs in crude extracts are presented in Farmer et al. [36]. The procedure of PM_{10} sampling, EOM extraction and chemical analyses has already been published [37]. In general, the organic fraction extracted from PM_{10} was 3-fold higher in winter than in summer, and carcinogenic PAHs over 10-fold higher in winter air than summer air (Fig. 1). Detailed information about the local- and season-related variations in the PAH concentrations in crude extracts has already been published [24].

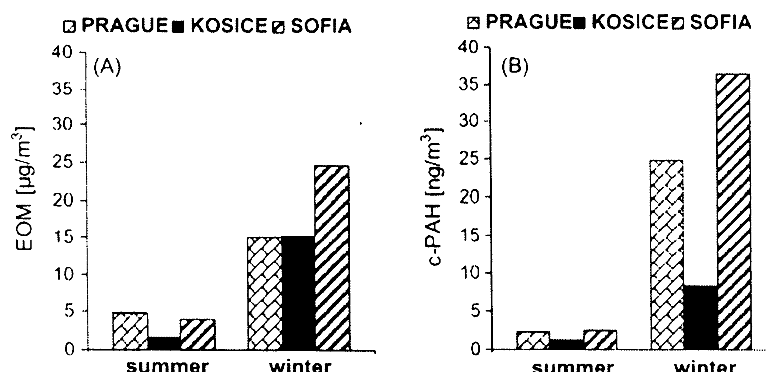

Fig. 1. Concentrations of EOM (μg/m³) (A) and c-PAHs (ng/m³) (B) in the individual localities in the summer and winter seasons.

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Fig. 2. Time-dependent increase of DNA adduct levels induced by B[a]P (7.5 μM) in Hep G2 cells; a representative experiment.

Fig. 3. DNA adduct levels induced by EOM samples at equivalent concentration 50 μg/ml in Hep G2 cells; *significantly different from Prague-EOM at $p < 0.05$.

3.2. Genotoxicity of extractable organic matter (EOM)

3.2.1. DNA adduct formation

The preliminary experiments were aimed to standardize the treatment conditions for DNA adduct assessment in Hep G2 cell exposed to EOM. In these experiments, B[a]P, a known carcinogen, was used. Hep G2 cells were exposed to 7.5 μM B[a]P for different time intervals ranging from 2 to 24 h and then processed to measure the total DNA adduct levels. Control cells were exposed to 0.5% DMSO alone under the same condition. A linear time-dependent increase in the DNA adduct levels were found in the exposed cells (Fig. 2). While 2- and 4 h cell exposure resulted in a very low level of DNA adducts (2.9 and 25.8 adducts/10^8 nucleotides, respectively), a 6 h exposure produced around 134 DNA adducts and 24 h treatment generated 1189 adducts per 10^8 nucleotides. Based on these preliminary experiments, a 24 h treatment interval of cells was chosen to assess the genotoxic potential of EOM samples.

Unlike using the discrete adduct spots formed by single genotoxic compounds, only diagonal radioactive zones of various intensities were determined in autoradiograms of ^{32}P-postlabelled DNA digest from Hep G2 cells exposed to EOMs. The total DNA adduct number induced by individual EOMs ranged from approximately 60 to 200 DNA adducts per 10^8 nucleotides. No substantial location- or season-related variations in the total DNA adduct levels were found in the localities Košice and Sofia (~100–140 adducts/10^8 nucleotides). In contrast, nearly three-fold higher total DNA adduct level was determined in cells exposed to EOM from winter sampling in comparison to summer sampling collected in the monitoring site Prague (Fig. 3). In addition, Prague-EOM, summer sampling, induced significantly lower level of DNA adducts than EOMs from Košice or Sofia ($p < 0.05$). On the contrary, Prague-EOM from winter sampling produced significantly higher DNA adduct level in comparison to EOMs from the localities Košice and Sofia ($p < 0.05$).

3.2.2. Single cell gel electrophoresis

Similarly as for the DNA adduct experiments the preliminary examinations were focused on standardization of treatment conditions of Hep G2 cells. Semi-confluent cultures of cells were exposed to B[a]P (7.5 μM) for different time intervals ranging from 20 min to 48 h. Control cells were exposed to 0.5% DMSO alone under the same condition. A linear time-dependent increase of DNA strand breaks was determined in B[a]P-treated Hep G2 cells within 20–120 min time exposure interval, and then the strand break level reached a plateau. Approximately the same strand break levels were found after 24 and 48 h exposure time interval (Fig. 4); at the same time, however, a notable decrease in cell viability was detected (data not shown). In agreement with this phenomenon, no significant differences in cell response to EOM treatment in concentration range from 5 to 150 μg/ml were found whether a 2, 24 or 48 h time exposure interval was used (Fig. 5A). Nearly the same DNA strand break levels were determined in treated cells; however, a substantial reduction of cell viability due to EOM toxicity was detected at the same time (Fig. 5B).

Based on these preliminary experiments, an exposure time of 2 h was chosen to assess the genotoxic potential of EOMs. Treatment of Hep G2 cells with indi-

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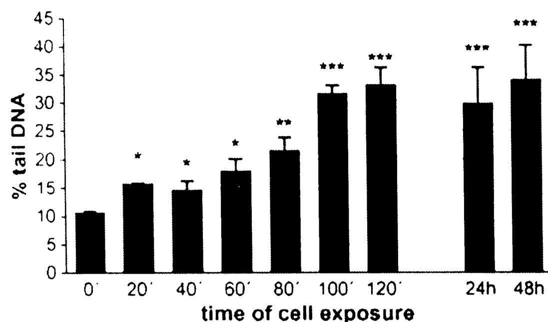

Fig. 4. Time-dependent increase of DNA strand break levels in Hep G2 cells exposed to B[a]P (7.5 μM); significantly different from untreated cells: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

vidual EOMs resulted in approximately the same levels of strand breaks expressed as 'percentage of DNA in the tail' (25–30% of tail DNA). No significant local- or season-related differences in DNA strand break levels were determined among individual EOM samples (Fig. 6).

3.3. Air genotoxicity

Substantial location- and season-related differences in the amount of both EOM and c-PAHs were estimated [36]. The highest c-PAH concentration was detected in Sofia, winter sampling (36.44 ng/m^3), while the lowest one in Košice, summer sampling (1.2 ng/m^3) (Fig. 1). It is reasonable to suppose that the higher the concentration of c-PAHs is in the ambient air, the higher is the health risk for man. In order to be closer to the in vivo situation DNA adduct and DNA strand break levels were expressed in relative units based on the amount of c-PAHs associated with particulate matter per unit volume of air (c-PAH, ng/m^3). The ambient air genotoxicity is shown in Fig. 7A (DNA adducts) and Fig. 7B (DNA strand breaks). Both DNA adduct

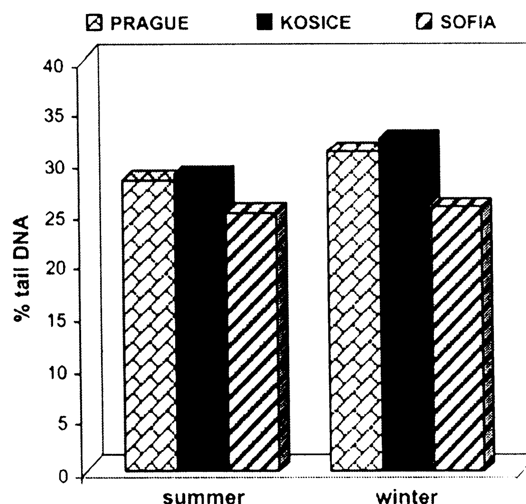

Fig. 6. DNA strand break levels induced by EOM samples at equivalent concentration 50 μg/ml in Hep G2 cells.

and DNA strand break determination manifested significantly higher genotoxicity of winter air than summer air. In general, the ambient air genotoxicity was at least six-fold higher during the winter season as compared to the summer season. While the summer air genotoxicity based on DNA adduct assessment increased in the order Košice ~ Prague < Sofia, using DNA strand break measurement this order was Košice ~ Sofia < Prague. The winter air genotoxicity determined by each particular endpoint, however, increased in the same order: Košice ≪ Prague < Sofia.

3.4. Expression of p53 protein

The p53 protein levels in EOM-treated cells were analyzed after 24 h postcultivation of exposed cells in fresh medium. Cells were treated with individual EOMs for 24 h. Control cells were exposed to 0.5% DMSO alone

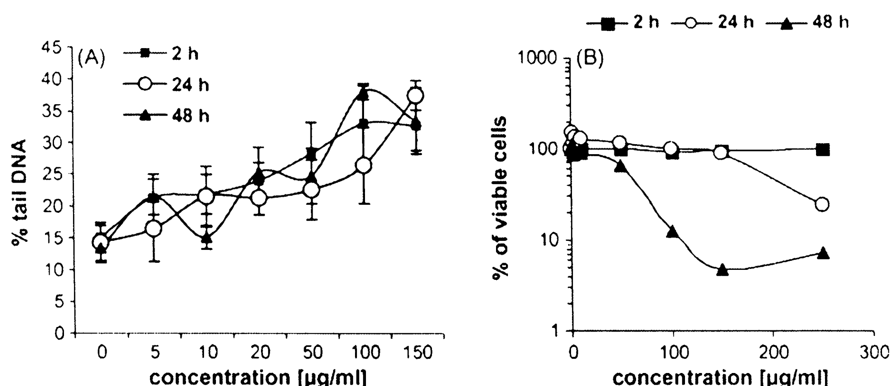

Fig. 5. DNA strand breaks (A) and cytotoxicity (B) of EOM from Prague, summer sampling after 2, 24 and 48 h of cell exposure.

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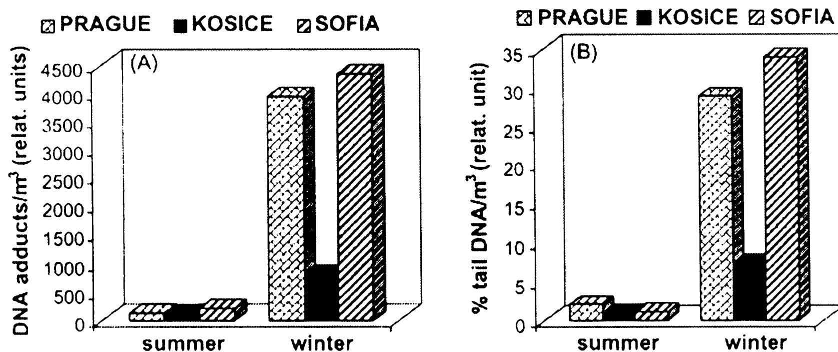

Fig. 7. Ambient air pollution genotoxicity expressed in terms of c-PAHs quantity per cubic meter air (ng c-PAH/m³) in individual monitoring sites according to Table 1: comparison of the summer and winter seasons: (A) DNA adduct determination (DNA adducts/m³); (B) DNA strand break determination (% tail DNA/m³).

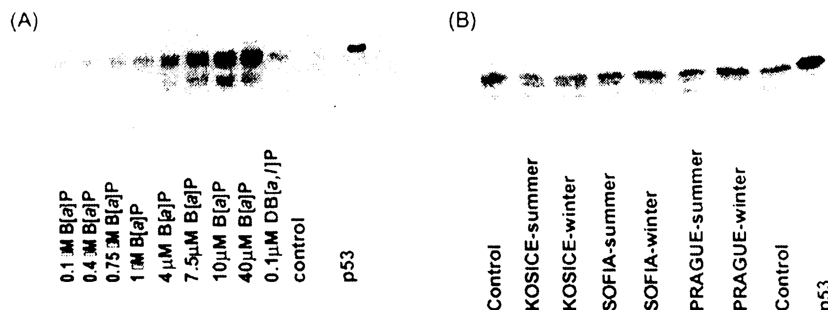

Fig. 8. p53 protein levels in Hep G2 cells exposed for 24 h to different B[*a*]P concentrations, 0.1 μM DB[*a*,*l*]P (A) and 50 μg EOM/ml collected in localities Košice, Prague and Sofia during the summer and winter seasons (B). The p53 protein level was followed after 24 h postcultivation of exposed cells in a fresh medium as described in Section 2.7. Fifteen micrograms of total protein from each sample in 10 μl of sample buffer were resolved by SDS-PAGE using 12.5% mini gel. P53 Western blotting standard from Oncogene (cat. #WB21) was used as a positive control for p53 protein. (Control) Cells treated with 0.5% DMSO alone under the same condition.

under the same condition. B[*a*]P (concentrations ranged from 0.1 to 40 μM) and DB[*a*,*l*]P (0.1 μM) were used as positive controls in these experiments. A significant up-regulation of p53 protein levels were determined only in cells exposed to B[*a*]P at concentrations 7.5–40 μM (Fig. 8A), however, no substantial rise of p53 protein levels were detected in Hep G2 cells exposed to EOMs regardless of the season and locality (Fig. 8B).

4. Discussion

Environmental air pollution is a matter of great interest because millions of people are chronically exposed to low doses of potentially toxic and genotoxic chemicals, frequently present in the complex mixtures. Numerous epidemiological and experimental studies have consistently demonstrated that urban air pollution may be responsible for increased incidence of respiratory and cardiovascular mortality and morbidity [39,40], intrauterine growth retardation [41] and increased rates of lung cancer [42–44]. Nearly 3000 chemical compounds have been identified in ambient air [45], many of which are known or suspected human mutagens and carcinogens [46,47]. Fractionation of the crude organic complex mixtures clearly demonstrated that the highest mutagenic potential is associated with fractions containing the PAHs and nitro-PAHs derivatives [37,48,49]. Therefore the first step towards lowering the risk of ambient air pollution on human health might be a biologically based characterization of complex mixtures using short-term in vitro assays, which are routinely used to measure the effects of single chemicals. Such an approach allows direct measurement of a specific toxicological endpoint in a biological system. In this respect, the selection of reliable and sensitive biomarkers of toxic and genotoxic activity is very important.

The major objective of this study was to compare the sensitivity and correlations of three endpoints to assess the genotoxic potential of organic complex mixtures extracted from airborne particles collected in three European cities, Prague (Czech Republic), Košice (Slovak Republic) and Sofia (Bulgaria) during the summer and winter seasons. Different approaches, ^{32}P-postlabelling, single cell gel electrophoresis and immunoblotting were

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employed in this study. Three endpoints, DNA adduct formation, DNA strand break induction and protein p53 up-regulation, were measured in Hep G2 cells exposed to EOMs at identical concentrations of 50 μg/ml.

Based on the preliminary experiments (Figs. 3 and 4), a 24 h cell treatment interval was chosen to assess the genotoxic potential of EOM samples using the ^{32}P-postlabelling technique and a 2 h time exposure using the SCGE. The total DNA adduct levels produced by EOMs ranged from 60 to 180 adducts per 10^8 nucleotides (Fig. 3); using the same treatment conditions B[*a*]P (7.5 μM) produced approximately 1200 adducts per 10^8 nucleotides (Fig. 2). If adjusted for the amount of B[*a*]P, the dose of 50 μg EOM/ml corresponded to the concentration ~0.05 μM B[*a*]P [50]. Although no local- and season-related differences in EOM genotoxicity were determined between Košice and Sofia, Prague-EOM, summer season, produced significantly lower level of DNA adducts than EOMs from Košice and Sofia (Fig. 3). Opposite results were determined for EOMs from winter sampling; Prague-EOM generated substantially more DNA adducts than EOMs from Košice and Sofia ($p < 0.05$).

Two-hour cell exposure to EOM resulted in a significant rise of DNA strand breaks in EOM-treated cells (Fig. 6), however, no substantial location- or season-related differences in in EOM genotoxicity were found. In agreement with these results neither Cerna et al. [48] nor Binkova et al. [37,51] and Topinka et al. [49] found any significant differences in EOM genotoxicity in dependence on the locality and season. A lack of time-dependent increase of strand breaks due to B[*a*]P or EOM exposure (Figs. 4 and 5A) might be explained by saturation of DNA repair mechanisms involved in DNA damage incision and removal at longer time treatment intervals. This assumption is supported by the fact that a time-dependent increase of DNA strand breaks was found up to 120 min of cell exposure to B[*a*]P and then the strand break level reached a plateau (Fig. 4). Under the same treatment conditions, a 2 and 4 h cell exposure to B[*a*]P resulted in very low levels of DNA adducts (2.9 and 25.8 adducts/10^8 nucleotides, respectively) probably due to efficient removal of damage from DNA. Extension of treatment intervals to 24 and 48 h might lead to depletion of DNA repair enzymes which allowed accumulation of damage in DNA. A time-dependent increase of DNA adduct levels with a maximum after 24 h exposure interval was found in B[*a*]P-treated cells (Fig. 2). The DNA damage accumulation in treated cells resulted subsequently in decreased cell viability, a phenomenon observed in both B[*a*]P- and EOM-treated cells (Fig. 5B). However, other explanations including a saturation of activating enzymes or competition of individual components (genotoxic and non-genotoxic) for the biotransformation enzymes cannot be excluded.

Western blot analyses did not demonstrate any up-regulation of protein p53 due to cell exposure to EOMs (Fig. 8B). Lack of protein p53 induction over the basal level due to exposure to artificial and environmental mixtures was reported also by Binkova and Sram [50]. Experiments with model carcinogens have suggested that a minimal DNA adduct level of ~200 adducts per 10^8 nucleotides is required to detect up-regulation of p53 protein [9]. The DNA adduct levels determined in exposed Hep G2 cells did not reach these values; therefore it is reasonable to suppose that the levels of p53 protein in treated cells were below the limit of detection.

A very good correlation of ambient air genotoxicity was found using DNA adduct determination and DNA strand break measurement (Fig. 7A and B). Evaluation of the concentration of c-PAHs (ng/m^3) in individual monitoring sites was taken into account (Table 1). Over 10-fold higher ambient air genotoxicity was determined in winter air than summer air using both endpoints. This

Table 1
Characteristics of HiVol samples of PM_{10} collected during the winter and summer seasons in three cities: Prague (Czech Republic), Košice (Slovak Republic) and Sofia (Bulgaria)

Monitoring site	Season	PM_{10} [μg/m^3]	EOM[a] [μg/m^3]	B[*a*]P [ng/m^3]	c-PAHs[b] [ng/m^3]
Prague	Winter	62.59	14.93	3.50	24.69
	Summer	36.91	4.96	0.28	2.29
Košice	Winter	57.99	15.30	1.37	11.87
	Summer	24.30	1.67	0.15	1.20
Sofia	Winter	89.88	24.60	4.84	36.44
	Summer	29.72	3.95	0.36	2.43

[a] Percentage of extractable organic matter in PM_{10} air particles.
[b] Sum of carcinogenic PAHs (c-PAHs): (benz[*a*]anthracene (B[*a*]A), chrysene (CHRY), benzo[*b*]fluoranthene (B[*b*]F), benzo[*k*]fluoranthene (B[*k*]F), benzo[*a*]pyrene (B[*a*]P), dibenzo[*a,h*]anthracene (DB[*a,h*]A), benzo[*g,h,I*]perylene (B[*g,h,I*]P), indeno[1,2,3/*cd*]pyrene (I[*c,d*]P)).

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finding is in agreement with observation of Topinka et al. [49] reporting 10-fold seasonal variability of ambient air genotoxicity in different localities in Czech Republic measured by DNA adduct analysis using primary rat hepatocytes. Moreover, quantitatively comparable seasonal increase in DNA adduct levels found in Hep G2 cells and primary rat hepatocytes indicate that Hep G2 cells are a relevant surrogate for primary hepatocyte cultures in terms of metabolic capacity. Finally, we found that both biomarkers of exposure used in this study (DNA adduct and strand break measurements) exhibited the same order of winter air genotoxicity which rose in the order: Košice ≪ Prague < Sofia.

The assessment of mutagenic/carcinogenic hazards posed by organic complex mixtures of anthropogenic emissions is not a simple task. In contrast to a single chemical, mixtures are likely to exhibit various interactions (additive or synergistic) that cannot be predicted by the concentration of any individual component. A comprehensive assessment of genotoxic potential of the complex organic mixtures requires the use of a battery of reliable and sensitive assays measuring different relevant endpoints. Results obtained in this study clearly demonstrated that both DNA adduct formation and DNA strand break estimation are sensitive in vitro biomarkers of complex mixture genotoxicity contributing to the risk assessment of ambient air pollution. However, one should keep in mind that both methods as used in this study are not specific enough to identify the significance of individual components in the final genotoxicity of the complex environmental mixtures. While the ^{32}P-postlabelling technique measures the amount of covalent DNA adducts (P1 nuclease modification) produced by EOMs, the SCGE assay estimates DNA strand break levels produced as a consequence of DNA repair or due to spontaneous release of unstable DNA adducts resulting in alkali labile sites. Based on this fact, the individual approaches provide complementary information about the total genotoxic potency of the mixtures, which is basic information to identify carcinogenic risk. The assessment of tumour suppressor protein p53 up-regulation is a less efficient biomarker of genotoxicity requiring probably a threshold extent of DNA damage. Protein p53 up-regulation could be caused by several mechanisms including those which are independent from DNA damage induction, e.g. by inhibition of p53 degradation via ubiquitin pathway [52] or ribonucleotidyl depletion [53].

The in vitro short-term assays such as DNA adducts by ^{32}P-postlabeling and DNA strand breaks by SCGE analyses provide a valuable tool to evaluate the genotoxic potential of complex mixtures. These assays can be used not only to assess the impact of air pollution on the human health but they contribute to a better understanding of the mechanisms underlying air pollution induced adverse effects on biological systems.

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Appendix 4

O.Sevastyanova, Z.Novakova, K.Hanzalova, B.Binkova,
R.J.Sram, J.Topinka

Seasonal variability in the genotoxic potential of urban air particulate matter

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Seasonal variability in the genotoxic potential of urban air particulate matter

O. Sevastyanova, Z. Novakova, K. Hanzalova, B.Binkova, R.J. Sram, J.Topinka

Institute of Experimental Medicine AS CR and Health Institute of Central Bohemia, Prague, Czech Republic

To whom correspondence should be addressed:
Jan Topinka
Institute of Experimental Medicine
Academy of Sciences of the Czech Republic
Vídeňská 1083
142 20 Prague 4
Czech Republic
Tel. +420-2-4106-2675
Fax. +420-2-4106-2785
e-mail: jtopinka@biomed.cas.cz

Abstract

The main aim of this study was to compare the genotoxic potential of organic extracts from urban air particles collected in various seasons in the center of Prague (Czech Republic). For this purpose, we analyzed the DNA adduct forming activity of extractable organic matter (EOM) from urban air particles <10 μm (PM10) in the human hepatoma cell line HepG2. DNA adducts were analyzed by ^{32}P-postlabelling with nuclease P1 enrichment. PM10 concentrations were 36.9 μg/m^3, 62.6 μg/m^3 and 39.0 μg/m^3, in summer 2000, winter 2001 and winter 2005, respectively. The corresponding EOM contents were 5.0 μg/m^3 (13.9% of PM10), 14.9 μg/m^3 (23.8%) and 6.7 μg/m^3 (17.2%). The total DNA adduct levels induced by 10 μg EOM/ml were 4.7, 19.5 and 37.2 adducts/10^8 nucleotides in summer 2000, winter 2001 and winter 2005, respectively. However, when the EOM quantities per m^3 of air were taken into consideration, the summer sample exhibited a 10-fold lower genotoxicity than did those of winter, while the difference between the winter samples was not significant: 23.4 in summer 2000, 291 in winter 2001 and 249 in winter 2005 (in relative units). Although the PM10 concentration in air and the EOM content in particles in winter 2005 were significantly lower than in winter 2001, the genotoxic potential of the ambient air in these samples was almost equal. There were significant positive correlations between the B[a]P and c-PAH content in EOM from various sampling periods and the total DNA adduct levels detected in the EOM treated samples. These findings support the hypothesis that the B[a]P and c-PAH content in EOM is the most important factor in its genotoxic potential. Thus, estimating

the genotoxic potential of the ambient air and predicting health risk should be based mainly on the c-PAHs concentration and the biological activity of the extracts, while the number of particles and the EOM content do not seem to be crucial determinants of ambient air genotoxicity.

Abbreviations: B[a]A, benz[a]anthracene; B[a]P, benzo[a]pyrene; BPDE, benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±]; B[b]F, benzo[b]fluoranthene; B[ghi]P, benzo[ghi]perylene; B[k]F, benzo[k]fluoranthene; CHRY, chrysene; DB[ah]A, dibenz[ah]anthracene; DRZ, diagonal radioactive zone; c-PAH, carcinogenic polycyclic aromatic hydrocarbons; DB[al]P, dibenzo[al]pyrene; 7,12-DMBA, 7,12-dimethylbenz[a]anthracene; DMSO, dimethylsulphoxide; EOM, Extractable Organic Matter adsorbed to respirable air particles; HiVol, high volume; I[cd]P, indeno[cd]pyrene; PM2.5, air particles < 2.5 μm; PM10, air particles < 10 μm; PAH, polycyclic aromatic hydrocarbons; RAL, relative adduct labelling; TLC, thin layer chromatography; VAPS, versatile air pollution samplers.

Key words: DNA adducts, carcinogenic PAHs, air particles, genotoxicity, ^{32}P-postlabelling.

1. INTRODUCTION

Air pollution consists of a very complex mixture of gases and particles with condensed organic matter. Currently, more than 500 compounds present in the ambient air have been identified as mutagenic [1]. Since the general population is exposed to many agents simultaneously, predicting health risk represents a very complicated problem, because a mixture of genotoxic chemicals may undergo a variety of interactions, which can affect the transport, metabolism or molecular binding of the components [2,3]. Thus, the biological effects of complex environmental mixtures, especially genotoxicity, which represents the interaction of xenobiotics with DNA, seem to be more informative for risk assessment than circumstantial chemical analysis [4,5].

Particulate ambient air pollution has proven to be of major importance in increased cancer risk as well as the morbidity and mortality of people with cardiovascular and pulmonary diseases [6-8]. For this reason, ambient air quality assessment is usually based on PM10 concentrations, and reducing PM10 concentration in the ambient air is thought to be one of the most important tasks. There is growing evidence that the health effect of air pollution depends not only on PM concentration, but also on the aerodynamic diameter of the particles [9-11], their sources and chemical composition [12,13]. Among the chemicals absorbed on air particles, the major role in genotoxic effects is played by carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) [14,15]. Previous study suggests that their concentrations may be considered as an index of the biologically active components in air particulate samples [16]. The aim of this study was to compare the genotoxicity of the ambient air in the center of Prague at 5-year intervals and to assess

seasonal variabilities in the genotoxic potential. For this purpose, we compared the DNA adduct forming activity of organic extracts from urban air particles (PM10) collected in various seasons in 2000/2001 and in 2005 in the center of Prague (Czech Republic). Based on our previous work [17] we chose the human hepatoma cell line HepG2, equipped with enzyme activities to metabolize c-PAHs and their derivatives in DNA reactive intermediates, to perform this study.

2. MATERIALS AND METHODS

2.1. Chemicals and biochemicals

Spleen phosphodiesterase was purchased from ICN Biomedicals, Inc.; proteinase K, RNases A and T1, micrococcal nuclease and nuclease P1 from Sigma (Deisenhofen, Germany); polyethylene-imine cellulose TLC plates (0.1 mm) from Macherey-Nagel (Düren, Germany); c-PAHs (99% pure) from Supelco, Inc; T4 polynucleotide kinase (USB Corporation, USA); γ-^{32}P-ATP (3000 Ci/mmol, 10 µCi/µl) from Amersham Biosciences. All other chemicals and solvents were of HPLC or analytical grade.

2.2. Air sampling, EOM extraction and chemical analysis

Air particles < 10 µm (PM10) were collected in the center of Prague (stationary monitoring by the Czech Hydrometeorological Institute, Prague) by HiVol air samplers (Anderson) 24 hours daily in summer 2000 (15.6.2000 - 15.9.2000), winter 2000/2001 (4.12. 2000 - 7.3. 2001) and winter 2005 (7.11. - 22.12. 2005). Air particles < 2.5 µm

(PM2.5) and < 10 µm (PM10) were collected simultaneously by versatile air pollution samplers (VAPS) at the same monitoring site.

Dichloromethane extraction of the organic complex mixture (EOM) from 20x20 cm (T60A20) Pallflex filters with the collected PM10 samples from the HiVol samplers and the quantitative chemical analysis of PAHs were performed by HPLC with fluorimetric detection in the laboratories of the certified company ALS Global (EN ISO CSN IEC 17025). All procedures were performed according to US EPA methods [18,19].

The concentrations of eight PAHs regarded, according to IARC [20], as carcinogenic PAHs (c–PAHs), namely, benz[a]anthracene (B[a]A), benzo[a]pyrene (B[a]P), benzo[b]fluoranthene (B[b]F), benzo[g,h,i]perylene (B[ghi]P), benzo[k]fluoranthene (B[k]F), chrysene (CHRY), dibenzo[a,h]anthracene (DB[ah]A) and indeno[1,2,3-cd]pyrene (I[cd]P), were analyzed in each EOM sample. For the *in vitro* experiments, EOM samples were evaporated to dryness under a stream of nitrogen and the residue re-dissolved in dimethylsulfoxide (DMSO). The stock solution of each EOM sample contained 50 mg of EOM/ml DMSO.

2.3. **Cell culture conditions and treatment**

The human HepG2 cell line was originally established from a human liver biopsy [21] and was originally obtained from A. Gabelova (Cancer Research Institute, Bratislava, Slovakia). Cells (2-4x10^6) were cultured in 75 cm^2 flasks. The cells were grown in William's medium (Gibco) containing 10% FBS (Gibco), 2 mmol/l glutamin and supplemented with penicillin (50 U/ml) for 3-4 days until confluency, prior to treatment with EOMs. The EOMs were diluted in DMSO immediately before use and added to

serum-free medium to reach the required concentrations. The concentration of DMSO never exceeded 0.5% (v/v). After 24-hour exposure, the medium was aspirated off and the cells washed twice with physiological saline. The cells were harvested into 15 ml centrifugation tubes using 4 ml physiological saline and centrifuged for 5 min. Cell pellets were washed twice with 13 ml physiological saline. Finally, the cells were resuspended in 3 ml physiological saline. Two ml aliquots of all suspensions were frozen in liquid nitrogen for the analysis of DNA adducts.

2.4. DNA isolation

The cell pellets were homogenized in a solution of 10 mM Tris-HCl, 100 mM EDTA and 0.5% SDS, pH 8.0. DNA was isolated using RNAses A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol as previously described [22]. The DNA concentration was estimated spectrophotometrically by measuring the UV absorbance at 260 nm. DNA samples were kept at -80° C until analysis.

2.5. DNA adduct analysis

^{32}P-postlabelling analysis was performed as previously described [23,24]. Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37°C. Nuclease P1 was used for adduct enrichment. The labelled DNA adducts were resolved by two-directional thin layer chromatography on 10x10 cm PEI-cellulose plates. Solvent systems used for TLC were the following: D-1: 1M sodium phosphate, pH 6.8; D-2: 3.8M lithium formate, 8.5M urea, pH 3.5; D-3: 0.8M lithium chloride, 0.5M Tris, 8.5M urea, pH 8.0. Autoradiography was carried out at -80°C

for 1 to 24 h. The radioactivity of distinct adduct spots was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A BPDE-DNA adduct standard was run in triplicate in each postlabelling experiment to control for interassay variability and to normalize the calculated DNA adduct levels.

3. RESULTS

3.1. Air sampling, EOM extraction and chemical analysis

The sampling data from HiVol samplers in Prague in summer and winter 2000/2001 and in winter 2005 are summarized in Table 1. Total air volume and the number of filters with collected PM10 were ~2-fold higher in summer and winter 2000/2001 than in winter 2005, corresponding to the longer sampling period in 2000/2001. PM10 concentration and EOM content per m^3 of air in winter 2000/2001 were approximately 2-3-fold higher than in summer 2000. Comparing these parameters for winter 2005 and summer 2000 (Table 1), almost no differences were observed. In contrast, the B[a]P and c-PAHs concentrations in EOM in the winter 2005 sample were significantly higher than in the sample from winter 2001. The concentrations of B[a]P and c-PAHs per m^3 in both winter sampling periods were comparable and were almost 10-fold higher than those in summer 2000. The proportions of PM2.5 in the total volumes of PM10 in each sampling period

were calculated accordingly to the results from VAPS (Fig. 1). The mean values of PM2.5 and PM10 during the sampling periods were used. The maximum percentage of PM2.5 was found in winter 2005 (87.6% of PM10), followed by winter 2001 (74.4%) and the minimum percentage in summer 2000 (51.5%).

Different relative abundances of individual c-PAHs were seen in the summer sample as compared to the winter samples (Table 2). The lighter PAHs (B[a]A and CHRY) were more abundant in the winter samples, whereas heavier PAHs (B[ghi]P and I[cd]P) were relatively more abundant in the summer sample. Interestingly, the winter samples varied in B[a]A and B[b]F concentrations, which were lower in winter 2005 by 40% and 60%, respectively.

3.2. Genotoxicity of extractable organic matter (EOM)

The DNA adduct patterns obtained in HepG2 cells incubated with EOMs from all seasons were qualitatively similar and exhibited diagonal radioactive zones of various intensities with a dominating B[a]P-like DNA adduct spot (Fig. 2). A diagonal radioactive zone represents the overlapping spots of individual DNA adducts and is the typical adduct pattern for exposure to complex mixtures of genotoxic compounds in various experimental systems.

The total DNA adduct levels induced after 24h exposure of HepG2 cells to different EOM concentrations from all seasons are shown in Table 3. At the lowest EOM concentration (10 μg /ml), the strongest genotoxic effect was induced by EOM from winter 2005, followed by winter 2001 and summer 2000. At a dose of 50 μg EOM/ml, both winter samples induced maximum DNA adduct levels, and EOM from winter 2001

was 30% more genotoxic than that from winter 2005. These findings suggest the presence of a toxic effect in the winter samples, especially in winter 2005. This effect was more pronounced at a dose of 100 μg EOM/ml, where the total DNA adduct levels induced by the winter samples significantly decreased.

To compare the genotoxicity of the ambient air particles from various sampling periods, the amount of EOM per m^3 was taken into consideration. DNA adducts/10^8 nucleotides induced by 10 μg EOM/ml in HepG2 cells were multiplied by a correcting factor for differences in EOM/m^3 of air (Fig. 3). Under these conditions, the summer sample exhibited 10-fold lower genotoxicity (23.4 relative units) than those of winter, while the difference between the winter samples was not significant (290.6 and 249.2 relative units for 2001 and 2005, respectively).

Significant positive correlations were found between B[a]P or c-PAH concentrations in the ambient air in various sampling periods and the DNA adduct forming activity of various EOMs detected in HepG2 cells (Fig. 4). Differences in EOM concentrations between seasons were taken into consideration. DNA adducts/10^8 nucleotides were multiplied by a correcting factor for differences in EOM/m^3 of air.

4. DISCUSSION

The most important finding of this study is that the genotoxic potential of ambient air particles is not directly proportional to the PM10 or PM2.5 concentration, but rather that it depends predominantly on the quantity of c-PAHs bound to the particles. This result is based on the long-term measurement of PM10, PM2.5 and c-PAHs concentrations in the city center of Prague (Czech Republic) and the estimation of ambient air genotoxicity via

measurements of the DNA adducts induced by organic extracts from urban air particles (PM10) in the human hepatoma cell line HepG2. This finding is further supported by our recent study dealing with the genotoxicity of ambient air particles in several European cities [25].

Organic extracts were obtained from PM10 collected in summer and winter 2000/2001 [26] and in winter 2005 by HiVol air samplers. PM10 concentration and EOM content in winter 2005 were significantly lower than in winter 2001 (62.6 mg/m^3, 14.9 $\mu g/m^3$ and 39.0 mg/m^3, 6.7 $\mu g/m^3$, respectively). In contrast, comparable concentrations of B[a]P and c-PAHs per m^3 of ambient air were detected in both winter periods. PM10 concentration and EOM content did not differ significantly between summer 2000 and winter 2005, but B[a]P and c-PAH concentrations per m^3 were 8 times higher in winter 2005 than in summer 2000. The absence of a correlation between PM10 and c-PAH concentrations could be partially explained by different proportions of PM2.5 in PM10 in the different sampling periods. The maximum percentage of PM2.5 was found in winter 2005 (87.6%), the minimum in summer 2000 (51.5%). Comparable concentrations of PM2.5 in the winter periods correlate with the comparable c-PAH concentrations at these periods. However, the PM2.5 concentration in summer is approximately 2-fold lower than in the winter periods, while the difference in c-PAH concentration is more than 8-fold. This comparison indicates that seasonal variability in PM2.5 content can hardly explain the huge difference in cPAH levels. These results are consistent with a study of Georgiadis et al. [27] in which no correlation between PM2.5 and c-PAH concentrations was observed in two Greek cities.

The varied relative abundance of PM2.5 and individual c-PAHs may suggest different ambient air pollution sources in various seasons and/or the influence of meteorological conditions - increased atmospheric inversions, which retain pollution close to the surface, and/or changes in atmospheric chemical transformation processes. However, the almost equal average temperatures during the winter sample periods (1.4°C and 2.4 in 2001 and 2005, respectively) exclude a significant impact of meteorological conditions on these sampling results. Thus, we can conclude that the differences in PM10 and individual c-PAH concentrations are probably determined mostly by the contribution of different air pollution sources. Higher proportions of benz[a]anthracene and chrysene in the winter samples, and lower proportions of benzo[ghi]perylene and indeno[1,2,3-cd]pyrene, were also detected by Binkova et al. in Northern Bohemia and Prague [16] as well as by Georgiadis et al. in Athens [27]. The most probable explanation for these observations is a predominant effect of traffic (gasoline-powered vehicles) air pollution in the summer period and residential heating in the winter [16,28].

The higher concentrations of carcinogenic PAHs, and particularly B[a]P, in EOM from winter 2005 compared to winter 2001 (c-PAHs: 2.91 vs. 1.65 ng/mg and B[a]P 0.49 vs. 0.24 ng/mg EOM) correlate with the total DNA adduct levels induced by the lowest EOM concentration tested (10 μg EOM/ml) – 37.2 ± 6.1 vs. 19.5 ± 3.2 adducts / 10^8 nucleotides. This finding indicates a crucial role for c-PAHs and B[a]P in the total DNA adduct forming activity of extractable organic matter. The significance of benzo[a]pyrene for DNA adduct induction is readily apparent on autoradiographs obtained from HepG2 cells incubated with EOM from all seasons, where B[a]P-like DNA adduct spots (spots

on the autoradiograph in the identical position as the major B[a]P-derived adduct) are clearly exhibited (Fig. 2).

The concentration of 50 μg EOM/ml derived from winter samples induced DNA adduct levels that did not correlate with c-PAH concentrations: 80.4 ± 3.5 for winter 2001 and 58.8 ± 14.7 adducts / 10^8 nucleotides for winter 2005. The DNA adduct levels detected in HepG2 cells incubated with the highest EOM concentration tested, 100 μg EOM/ml (and partially also 50 μg EOM/ml), indicate cell toxicity caused by winter samples, especially for EOM sampled in winter 2005. Consequently, a linear dose-response relationship of adduct forming activity was detected only for the summer sample, suggesting its lower genotoxicity and also cell toxicity. This suggestion was further confirmed by the comparison of the DNA adduct forming activity of extracts when EOM quantities per m^3 were taken into consideration (Fig. 3). Under such conditions, the summer sample exhibited 10-fold lower genotoxicity than those of winter, while the difference between the winter samples was not significant.

Although PM10 concentration and EOM content in winter 2005 were significantly lower than in winter 2001, the genotoxic potential of the ambient air in both samples was almost equal. Conversely, almost equal PM10 concentrations and EOM content in winter 2005 and summer 2000 were accompanied by a significant difference in the genotoxic potential of the ambient air in these seasons. The concentration of PM2.5 better reflects the genotoxic potential of the ambient air, but a direct correlation between PM2.5 concentration and the genotoxic potential of the ambient air was not detected. Thus, we can conclude that the PM10 concentration, EOM content and even the PM2.5

concentration in the ambient air do not correlate with its genotoxic potential and so cannot reflect its real hazard.

The significant positive correlation between B[a]P or c-PAH (r^2=0.9) concentrations in the ambient air in various sampling periods and the DNA adduct forming activity of various EOMs detected in HepG2 cells (Fig. 4) supports the hypothesis that B[a]P and c-PAH concentrations in the ambient air are the most important predictors of its genotoxic potential. Thus, we may conclude that the concentration of PM10 particles and the EOM content do not seem to be crucial determinants of DNA adduct forming activity. Estimations of the genotoxic potential of the ambient air and predictions of health risk should be based mainly on the measurement of c-PAH concentrations.

5. ACKNOWLEDGEMENTS

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Legends to figures:

Fig. 1: Proportion of PM2.5 in the total volume of PM10 in different sampling periods.

Fig. 2: Autoradiographs of TLC maps of ^{32}P-labelled DNA digests after the incubation of HepG2 cells with 10 μg/ml or 50 μg/ml extractable organic matter (EOM) from Prague (Czech Republic) in different seasons (summer 2000, winter 2001, winter 2005).

Fig. 3: Relative comparison of the DNA adduct forming activity in HepG2 cells for various EOM. Differences in EOM concentrations between seasons were taken into consideration. DNA adducts/10^8 nucleotides were multiplied by a correcting factor for differences in EOM/m^3 of air.

Fig. 4: Correlation between the B[a]P or c-PAH concentrations in the ambient air in various sampling periods and the DNA adduct forming activity of various EOMs (induced by 10 μg EOM/ml) in HepG2 cells.

Table 1.

Characteristics of Hi-Vol samples of PM10 collected in Prague during various seasons

Season	number of filters	total volume (m^3)	PM10 (mg/m^3) mean	EOM ($\mu g/m^3$) mean	EOM (%)	B[a]P (ng/m^3) mean	c-PAH (ng/m^3) mean	c-PAH (ng/mg EOM)
summer 2000	90	125064	36.9	5.0	13.4	0.3	2.3	0.5
winter 2001	94	147699	62.6	14.9	23.9	3.5	24.7	1.7
winter 2005	41	65150	39.0	6.7	17.2	3.3	19.5	2.9

Table 2.

Proportion of individual c-PAHs in EOM samples

	Summer 2000		Winter 2001		Winter 2005	
	(ng/m^3) mean	%	(ng/m^3) mean	%	(ng/m^3) mean	%
Benz[a]antracene	0.17	7.3	5.2	21.1	3.2	16.4
Chrysene	0.19	8.3	4.7	19.0	4.6	23.6
Benzo[b]fluorantene	0.37	16.0	4.6	18.6	2.0	10.3
Benzo[k]fluorantene	0.18	7.7	2.0	8.1	1.5	7.7
Benzo[a]pyrene	0.25	10.8	3.5	14.2	3.3	16.9
Dibenz[a,h]antracene	0.05	2.0	0.29	1.2	0.27	1.4
Benzo[ghi]perylene	0.66	28.5	1.9	7.7	2.3	11.8
Indeno[1,2,3-cd]pyrene	0.43	18.8	2.5	10.1	2.3	11.8

Table 3.

Total DNA adduct levels induced by different concentrations of EOM extracted from PM10 particles collected during various seasons

EOM	DNA adducts / 10^8 nucleotides		
Concentration (μg/ml)	summer 2000 mean (SD)	winter 2001 mean (SD)	winter 2005 mean (SD)
10	4.67 (0.7)	19.5 (3.17)	37.2 (6.05)
50	51.55 (28.5)	80.43 (3.50)	58.84 (14.73)
100	85.87 (11.8)	38.01 (2.16)	19.26 (2.73)

Fig.1

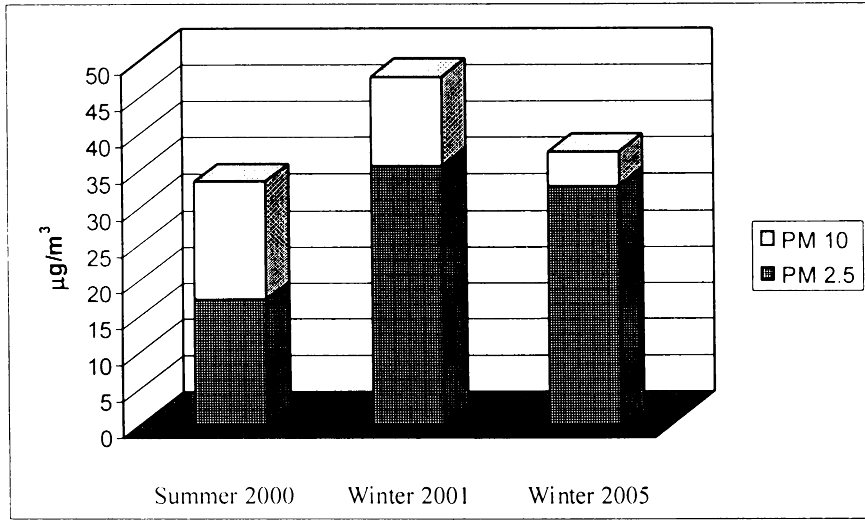


Fig.2

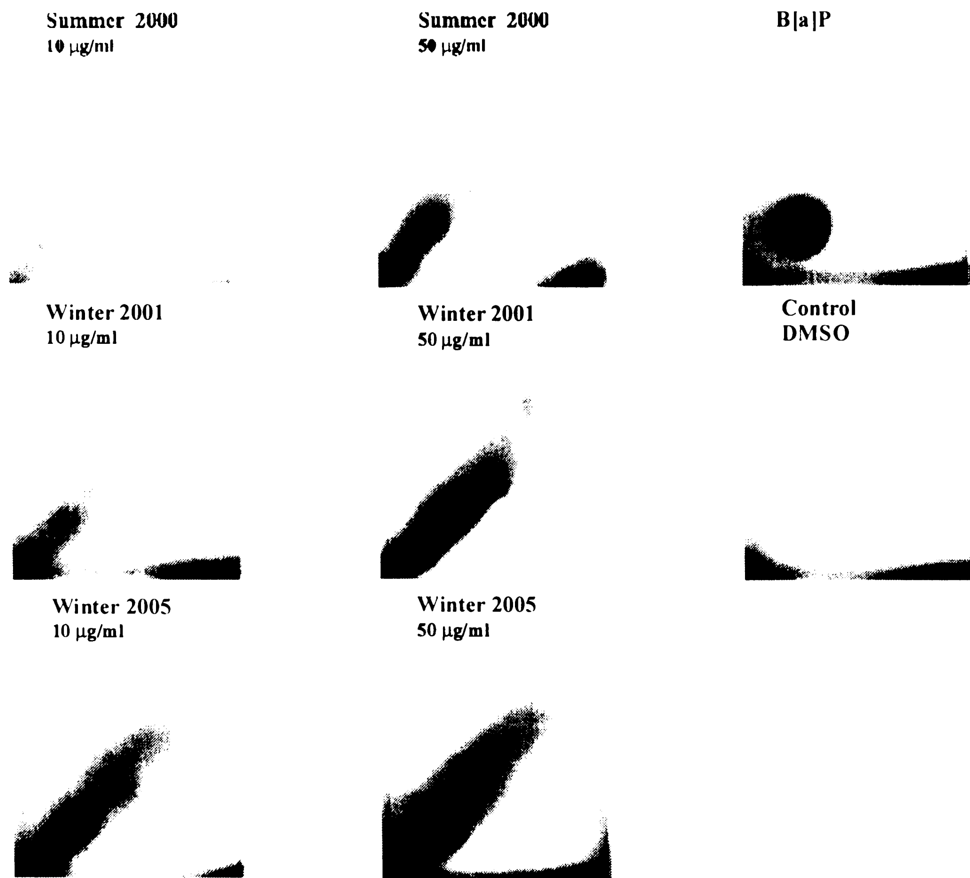


Fig.3

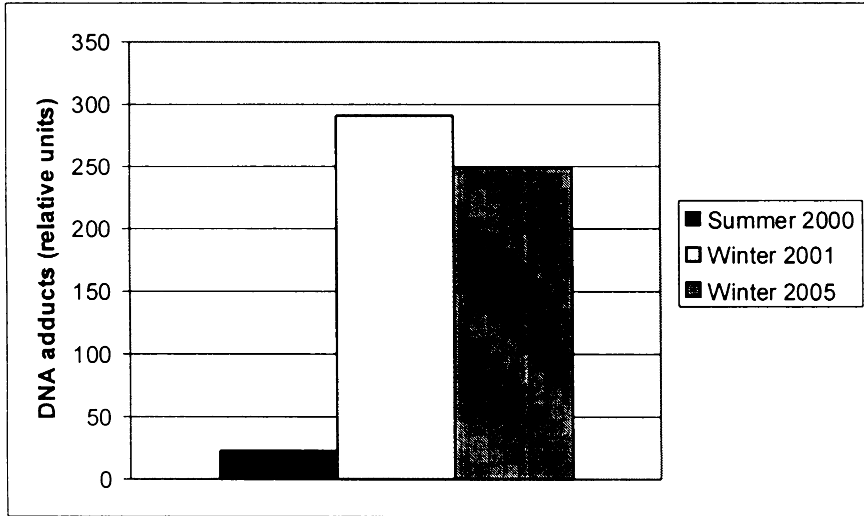
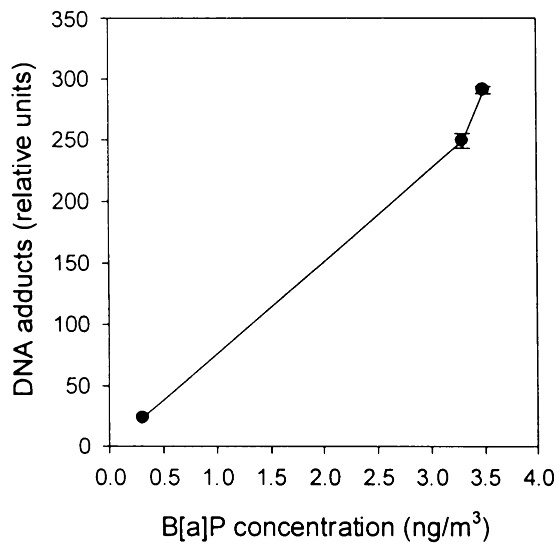
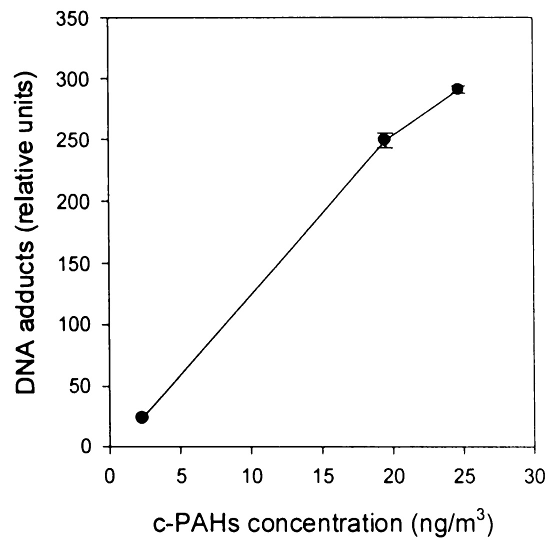


Fig. 4





Appendix 5

J.Topinka, O.Sevastyanova, B.Binkova, I.Chvatalova,
A.Milcova, Z.Lnenickova, Z.Novakova, I.Solansky,
R.J.Sram

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Biomarkers of air pollution exposure—A study of policemen in Prague

J. Topinka*, O. Sevastyanova, B. Binkova, I. Chvatalova, A. Milcova, Z. Lnenickova, Z. Novakova, I. Solansky, R.J. Sram

Laboratory of Genetic Ecotoxicology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic and the Health Institute of Central Bohemia, Vídeňská 1083, 14220 Prague 4, Czech Republic

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Abstract

The effect of exposure to organic compounds adsorbed onto respirable air particles (<2.5 μm) on DNA adducts in lymphocytes was studied in a group of non-smoking policemen ($N = 109$, aged 35 ± 0.9 years) working in the downtown area of Prague and spending >8 h daily outdoors. Personal exposure to carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) adsorbed on respirable particles was monitored in each subject for 48 h before biological sampling. DNA adducts were analyzed by a ^{32}P-postlabelling assay, and total DNA adduct levels and B[a]P-like spots were determined. Further biomarkers included cotinine levels in urine to control for exposure to tobacco smoke, plasma levels of vitamins A, E and C and polymorphisms of metabolic genotypes (GSTM1, GSTP1, GSTT1, CYP 1A1-Msp I and Ile/Val, MTHFR, MS), DNA repair genotypes (XRCC1, hOGG1 and XPD exons 6 and 23) and the p53 gene (p53 Msp I and BstU I). All the biomarkers of exposure and effect were analyzed repeatedly during a period of one year at 2–3 month intervals (January, March, June, September 2004) to cover periods with high (winter) and low (summer) levels of air pollution. The highest personal exposure to c-PAHs was found in January (8.1 ± 8.8 ng/m^3), while the other three sampling periods exhibited 3–4-fold lower c-PAH exposure. The total DNA adducts were only slightly elevated in January (2.08 ± 1.60) compared to March (1.66 ± 0.65), June (1.96 ± 1.73) and September (1.77 ± 1.77). B[a]P-like DNA adducts, however, were significantly higher in January than in the March and June sampling periods (0.26 ± 0.14 vs. 0.19 ± 0.12 and 0.22 ± 0.13, respectively; $p < 0.0001$ and $p = 0.017$) indicating that c-PAH exposure probably plays a crucial role in DNA adduct formation in lymphocytes. No effect of individual metabololic or DNA repair genotypes on DNA adduct levels was observed. However, the combination of two genotypes encoding enzymes metabolizing c-PAHs – CYP 1A1 and GSTM1 – was associated with the levels of total and B[a]P-like DNA adducts under conditions of increased exposure to c-PAHs. Our study suggests that DNA adducts in the lymphocytes of subjects exposed to increased c-PAH levels are an appropriate biomarker of a biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to an increased mutagenic and carcinogenic risk.

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Keywords: Air pollution; DNA adducts; PAHs; Complex mixtures; Genotypes

Abbreviations: B[a]P, benzo[a]pyrene; B[b]F, benzo[b]fluoranthene; B[k]F, benzo[k]fluoranthene; B[a]A, benz[a]anthracene; B[ghi]P, benzo[ghi]perylene; BPDE, benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±]; c-PAHs, carcinogenic polycyclic aromatic hydrocarbons; CHRY, chrysene; DRZ, diagonal radioactive zone; DB[al]P, dibenzo[al]pyrene; DB[ah]A, dibenz[ah]anthracene; DCM, dichlormethane; 7,12-DMBA, 7,12-dimethylbenz[a]anthracene; HPLC, high performance liquid chromatography; I[cd]P, indeno[cd]pyrene; PM2.5, particulate matter < 2.5 μm; RAL, relative adduct labelling; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography; VAPS, versatile air pollution sampler

* Corresponding author at: Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Tel.: +420 2 4106 2675; fax: +420 2 4106 2785.
E-mail address: jtopinka@biomed.cas.cz (J. Topinka).

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1. Introduction

It is well established that ambient air pollution is related to human health. Increased exposure to respirable particulate matter correlates with increased mortality caused by lung cancer and cardiovascular diseases [1–3]. Pope et al. [4] suggested that a long-term increase in PM2.5 of 10 $\mu g/m^3$ is connected with an 8% increase in lung cancer mortality in adult men. Despite the fact that other factors related to cancer incidence, such as smoking habit or inappropriate diet, are probably stronger influences, the absolute number of cancer cases related to air pollution is high due to the high prevalence of exposure [5].

Although the quantitative health risk related to air pollution is assessed by epidemiological studies [6–9], alternative types of studies are necessary for the purposes of primary prevention. On the level of human populations, such studies are first of all molecular epidemiological studies evaluating quantitative relations between external exposure and measurable biological events (biomarkers). These biomarkers form a chain from exposure to disease [10–12]. One of the most frequently used biomarkers are DNA adducts, quantifying the biologically effective dose of genotoxic compounds that were bound to DNA as a target molecule of carcinogenesis [13–17]. If DNA adducts are not effectively repaired, they might be fixed as mutations during replication. According to the well known scheme of the multi-step process of chemical carcinogenesis, an accumulation of mutations may lead to carcinogenesis. Thus, DNA adduct levels have a direct relation to mutagenesis and carcinogenesis. Data are accumulating about the relation of DNA adducts to environmental exposure to complex mixture components such as carcinogenic PAHs (c-PAHs) [18] and to malignant tumors and other degenerative diseases [19,20]. Another important aspect demonstrating the advantages of molecular epidemiology studies over classical epidemiology is the possibility of identifying the genetic susceptibility of individuals to the action of various compounds [12]. The role of genetic polymorphisms on the metabolic activation of xenobiotics (oxygenases of cytochromes P450 such as CYP 1A1) and also their detoxification (glutathione-S-transferases) is well known. Further biomarkers of individual susceptibility are polymorphisms in genes encoding DNA repair enzymes (XRCC1, XPD, hOGG1) [21–23]. Another factor affecting susceptibility to the genotoxic and carcinogenic effects of xenobiotics is the saturation of the organism by vitamins A, C, E, folic acid, etc., which are known to play a significant role as free radical scavengers and antioxidant agents and which also affect the synthesis of DNA repair enzymes [24–27].

The major aim of this study was to evaluate the relation between external exposure to ambient air pollutants and the main biomarker of a biologically effective dose—DNA adducts. Simultaneously, we studied the effects of genetic and acquired susceptibility on this relation. This cohort study was performed in non-smoking city policemen, and all biomarkers were measured repeatedly during one year to evaluate the dynamics of the observed changes and seasonal variability.

2. Materials and methods

2.1. Study population

The study population consisted of city policemen working in the downtown area of Prague and spending their working shifts outdoors. The total number of study participants was 109. All of the study participants were non-smokers. Another selection criterion was a duration of employment of at least one year. Information about other potential factors was surveyed by means of questionnaires (education, diet, alcohol intake, socio-economic factors, leisure/recreational activities, etc.).

2.2. Inhalation exposure

The level of ambient air pollution and the external exposure of subjects was determined from two sources. First, data from stationary measuring stations in Prague were used. The long-time average concentrations of air pollutants, including c-PAHs, during separate periods of the study were monitored daily (for 30 days before biological sampling) using versatile air pollution samplers (VAPS). Second, before each collection of biological material, individual exposure to air pollutants was measured for 48 h using personal sampling devices (URG Corp, USA). Pallflex glass fibre filters with collected PM2.5 particles were extracted by DCM, and c-PAHs (benzo[a]pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[ah]-antracene, indeno[cd]pyrene) were determined by a certified laboratory (ALS, Prague) using HPLC with fluorimetric detection according to the standard operating procedure (EPA Method 610).

2.3. Chemicals and biochemicals

Spleen phosphodiesterase was purchased from ICN Biomedicals Inc. (Eschwege, Germany); ribonuclease A and T1, proteinase K, micrococcal nuclease and the protein assay kit (No. 5656) from Sigma (Deisenhofen, Germany); polyethylene-imine cellulose TLC plates (0.1 mm) from Macherey-Nagel (Düren, Germany); nuclease P1 from the Japan Institute for the Control of Aging (Shizuoka, Japan);

T4 polynucleotide kinase from USB (Cleveland, USA); and γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Amersham Biosciences (Amsterdam, The Netherlands). All other chemicals and solvents were of HPLC or analytical grade.

2.4. DNA isolation

Lymphocytes were isolated from whole blood by Ficoll 400 gradient centrifugation. The cell pellets were homogenised in a solution of 10 mM Tris–HCl, 100 mM EDTA and 0.5% SDS, pH 8.0. DNA was isolated using RNAses A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol as previously described [28]. DNA concentration was estimated spectrophotometrically by measuring the UV absorbance at 260 nm. DNA samples were kept at −80 °C until analysis.

2.5. DNA adduct analysis

^{32}P-postlabelling analysis was performed as previously described [29,16]. Briefly, DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37 °C. Nuclease P1 was used for adduct enrichment. The labelled DNA adducts were resolved by two-directional thin layer chromatography on 10 cm × 10 cm PEI-cellulose plates. Solvent systems used for TLC were the following: D-1: 1 M sodium phosphate, pH 6.8; D-2: 3.8 M lithium formate, 8.5 M urea, pH 3.5; D-3: 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. Autoradiography was carried out at −80 °C for at least 72 h. Total DNA adduct levels were evaluated from the diagonal radioactive zones (DRZ) on thin layer chromatograms. Additionally, B[a]P-like DNA adducts were determined using radioactivity detected in the area of the chromatograms corresponding to a major B[a]P-derived DNA adduct. The radioactivity of distinct adduct spots was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of the DNA enzymatic digest (1 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection, which simultaneously allowed for controlling the purity of the DNA. DNA adduct levels were expressed as adducts per 10^8 nucleotides. A major B[a]P-derived adduct obtained following the oral administration of B[a]P (100 mg/kg body weight) to rats (DNA from the lung tissue) served as a positive DNA control and was analyzed in each experiment to check the variability between experiments.

2.6. Genetic polymorphisms

Polymorphisms of metabolic genes (CYP1A1, GSTM1, GSTP1, GSTT1), folic acid metabolism (MS, MTHFR), DNA repair genes (XRCC1, XPD6, XPD23, hOGG1) and the p53 gene (BstU I, Msp I) were determined by PCR and RFLP methods [30].

2.7. Vitamins

The levels of vitamins A, C and E in plasma were analyzed by HPLC [31]. Folic acid was measured by a commercial kit from Cedia Folate Roche (Mijdrecht, The Netherlands) according to the manufacturer's protocol [32].

2.8. Cotinine

Cotinine, as the major nicotine metabolite, was analyzed in urine by an RIA assay [33] to check the tobacco smoke exposure reported in the lifestyle questionnaires. This marker is highly sensitive for distinguishing between smokers and non-smokers.

2.9. Statistical analysis

Bivariate and multivariate statistics were used to evaluate the association between exposure and various biomarkers. The comparison of different sampling periods was done using Student's t-test on log transformed values and the detailed impact of metabolic and DNA repair gene polymorphisms on DNA adduct levels by multivariate statistics.

3. Results

The stationary measurements of B[a]P and c-PAHs (bound on respirable PM2.5 particles) in the city centre of Prague during the sampling periods indicated that the highest air pollution level occurred during the 1st sampling period in January 2004 (Table 1). Personal exposure monitoring data confirmed that the highest exposure to c-PAHs (the same PAHs as in the stationary monitoring) was found during the first sampling in January. This exposure was 3–4-fold higher than that found during the three other sampling periods: March, June and September (Table 2). Urinary cotinine levels

Table 1
Results of stationary monitoring of carcinogenic PAHs in the city centre of Prague during various sampling periods

	1st sampling Mean ± S.D. (range)	2nd sampling Mean ± S.D. (range)	3rd sampling Mean ± S.D. (range)	4th sampling Mean ± S.D. (range)
B[a]P (ng/m^3)	2.65 ± 2.86 (0.51–9.1)*	0.63 ± 0.35 (0.25–1.2)	0.48 ± 0.56 (0.14–1.73)	1.15 ± 1.15 (0.14–3.61)
cPAHs (ng/m^3)	16.6 ± 17.9 (3.9–61.7)*	3.7 ± 1.8 (3.6–6.2)	3.5 ± 2.8 (1.8–9.6)	8.6 ± 7.3 (1.7–24.3)

* $p < 0.001$ vs. all other samplings.

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Table 2
Mean age, personal exposure and urinary cotinine levels of the subjects in the study

	1st sampling		2nd sampling		3rd sampling		4th sampling	
	N	Mean ± S.D. (range)	*N*	Mean ± S.D. (range)	*N*	Mean ± S.D. (range)	*N*	Mean ± S.D. (range)
Age (years)	78	33.7 ± 7.9 (22–52)	85	34.5 ± 8.7 (22–55)	85	34.5 ± 8.9 (22–55)	87	34.7 ± 8.9 (23–56)
Personal exposure B[a]P (ng/m^3)	78	1.56 ± 1.33 (0.13–5.5)*	85	0.38 ± 0.62 (0.13–3.6)	85	0.19 ± 0.23 (0.13–1.6)	87	0.43 ± 0.40 (0.15–3.1)
Personal exposure c-PAHs (ng/m^3)	78	8.1 ± 8.8 (1.5–46.0)*	85	3.2 ± 4.3 (1.5–32)	85	1.9 ± 1.1 (1.5–8.6)	87	2.9 ± 3.0 (1.5–25)
Cotinine (ng/mg creatinine)	78	19.5 ± 18.2 (3–141)	85	16.6 ± 12.0 (2–84)	85	16.4 ± 24.2 (2–199)	87	14.4 ± 18.0 (2–160)

* $p < 0.001$ vs. all other samplings.

(Table 2) confirmed the questionnaire data that all the subjects in the study were non-smokers. Mean levels ranged between 14 and 20 ng cotinine/mg creatinine, and individual levels did not exceed 200 ng, which is still under the limit for active smoking.

As described in Section 2, we measured the total DNA adduct levels corresponding to the total ^{32}P-radioactivity on the diagonal radioactive zone (DRZ) (Fig. 1A) and also the so-called B[a]P-like DNA adducts corresponding to the chromatographic mobility of the major B[a]P-induced spot (Fig. 1C) derived from a major B[a]P-metabolite, BPDE. Similarly to finding the highest exposure to c-PAHs during the 1st sampling period, we observed the highest total (2.08 ± 1.60 adducts/10^8 nucleotides) and B[a]P-like (0.26 ± 0.14 adducts/10^8 nucleotides) adduct levels in subjects sampled within this 1st sampling (Table 3). The total DNA adduct levels detected during the other three sampling periods ranged from 1.66 to 1.96 adducts/10^8 nucleotides and B[a]P-like DNA adducts from 0.19 to 0.25 adducts/10^8 nucleotides.

The plasma levels of selected natural antioxidants (Table 4) did not indicate any substantial differences among the various samplings with the exception of higher levels of vitamin C in September 2004 (4th sam-

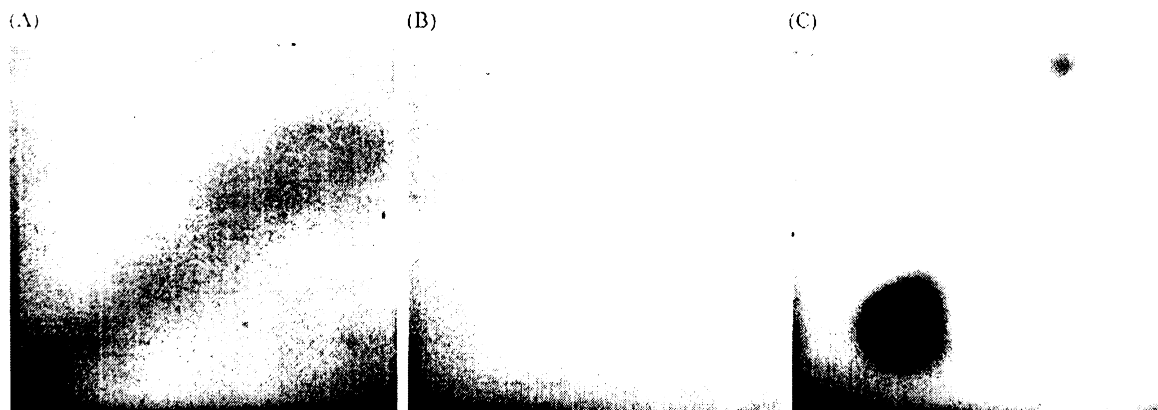

Fig. 1. Representative autoradiographs of thin layer chromatograms with the DNA adduct pattern of: DNA isolated from the lymphocytes of a subject sampled in January 2004 (A); water blank (B); positive control (DNA isolated from the lung of rats treated intraperitoneally with 100 mg B[a]P/kg b.w.) (C). DNA (5 μg) was analyzed using the nuclease P1 method of sensitivity enhancement. Screen enhanced autoradiography was performed at −80 °C for 72 h.

Table 3
DNA adduct levels in lymphocytes

Adducts × 10^8/nucleotides	1st sampling		2nd sampling		3rd sampling		4th sampling	
	N	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.
Total DNA adducts	78	2.08 ± 1.60	85	1.66 ± 0.65	85	1.96 ± 1.73	87	1.77 ± 1.15
B[a]P-like DNA adducts	78	0.26 ± 0.14*	85	0.19 ± 0.12	85	0.22 ± 0.12	87	0.25 ± 0.09

* $p < 0.001$ vs. 2nd sampling and $p = 0.017$ vs. 3rd sampling.

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Table 4
Plasma levels of vitamins and lipids

	1st sampling		2nd sampling		3rd sampling		4th sampling	
	N	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.
Vitamin C (mg/l)	77	11.2 ± 2.7	84	10.7 ± 2.2	85	11.9 ± 3.8	86	15.4 ± 3.0*
Vitamin A (mg/l)	78	1.0 ± 0.3	85	1.1 ± 0.3	85	1.3 ± 0.3	86	1.1 ± 0.2
Vitamin E (mg/l)	78	10.4 ± 3.7	85	12.3 ± 4.2	85	13.9 ± 4.6	86	12.9 ± 3.4
Folates (ng/ml)	78	11.8 ± 9.3	84	14.2 ± 8.4	85	15.6 ± 7.4	–	–

* $p < 0.01$ vs. all other sampling periods.

pling), which might be a consequence of an increased intake of fruits and vegetables during the summer period.

The effect of personal exposure and other biomarkers on DNA adduct levels was analyzed separately for the 1st sampling (highest exposure) and also for all samplings together (Table 5). Bivariate correlations of the total DNA adduct levels with personal exposure to B[a]P and c-PAHs indicated a weak association ($R = 0.16$–0.17; $p = 0.07$–0.09) between the mean values of these parameters in all four samplings. The correlation was slightly more significant for B[a]P-like DNA adducts related to B[a]P and c-PAH exposure ($p = 0.04$–0.05).

As factors of individual susceptibility to the action of c-PAHs, genetic polymorphisms of several enzymes involved in PAH metabolism, DNA repair and p53 protein were studied. The distribution of genotypes within the population studied corresponded to the distribution in the Caucasian population (Table 6). No significant association between DNA adduct levels and individual genotypes was observed for either individual samplings or for mean adduct levels from all four samplings. However, the combination of two genotypes encoding enzymes metabolizing c-PAHs – CYP 1A1 and GSTM1 – was associated with the levels of total and B[a]P-like DNA adducts (Figs. 2 and 3), but only under conditions of increased exposure to c-PAHs. The combination of genotypes for these enzymes might be the reason for inter-individual variability in the ability to activate or

Table 6
The distribution of genotypes in the study population

Gene	Variant *N* (%)		
	WW	WM	MM
GSTM1	53 (48.6%)	–	56 (51.4%)
GSTP1	47 (43.1%)	50 (5.9%)	12 (11.0%)
GSTT1	95 (87.2%)	–	14 (12.8%)
CYP 1A1 Msp I	89 (81.7%)	18 (16.5%)	2 (1.8%)
CYP 1A1 Ile/Val	102 (93.6%)	7 (6.4%)	–
MTHFR	50 (45.9%)	47 (43.1%)	12 (11.0%)
MS	75 (68.8%)	28 (25.7%)	6 (5.5%)
XRCC1	43 (39.4%)	49 (45.0%)	17 (15.6%)
hOGG1	68 (62.4%)	33 (30.3%)	8 (7.3%)
XPD, exon 6	31 (28.4%)	57 (52.3%)	21 (19.3%)
XPD, exon 23	37 (33.9%)	52 (47.7%)	20 (18.3%)
P53 Msp I	81 (74.3%)	27 (24.8%)	1 (0.9%)
P53 Bstu I	55 (50.5%)	48 (44.0%)	6 (5.5%)

Table 5
Bivariate correlations of DNA adduct levels with personal exposure and plasma vitamin levels (Spearman rank test)

	Total DNA adducts				BaP-like DNA adducts			
	1st sampling		All samplings		1st sampling		All samplings	
	N	Spearman R	*N*	Spearman R	*N*	Spearman R	*N*	Spearman R
Personal exposure – B[a]P	78	0.12 ($p = 0.30$)	109	0.16 ($p = 0.09$)$^{+}$	78	0.15 ($p = 0.20$)	109	0.19 ($p = 0.04$)*
Personal exposure – c-PAHs	78	0.20 ($p = 0.08$)$^{+}$	109	0.17 ($p = 0.07$)$^{+}$	78	0.21 ($p = 0.06$)	109	0.19 ($p = 0.05$)
Cotinine	78	0.004 ($p = 0.97$)	109	−0.12 ($p = 0.22$)	78	0.003 ($p = 0.98$)	109	−0.17 ($p = 0.07$)$^{+}$
Vitamin C	77	−0.03 ($p = 0.80$)	108	0.004 ($p = 0.97$)	77	−0.03 ($p = 0.82$)	108	−0.08 ($p = 0.43$)
Vitamin A	78	−0.31 ($p = 0.006$)**	109	−0.1 ($p = 0.28$)	78	−0.27 ($p = 0.018$)*	109	−0.05 ($p = 0.61$)
Vitamin E	78	−0.03 ($p = 0.78$)	109	−0.04 ($p = 0.68$)	78	−0.05 ($p = 0.64$)	109	0.05 ($p = 0.65$)
Folates	78	−0.006 ($p = 0.96$)	98	−0.01 ($p = 0.89$)	78	0.03 ($p = 0.77$)	98	−0.1 ($p = 0.34$)

$^{+}$ $p < 0.1$.
* $p < 0.05$.
** $p < 0.01$.

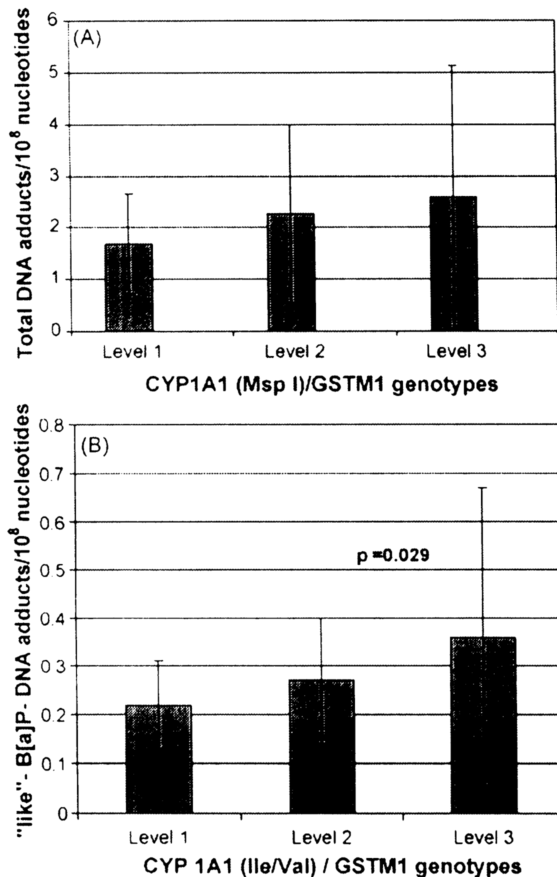

Fig. 2. The effect of combined CYP 1A1 (Msp I) + GSTM1 genotypes on total DNA adduct levels (A) and B[a]P-like (B) DNA adduct levels in lymphocytes. Genotypes combinations were: Level 1 ($N = 27$): CYP 1A1 (Msp I) = WW and GSTM1 = Wx; Level 2 ($N = 44$): CYP 1A1 (Msp I) = Mx and GSTM1 = Wx or CYP 1A1 (Msp I) = WW and GSTM1 = MM; Level 3 ($N = 6$): CYP 1A1 (Msp I) = Mx and GSTM1 = MM. W – wild type; M – mutated; x – wild type or mutated.

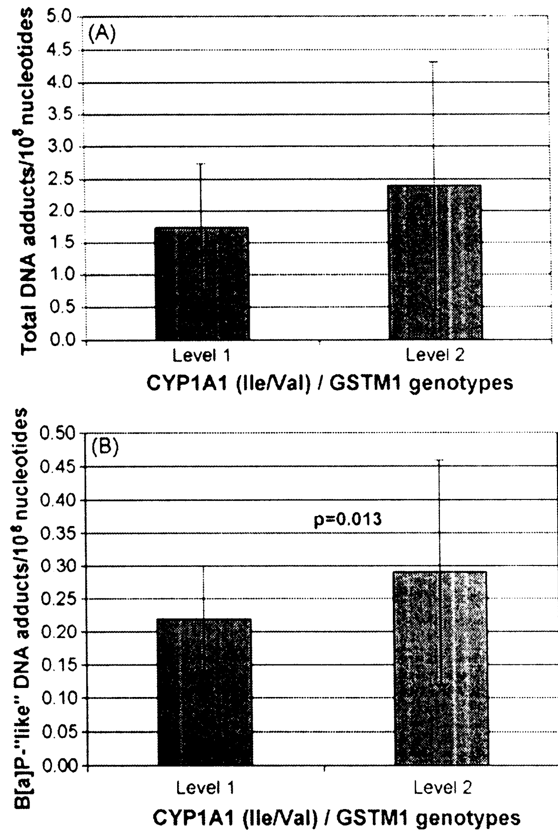

Fig. 3. The effect of combined CYP 1A1 (Ile/Val) + GSTM1 genotypes on total DNA adduct levels (A) and B[a]P-like (B) DNA adduct levels in lymphocytes. Genotypes combinations were: Level 1 ($N = 33$): CYP 1A1 (Ile/Val) = WW and GSTM1 = Wx; Level 2 ($N = 42$): CYP 1A1 (Ile/Val) = Mx and GSTM1 = Wx or CYP 1A1 (Ile/Val) = WW and GSTM1 = MM; Level 3 ($N = 2$): CYP 1A1 (Msp I) = Mx and GSTM1 = MM. W – wild type; M – mutated; x – wild type or mutated.

229 detoxify c-PAHs, expressed as various "levels" of enzy-
230 matic activities. A detailed description of CYP 1A1 and
231 GSTM1 allelic combinations is shown in the legends of
232 Figs. 2 and 3.

233 4. Discussion

234 One of the major findings of this study is a direct
235 association between personal exposure to c-PAHs and
236 the level of total and B[a]P-like DNA adducts. We con-
237 firmed that the relation between c-PAH exposure and
238 DNA adduct levels is not linear as proposed by Lewtas
239 et al. [34]: a substantial increase in exposure (3–4-fold) is
240 associated with a moderate increase in DNA adduct lev-
241 els (~20%). We repeatedly observed a similar result in
242 earlier studies, e.g. in coke oven workers [15], a10-fold
243 increase in exposure to c-PAHs caused only a ~2-fold
244 elevation of total DNA adduct levels in lymphocytes,
245 indicating that DNA adduct levels were approximately
246 proportional to the logarithm of the c-PAH dose. It seems
247 likely that such a relation is associated with efficient
248 DNA repair that eliminates a substantial quantity of DNA
249 adducts after c-PAH exposure. This adduct elimination
250 is one of the basic prerequisites of genomic stability.

251 According to our results, we can conclude that DNA
252 adduct measurements probably have limited sensitiv-
253 ity, since a significant increase in such adducts can
254 only be detected under conditions of increased expo-
255 sure to genotoxic compounds. However, this apparent
256 disadvantage is compensated for by the fact that DNA
257 adduct measurements include DNA repair and provide us
258 with information about unrepaired lesions after exhaust-
259 ing the individual's DNA repair capacity. Therefore,
260 it is not surprising that within the 2nd–4th samplings,
261 we observed low DNA adduct levels that are almost

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unrelated to external c-PAH exposure and are probably efficiently eliminated by DNA repair. However, under conditions of increased c-PAH exposure (1st sampling), an increase in DNA adduct levels was observed because the DNA repair capacity of at least some of the individuals was, in all likelihood, exhausted and thus DNA adducts could accumulate. The ability of a similar concentration of c-PAHs to decrease the DNA repair capacity of another group of city policemen was already demonstrated by Cebulska-Wasilewska et al. [35]. We may expect that an increase in DNA adducts observed in human studies truly indicates significant genetic damage. The implication of such an observation for human health and risk assessment should be further explored.

The more significant correlation of B[a]P-like DNA adducts with B[a]P and c-PAH exposure than that of the total adduct levels suggests that B[a]P-like DNA adducts better reflect specific exposure to B[a]P and c-PAHs than do total DNA adduct levels derived from the DRZ on TLC chromatograms, reflecting exposure to many other genotoxic compounds contained in the ambient air.

In contrast with our previous study [18], which also included smokers, the current study did not find any association between DNA adduct levels in lymphocytes and plasma vitamin C levels. This difference might be due to the fact that the need for vitamin C is known to be greater in smokers than in non-smokers and that vitamin C eliminates some of the adverse effects of tobacco smoke exposure in smokers. The current study (performed exclusively in non-smokers) suggests a protective effect of vitamin A under conditions of elevated exposure to c-PAHs (1st sampling).

Analyzing the association of DNA adduct levels with various metabolic and DNA repair genotypes, we found no effect when individual genes were considered. When the effects of different combinations of genotypes were assessed, an association was found with certain combinations of CYP 1A1 and GSTM1 gene allelic forms encoding enzymes primarily involved in c-PAH metabolism. This combination of phase I and II metabolic gene polymorphisms has been extensively studied in many cancer susceptibility studies [36–39]. One can speculate as to which allelic combinations of CYP1A1 and GSTM1 are most effective in detoxifying compounds such as PAHs. In our study, the subjects were categorized into three groups (levels) according to their combination of CYP1A1 and GSTM1 genotypes. The subjects carrying the wild type allele for both CYP1A1 polymorphisms (Msp I, Ile/Val) together with an active GSTM1 allele exhibited the lowest DNA adduct levels, suggesting quick metabolic elimination (glutathione conjugation) of the DNA reactive intermediates formed by CYP1A1 activity. The DNA adduct levels in the lymphocytes of other subjects carrying variant alleles for CYP1A1 Msp I and Ile/Val were higher, independent of the GSTM1 genotype. This effect was stronger in mutated homozygotes than in heterozygotes. It should be noted that the association of the DNA adduct levels with both genotypes was observed specifically under conditions of higher exposure to c-PAHs (1st sampling period), which is in agreement with our previous observation [40]. The results of other studies dealing with environmentally exposed populations [18] indicate that the association of biomarkers such as DNA adducts or chromosomal aberrations with external exposure is more complicated than might be explained by external personal exposure monitoring of basic air pollutants. It may be affected by many factors such as environmental tobacco smoke exposure and diet. We found greatly increased personal exposure to c-PAHs in the winter sampling period, leading to increased DNA adduct levels in lymphocytes. The strength of this association is probably limited by the fact that 48 h of personal exposure monitoring is too short to accurately reflect the exposure responsible for the observed DNA adduct levels. Long-term stationary monitoring of c-PAHs for 30 days before biological sampling is not representative of the actual personal exposure of the subjects, which is the relevant factor for DNA adduct formation. Therefore, no correlation was found between DNA adduct levels and c-PAHs as measured by VAPS. The effects of other c-PAH sources such as diet should be taken into consideration. Passive smoking was not associated with DNA adduct levels in our study.

In conclusion, this study suggests that DNA adducts in the lymphocytes of subjects exposed to increased c-PAH levels in polluted air are an appropriate biomarker of a biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to an increased mutagenic and carcinogenic risk.

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