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Ph.D. Thesis

In Biomedicine

Genotoxicity of organic air pollutants studied by DNA adduct detection

Oksana Sevastyanova, M.D.

Institute of Experimental Medicine AS CR, v.v.i.
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1. Introduction

The negative effects of environmental pollutants on human health are of serious concern worldwide. Except for a few specialized industrial settings, most people are exposed to many potentially harmful agents at the same time. Exposure to such complex mixture poses especially difficult regulatory and scientific problems. The earliest studies on the genotoxicity of complex mixtures began in the 1940s, when the extractable organic matter (EOM) from air particles was found to be carcinogenic in rodents (Leiter et al., 1942; Hueper et al., 1962) and later short-term bioassays demonstrated that similar organic extracts induced oncogenic transformation in mammalian cells (Freeman et al., 1971; Gordon et al., 1973) and mutations in bacteria (Tokiwa et al., 1976; Pitts et al., 1977).

1.1. Ambient air particles

Humans are exposed to a broad range of complex mixtures in air, food and water. Air pollution consists of a very complex mixture of gases and particles with condensed organic matter. The size, chemical composition, and other properties of particulate matter depend on the sources of the particles and the changes the particles undergo in the atmosphere. In urban environments, these particles derive mainly from combustion, including mobile sources such as motor vehicles and stationary sources such as power plants and local heating.

Ambient air particles (particulate matter (PM)) range in size from 0.01 to 50 μm (Phillips, 1999). Particles that are > 10 μm diameter are deposited primarily in the nasal and oral cavities. Particles within the range of 2.5-10 μm diameters are deposited mainly in the upper respiratory tree; whereas, PM <2.5 μm can penetrate deep into the alveolar sacks of the lung (Claxton et al., 2004). Fine and ultrafine particles (<2.5 μm) are dominated by emissions from combustion processes, while coarse particles (>2.5 μm) are mostly generated by mechanical processes from a variety of noncombustion sources. A number of epidemiological studies indicated that human exposure to high concentrations of PM had adverse effects on human health, such as increases in mortality and morbidity, particularly in children, the elderly, and people with respiratory and cardiovascular diseases (Dockery et al.,

1993; Laden et al., 2000; Pope et al., 2002) as well as on pregnancy outcome (Sram et al., 1999).

Epidemiological studies indicate that mutagenic activity varies significantly between size fractions. It increases with decreasing particle size (Hayakawa et al., 1995; Pagano, 1996). There is a number of potential explanations for this observation. First, smaller aerosols may arise from sources that emit a larger proportion of mutagens. For example, many combustion emissions contain particles with small aerodynamic size and they may contain higher levels of mutagenic extractable matter. In contrast, larger particles, containing very small amounts of organic matter, are often produced by mechanical forces (e.g., reentrainment of soil particles during storms, release of limestone particles during excavation, sea water spray, etc.) (Graedel et al., 1986; Pagano, 1996). Second, smaller aerosols penetrate deeper in the lung, so they can induce more substantial harm.

The mutagenicity of airborne particulate organics is due to at least 500 identified compounds from varying chemical classes (Claxton et al., 2004). These pollutants are emitted from many sources and may undergo chemical and physical changes in the atmosphere. Long wavelengths of natural sunlight could be responsible for the conversion of certain promutagens in air particles into direct-acting mutagens (Al-Khodairy et al., 1997). Investigators have shown that photochemical transformation of a number of source emissions (including wood smoke and automotive exhaust) produce mutagenic products (Kleindienst et al., 1986 and 1992). It was also proved that single non-mutagenic organic compounds (e.g., ethylene, propylene, toluene) exposed to reactive gases (e.g. NO_x and O_3) under photoactivating conditions produce mutagenic products (Kleindienst et al., 1985; Shepson et al., 1985). Reactions that occur in the atmosphere, therefore, can have a profound effect on the genotoxic burden of ambient air. Several studies (Sato et al., 1995; Morozzi et al., 1997) have shown that mutagenicity inversely correlates with ambient temperature tending to peak during winter. Possible explanations include increased levels of particle-associated mutagens due to domestic heating and other combustion emissions, increased atmospheric inversions that retain pollution close to the surface, increased deposition of vapor-phase mutagens onto particles during cold

months, and/or changes in atmospheric chemical transformation processes due to changes in ozone and NO_x levels (Nikolaou et al., 1984).

Long-range transport and/or atmospheric transportation processes affect the level and type of mutagens associated with airborne gases and particles. Fine particulate matter from air pollution sources can be transported many thousands of kilometers through the atmosphere and may remain airborne for long periods of time. Jaffe et al. (1999) showed that a number of primary industrial pollutants (e.g., aerosols) and secondary pollutants (e.g. peroxyacetyl nitrate) were transported across the Pacific to the US northwest coast from Asia.

The studies of airborne mutagens, especially their relative amounts, provide information about the source of contamination. This approach is based on observation that different sources of polycyclic aromatic hydrocarbons (PAHs) are characterized by particular PAH profiles. Because the benzo[g,h,i]perylene (B[g,h,i]P) content of gasoline exhaust gases is relatively high, it has been suggested that B[g,h,i]P/B[a]P (benzo[a]pyrene) ratio reflects the contribution of gasoline-driven vehicles to air pollution (Nielsen et al., 1996). The prevalence of light PAHs, such as benz[a]anthracene (B[a]A) and chrysene (CHRY), was supposed to reflect the contribution of industrial pollution and local heating (Valerio et al., 1992).

Although most human exposure to mutagens is in the form of complex mixtures, much of the mutagenic activity of a particular complex mixture is due to a single chemical class within that mixture (DeMarini, 1998). Carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) and nitroarenes likely accounted for much of the observed mutagenic activity and mutation spectrum of the whole environmental mixture.

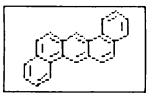
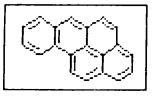
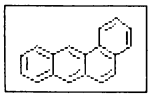
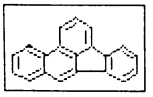
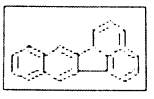
1.2. Polycyclic aromatic hydrocarbons (PAHs)

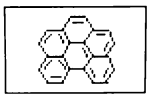
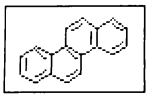
PAHs are a class of very stable organic molecules consisting of multiple interconnected 6-carbon (benzene) rings. Complex PAHs may contain elements other than carbon and hydrogen, including nitrogen and sulfur. Pure PAHs usually exist as colorless, white, or pale yellow-green solids. PAHs of three or more rings have low solubility in water and a low vapor pressure. As molecular weight increases, solubility and vapor pressure decrease. As molecular weight increases, the

carcinogenicity of PAHs also increases, and acute toxicity decreases. PAHs are found in coal tar, crude oil, creosote, and roofing tar, a few are used in medicines or in dyes, plastics, and pesticides production.

PAHs are released to the environment through natural and anthropogenic sources with emission largely to the atmosphere. Natural sources include emission from volcanoes and any naturally occurring fire, such as bush or forest fires. Anthropogenic sources provide much greater release volume than natural sources and include the incomplete burning of organic material such as coal, oil, gasoline and garbage. The primary source of many PAHs in air is an incomplete combustion of wood and fuel (Perwak et al., 1982). In air PAHs are found sorbed to particulates and as gases. Particle-bound PAHs can be transported long distances and are removed from the atmosphere through precipitation and dry deposition. The major processes for degradation of PAHs are microbal metabolism, photooxidation and chemical oxidation.

Polycyclic aromatic hydrocarbons are a group of over 100 chemicals (without derivatives). Many PAHs have been identified as carcinogenic chemicals for animals and/or humans (IARC, 1983). The toxicity equivalence factors of the most common and potent c-PAHs (based on their relative potency to benzo[a]pyrene) according to Nisbet and LaGou (1992) are as follows:

Compound		Toxicity Equivalency Factor (TEF)
	Dibenz[a,h]anthracene	5
	Benzo[a]pyrene	1
	Benz[a]anthracene	0.1
	Benzo[b]fluoranthene	0.1
	Benzo[k]fluoranthene	0.1

	Indeno[1,2,3-c,d]pyrene	0.1
	Benzo[g,h,i]perylene	0.01
	Chrysene	0.01

In humans, the major routes of PAH uptake are thought to be trough:

(1) the lungs and the respiratory tract after inhalation of PAH-containing aerosols or of particulates to which PAHs, in the solid state, has become to be absorbed;

(2) the gastro-intestinal tract after ingestion of contaminated food or water;

(3) the skin as a result of contact with PAH-bearing materials.

Owing to the high lipophilicity of this class of compounds, their bioavailability after ingestion and inhalation must be considered to be significant. Investigations have shown that detectable levels of PAHs occur in almost all internal organs; organs rich in adipose tissue can serve as storage depots from which the hydrocarbons are gradually released, and the gastrointestinal tract contains high levels of hydrocarbon and metabolites, even when PAHs are administered by other routes, as a result of mucociliary clearance and swallowing or hepatobiliary excretion (IPCS, 1998). Most metabolites of PAHs are excreted in faeces and urine. Recent extensive study (Gerde et al., 2001) indicates that bioavailability of c-PAHs after inhalation depends on the deposition of PM in the respiratory tree. The highest bioavailability is in lymph nodes, followed by alveolar region from which larger fraction of c-PAHs are absorbed mostly unaltered into the blood and metabolized systematically. In conducting airways absorbed c-PAHs slowly penetrate the capillary bed and are rapidly metabolized in airway epithelium, due to this fact the substrate levels of c-PAHs in the epithelium of conducting airways exceeded the systemic levels by up to two orders of magnitude.

The mechanism of action of most c-PAHs involves covalent binding to DNA nucleophilic sites by c-PAH electrophilic metabolites with DNA adduct formation.

Brookes and Lawley (1964) demonstrated positive correlation between the interaction of PAHs with DNA and their carcinogenic potency. Since than, many individual PAHs have been tested for tumorigenicity. It was observed that PAHs with bay regions were likely to be potent carcinogens (Figure 1), and those with fjord regions such as DB[a,l]P were even more potent (Figure 2) (Dipple, 1994; Szeliga and Dipple, 1998).

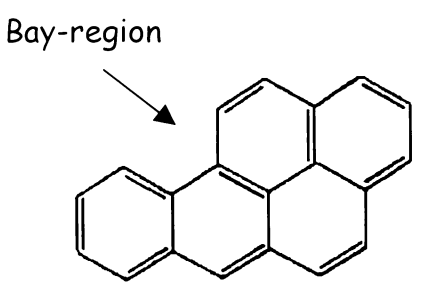


Figure 1. Benzo[a]pyrene

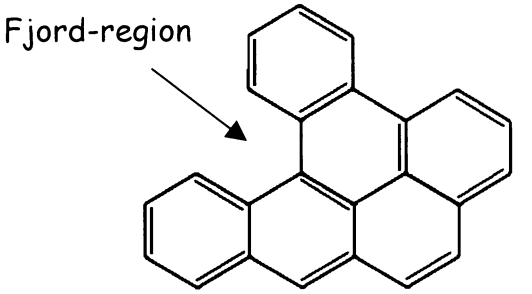


Figure 2. Dibenzo[a,l]pyrene

For interaction with cellular macromolecules multistep metabolic activation of c-PAHs is necessary. Metabolism of c-PAHs occurs primarily by enzymatic action of cytochrome P450 (CYP) and epoxide hydrolase. Cytochrome P450 enzymes are a superfamily of haem-containing monooxygenases, responsible for the phase I metabolism of a wide range of substrates (drug, xenobiotics and steroid metabolism). Cytochromes P450 insert an atom of molecular oxygen into their substrates, which, in most cases, leads to an increase in hydrophilicity of the substrates and its excretion from the cell. But in this process of detoxification active PAH metabolites capable to bind DNA may arise. Cytochromes P450 are predominantly expressed in the liver, but a remarkable amount is also found in extrahepatic tissues (lung, small intestine). The two predominant CYP isozymes that metabolize PAHs are CYP1A1 and CYP1B1 (Hemminki et al., 1997). CYPs first add an epoxide group to the PAHs, which can be converted to a dihydrodiol by epoxide hydrolase. This PAH metabolite may be further metabolized by CYPs to form a diol epoxide that reacts and form bulky adducts primarily with exocyclic aminogroup of guanine (N^2). To a lesser degree, PAHs may be activated by prostaglandin synthase, lipoxygenase, or one-electron oxidation (Miller and Ramos, 2001).

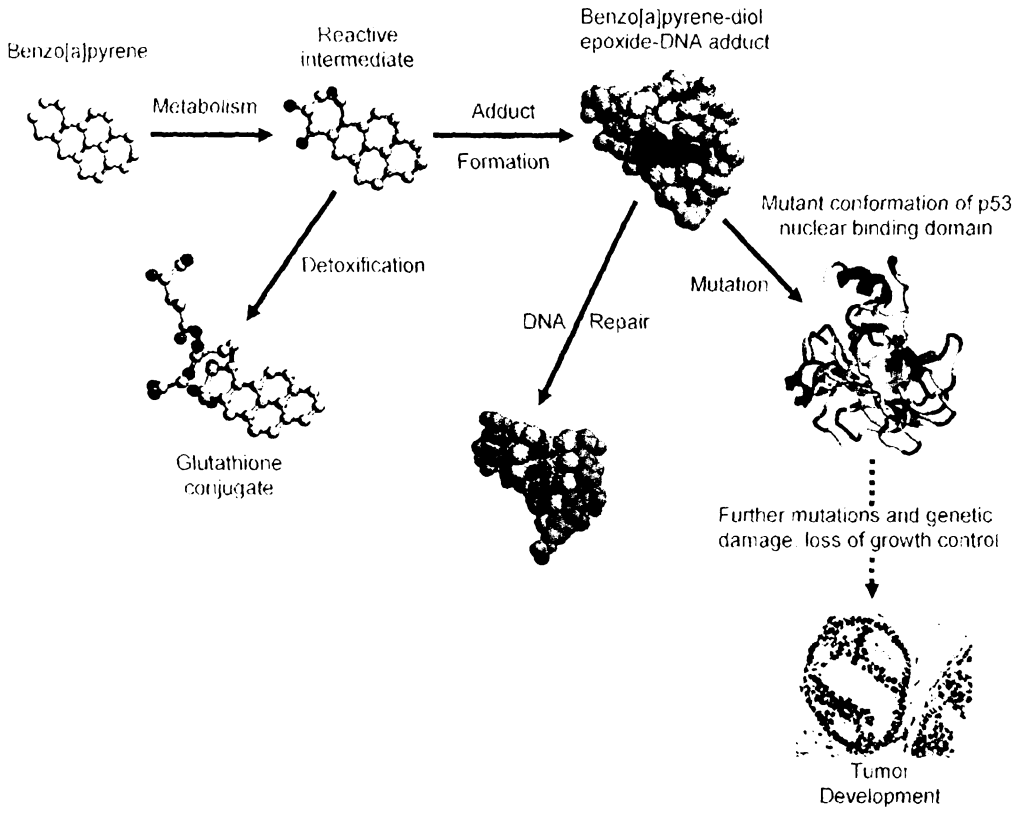


Figure 3. A schematic diagram showing the mechanism of carcinogenic action of polycyclic aromatic hydrocarbons (from Rundle, 2006).

PAH epoxides can be conjugated with glutathione. This conjugation is regarded as a true detoxification reaction and is mediated by glutathione transferase (GSTM1) (Casarett and Doulls, 1991; Hemminki et al., 1997). It has been established by Blaha et al. (2002) that PAHs are also cause changes in cellular gap-junction communication, similar to those caused by the well-studied tumor promoter 12-O-tetradecanoylphorbol-13-acetat. Thus, in addition to their genotoxic mechanism of inducing mutations, PAHs have the ability to act as tumor promoters.

1.3. Carcinogenic PAH artificial mixtures

Since humans are rarely exposed to single substances, knowledge about the potential interactions of the components of environmental mixtures is highly important for human health risk assessment. Mixtures of genotoxic chemicals may undergo a variety of interactions, which may be expressed as synergistic, antagonistic, or additive interactions and may have a significant influence on the ultimate toxicity of a complex mixture. The mechanism of chemical interactions can involve changes in transport, metabolism or molecular binding of the components.

Most studies of the mutagenicity of binary combinations of organic chemicals have been performed in the Salmonella plate-incorporation assay (Taylor et al., 1995). Although a common assumption for risk assessment regarding chemical mutagens is that the mutagenic potency of combination of two or more mutagens is additive (Smyth et al., 1969), nonadditive responses for binary combinations of mutagens have been observed more frequently (Lewtas et al., 1997). Hass et al. (1981) found that the presence of 1.5 μg benzo[e]pyrene (B[e]P) per plate increased the mutagenic response of 0.3 μg benzo[a]pyrene by approximately 25 times in the Salmonella/microsome assay. Using an animal model, DiGiovanni et al. (1982) demonstrated that B[e]P enhanced B[a]P genotoxicity but decreased tumor initiation by dimethylbenz[a]anthracene (DMBA). Therefore, B[e]P can act as either a co-carcinogen or anti-carcinogen depending on the carcinogenic hydrocarbon it is applied with. Donelly et al. (1998) found that bacterial mutagenicity of B[a]P is reduced or eliminated in the presence of 2,4,6-trinitrotoluene, and concentration of rat liver microsomal S9 fraction has minimal effect on the mutagenicity of this binary mixture.

Several studies have obtained different responses to binary combinations of mutagens depending on the doses of the compounds (Hermann, 1981; Ogawa et al., 1985; Taylor et al., 1995; White, 2002) or the concentrations of S9 (Yoshida et al., 1979; Taylor et al., 1995). At low doses of the mutagens most combinations of agents gave additive responses but higher doses (greater than 1 μg/ml) frequently produced subadditive effects. For the plate Salmonella assay for those agents requiring S9, low

concentrations of S9 generally gave subadditive responses, but high concentrations of S9 produced additive results; the opposite was generally observed in the spiral Salmonella assay.

Studies documenting antagonistic interactions (Hermann, 1981; Cherng et al., 1996) suggest that the antagonism is likely caused by competitive inhibition of enzymes required for metabolic activation and reduction in conversion of pro-mutagens into reactive metabolites. Other studies present evidence that antagonism can be driven by a shift in metabolism from conversion to reactive metabolites to detoxification or inhibition cellular uptake and binding to DNA (Shepson et al., 1985; Hattemer-Frey and Travis, 1991). *In vivo* less-than-additive phenomenon is also suggested to be driven by saturation of metabolic activation pathways or induction of DNA repair processes (Lewtas et al., 1997).

It has been also demonstrated that many non-mutagenic substances (e.g. naphthalene, anthracene) can modulate (e.g. enhance or diminish) the effects of mutagens such as B[a]P (Hermann, 1981; Donelly et al., 1990). Olson et al. (1995) shows that ferrous and germanium ions reduce the mutagenic activity of B[a]P with iron showing a stronger effect in this regard. Since the mutagenicity of B[a]P activated by S9 is due to the production of primary and secondary metabolites, which are direct-acting mutagens themselves, ferrous ions may interfere with their production by blocking an enzyme system necessary for their production or favor the synthesis of metabolites that are not carcinogens. In addition, the ferrous ion may change the activity of an enzyme such as aryl hydrocarbon hydroxylase in the S9 mixture (Olson et al., 1995). There is evidence that trace element selenium can reduce the mutagenicity of B[a]P (Lin et al., 1984).

The results of *in vitro* and animal studies related to DNA adduct and tumor forming potencies are, in some cases, contradictory. Smolarek et al. (1987) showed that benzo[e]pyrene increased B[a]P-induced DNA adduct levels and decreases dimethylbenz[a]anthracene (DMBA) induced DNA adduct levels in Sencar mouse epidermis. These effects of B[e]P correlated with the co-carcinogenic and anti-carcinogenic effects of B[e]P on B[a]P and DMBA, respectively, in a mouse skin initiation-promotion assay (DiGiovanni et al., 1982). Thus, they suggested that the mechanism of the pro- and anti-carcinogenic action of B[e]P involves the alteration of

c-PAH binding to DNA and proposed the measurement of PAH-DNA adducts as a rapid method for predicting the pro- and anti-carcinogenic effect of PAHs in a mixture. The results of Lau and Baird (1992) indicate that the increase in B[a]P-DNA adduct levels caused by co-treatment with B[e]P results from increased metabolism of B[a]P to the proximate carcinogen B[a]P-7,8-dihydrodiol.

Hughes and Phillips (1990) investigated the synergistic and inhibitory interactions of dibenzopyrenes and benzo[a]pyrene when applied in combination to mouse skin. The relative binding potencies of the compounds in both skin and lungs were: DB[a,l]P >>B[a]P > DB[a,h]P > DB[a,i]P > DB[a,e]P, in good agreement with their reported carcinogenicities in mouse skin. The majority of adducts was removed from DNA within 21 days of treatment, but low levels of adducts were found to persist for at least 3 months in both tissues. When DB[a,l]P, DB[a,e]P and B[a]P were applied together to mouse skin, a total binding 31% lower than expected was detected, while with a mixture of DB[a,e]P and B[a]P the binding to DNA in skin was 65% higher than expected from the binding levels of the carcinogens when applied individually. Other binary combinations of these three PAHs gave adduct levels similar to the sum of the binding levels of each component applied individually.

Topinka et al. (1998) in their study on primary cultures of rat hepatocytes found that equimolar mixture of 3 c-PAHs (B[a]P, B[a]A, and B[b]F) as well as 8 c-PAHs (B[a]A, B[a]P, B[b]F, B[k]F, CHRY, DB[a,h]A, I[c,d]P and B[g,h,i]P) induced the total adduct levels closely similar to the sum of adduct levels resulting from incubations with individual compounds in this cells.

Nesnow et al. (1998) investigated lung tumorigenic interactions of five environmental c-PAHs (benzo[a]pyrene, benzo[b]fluoranthene, 5-methylchrysene, dibenz[a,h]anthracene, and cyclopenta[c,d]pyrene) in strain A/J mice. The binary interaction functions were dominated for the most part by DB[a,h]A and were inhibitory.

Binkova and Sram (2004) investigated the genotoxic effect of single carcinogenic polycyclic aromatic hydrocarbons, their binary mixtures, artificial mixture of 8 c-PAHs and real EOM mixture using normal diploid lung fibroblasts. The DNA binding potencies of c-PAHs were as follows: DB[a,l]P>>B[a]P>> B[b]F~ CHRY~B[k]F~ B[a]A~ DB[a,h]A~ I[c,d]P> B[g,h,i]P. Co-treatment of DB[a,l]P and

B[a]P with other c-PAHs resulted in significantly lower DNA adduct levels as compared with adduct levels resulted from single compounds. The highest inhibitory effect was found in the presence of DB[a,h]A followed by B[k]F. In both artificial and EOM mixtures from air particles a strong inhibitory effect of other compounds on benzo[a]pyrenedihydrodiolepoxide (BPDE)-induced DNA adducts was observed. BPDE-DNA adduct levels were up to five-fold lower in artificial c-PAH mixtures and up to 10-fold lower in EOM samples than expected from exposure to B[a]P alone at the same concentration.

1.4. DNA adducts as biomarker of exposure and risk

Human exposure to carcinogens may be determined using different sources of material for analysis:

- stationary and personal monitoring;
- human blood, urine and other tissue for determining the concentration of carcinogen or its metabolites;
- cellular macromolecules (proteins and DNA) containing covalently bound carcinogens.

Monitoring of external concentration of carcinogens in air, diet and occupational environment has played an important role in epidemiological studies, but does not affect directly the toxicological impact of such exposures to individuals.

The measurements of the genotoxin or its active metabolites and adducts in body fluids are highly sensitive and specific to the exposure. These data can be used to calculate the internal exposure doses of the chemicals and to determine the dose-response relationship, which is used to consider the biological significance of the exposure. The measurement of adducts is particularly useful for identifying the chemicals that form bulky adducts with macromolecules (e.g. protein and DNA adducts). The most relevant adducts for carcinogenic risk assessment are those formed at the target site in DNA.

DNA adducts are useful markers of carcinogen exposure, providing an integrated measurement of carcinogen intake, metabolic activation, and delivery to the target macromolecule in target tissues (Phillips, 2005). Molecular epidemiological research has demonstrated significant interindividual variation in carcinogen-DNA

binding among subjects with comparable exposure, suggesting that DNA adducts have the potential to identify persons with an enhanced response due to the nature of their metabolic/detoxification and DNA repair pathways (Phillips, 2005).

The ability of DNA adducts to predict the cancer risk has been tested within the prospective Physicians' Health Study (Tang et al., 2001). Blood samples were collected between 1982 and 1984 from approximately 15.000 males, subsequently, 89 cases of lung cancer occurred, and these were matched on smoking, age, and duration of follow-up with 173 controls. These analyses demonstrated an elevated level of DNA adducts in smokers, compared with nonsmokers, confirming the validity of DNA adducts as biomarker of exposure. "Healthy" (at the time of sampling) current smokers who had elevated levels of aromatic DNA adducts in WBCs were approximately three times more likely to be diagnosed with lung cancer 1-13 years later than current smokers with lower adducts concentrations. This study indicate that using DNA adducts as a measure of exposure, several years prior to the onset (or clinical manifestation) of disease, can identify individuals at high probability of developing cancer subsequently. This demonstrates the potential of DNA adducts as biomarker of risk. Several recent studies (Peluso et al., 2005 and Bak et al., 2006) also indicate that bulky adducts are a good indicator of cancer risk.

The main analytical procedures currently used for DNA adducts detection include immunoassay, ^{32}P-postlabelling and accelerator mass spectrometry. The sources of the DNA are usually lymphocytes (or the whole fraction of nucleated white blood cells), although buccal mucosa, placenta, bladder epithelial cells, lung, cervix, sperm and hair roots have also been used. While lymphocytes are not the target cells for malignancy, they are useful surrogates and are known to display evidence of genotoxic exposure (Phillips, 2005). A significant correlation between DNA adducts levels in mononuclear WBCs and lung tissue was found by Wiencke et al. (1995). They analyzed a major adducts in WBCs and in non-involved lung tissue samples from patients undergoing surgery for lung cancer. The same results were obtained by Tang et al. (1995).

The adduct lifetime is not always clear. Repair processes remove with different efficiencies many adducts. Also the lifetime of the DNA adducts *in vivo* is variable according to the tissue from which it was derived (Farmer, 2004). PAH-DNA

adducts persist longer in lung tissues than in liver or peripheral blood lymphocytes (PBLs). The half-lives of B[a]P and B[b]F DNA adducts in PBLs are 8-16 days (Nesnow et al., 1993).

Carcinogen adducts are formed at all the nucleophilic sites on the DNA bases with different specificities according to the nature of the genotoxic compound. Adducts from PAHs include guanine N^7 and N^2. Adducts at N^7 of guanine are unstable to chemical and enzymatic hydrolysis of the glycolytic bond, they are removed from the DNA by glycosylases and some have been shown to be excreted unchanged in the urine during the next few days (Shuker and Farmer, 1992). Analysis of these repair products offers an attractive non-invasive way to detect the amount of nucleic acid damage that has occurred (Farmer, 2004).

DNA adducts can be removed by DNA repair processes or by cell death, but during chronic exposures they often reach steady-state levels in carcinogen-target tissues. During cell replication, the DNA adducts can result directly in mutations in genes that control cell growth and thus can lead to neoplasia.

1.4.1. Carcinogenic PAH-DNA adduct formation in model systems

For investigation of c-PAH-DNA adduct formation can be used a lot of experimental systems such as *in vitro* acellular assay (Savela et al., 1996; Binkova et al., 1999; Binkova et al., 2003), cell cultures (Topinka et al., 1998 and 2000; Binkova et al., 2000 and 2004; Vakharia et al., 2001; Wu et al., 2003) and animal models (Marston et al., 2001). Each of these systems has its advantages and disadvantages and thus its own purposes in investigation of compounds health effects. Acellular assay of calf thymus DNA provides an early assessment of the genotoxic potential of compounds. The calf thymus DNA/ ^{32}P-postlabelling adduct assay detect direct DNA damage produced by a variety of chemical agents and may provide valuable mechanistic insight for positive responses in cell-based genetic toxicology tests (Adams et al., 1996). Savela et al. (1996) characterized the nature of DNA reactive chemicals in foundry air samples through incubating the foundry filter extract with calf thymus DNA with rat liver activation mixture (S9 mix). Binkova et al. (1999 and 2003) used acellular assay for the evaluation of DNA adduct-forming potency of complex mixture of organic compounds adsorbed onto ambient air particles. The

major DNA adducts resulting from the crude extracts were identical to those derived from aromatic fraction. DNA adduct maps were similar for the EOM samples regardless of monitoring site (polluted and control districts in the Czech Republic) and season, and distinct aromatic-DNA spots accounted for approximately 80% of the total radioactivity were detected along the diagonal zone.

In their study Jankowiak et al. (1998) compared DNA adducts formed by dibenzo[a,l]pyrene in the presence of rat liver microsomes in calf thymus DNA *in vitro* and in mouse skin *in vivo*. Identical profile of adducts was found in these systems, activation by rat liver microsomes introduce two additional spots.

Cell cultures present a more complex experimental system that can be used as a next stage in investigation of genotoxic potential of compounds or their mixtures. There are several main groups among cell cultures that are used for investigating of DNA adduct formation: animal and human-derived cells, normal and tumor cell lines. Animal cells are easily to access, so they can be widely used in primary cultures, which are closer to *in vivo* conditions than transformed cell lines, but human-derived cell lines are more suitable for health risk assessment. It is easily to maintain tumor cell lines, but they are likely to contain multiple mutations that may affect the DNA damage response (Leonardo et al., 1994; Venkatachalan et al., 1997). Cell cultures were proved to be suitable models in investigating the genotoxic effect of single c-PAHs, artificial and environmental mixtures. Binkova and Sram (2004) used human diploid lung fibroblasts in confluent state to investigate the genotoxic effect of single c-PAHs, their binary mixtures, artificial mixture of 8 c-PAHs and real EOM mixture. They found that human diploid lung fibroblasts possess lower metabolic capacity than other human target cells and it may be easier to competitively inhibit the metabolizing enzymes in this cell system. They suggest that this finding can explain the generally low c-PAH-DNA adduct levels detected in human studies using surrogate cells such as blood WBC or lymphocytes that also posses low metabolic capacity.

Combined use of cells equipped with different enzymatic systems and thus with different capability of activating pro-genotoxic chemicals provide a suitable tools for discriminating the contribution of different compounds to the genotoxicity of complex mixture (Topinka et al., 2000). Cell cultures can also be used for hazard

identification of occupational exposure to mutagens and carcinogens (Topinka et al., 1998).

Animal models are widely used for investigation of the carcinogenic and genotoxic effects of PAHs (Smolarek et al., 1987; Hughes et al., 1993; Jankowiak et al., 1998). They enable evaluation of DNA adduct formation *in vivo* in different organs including target organs for carcinogenesis in response to c-PAH exposure, but it is difficult to extrapolate these results on humans. Within this study animal models were not used.

1.4.2. Carcinogenic PAH-DNA adduct formation in humans

The advent of very sensitive methods for detecting DNA adducts has made it possible to monitor DNA isolated from human tissues for evidence of prior exposure to carcinogens. Several studies have investigated DNA adduct formation in workers in heavy industries, petrol refinery workers, traffic police and bus maintenance workers. In general, the results of such studies demonstrated statistically significant increases in the level of DNA adducts in the exposed workers, compared with controls. Nielsen et al. (1996) studied aromatic DNA adduct levels in peripheral blood lymphocytes of bus drivers from the Copenhagen area. A significantly higher adduct level was observed in bus drivers from the center of the city than from suburban and dormitory areas. All three exposure groups (non-smoking bus drivers from central Copenhagen, suburban residential Copenhagen and dormitory village area) had higher adduct levels than rural controls.

A biomonitoring study in Genoa, (Italy) involved 34 nonsmoking police officers and 36 nonsmoking office workers, as control group. The police officers were exposed to significantly higher levels of B[a]P than were office workers (3.3 ng/m^3 and 0.03 ng/m^3, respectively), no seasonal variation of B[a]P concentrations was found in either group. Although the DNA adduct levels of police officers were higher than those of controls, the difference was entirely due to the summer difference (median values 2.8 and 0.80, respectively). In winter the DNA adduct levels were almost identical, and in midseason there was no significant increase in police officers, with respect to controls. More significant seasonal variation of bulky aromatic DNA

adduct levels was observed in WBC DNA samples of police officers ($P < 0.05$) compared to those of controls (Peluso et al., 1998).

Environmental exposure to industrial sources of carcinogens has been investigated in polluted areas in comparison with rural areas of the same countries in several studies. In 1990 Hemminki and colleagues reported the results of a case-control study from Upper Silesia, Poland, which is a heavily industrialized area of black coal mines, smelters, cookeries, chemical works and electrical power plant that operate on black coal with heavy vehicle exhaust and coal burning for domastic heating. Concentration of B[a]P in urban areas ranged from 60 to 90 ng/m^3 in winter and 5 to 20 ng/m^3 in summer. Total organic extracts of small particulate matter exhibited both direct and indirect mutagenic activity. Residents of Upper Silesia, from the vicinity of a coke oven and countryside controls from northeastern Poland were involved in a cross-sectional study to determine DNA adducts in white blood cells. Adduct levels of the Silesian residents were nearly as high as of the coke oven workers, and about 2-fold higher than of the countryside controls, which indicated substantial exposure in the surroundings of the coke oven. The ^{32}P-postlabelling adduct patterns were similar for coke oven workers and Silesian residents, but the countryside controls exhibited no uniform adduct patterns. Seasonal variation in WBCs DNA adducts levels was more significant in environmentally exposed Silesian residents in comparison to the countryside controls and paralleled with the seasonal change of air pollution (Hemminki et al., 1990; Chorazy et al., 1994).

Binkova et al. (1995) analyzed effect of personal exposure to air pollution on DNA adducts in humans in a group of woman working outdoors as a postal workers or gardeners in the city of Teplice, one of the mining districts in northern Bohemia. Personal exposure monitoring for respirable particles (<2.5 μm) was conducted for the 24 h period prior to collection of blood and urine. Particle extracts were analyzed for c-PAHs. A highly significant correlation between short-term personal exposure to c-PAHs and DNA adducts was found (Binkova et al., 1996).

Bulky DNA adduct levels have been determined in human placentas from women in the polluted Teplice region and the control Prachatice region of the Czech Republic to evaluate the effect of environmental pollution on reproductive outcomes.

Air pollution monitoring was carried out continuously during this study at both regions, in Teplice and Prachatice. Air pollution level in the polluted region (namely PM10, c-PAHs) was higher during winter than in summer (Sram et al., 1999). The total DNA adduct levels were significantly increased in persons living in polluted areas in comparison with controls– 2.12 and 1.48 adducts per 10^8 nucleotides, respectively. Elevated DNA adduct levels were found in smoking mothers (10 or more cigarettes per day) 3.21 adducts per 10^8 nucleotides in comparison with non-smoking 1.32 adducts per 10^8 nucleotides. Placental DNA adduct levels in smokers correlated with cotinine measured in plasma. DNA adduct levels were evaluated separately for non-smokers (1.50 and 1.09 adducts per 10^8 nucleotides for the Teplice and Prachatice districts, respectively) and smokers (3.35 and 2.91 adducts per 10^8 nucleotides for the Teplice and Prachatice districts, respectively). This findings indicate that the effect of smoking on adduct level was stronger than that of the ambient exposure.

The high lung cancer rate in Xuan Wei, China, is associated with smoky coal use in unvented homes. DNA adducts were determined by enzyme-linked immunosorbent assays (ELISA) and ^{32}P-postlabelling in placentas and peripheral and cord white blood cells (WBC) from Xuan Wei women burning smoky coal or wood and from Beijing women using natural gas. Indoor B[a]P concentrations were in the magnitude of 2000 (smoky coal), 500 (wood) and 20 ng/m^3 (natural gas). PAH-DNA adducts were detected in a higher percentage of placentas from Xuan Wei women living in houses exposed to smoky coal or wood emissions than from those of the Beijing controls. No dose-response relationship was observed between the air benzo[a]pyrene concentrations and DNA adduct levels or percentage of detectable samples. The results suggest that DNA adducts can be used as a qualitative biomarker to assess human exposure to combustion emissions (Mumford et al., 1993).

There is evidence that dietary exposure is a possible confounding factor in biomonitoring studies of occupational exposure to c-PAHs. In the Persian Gulf War 605 oil wells were set on fire, which caused an unprecedented environmental pollution in a large geographical region. Biomarkers of c-PAH exposure were determined in a group of 61 United States Army soldiers who were deployed to Kuwait and Saudi Arabia in 1991 in the aftermath of the Persian Gulf War (Poirier et

al., 1998). DNA adduct levels in the blood of the personnel were found to be lower while soldiers were putting out fires in the desert than they were both before and after the tour of duty when the soldiers were stationed in Germany. One possible explanation is that during working in Kuwait the soldiers ate a diet that contains much lower levels of c-PAHs than their diet at the European base (Phillips, 2005).

On the example of dietary exposure to c-PAHs we can see that final results of human epidemiological studies of health impact of ambient air pollution can be influenced by a lot of external and internal confounding factors. Human studies should be based on sensitive biomarkers detection and take into consideration individual characteristics, which can affect studied biomarker level. In the case of DNA adduct determination, smoking habits, diet and individual genetic susceptibility should be considered.

2. Aims of study

The main aim of this study was **complex investigation of the genotoxicity of organic air pollutants adsorbed onto urban air particles by means of DNA adduct analysis in different *in vitro* systems and in humans.**

In order to achieve the main goal following tasks were defined:

- to assess whether 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;

- to find a relevant human derived cell line to investigate the genotoxic potential of PAH-containing complex mixtures;

- to use this cell system for the analysis of DNA adduct forming activity of organic compounds bound onto PM10 particles;

- to compare the sensitivity and correlations of three endpoints (DNA adduct formation, DNA strand break induction, and protein p53 up-regulation) to assess the genotoxic potential of the complex mixture of organic compounds extracted from ambient air particles;

- to compare the genotoxic potential of organic extracts from urban air particles collected in various seasons in the center of Prague;

- to study the effect of exposure to organic compounds adsorbed onto respirable air particles on DNA adducts in lymphocytes in exposed group of non-smoking policemen working in the downtown area of Prague.

3. Results (see Appendixes 1-5)

In order to study the biological effects of organic extracts from ambient air particulate matter we used different *in vitro* systems and conducted an epidemiological study in exposed group of non-smoking policemen working in the downtown area of Prague.

In our first study (**Appendix 1**) genotoxicity of complex mixtures of environmental pollutants adsorbed onto respirable air particles was investigated using acellular assay of calf thymus DNA +/- rat liver microsomal S9 fraction. The study has shown that a cell-free system in conjunction with the sensitive ^{32}P- postlabelling is a 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.

Then we tested 3 human-derived cell lines to find an appropriate cell line to investigate the genotoxic potential of c-PAH-containing complex mixtures and used this cell system for the analysis of DNA adduct forming activity of organic compounds bound onto PM10 particles (**Appendix 2**). We have shown that out of 3 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.

In order to evaluate the sensitivity and correlation among three biomarkers of exposure, assessing the genotoxic potential of the complex mixture of organic compounds, three endpoints: DNA adduct formation (^{32}P-postlabelling), DNA strand break induction (single cell gel electrophoresis) and protein p53 up-regulation (immunoblotting) were tested in HepG2 cells exposed to EOMs (**Appendix 3**). Strong correlation between DNA adduct formation and DNA strand breaks was established. Western-blot analyses did not demonstrate any up-regulation of protein p53 due to cell exposure to EOMs.

In our further study (**Appendix 4**) we used HepG2 cells to compare the genotoxic potential of organic extracts from urban air particles collected in various seasons in the center of Prague expressed as DNA adducts. Organic extracts were obtained from PM10 collected in summer and winter 2000/2001 and in winter 2005 by HiVol air samplers. The summer sample exhibited a 10-fold lower genotoxicity than did those of winter, while the difference between winter samples was not significant: 23.4 (DNA adducts in relative units) in summer 2000, 291 in winter 2001 and 249 in winter 2005. 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. So that 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 it depends predominantly on the quantity of c-PAHs bound to the particles. Thus estimations of the genotoxic potential of the ambient air and predictions of health risk should be based preferably on the measurement of c-PAH concentrations.

The effect of exposure to c-PAHs adsorbed onto respirable air particles (<2.5 μm) on DNA adducts in lymphocytes was studied in a group of non-smoking policemen working in the downtown area of Prague and spending >8 h daily outdoors (**Appendix 5**). 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. One of the major findings of this study is a direct association between personal exposure to increased c-PAH levels and the level of total and B[a]P-like DNA adducts under conditions of increased c-PAH exposure. No effect of individual metabololic and DNA repair genotypes on DNA adduct levels was observed. However, the combination of 2 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. This study suggests that DNA adducts in lymphocytes of subjects exposed to increased c-PAHs levels in polluted air are an appropriate biomarker of biologically effective dose, directly indicating whether or not the extent of exposure to these compounds is related to the increased mutagenic and carcinogenic risk.

4. Discussion

4.1 *In vitro* genotoxicity of PAH mixtures and organic extract from urban air particles

4.1.1. Acellular assay (Appendix 1)

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 (Nesnow et al., 2002). 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. (1999). The question concerning the interaction of c-PAHs in the process of bioactivation (competition for activating enzymes in S-9 fraction) as well as in the process of DNA adduct formation, was addressed. We observed lower DNA adduct levels in c-PAH mixture treated samples, suggesting a strong inhibitory effect, also observed by others (White, 2002). 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 (Appendix 2). 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.

Several studies have demonstrated that EOMs extracted from ambient air particles consist of many diverse classess of compounds, such as PAHs, nitro- and alkyl-substituted PAHs, and heterocyclic compounds, that have both mutagenic and carcinogenic activity (Lewtas et al., 1992, 1993). The question is whether majority of these EOM components induces DNA adducts. 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 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 for 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 were taken into consideration. This correlation supports our earlier hypothesis 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.

In order to get more insight into the real human exposures resulting from the air pollution in all 3 localities (Prague, Kosice and Sofia) and both sampling periods (summer and winter), the results of total DNA adduct levels were normalized to the amounts of EOMs per m^3. Such a comparison underlines the difference between summer and winter sampling periods, 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. 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 period.

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

4.1.2. Human cell lines (Appendix 2)

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 3 European cities (Prague, Kosice and Sofia) during both summer and winter sampling periods.

It is well established that c-PAHs are probably the most important components of complex environmental mixtures (Warshawsky, 1999). 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 c-PAH mixtures as indicated by changes in BPDE-derived adduct spots caused by the presence of the second c-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 c-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 (Topinka et al., 1998), HepG2 cells contain inducible CYP 1A1 (Burczynski et al., 1999; Vakharia et al., 2001), 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 and c-PAH concentrations), as is in the case of HEL cells (Binkova et al., 2004).

Approaching further the situation of exposure to real EOMs, we analyzed the DNA adduct forming activity of an artificial c-PAH mixture containing 8 c-PAHs. The mixture was prepared according to the chemical analysis of c-PAHs in EOM obtained in Prague during the winter sampling period. 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 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 (DeMarini, 1998; Binkova et al., 1999; Warshawsky, 1999) and may be therefore used as the indicator of c-PAH concentrations and biological activity of mixtures.

Linking the results of the DNA adduct analysis induced by various EOMs in this study and chemical analyses of c-PAHs reported earlier (Farmer et al., 2003), 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 the most of its genotoxicity. A direct correlation was found between the B[a]P content in EOMs and B[a]P-like adduct spots ($R = 0.90$; $p = 0.016$). A similar direct correlation exists between c-PAH content and the total DNA adduct levels in HepG2 cells incubated with 50 μg EOM/ml ($R = 0.88$; $p = 0.0192$) for all EOMs in this study. 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 (Binkova et al., 1999; Topinka et al., 2000), 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 spectrum 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 3 localities are even more pronounced as demonstrated for HepG2 cells. Differences among localities are less pronounced (Sofia ~ Prague > Kosice).

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 3 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 3 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.

4.2. Sensitivity of different endpoints for *in vitro* measurement of genotoxicity of extractable organic matter associated with ambient airborne particles (Appendix 3)

The major objective of this study was to compare the sensitivity and correlations of three endpoints to assess the genotoxic potential of the complex mixture of organic compounds extracted from ambient air particles collected in three European cities (Prague, Kosice and Sofia) during summer and winter seasons. Three endpoints, DNA adduct formation (^{32}P-postlabelling), DNA strand break induction (single cell gel electrophoresis) and protein p53 up-regulation (immunoblotting) were tested in HepG2 cells exposed to EOMs at identical concentrations of 50 μg/ml (Appendix 3). A very good correlation of ambient air genotoxicity was found using DNA adduct determination and DNA strand break measurement. Over 10-fold higher ambient air genotoxicity was determined in winter air than summer air using both endpoints. This finding is in agreement with observation of Topinka et al. (2000) reporting 10-fold seasonal variability of ambient air genotoxicity in different localities in the Czech Republic measured by DNA-adduct analysis using primary rat hepatocytes. Moreover, quantitatively comparable seasonal increase in DNA adduct levels found in HepG2 and primary rat hepatocytes indicates that HepG2 cells are relevant surrogate for primary hepatocyte cultures in terms of metabolic capacity. Finally, we found that both biomarkers of exposure (DNA adduct and strand break measurement) exhibited the same order of winter air genotoxicity, which rose in order: Kosice<<Prague<Sofia.

Western-blot analyses did not demonstrate any up-regulation of protein p53 due to cell exposure to EOMs. Lack of protein p53 induction over the basal level due to exposure to artificial and environmental mixtures reported also Binkova and Sram (2004). Experiments with model carcinogens have suggested that minimal DNA adduct level of ~200 adducts per 10^8 nucleotides is required to detect up-regulation of p53 protein (Binkova et al., 2000). The DNA adduct levels determined in exposed HepG2 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.

Results obtained in this study demonstrated that DNA strand break estimation and DNA adduct formation are sensitive *in vitro* biomarkers of complex mixture genotoxicity

contributing to the risk assessment of ambient air pollution. A very good correlation between DNA adduct formation and DNA strand breaks was established.

4.3. Seasonal variability in the genotoxic potential of urban air particulate matter (Appendix 4)

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 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.

Organic extracts were obtained from PM10 collected in summer and winter 2000/2001 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 $\mu g/m^3$, 14.9 $\mu g/m^3$ and 39.0 $\mu g/m^3$, 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 c-PAH levels. These results are consistent with a study of Georgiadis et al. (2001) 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°C in 2001 and 2005, respectively) exclude a significant impact of meteorological conditions on these sampling results. 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[g,h,i]perylene and indeno[1,2,3-c,d]pyrene, were also detected by Binkova et al. (2003) in Northern Bohemia and Prague as well as by Georgiadis et al. (2001) in Athens. 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 (Westerholm and Egeback, 1994; Binkova et al., 2003).

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 reflects the genotoxic potential of the ambient air better than the concentration of PM10, but a direct correlation between PM2.5 concentration and the genotoxic potential of the ambient air was not detected. 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 supports the hypothesis that B[a]P and c-PAH concentrations in the ambient air are the most important predictors of its genotoxic potential. Estimations of the genotoxic potential of the ambient air and

predictions of health risk therefore should be based preferably on the measurement of c-PAH concentrations.

4.4. Biomarkers of air pollution exposure – A study of policemen in Prague (Appendix 5)

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 c-PAHs adsorbed on respirable particles was monitored in each subject for 48 hours before biological sampling. DNA adducts were analyzed by a ^{32}P-postlabelling assay, and the 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.

One of the major findings of this study is a direct association between personal exposure to c-PAHs and the level of total and B[a]P-like DNA adducts under conditions of increased c-PAH exposure. We confirmed that the relationship between c-PAH exposure and DNA adduct levels is not linear as proposed by Lewtas et al. (1997), a substantial increase in exposure (3-4-fold) was associated with a moderate increase in DNA adduct levels (~ 20%). It seems likely that such a relation is associated with efficient DNA repair that eliminates a substantial quantity of DNA adducts after c-PAH exposure. This adduct elimination is one of the basic prerequisites of genomic stability. Thus DNA adduct measurements include DNA repair and provide us with information about unrepaired lesions after exhausting the individual's DNA repair capacity. Therefore, it is not surprising that within the 2nd - 4th samplings (with low c-PAH exposure), we observed low DNA adduct levels. However, under conditions of increased

c-PAH exposure (1st sampling), an increase in DNA adduct levels was observed. The reason might be that the DNA repair capacity of at least some of the individuals was exhausted and 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. (2005). The results of these studies suggest that the concentration of 1 ng B[a]P/m^3 of the ambient air is critical, and its exceeding can lead to exhaustion of DNA repair capacity and increased level of DNA adducts in human tissues. 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.

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. In our study, the subjects were categorized into 3 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 previous observation (Topinka et al., 1997).

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

5. Summary and Conclusions

In vitro studies

- Cell-free system in conjunction with the sensitive ^{32}P- postlabelling is a suitable approach 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.
- HepG2 cells are an appropriate *in vitro* model to test genotoxic potential of complex mixtures since they are metabolically competent to activate c-PAHs as the most important genotoxic EOM components and yield dose-response relationship of adduct forming activity in a wide range of EOM concentrations.
- HEL cells are sensitive enough to detect DNA adducts of individual c-PAHs, but strong toxicity occurs when artificial c-PAH mixtures and real EOMs are employed.
- DNA adduct patterns derived from the different localities and sampling periods resemble each other. This fact suggests that the spectra of genotoxic EOM components in studied cities are similar.
- c-PAHs contribute predominantly to the total genotoxicity of various EOMs.
- B[a]P is of outstanding importance as a reference c-PAH for many environmental complex mixtures and may be used as the indicator of c-PAH concentrations and biological activity of these mixtures.
- The estimation of the genotoxic potential of ambient air and prediction of health risk should be based preferably on c-PAH concentrations.

Human epidemiological study

- DNA adduct levels were not associated with the individual metabolic and DNA repair gene polymorphisms, but correlation was found with combinations of genes, encoding enzymes, primarily involved in c-PAH metabolism, under conditions of increased exposure to c-PAHs.
- Concentration of 1 ng B[a]P /m^3 of the ambient air seems to be critical, and its exceeding can lead to exhaustion of DNA repair capacity and increased level of DNA adducts in human tissues.

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

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7. Abbreviations

B[a]A - benz[a]anthracene;
B[a]P - benzo[a]pyrene;
B[b]F - benzo[b]fluoranthene;
B[g,h,i]P - benzo[g,h,i]perylene;
B[k]F - benzo[k]fluoranthene;
BPDE - benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide[±];
CHRY - chrysene;
c-PAHs - carcinogenic polycyclic aromatic hydrocarbons;
CYP 1A1 – cytochrome P450, subfamily I (aromatic compound inducible), polypeptide;
DB[a,h]A - dibenz[a,h]anthracene;
DB[a,l]P - dibenzo[a,l]pyrene;
DMBA - 7,12-dimethylbenz[a]anthracene;
DMSO - dimethylsulphoxide;
DRZ - diagonal radioactive zone;
EOM - Extractable organic matter adsorbed to respirable air particles;
GSTM1 - glutathione S-transferase Mu 1;
GSTP1 - glutathione S-transferase P 1;
GSTT1 - glutathione S-transferase theta 1;
HEL - human embryonic lung fibroblasts;
HepG2 – human hepatoma cell line;
HiVol- high volume;
hOGG1 - human 8-oxo-guanine DNA glycosylase I;
IARC - International Agency on Research on Cancer;
I[c,d]P - indeno[c,d]pyrene;
MS – methionine synthase;
MTHFR – methylenetetrahydrofolate reductase;
PAHs - polycyclic aromatic hydrocarbons;
PM2.5 - air particles < 2.5 μm;
PM10 - air particles < 10 μm;
p53 – protein p53;
RAL - relative adduct labelling;
TLC - thin layer chromatography;
VAPS - versatile air pollution samplers;
XPD - xeroderma pigmentosum group D;
XRCC1 – X-ray cross-complementing gene 1.

8. Appendixes 1-5

Appendix 1:

B.Binkova, J.Topinka, R.J.Sram, **O.Sevastyanova**, Z.Novakova, J.Schmuczerova, I.Kalina, T.Popov, P.B.Farmer: In vitro genotoxicity of PAH mixtures and organic extract from urban air particles Part I: Acellular assay. Mutation Research: Fundam. Mol. Mech. Mutagen. (2007), doi:10.1016/j.mrfmmm.2007.03.001

Appendix 2:

O.Sevastyanova, B.Binkova, J.Topinka, R.J.Sram, I.Kalina, T.Popov, Z.Novakova, P.B.Farmer: In vitro genotoxicity of PAH mixtures and organic extract from urban air particles Part II: Human cell lines. Mutation Research: Fundam. Mol. Mech. Mutagen. (2007), doi:10.1016/j.mrfmmm.2007.03.002

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}). Mutation Research: Fundam. Mol. Mech. Mutagen. (2007), doi:10.1016/j.mrfmmm.2007.02.026

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. Mutation Research (2007) - submitted

Appendix 5:

J.Topinka, **O.Sevastyanova**, B.Binkova, I.Chvatalova, A.Milcova, Z.Lnenickova, Z.Novakova, I.Solansky, R.J.Sram: Biomarkers of air pollution exposure – A study of policemen in Prague. Mutation Research: Fundam. Mol. Mech. Mutagen. (2007), doi:10.1016/j.mrfmmm.2007.02.032