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Molecular epidemiology studies of carcinogenic environmental pollutants
Effects of polycyclic aromatic hydrocarbons (PAHs) in environmental pollution on exogenous and oxidative DNA damage

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Abstract

Exposure to high levels of environmental air pollution is known to be associated with an increased carcinogenic risk. The individual contribution to this risk derived from specific carcinogenic chemicals within the complex mixture of air pollution is less certain, but may be explored by the use of molecular epidemiological techniques. Measurements of biomarkers of exposure, of effect and of susceptibility provide information of potential benefit for epidemiological and cancer risk assessment. The application of such techniques has been mostly concerned in the past with the carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) that are associated with particulate matter in air pollution, and has showed clear evidence of genotoxic effects, such as DNA adducts, chromosome aberrations (CA) and *ras* oncogene overexpression, in environmentally exposed Czech and Polish populations. We are currently extending these studies by an investigation of populations exposed to environmental pollution in three European countries, Czech Republic, Slovak Republic and Bulgaria. This pays particular attention to PAHs, but also investigates the extent of radically induced (oxidative) DNA damage in the exposed populations. Policemen, bus drivers and controls, who carried personal monitors to determine their exposures to PAHs have been studied, and blood and urine were collected. Antioxidant and dietary status were assessed in these populations. Stationary monitors were also used for ambient air monitoring. Amongst the parameters studied in the biological samples were: (a) exposure biomarkers, such

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as PAH adducts with DNA, p53 and $p21^{WAF1}$ protein levels, (b) oxidative DNA damage, (c) the biological effect of the exposure by measurement of chromosome damage by fluorescence in situ hybridisation (FISH) or conventional methods, and (d) polymorphisms in carcinogen metabolising and DNA repair enzymes. Repair ability was also measured by the Comet assay. In vitro systems are being evaluated to characterise the genotoxicity of the organic compounds adsorbed to air particles.

Keywords: Molecular epidemiology; Environmental pollution; PAH; Oxidative DNA damage; Genetic susceptibility

1. Introduction

Respirable ambient particulate matter of an aerodynamic diameter $<10\,\mu m$ (PM_{10}) comprises a complex mixture consisting of a large number of chemicals, many of which are toxic. Epidemiological studies conducted in metropolitan areas have consistently demonstrated that exposure to particulate matter is associated with increases in mortality and/or morbidity from respiratory and cardiovascular diseases [1–4]. High exposures are associated with an increased risk of cancer [5], and the presence of a wide variety of genotoxic compounds in environmental air pollution has been demonstrated. Notable amongst these are the carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), which are associated with the particulate matter, and which have been the basis of several molecular epidemiological investigations of populations exposed to environmental pollution [6,7]. Three extensive studies of this type have taken place in Europe, in Poland, the Czech Republic and Greece. In Poland, populations exposed to environmental pollution showed increased levels of several markers of genotoxicity, including PAH DNA adducts, chromosome aberrations (CA), sister chromatid exchanges (SCE), and *ras* oncogene overexpression [8,9]. In the Teplice Program in the Czech Republic, there was a significant increase in the level of PAH DNA adducts in the polluted compared to the control region. In Greece however bulky DNA adducts were significantly higher in an area of lower PAH exposures, although there were no differences in cytogenetic effects in the two areas studied [6].

A consistent relationship between maternal exposure to fine particles during early gestation and the intrauterine growth retardation (IUGR) was observed in a highly polluted district of northern Bohemia [10]. Extracts of particulate matter were able to preferentially produce DNA PAH adducts in calf thymus DNA and they were also embryotoxic using the chick embryotoxicity screening test. The highest activity was found for fractions containing mainly PAHs [11]. In addition another study using the same population suggested that IUGR was positively related to the level of DNA PAH adducts in placenta [12]. Perera et al. [13] showed in studies in Poland that ambient air pollution was significantly associated with the level of PAH DNA adducts in white blood cells from both maternal and infant cohorts. Newborns with elevated DNA adducts in cord blood had significantly decreased length, birthweight and head circumference compared to newborns with lower DNA adducts levels detected in the cord blood. These results indicate that at least in some regions PAHs are a major source of the genotoxic and embryotoxic activities of organic mixtures associated with air pollution.

In addition to the formation of DNA damage by exogenous exposures to carcinogens, such as PAHs, it is known that DNA is modified, often oxidatively, by radicals. These radicals may arise as a product of endogenous processes, but alternatively may arise from oxidative stress generated as a by-product of metabolism of xenobiotics, or from particle-induced inflammation. Endogenous or oxidative damage to human DNA is more abundant than DNA damage caused by exogenous agents, but the relative biological significance of the two types of damage is unknown. Little is known about whether an increase in DNA damage caused by exposure to components of environmental pollution is reflected by an increase in oxidative DNA damage, although a recent study by Sorensen [14] showed that personal $PM_{2.5}$ exposure was found to be a predictor of a marker of DNA oxidative damage, 8-oxo-7,8-dihydro-2′-deoxyguanosine in lymphocytes.

Although there is a dose related effect on the production of genotoxic effects from air pollution, the shape of the dose response curve for DNA adduct formation does not appear to be linear, and appears to show saturation at high exposure levels [15,16]. Also, as is the case for many toxic compounds, the shape of the dose response relationship at low, environmentally relevant, concentrations is unclear, leading to a requirement for further studies to investigate the relationship between biomarkers and exposures at normal environmental levels.

Another parameter affecting individual susceptibility to environmental carcinogens is genetic polymorphism. Genes involved in carcinogen metabolism and detoxification such as *CYP1A1, GSTM1, GSTT1* and *NAT2* have so far been investigated. These studies have demonstrated for example that being null in GSTM1 or slow in NAT2 increased frequencies of cells with CAs in Danish bus drivers [17] that placental PAH DNA adducts were higher in mothers who were GSTM1 null [18], and that CYP1A1 affected DNA adducts in newborns and placenta [9]. Other enzymes such as those involved in DNA repair have not so far been well studied.

In summary, the assessment of an individual's risk from exposure to environmental pollution is currently hindered by the following uncertainties: (a) shape of dose response relationship at low doses, (b) identification of the critical genotoxic/mutagenic lesions in DNA, and (c) genetic susceptibility of the exposed subjects. In order to partially explore these phenomena, the EC has funded a study in which environmentally exposed populations have had their exposure determined by the use of personal monitors, and have been extensively monitored for biomarkers of exposure, effect and susceptibility to genotoxic agents. An overview of how the currently available techniques for the molecular epidemiological study of the genotoxic effect of environmental pollutants are being applied to the populations in this study will be presented in this paper.

2. Human populations currently under study

There is a constant improvement in the urban environment because of more technically advanced petroleum and diesel engines in new vehicles, and through the reduction in industrial and heating emissions. However, it is a time-consuming process to generate a major decrease in air pollution because of the life span of the existing vehicle fleet, this being particularly apparent in some Third World countries. Even in 'cleaner' cities, the ambient concentration of PM may be above 100 $\mu g/m^3$ and the PAH concentration above 50 ng/m^3. The study currently being undertaken is in three European cities, Prague (Czech Republic), Košice (Slovak Republic) and Sofia (Bulgaria). This involved in each site an exposed group (policemen or bus drivers) who are usually working through busy streets in 8–10 h shifts, and a control group matched by age, sex and length of employment. The total number of subjects was 200 exposed and 150 controls. The populations were followed in winter, owing to the highest exposure during this season at the selected sites. Each participant completed a questionnaire for demographic, smoking and dietary information. The dietary section of the questionnaire included questions on meat consumption, including methods of preparation (roasting, frying, etc.). Demographic variables include age, sex, race; also weight, height, medical history, medications. The questionnaire was translated into the native language, and slightly modified to fit the local habits when necessary. In the period of the experiment blood samples were collected at the end of the working shift, and urine samples were collected before and after this shift. Blood samples (total 80 ml) were processed into plasma, erythrocytes and lymphocytes.

3. Exposure assessment

As part of a critical assessment of biomarkers for PAH exposure it is necessary to establish in an experimental system the relationship between these biomarkers and the actual exposure. As the carcinogenic PAHs are mostly associated with particulate matter this requires the collection of such particles. Stationary monitoring, using for example a versatile air pollution sampler (VAPS) allows the quantification of PM_{10}, $PM_{2.5}$ and associated PAHs on a continuous basis in the ambient air. In our current study, such measurements were carried out over a 12 months period in each city. Carcinogenic PAHs, (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene,

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

Monitoring site	Season	PM_{10} ($\mu g/m^3$)	EOM ($\mu g/m^3$)	EOM[a] (%)	B[*a*]P (ng/m^3)	c-PAHs[b] (ng/m^3)
Prague-SM	Winter	62.59	14.93	23.9	3.50	24.69
Prague-LB	Winter	38.97	10.86	27.9	2.90	20.36
Košice	Winter	57.99	15.30	26.4	1.37	11.87
Sofia	Winter	89.88	24.60	27.37	4.84	36.44
Prague-SM	Summer	36.91	4.96	13.4	0.28	2.29
Prague-LB	Summer	26.39	3.72	14.1	0.17	1.32
Košice	Summer	24.3	1.67	6.9	0.15	1.20
Sofia	Summer	29.72	3.95	13.3	0.36	2.43

[a] Percentage of extractable organic matter in PM_{10} air particles.
[b] Sum of carcinogenic PAHs.

benzo[*k*]fluoranthene, benzo[*a*]pyrene (B[*a*]P), dibenzo[*a*,*h*]anthracene, benzo[*g*,*h*,*i*]perylene and indeno[1,2,3-*cd*]pyrene) were determined in the filter extracts by HPLC with fluorescence detection.

Additionally PM_{10} filters were collected from high volume (HiVol) samplers in the winter and summer periods, to produce sufficient extracts for in vitro studies of the pollution mixture (see below). The characteristics of the HiVol samples are shown in Table 1 (fuller details in Binkova et al. [19]).The individual exposures of the subjects under study were determined during their working shift by the use of personal monitors, which collected PM_{10} from which the carcinogenic PAHs were extracted. Personal monitoring results for the populations are shown in Table 2.

Table 2
Personal monitoring (ng/m^3)

	Exposed	Controls
Prague		
c-PAHs	12.04 ± 11.10	6.17 ± 3.48
B[*a*]P	1.79 ± 1.67	0.84 ± 0.60
Košice		
c-PAHs	21.72 ± 3.12	6.39 ± 1.56
B[*a*]P	2.94 ± 1.44	1.07 ± 0.66
Sofia		
c-PAHs	93.84 ± 55.0 (policemen) 94.74 ± 120.34 (bus drivers)	41.65 ± 33.36
B[*a*]P	4.31 ± 2.6 (policemen) 5.4 ± 3.18 (bus drivers)	1.96 ± 1.53

4. Analysis of biological samples

4.1. Biomarkers of exposure

The extent of modification of lymphocyte DNA by PAHs was determined by a ^{32}P-postlabeling assay using the nuclease-P1 enrichment procedure combined with a TLC (or HPLC) separation of ^{32}P-labeled DNA adducts [20,21]. The induction of protein $p21^{WAF1}$ and p53 in plasma was determined by ELISA and Western blot immunoassays as an indicator of cell response to DNA damage. Cotinine was measured in urine by radioimmunoassay to evaluate possible confounding exposures to tobacco smoke. The antioxidant status of the subjects was assessed by analysis of Vitamins A, C, and E in plasma. Dietary and genetic status were checked by measurements of high and low density lipoproteins (HDL and LDL), total cholesterol and triglycerides in plasma samples.

4.2. Biomarkers of effect

Chromosome aberrations are being measured by the conventional method [22] and by fluorescence in situ hybridisation (FISH) [23,24]. For chromosome painting using FISH, whole chromosome-specific DNA probes (Cambio, UK) specific for chromosomes 1 and 4, as well as pericentromeric probes, are used.

4.3. Biomarkers of susceptibility

Genotype analysis on WBC-DNA for metabolic polymorphisms was performed by a PCR-based

method. The genotypes considered were: *N*-acetyltransferase 2 (*NAT2*), glutathione-*S*-transferase (GST) *GSTM1*, *GSTT1*, *GSTP*, the cytochromes *CYP1A1*, *CYP1B1*, epoxide hydrolase *EPHX*, and other genes relevant to the metabolism of carcinogens. Polymorphisms in genes coding for DNA repair function were analysed, including *XPD*, *hOGG1*, *XRCC1*. Gene expression at the mRNA level will be determined from blood. Quantitative RT-PCR will be used to determine relative levels of expression of significant genes such as *CYP1A1*.

4.4. Biomarkers of oxidative DNA damage

Malondialdehyde/guanine adducts in lymphocyte DNA from the subjects under study were determined by an immunoslot blot procedure [25]. The measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) was also used as a monitor of DNA oxidation, using a novel LC-MS/MS technique using immunoaffinity or HPLC purification of 8-oxo-dG [26]. The single cell gel electrophoresis assay (SCGE, Comet) was also used to detect oxidative DNA damage. The alkaline version of the SCGE assay was applied to evaluate the DNA damage present in the human lymphocytes and also that induced by a challenging dose of X-rays (2 Gy), which induces oxygen radicals and oxidative DNA damage. The efficiency of the repair process was evaluated after the exposure.

4.5. In vitro studies

In vitro systems were established to produce a suitable model system for the characterisation of the genotoxic properties of the air pollution mixture.

For these studies, particulate matter collected from high volume samplers in each city and seasons were extracted by dichloromethane (extractable organic matter, EOM) and chemically analysed for PAH contents (US EPA HPLC method with fluorimetric detection). The EOM (re-dissolved in DMSO for evaluation of biological activities) was incubated with human cells, and DNA damage was analysed using the modified single cell gel electrophoresis (Comet) assay, ^{32}P-postlabeling, and other techniques described in ‘Biomarkers of effect’ above. Oxidative DNA damage was also determined.

5. Status of study and preliminary conclusions

The data gathering exercise for this study is near completion, when statistical analysis will take place. In brief, in the samples analysed so far, it appears that:

(a) extractable organic matter per PM_{10} was at least two-fold higher in winter than in summer, and carcinogenic PAHs over 10-fold higher in winter air than summer air (Table 1);
(b) personal exposure to B(*a*)P and to total carcinogenic PAHs in Prague was ca. two-fold higher in the exposed group compared to the control group, in Košice ca. three-fold higher, and in Sofia ca. 2.5-fold higher (Table 2).

Thus, these populations seem very suitable for the use of biomarkers of exposure, effect and susceptibility to study the effect of environmental pollution. Full analysis of these results, and the impact of genotyping data, will be reported shortly; and ultimately the appropriateness of each of the biomarkers studied for risk assessment of PAHs in environmental mixtures will be determined.

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