TOXIC EFFECTS OF INDOOR AND OUTDOOR AIRBORNE PARTICLES RELEVANT TO CARCINOGENESIS

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TOXIC EFFECTS OF INDOOR AND OUTDOOR AIRBORNE PARTICLES RELEVANT TO CARCINOGENESIS

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STELLINGEN

1. Bij de beoordeling van de bijdrage van aerosolen aan het ontstaan van kanker moet niet alleen worden gekeken naar de mutagene eigenschappen van aerosolen, maar ook naar de mogelijk tumor promoverende en co-carcinogene eigenschappen.
   [dit proefschrift]

2. De remming van de intercellulaire communicatie via gap-junctions is een sterke aanwijzing voor een tumor promoverende potentie van aerosolen.
   [dit proefschrift]

3. Blootstelling aan aerosolen leidt tot een depletie van vitamine A in de long en derhalve mogelijk tot een verhoogde gevoeligheid voor het ontstaan van longkanker.
   [dit proefschrift]

4. Aangezien Lewtas in de door haar gebruikte 'comparative potency method' een kanker-risico van complexe aerosolmengsels mede berekent op grond van gegevens uit huidtumor initiatieproeven en daarbij geen rekening houdt met de mogelijk tumor promoverende eigenschappen van aerosolen, wordt het risico waarschijnlijk onderschat.
   [J. Lewtas (1992) Conference on chemical risk assessment in the Department of Defense: science, policy, and practice, Cincinnati]
   [dit proefschrift]

5. Daar vlees een belangrijke bijdrage levert aan de vetconsumptie moet de op slechts enkele jaren epidemiologisch onderzoek gebaseerde conclusie van van den Brandt en Bausch-Goldbohm dat vlees geen risicofactor is voor colonkanker als voorbarig worden beschouwd.
   [P. van den Brandt, S. Bausch-Goldbohm (1993) Thesis, Maastricht]
   [T. Hirayama (1990) Contributions to Epidemiology and Biostatistics Vol. 6, Basel]

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6. Er wordt nog teveel toxicologisch onderzoek uitgevoerd met irreëel hoge doses en niet relevante toedieningswegen.

7. Voor de preventie van beroepsmatige huidaandoeningen is de ontwikkeling van grenswaarden voor de dagelijkse huidbelasting aan stoffen op de werkplek gewenst.
[C.L. Maas (1993) Tijdschrift voor Sociale Geneeskunde 71(2):124-125]

8. Aangezien planten van nature een scala aan mutagene stoffen bevatten is de conclusie van Suzuki et al. dat de door hen gevonden mutageniteit in plantenbladeren afkomstig is van depositie van mutagenen uit de lucht voorbarig.
[J. Suzuki, K. Kuwayama, S. Suzuki (1992) Mutation Research 271:89-96]

9. Een dubbelfunctie als tweede kamerlid én Europarlementariër is een ongewenste vorm van deeltijdarbeid in een periode van oplopende werkloosheid.
[Volkskrant 16/02 en 16/03/1993]

10. De pakkans van PAKs in binnen- en buitenlucht is bijzonder groot.

11. Hoogtevrees is tot op zekere hoogte geen belemmering om bergsport te beoefenen.

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Henri Heussen, Wageningen 21 mei 1993.

Voor Beitj

en mijn ouders

Wat wij het diepst in ons hart begeren en nodig hebben, bestaat niet uit plezier, afleiding of gemak, maar uit vrede met de wereld en met onszelf.
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ABBREVIATIONS

APM	Airborne Particulate Matter
BaP	Benzo(a)pyrene
BPDE	Benzo(a)pyrene-7,8-diol-9,10-epoxide
CSC	Cigarette Smoke Condensate
DMNA	Dimethylnitrosamine
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetate
ETS	Environmental Tobacco Smoke
FT4	Free Thyroxin
GJIC	Gap-junctional Intercellular Communication
IC	Intercellular Communication
i.p.	intraperitoneal
i.t.	intratracheal
LDH	Lactate Dehydrogenase
NF	2-Nitrofluorene
PAHs	Polycyclic Aromatic Hydrocarbons
PAKs	Polycyclische Aromatische Koolwaterstoffen
PBS	Phosphate Buffered Saline
PCBs	Polychlorinated Biphenyls
PCP	Pentachlorophenol
PG	Propylene Glycol
PKC	Protein Kinase C

RBP	Retinol Binding Protein
RWC	Residential Wood Combustion
SCE	Sister Chromatid Exchange
T3	Triiodothyronin
T4	Thyroxin
TT3	Total Triiodothyronin
TT4	Total Thyroxin
TBG	Thyroxin Binding Globulin
TPA	12-tetradecanoylphorbol-13-acetate
TTR	Transthyretin
WBC	White Blood Cells

CHAPTER 1

GENERAL INTRODUCTION

Airborne particulate matter (APM) is a common constituent of outdoor and indoor air and because of this global occurrence every human being is continuously exposed to APM, although the concentrations vary considerably from place to place and from time to time. According to current knowledge APM should be considered as a potential threat towards human health, especially with regard to the functions of the respiratory system. Considerable evidence stems from epidemiological studies implicating outdoor APM as contributing to the incidence and severity of respiratory diseases (e.g. American Thoracic Society, 1978; Braun-Fahrländer et al., 1992; Burema et al., 1964; Ostro, 1989). Associations between decreased pulmonary functions and increased APM concentrations have been reported (e.g. Dassen et al., 1986; Hoek and Brunekreef, in press; Schwartz, 1989). In all the above-mentioned studies high APM concentrations were accompanied by high concentrations of other pollutants, which could mean that not APM alone is responsible for the observed effects. However, recently a decline in children's pulmonary function was found on days when concentrations of fine particulate matter -but not of other air pollution parameters- were increased (Pope III et al., 1991).

Exposure to APM is not only an outdoor air problem. Indoor exposure also occurs, the most important sources being cigarette smoking and residential heating appliances. Involuntary smoking by children has been correlated with increased occurrence of respiratory disease related symptoms (for a review see Samet et al., 1987). The mechanism of injury has not been elucidated and it is also not known of these symptoms are caused by APM alone or by a combination of APM with other compounds present in cigarette smoke. Finally, several studies implicate wood smoke exposure as a risk factor for the development of respiratory disease in less developed countries, but for western societies data are limited (for a review see Samet et al., 1987).

Historically it is known that one of the most serious respiratory diseases, lung cancer, is consistently more common in urban areas than in rural (Biersteker and Zielhuis, 1975; Carnow, 1978; Doll, 1978; Henderson et al., 1975; Kotin and Falk, 1959; Menck et al., 1974; Walker

et al., 1982). This so called "urban factor" may be due to different life stile factors (eg. cigarette smoking), occupational exposures and general air pollution (IRCP, 1966). The risk of lung cancer attributable to air pollution was in earlier studies estimated to be 2% (range 1-5%; Doll and Peto, 1981), while in later studies, due to decreased levels of urban air pollution, the estimated risk was decreased to 1% (Peto, 1985; Speizer, 1983). Studies already started in the early 1940s suggested a causal relationship between APM and the urban factor, because of the fact that extracts of urban outdoor APM induced tumors in mice (Leiter and Shear, 1942; Leiter et al., 1942). Later studies confirmed these findings (Asahina et al., 1972; Epstein et al., 1966, 1979; Pott and Stöber, 1983; Sasaki et al., 1987).

Despite such epidemiological and toxicological studies, it is still unknown if exposure to APM poses a carcinogenic risk to humans. This uncertainty is mainly caused by the fact that the scientific question concerning a causal relationship between APM and carcinogenic risk is in principle difficult to answer. First of all it should be recognized that the analytical potential of epidemiological studies to establish a causal relationship between APM exposure and a particular disease is relatively small, because there are many so called confounding variables including e.g. life stile factors, nutritional status and smoking habits, the latter being the most important factor in this particular case. Consequently a low incidence of cancer of the respiratory system caused by exposure to APM will not be detected. Secondly, the predictive value of the existing experimental toxicological models is still very limited. Finally, the chemical composition and physical properties of APM are extremely variable (both quantitatively and qualitatively) due to changing source emissions, meteorological and atmospheric conditions.

One may wonder what type of approach should be followed to further identify the possible carcinogenic impact of APM. In general, the understanding would be improved if one would better understand the mode of action of APM in the pathogenesis of lung cancer. Knowing these mechanisms would greatly improve epidemiological methods. The present thesis is an attempt to a) contribute to the development of experimental techniques both in vitro and in vivo and b) better understand the toxicological effects which APM may have on processes known to be relevant for carcinogenesis.

Aerosols: nature and occurrence

The term aerosol was introduced by Donnan in the beginning of this century in analogy to colloidal suspensions in water (hydrosols). By definition, aerosols describe a gas particle suspension with colloidal stability to gravitational settling. Due to this settling, on earth the particle size is restricted to particles of 10-20 μm diameter or less (Hidy, 1986).

According to their origin aerosols can be classified as primary or secondary. Primary particles are brought directly into the atmosphere, and can be man-made or of natural origin. Industrial processes, e.g. transport and handling of bulk materials accounted in 1990 for 59% of the anthropogenic emissions (Table 1.1). Combustion related processes comprising stationary (combustion of fossile fuels, refuse burning) and mobile (traffic) sources accounted for the remaining 41%. It was estimated that in The Netherlands 73 kt of aerosol were emitted in 1990 by these sources (Netherlands Central Bureau of Statistics, 1992). Wind blown dust, sea spray, volcanoe- and forest fires, and emission by vegetation are examples of particles of natural origin. For example, by mechanical agitation of plant wax surfaces up to 50% of the wax may be lost directly to the atmosphere as particles (see review, Simoneit and Mazurek, 1981).

Secondary aerosols are produced by complex chemical reactions in the atmosphere. By gas-to-particle conversions gaseous compounds can be transformed into secondary aerosols like acid sulfates, nitrates and organic matter. Nitrate, for example, is found in large quantities in ambient aerosol, while for nitrate direct sources are not known (Van Vate and Ten Brink, 1986). From Table 1.2, in which production rates of global tropospheric aerosols are given, it can be concluded that anthropogenic sources make up about 6% by mass to the total atmospheric burden.

Table 1.1 Estimates of primary anthropogenic aerosol production rate in 1990 in the Netherlands[a]

Source	Production rate (kt)	weight of total (%)
1) Industrial processes	43	59
2) Combustion processes		
* Fossile fuel combustion	9	
* Road traffic	16	
* Other mobile sources	5	
Subtotals	30	41
Totals	73	100

[a] Adapted from CBS, 1992.

People in western societies spent on average 73% of their time at home, 23% in other indoor locations while only 4% is spent outdoors (Boleij and Brunekreef, 1982; Chapin, 1974; Knulst, 1977; Szalai, 1972). Therefore, total human exposure to air pollution is largely determined by the air quality of the indoor environment.

Indoor air particles can in principle originate both from penetration of outdoor particles into indoor locations and from emission by indoor sources themselves. Cigarette smoking and residential heating, for example wood combustion are the major sources of indoor air particles (Alfheim and Ramdahl, 1984; Löfroth et al., 1983; Van Houdt et al., 1984, 1986, 1990).

Table 1.2 Estimates of tropospheric aerosol production rates[a]

	Source	production rate tons/day	weight of total in %
		A. natural sources	
1.	primary		
	wind blown dust	$2 \times 10^4 - 1 \times 10^6$	9.3
	sea spray	3×10^6	28
	volcanic	1×10^4	0.09
	forest fires	4×10^5	3.8
2.	secondary		
	vegetation	$5 \times 10^5 - 1 \times 10^6$	28
	sulfur cycle	$1 \times 10^5 - 1 \times 10^6$	9.3
	nitrogen cycle	2×10^6	14.8
	volcanic (gases)	1×10^3	0.009
	Subtotals	10×10^6	94
		B. man-made sources	
1.	primary combustion		
	and industrial dust	$1 \times 10^5 - 3 \times 10^5$	2.8
	from cultivation	$1 \times 10^2 - 1 \times 10^3$	0.009
2.	secondary		
	hydrocarbon vapors	7×10^3	0.065
	sulfates	3×10^5	2.8
	nitrates	6×10^4	0.56
	ammonia	3×10^3	0.028
	Subtotals	6.7×10^5	6
	Totals	10.7×10^6	100

[a]Taken from Van Houdt (1990)

Table 1.3 Examples of chemical classes of organic compounds detected in outdoor APM

Chemical class	References
Aliphatic acids	1,4,5
Aliphatic alcohols	4,5
Aliphatic aldehydes and ketones	4
Alkylated PAHs	1,6,7,9,13
Alkylsulfonic acids	5
Aromatic acids	1
Aza-heterocyclic polyaromatics	1
Di- and triterpenoids	4,13
Dioxins	15
Halogenated PAHs	12
Hydroxylated alkylated PAHs	8
Hydroxylated nitrated PAHs	8
Hydroxylated PAHs	1,8
Nitrated PAHs	1,7,9,10
Organic sulfur oxides	3
Oxygenated alkylated PAHs	7
Oxygenated PAHs	1,2,7,8
PAHs	1,2,7,9,10,13
PCBs	15
Pesticides	11,15
Phtalic acid esters	1,5
Saturated aliphatic hydrocarbons	1,4,13
Tryptophan pyrolysates	14

References: (1) Cautreels and Van Cauwenberghe 1976; (2) Salamone et al. 1978; (3) Lee et al. 1980; (4) Simoneit and Mazurek 1981; (5) Lamb and Adin 1983; (6) Ramdahl 1983; (7) Brorström-Lunden and Lindskog 1985; (8) Schuetzle et al. 1985; (9) Wise et al. 1985; (10) De Raat et al. 1987, 1988; (11) Foreman and Bidleman 1987; (12) Haglund et al. 1987; (13) Standley and Simoneit, 1987; (14) Manabe et al. 1989; (15) Nakano et al. 1990.

Chemical composition of aerosols

Aerosols consist of a variable mixture of elemental carbon and inorganic oxides and salts on which organic compounds are adsorbed. Carbon is responsible for 10-20% of aerosol mass in outdoor APM (Shah et al., 1986; Wolff, 1981) and organic carbon makes up 60-90% of this carbon mass (Bremond et al., 1989; Cadle and Dash, 1988; Grosjean, 1984; Japar et al., 1986; Shah et al., 1986). Outdoor APM is a complex mixture of thousands of organic compounds distributed over many chemical classes (Table 1.3). The sources of these compounds can, as for the particles themselves, be of anthropogenic (traffic, stationary combustion appliances) or of natural origin. The latter include emission by vegetation, biogenic detritus and forest fires. For example, a significant amount of the organic carbon in rural aerosols is derived from higher molecular lipids from flora (e.g. plant epicuticular wax, terpenes) (Simoneit and Mazurek, 1982).

Cigarette smoking and residential combustion are major sources of particle-associated indoor air pollutants. Environmental tobacco smoke (ETS) originates primarily from sidestream smoke emitted from the burning end of a cigarette. Exhaled mainstream smoke is of minor importance. Almost 4000 compounds have been identified in mainstream smoke and about 10% of these compounds have been found also in sidestream smoke (IARC, 1986; Löfroth, 1989).

The predominant organic components emitted by wood combustion which have been characterized thus far, are the polycyclic aromatic hydrocarbons (PAHs) and their derivatives (Alfheim et al. 1984; Boleij et al., 1989; Möller et al., 1985; Ramdahl, 1983). However, also n-alkanes, alkanoic acids, alkanones and alcohols have been found in patterns characteristic of plant wax signatures (Standley and Simoneit, 1987; 1990). The same authors further detected several thermally altered and unaltered di-and triterpenoid compounds.

Considering the enormous complexity of the aerosol mixture present in the environment, it will be virtually impossible to assess the individual toxicity experimentally of all the constituents which may be present. For the time being, the only feasible approach is to study the toxicity of complete mixtures or groups of compounds e.g. PAHs, nitroPAHs etc.. It can not be excluded that in some stage in the future hazard assessments of complex mixtures can also be based on structure-activity relationships.

Genotoxic and toxic properties of airborne particulate matter and their possible consequences for chemical carcinogenesis

Chemical carcinogenesis

Berenblum (1941, 1949) initiated the concept that the development of cancer is a multistage process (for a recent review see Goldsworthy and Hanigan, 1987), and this model is still the most widely adopted concept in chemical carcinogenesis. These stages have been defined as initiation, promotion and progression. Initiation is the first step of carcinogenesis and represents activation or derepression of certain key genes (oncogenes) involved in the regulation of normal cellular growth and differentiation. One of the mechanisms recognized is the reaction of a (metabolite of a) chemical carcinogen with cellular DNA (e.g. by formation of DNA adducts) and subsequent irreversible fixation of this interaction resulting in genetically stable changes (mutations). Initiation may in principle be achieved by a single exposure to a so called genotoxic agent.

Genotoxicity of outdoor and indoor airborne particulate matter

With the introduction of the Salmonella/microsome assay (Ames and Yanokofsky; 1971) a rapid, cheap and sensitive in vitro assay for the screening of mutagenicity of environmental samples became available. It was strongly felt that mutagenicity studies were relevant for carcinogenesis, and as a result a boom in mutagenicity testing occurred. Many studies were published concerning the mutagenicity not only of urban outdoor APM (Commoner et al., 1978; Dehnen et al., 1977; Pitts et al., 1977; Pitts, 1978; ; Talcott and Wei, 1977; Tokiwa et al., 1977), but also of industrial outdoor APM (Dehnen et al., 1977; De Raat, 1983; De Raat et al., 1985; Tokiwa et al., 1977, 1980) and rural outdoor APM (Alfheim et al., 1983; Alink et al, 1983; Van Houdt et al., 1984, 1986, 1987). Even nowadays, the Ames assay is still being used for determining mutagenicity of outdoor APM (Barale et al., 1989; De Flora et al., 1989; Miguel et al., 1990; Viras et al., 1990).

Besides being mutagenic, extracts of outdoor APM also proved to induce other genotoxic effects in vitro, like cell transformation, sister chromatid exchanges and chromosomal aberrations (Alink et al., 1983; De Raat, 1983; Hadnagy et al., 1986, 1987, 1989; Krishna et al., 1984; Seemayer et al., 1986).

In addition, it was found that outdoor APM may cause non-genotoxic effects relevant to carcinogenesis. For instance, recently a damaging influence on mitotic cell division (probably by disturbance of the spindle apparatus) of automobile exhaust (Hadnagy and Seemayer, 1986; Seemayer et al., 1987) and outdoor APM (Motykiewicz et al., 1991) was found. As a result of disturbances of the spindle apparatus, aneuploidy and polyploidy were induced, both suggested to play a role in carcinogenesis (Sandberg, 1983).

As scientists became aware that human exposure to air pollution occurs most frequently indoors, studies were performed on indoor air mutagenicity. Cigarette smoking and to a lesser extent residential heating (wood, coal or kerosene combustion) were shown to significantly increase background mutagenicity indoors (Alfheim and Rahmdahl, 1984; Van Houdt et al., 1984, 1986; Yamasaki and Muruoka, 1984). Furthermore, in a recent study in Shenyang, a highly polluted industrial city in China, significant increases in lung cancer risks were not only correlated with high outdoor air pollution levels (caused by industrial emissions), but also with high indoor air pollution levels (caused by residential coal combustion) (Xu et al., 1989). Also in the study of Mumford et al. (1987) lung cancer rates have been correlated with indoor air mutagenicity in the case of residential coal combustion.

Tumor promotion and progression

Tumor promotion is regarded as the second phase of chemical carcinogenesis, following initiation. It is thought that an initiated cell may phenotypically alter into a transformed cell and clonally expand by means of a selective growth advantage. A continuous exposure to promoting chemicals is necessary for an initiated cell to clonally expand. A number of mechanisms has been identified as relevant for tumor promotion such as induction of ornithine decarboxylase, induction of changes in cell membranes, stimulation of cell proliferation, cellular dedifferentiation, increased expression of oncogenes and deranged intercellular communication (IC). For deranged IC there is increasing evidence on the basis of both in vitro and in vivo studies, that it is probably one of the most important mechanisms in tumor promotion (Boreiko et al., 1987; Enomoto and Yamasaki, 1984, 1985; Mesnil and Yamasaki, 1988; Mehta et al., 1986; Rivedal et al., 1985; Yamasaki 1984a,b; Yamasaki and Katoh, 1988a,b; Yamasaki et al.,

1985, 1987). Most cells have two different ways to communicate with eachother: a) by cell contact-mediated IC or b) by growth factor- or hormone-mediated IC.

Among cell contact-mediated IC, gap-junctional intercellular communication (GJIC) is believed to play a crucial role in the maintenance of tissue homeostasis (Loewenstein, 1979). Gap junction channels allow the intercellular diffusion of ions and molecules (second messengers, metabolites) with sizes up to about 1 kDa. Murray and Fitzgerald (1979) and Yotti et al. (1979) were the first to discover that tumor promoters, in this case phorbol esters, were able to inhibit GJIC. Soon, several other tumor promoters were shown to be able to inhibit IC (Yamasaki, 1990, 1991, review). All these studies were performed in vitro using cell cultures, but recently studies have been reported on inhibition of GJIC in the liver of animals treated with tumor promoters (Klaunig et al., 1990; Krutovskikh et al., 1991).

Growth factor- or hormone-mediated IC is the second process by which cells communicate with eachother. There are several reports which show that certain oncogenes can modulate GJIC in various cell types (Yamasaki, 1990, review); for example, several studies reported that in src oncogene-containing cells communication was reduced. Conversely, there are also several lines of evidence which suggest that cell-cell interaction, including GJIC between normal cells and oncogene-containing cells can influence oncogene-mediated expression of transformed phenotypes. From the fact that several oncogenes encode for growth factors or growth factor receptors, it can be concluded that disturbance of this kind of IC is also important in carcinogenesis.

An important role of tumor promotion in lung carcinogenesis is suggested by the fact that upon smoking cessation the risk of lung cancer does not increase, as mathematical modeling suggests, but instead decreases. Upon smoking cessation the effects of nongenotoxic promoting agents abruptly stop. As a consequence preneoplastic lesions remain static or regress, whereas in the continuing smoker they progress (Weisburger, 1990).

Although carcinogenicity studies (which includes initiating and tumor promoting potential) have been performed in the case of urban APM, no information is available on the tumor promoting potential of APM.

Tumor progression is thought to be the final stage of carcinogenesis. In this stage one or more focal proliferated cells undergo a cellular evolution to malignant neoplasm. These cells acquire

the capacity for infinite growth, and furthermore they are characterized by (additional) structural chromosomal changes and invasive properties (Scherer, 1984). Of the process of carcinogenesis this stage is least understood, and no information is available on a possible interaction of APM with this stage.

Assessment of carcinogenic risk

Most of the research dedicated to the identification of specific genotoxic compounds in APM has focused on PAHs and all kinds of derivatives (see table 1.3 and Schuetzle and Daisey, 1990). However, most of the genotoxic activity of APM samples has not been accounted for.

Risk estimations concerning the attributable risk of air pollution to lung cancer are therefore very uncertain, because they are based on the levels of only one class of compounds, namely the PAHs. The fact that the Dutch National Health Council (Gezondheidsraad) concluded that the higher mortality rates of lung cancer in urban areas in comparison with rural areas are not necessarily caused by PAHs (Gezondheidsraad, 1984) is a striking example of this uncertainty. A lifetime risk of exposure to benzo(a)pyrene calculated from epidemiological studies on coke oven workers (WHO, 1987) is the basis for the risk estimations on PAHs. Exposure to benzo(a)pyrene is taken as an indicator of exposure to total PAHs, assuming that emitted PAH profiles of coke ovens resemble PAH profiles of urban air. This assumption is questionable and the Dutch National Health Council, approving such risk estimations because of lack of more appropiate data, recommended that more studies have to be performed on PAH profiles of outdoor air, in order to verify the above-mentioned assumption (Gezondheidsraad, 1990).

In addition to the above-mentioned uncertainties in risk estimations, there is a striking lack of knowledge on the tumor promoting potential of APM.

Objectives of the present study

Previous studies conducted at our department have identified the sources of mutagenic indoor APM (Van Houdt et al., 1984, 1986). Extracts of outdoor APM sampled in Wageningen showed the highest degree of mutagenicity if they were collected in the winter, especially with wind directions from south to east (Alink et al., 1983; Van Houdt et al., 1987). With these wind

directions air masses have passed industrialized areas, and levels of typical air pollution parameters are increased.

The above-mentioned studies were based on in vitro mutagenicity assays. In the present follow-up study the main objective was to expand the scope to the in vivo initiating, tumor promoting and carcinogenic potentials of APM.

The approach was as follows: 1) Evaluation of DNA adduct formation in human volunteers after exposure to APM derived from wood combustion (chapter 3).

2) Evaluation of the in vivo genotoxicity of APM (chapter 4).

3) Evaluation of the tumor promoting potential by a) the effect on gap-junctional intercellular communication (chapter 5) and b) the interaction with the kinetics of vitamin A, an important regulator of normal epithelial differentiation and proliferation (chapter 6).

4) Evaluation of the carcinogenicity of APM in an experimental animal study (chapter 7).

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CHAPTER 2

PRINCIPLES OF THE TEST METHODS

Sampling methodology and extraction of samples

The sampling procedure and the subsequent extraction of the samples has been described in detail by Van Houdt et al. (1984, 1986, 1987). Briefly, outdoor as well as indoor sampling was performed in Wageningen, a small city with about 30,000 inhabitants in a non-industrial area. Outdoor samples were taken using a standard high volume sampler operated at a flow rate of about 100 m^3/h. Sampling lasted for 24 hours. Two different outdoor sample locations situated in Wageningen were used, which were far away from traffic outside the built-up area. In the experiments described in chapter 3 and 5 the sample location was at the meteorological station of the Agricultural University. In the experiments described in the other chapters, the sample location was at the IMAG institute.

For indoor sampling low volume samplers were used, consisting of a centrifugal pump and a filter holder for 3 filters. Samplers were used upside-down at a flow rate of about 8 m^3/h, resulting in the collection of respirable particles. Sampling lasted for 1 week. Two indoor pollution sources were investigated. First, sampling was performed in living rooms polluted by wood combustion. Therefore two samples were taken per livingroom. One week no wood combustion took place (control sample) while in the other week an open fire place was burning for approximately 4 h/day (experimental sample). Second, sampling took place in a room (about 18 m^3) in our institute which was polluted by smoking cigarettes (60 cigarettes/week, experimental sample). Also control samples were taken in this room.

In both samplers particles were collected on Gelman GF/A (20*24 cm) glass fibre filters. After sampling the filters were extracted for 8 hours in a soxhlet apparatus with 200 ml of methanol. The extracts were evaporated to dryness and the residues were dissolved in dimethylsulfoxide (DMSO) and stored in liquid nitrogen.

Salmonella typhimurium/microsome assay

The mutagenicity of extracts of indoor and outdoor APM has been studied by means of the Salmonella typhimurium/microsome assay, the so called "Ames test" (Ames et al., 1975, Maron and Ames, 1983). APM sampled in Wageningen has been tested as reported earlier by Van Houdt et al. (1984, 1986, 1987). In this bacterial mutagenicity assay reverse mutations from histidine auxotrophy to prototrophy are measured in several specially constructed S. typhimurium strains. In the present studies strain TA 98 was used. This strain is able to detect frame shift mutations and is the most sensitive strain for the detection of mutagenicity of APM (Van Houdt et al., 1984). To mimic mammalian metabolism a metabolically active preparation of Aroclor induced liver homogenate (the so called S9-mix) was included in the test protocol.

DNA repair host-mediated assay

In the DNA repair host-mediated assay DNA damage is determined in E. coli cells present in various organs of mice exposed to genotoxic agents. Therefore a pair of derivatives of E. coli K-12 strain 343/113 which differs vastly in DNA repair capacity (uvr^+/rec^+ vs. uvrB/recA) is used as a means of genotoxicity (Mohn, 1984). The strains also differ in their capacity to ferment lactose (Lac^- vs. Lac^+), so that the individual survival of both strains can be determined on a single agar plate which contains neutral red as pH indicator. On these plates the Lac^+ strain forms red colonies, while the Lac^- strain forms white colonies. Finally, to prevent uncontrolled growth of the bacteria in the various organs of the host the strains were made streptomycin dependent. In the experiments described in this thesis a mixture of both strains was injected intravenously into mice, which were subsequently treated (intraperitoneal or intratracheal) with genotoxic agents. After 120 minutes different organs were removed, homogenized, diluted and the homogenate was plated. Two days after incubation differential survival was determined.

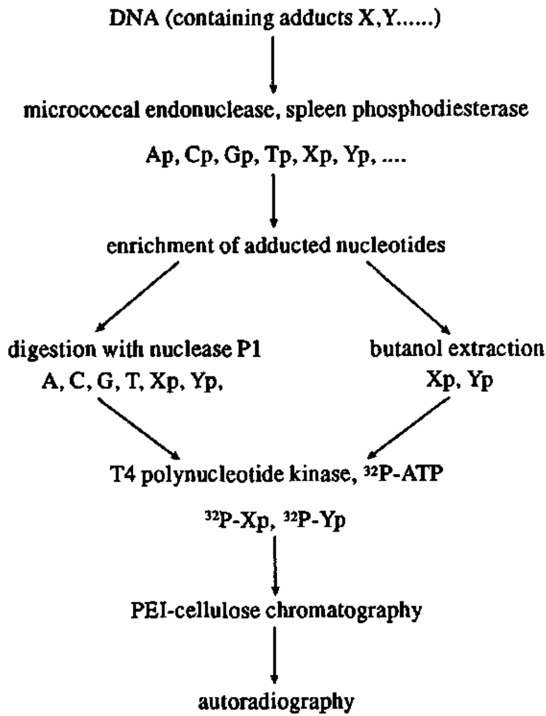

Figure 2.1 Procedure of the ^{32}P-postlabelling analysis

^{32}P-postlabelling analysis of DNA adducts in human peripheral white blood cells

^{32}P-Postlabelling analysis (Gupta et al., 1982) is a sensitive technique for the monitoring of prior exposure of humans to chemical carcinogens by examining their DNA for the presence of covalently-bound carcinogens (DNA adducts). This assay is particularly useful for monitoring exposure to complex mixtures, because prior identification of the chemical(s) in the mixtures that may form adducts is not necessary (Randerath et al., 1985).

In the present studies this technique was used to monitor the exposure of humans to carcinogens derived from residential wood combustion in open fire places. Therefore white blood cells were isolated from whole blood, and DNA was isolated. The procedure for postlabelling analysis of the DNA samples is shown in figure 2.1. Briefly, DNA was digested with micrococcal endonuclease and spleen phosphodiesterase to deoxyribonucleoside-3'-monophosphates. Enrichment of adducted nucleotides took place by nuclease P1 dephosphorylation of normal nucleotides or by butanol extraction of adducted nucleotides (Gupta, 1985; Reddy and Randerath, 1986). The enriched digest was labelled with [^{32}P]-ATP by incubation with T4-polynucleotide kinase to deoxyribonucleoside-3',5'-biphosphates. The reaction mixture was applied to the origin of a poly(ethylenimine)(PEI)-cellulose thin layer sheet. The thin layers were then developed sequentially with four solvent systems to produce a 2-D map of radiolabelled adducts. The adducts were localized by autoradiography, the spots were cut out and the radioactivity was determined by liquid scintillation counting. The level of DNA adducts was calculated on the basis of the analysis of standard samples carrying known amounts of BaP-DNA adducts.

Inhibition of gap-junctional intercellular communication

Various methods can be used to measure GJIC in cell culture, i.e. by means of a metabolic cooperation assay, by electrophysiological or by dye-transfer methods (Loewenstein, 1979). In the studies presented in this thesis GJIC was measured by means of dye transfer. This method has been accepted by the IARC (Barrett et al., 1986) as a screening assay for tumor promoting potential. In this assay the fluorescent dye Lucifer Yellow is injected into cells by means of a glass capillary tip with a diameter of $< 1\mu m$ with the use of a microinjector coupled to a phase contrast microscope. The transfer of this dye to neighbouring cells occurs via gap junctions, because it cannot diffuse through the membrane. Ten to fifteen minutes after the injection of the dye the transfer to surrounding cells is determined by counting fluorescent cells.

Effects on the kinetics of vitamin A in rats

Vitamin A is transported by its plasma carrier protein Retinol Binding Protein (RBP) (Goodman, 1984). This latter complex binds to transthyretin (TTR), a plasma transport protein for thyroxin (T4). Chemicals compete with T4-TTR binding and might weaken the RBP-TTR complex, resulting in an increased glomerular filtration of retinol-bound RBP and in a depletion of vitamin A and thyroid hormone levels (Brouwer et al., 1988, 1990).

Two different methods based on the same principle were used for studying competition of compounds with T4-TTR binding in vitro. In the first method compounds were incubated with a mix of TTR and T4, whereafter a modification of the gelfiltration procedure as described by Somack et al. (1982) was used for the separation of free and TTR-bound T4. Bound T4 was measured using a chemoluminescence immunoassay. This assay is based on the competition of (bound) T4 in a sample and a modified T4-peroxidase complex with anti-T4 antibodies. T4-peroxidase binding to the antibodies is measured by chemoluminescence and the binding is inversely related to the amount of T4 in the sample. In the second method compounds were incubated with a mix of TTR and ^{125}I-T4. After separating bound T4 by the same gelfiltration procedure as described above, quantification of bound T4 took place by measuring radioactivity in a gamma-counter.

For studies on in vivo effects on thyroid hormone and vitamin A metabolism, animals were intraperitoneally treated with APM extracts. At different time points blood, liver and lungs were collected. Plasma thyroid hormones were determined by means of a chemoluminescence immunoassay. Plasma retinol, hepatic and pulmonary retinol and retinyl esters were determined, after extraction of retinoids from plasma or organ homogenates in diisopropylether, by a reversed phase HPLC method, as descibed earlier (Brouwer and Van den Berg, 1984). Retinyl acetate was used as an internal standard.

Newborn mouse carcinogenicity assay

The newborn mouse lung adenoma bioassay is a 6-12 months during carcinogenicity assay. This assay is very sensitive for the detection of carcinogenicity of PAHs and nitroPAHs (Busby et al., 1985, 1988, 1989), compounds known to contribute to the

mutagenicity of outdoor APM.

In this assay male newborn NMRI mice were intraperitoneally treated at an age of 1, 8 and 15 days with respectively 1/7, 2/7 and 4/7 of the total dose of an outdoor APM extract. Mice were weaned at an age of 3 weeks and weights were recorded during the time of the experiment. After 1 year animals were sacrificed, macroscopic abnormalities were recorded and different organs were removed and fixed in neutral phosphate buffered 4% formaldehyde. Subsequently, lungs were prepared for histopathology and tumors were classified as alveolar type II cell adenomas or carcinomas or bronchiolar adenomas or carcinomas.

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CHAPTER 3

^{32}P-POSTLABELLING ANALYSIS OF DNA ADDUCTS IN WHITE BLOOD CELLS OF HUMANS EXPOSED TO RESIDENTIAL WOOD COMBUSTION PARTICULATE MATTER

Summary

Residential wood combustion (RWC) in open fire places poses a possible health risk because of the emission into the indoor air of mutagenic and carcinogenic compounds. In the present report it was investigated whether this emission leads to enhanced levels of DNA adducts in white blood cells (WBC) of exposed subjects. Under conditions that most likely reflect the Dutch pattern of use of open fire places, RWC increased both indoor air mutagenicity and levels of benzo(a)pyrene (BaP) and pyrene. The indirect mutagenicity showed a stronger increase than the direct mutagenicity. The increase in indirect mutagenicity was not directly correlated with the increase in the levels of BaP and pyrene. ^{32}P-postlabelling analysis of DNA adducts following nuclease P1 enrichment or butanol extraction revealed low adduct levels. No combustion-related increase in the amount of adducts was observed. Possible explanations for the lack of correlation between air monitoring data and WBC DNA adduct levels are discussed.

This chapter is based on: Heussen GAH, Bouman HGM, Roggeband R, Baan RA, Alink GM (submitted) ^{32}P-Postlabelling analysis of DNA adducts in white blood cells of humans exposed to residential wood combustion particulate matter.

Introduction

Many studies have been published on the genotoxic activity of outdoor airborne particulate matter (APM) (Pitts et al. 1977; Dehnen et al. 1977; Tokiwa et al. 1977; Talcott and Wei 1977; Alink et al. 1983). People in western societies spend on average 73% of their time at home, 23% in other indoor locations while only 4% of the time is spent outdoors (Szalai, 1972; Chapin, 1974; Boleij and Brunekreef, 1982). Therefore, total human exposure to APM is largely determined by the air quality of the indoor environment. Genotoxicity of indoor APM can in principle originate both from penetration of outdoor particles into indoor locations and from emission by indoor sources themselves. Cigarette smoking and residential heating, for example residential wood combustion (RWC), are the major sources of indoor APM-derived genotoxicity (Löfroth et al., 1983; Alfheim and Ramdahl, 1984; Van Houdt et al., 1984, 1986).

In The Netherlands residential heating occurs mainly through combustion of natural gas, while wood combustion is of minor importance. About 10% of the Dutch population is exposed to indoor APM emitted from open fire places during wood combustion (Slooff et al., 1989). Of these 10% most people use their open fire place not for residential heating, but for social purposes. This pattern of use implies that wood combustion mainly takes place during the evening. In our previous study the observed increase in indoor airborne mutagenicity due to RWC in the evening was significant when open fire places were used, but not when wood stoves were used (Van Houdt et al., 1986). The same phenomenon was reported by Alfheim and Ramdahl (1984).

Although RWC thus has been shown to increase indoor air mutagenicity, information on the actual internal body load of humans exposed to genotoxicants derived from RWC is rare. Such information is important for health risk assessment. Reddy and coworkers (1990) used ^{32}P-postlabelling analysis to determine DNA adducts in placentas and white blood cells (WBC) of women exposed during pregnancy to genotoxicants derived from RWC in wood stoves. However, in their experimental set-up no air monitoring was included, so that a relationship between external exposure and internal body load cannot be derived.

The aim of the present study was to investigate the external exposure and internal body load of humans exposed to indoor airborne genotoxicants derived from RWC in

open fire places. In five livingrooms RWC took place under circumstances that are most likely to reflect the Dutch pattern of use of open fire places. Air monitoring was carried out by measurement of mutagenicity of APM extracts in the Salmonella/microsome assay. Because polycyclic aromatic hydrocarbons (PAHs) are known to contribute to the genotoxicity of APM derived from combustion sources (Schuetzle and Daisey, 1990), concentrations of airborne pyrene and benzo(a)pyrene were determined as indicators of exposure to PAHs. ^{32}P-postlabelling analysis of DNA adducts in WBC of exposed people was used to determine the internal body load. This assay is particularly useful for monitoring exposure to complex mixtures, because prior identification of the chemicals in the mixtures that may form adducts is not necessary (Randerath et al., 1985). WBC have been used before as surrogate cells for the detection of exposure to complex mixtures of environmental (Hemminki et al., 1990a) and occupational (Phillips et al., 1988; Herbert et al., 1990; Reddy et al., 1991; Hemminki et al., 1990b) genotoxic compounds.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO) was obtained from Merck (Amsterdam, The Netherlands). Benzo(a)pyrene (BaP, purity approximately 98%) was obtained from Sigma (St. Louis, MO, USA). Analytical grade pyrene (purity > 99%) and 1-nitropyrene (purity 97%) were purchased from Serva (Heidelberg, Germany) and Aldrich (Bruxelles, Belgium), respectively.

Sampling strategy

Airborne particulate matter (APM) was collected simultaneously in 5 living rooms in Wageningen, as described in Chapter 2. Ventilation was kept to a minimum during three consecutive weeks, meaning that windows, flap-windows, skylights etc. were kept closed. One week (17-24 October 1991) no wood combustion took place (control sample) while in the following week (24-31 October 1991) an open fire place was kept burning for at least 4 h/day (combustion sample). Each day wood combustion started in the late afternoon or at the beginning of the evening and lasted the whole evening. Different kinds of wood

were used. Outdoor sampling was performed simultaneously at a location far away from traffic outside the built-up area in Wageningen, using the same type of sampler. The mutagenicity of outdoor APM was studied in order to correct for a possible infiltration of ambient air mutagens into the livingrooms. Meteorological data were obtained from the meteorological station of the Agricultural University, which also acts as a station of the Royal Dutch Meteorological Institute. Extraction of the filters took place as described in Chapter 2. A part of the methanol extract was stored at -20°C for chemical analysis. The remaining part was stored in DMSO at -20°C for mutagenicity testing.

At the beginning of the experiments the volunteers had not been exposed to wood combustion-derived APM for at least a month. In the experimental week they were asked to stay in the living room during the time that wood combustion took place. All volunteers were non-smoking individuals, not occupationally exposed to PAHs and not exposed to environmental tobacco smoke at home. They were asked to register: a) the time spent in the living room during the sampling periods (see Table 3.1), b) the burning time of the open fire place (see Table 3.1), c) exposure to environmental tobacco smoke at other locations than at home and d) consumption of roasted/fried meat (data of c) and d) not given).

From each volunteer blood samples were taken at different time points (one person per home): sample no. 1 when air sampling of the control week started (control); no. 2 one week later when air sampling of the control week ended and wood combustion started (control); no. 3 one week later when wood combustion ended (i.e. directly after exposure), and no. 4 again one week later (i.e. one week after the end of exposure). Each person thus served as his or her own control. Blood samples were collected at our laboratory between 7.30 and 9.00 a.m. Blood (20 ml) was drawn into heparinized tubes and WBC were immediately isolated by lysing red blood cells with a buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4. The volume ratio blood:buffer was 1:3. WBC were collected by centrifugation for 10 min at 250 g, and frozen at -20°C until DNA isolation.

Table 3.1 Personal data of volunteers

Person	Male/ female	Age	Profession	Time spent in living room[a]	Burning time[b]
1	male	42	gardener	35, 39	51
2	male	69	retired	68, 62	36
3	female	31	secretary	37, 47	44
4	female	43	housewife	60, 55	38
5	female	48	remedial teacher (at home)	40, 46	32

[a] Numbers of hours spent in living rooms during the control and combustion week respectively.
[b] Number of hours of wood burning during the combustion period of one week.

^{32}P-postlabelling analysis of DNA adducts in white blood cells

DNA of WBC was isolated by incubation of the cell pellets overnight at 37°C in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl, 1% SDS and 100 μg/ml proteinase K (Merck), followed by standard phenol/chloroform/isoamylalcohol extraction. DNA was precipitated from the aqueous phase by the addition of 0.1 volume of 3M NaAc, pH 6.0 and 3 volumes of ethanol at -20° C and washed with 70% ethanol in water. After drying *in vacuo* the DNA was suspended overnight in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and subsequently treated with RNAse A (final concentration 50 μg/ml, Sigma) and RNAse T1 (final concentration 50 units/ml, Boehringer Mannheim BV, Germany) for 1.5 hours at 37°C. Thereafter DNA was again incubated with proteinase K for 1 h (final concentration 100 μg/ml, 1% SDS), extracted and precipated as described above. DNA samples were stored at -20°C until postlabelling analysis.

For postlabelling DNA (15-20 μg) was digested with micrococcal endonuclease (in a total volume of 18 μl, final concentration 0.05 U/μl, Sigma) and spleen phosphodiesterase (final concentration 0.001 U/μl, Sigma). Enrichment of adducted nucleotides took place

by nuclease P1 (Boehringer) dephosphorylation (final concentration 0.33 U/μl) of normal nucleotides or by butanol extraction of adducted nucleotides (Gupta, 1985; Reddy and Randerath, 1986). The enriched digest was labelled with 4.6 MBq γ-[^{32}P]-ATP (Amersham, specific activity > 5,000 Ci/mmol) by incubation with 3.1 units T4-polynucleotide kinase for 30 min at 37°C. In the first experiment the postlabelled mixtures were applied to 10-cm x 10-cm poly(ethylenimine)(PEI)-cellulose thin layer sheets (GT Baker, Phillipsburg, N.J., U.S.A), in later experiments to 15-cm x 15-cm sheets. The thin layers were developed overnight in 1 M sodium phosphate, pH 6.0 (D1), washed two times with water, developed in 8.5 M urea, 3 M lithium formate, pH 3.5 (D3) for 6.5 h (10-cm x 10-cm thin layers) or 7.5 h (15-cm x 15-cm thin layers), washed again, and finally developed in 8.5 M urea, 0.8 M LiCl, 0.5 M Tris, pH 8.0 (D4) for 5.5 or 7 h and washed again. In order to remove remaining impurities, the chromatograms were developed overnight in 1.7 M sodium phosphate, pH 6.0 (D5) in the direction of D1. Adduct spots were detected by autoradiography at -70°C on Kodak XAR-5 film with an intensifying screen. Spots were cut out and radioactivity was determined by liquid scintillation counting (Hewlett Packard). The level of DNA adducts was calculated on the basis of the analysis of standard samples carrying known amounts of BaP-DNA adducts. The exact amount of input DNA and the absence of RNA were determined by chromatography of the DNA digest on an FPLC column (Steenwinkel et al., in press).

Mutagenicity

Extracts were tested for mutagenicity in the Salmonella microsome assay, as described by Ames et al. (1975) with minor modifications (Van Houdt et al., 1986) in strain TA 98 with and without Aroclor-induced rat liver S9. BaP and 1-nitropyrene were used as positive controls for indirect and direct mutagenicity, respectively. The number of spontaneous revertants varied from 20 to 39 (-S9) and 27 to 61 (+S9).

Benzo(a)pyrene and pyrene analysis

BaP and pyrene were determined with an HPLC gradient system (Kipp Analytica, Kipp and Zonen, Delft, The Netherlands) on a reversed phase Lichrosphere 100 RP-18 column. The flow rate was 1 ml/min and the solvent gradient was as follows: 5 min 70%

solvent A (60% distilled water, 40% methanol), in 37 min to 75% solvent B (100% methanol), and finally in 8 min to 90% solvent B. A Perkin Elmer type LS4 fluorescence spectrophotometer was used for analysis of the eluate at the following fluorescence wavelengths: emission 381 and 407 nm, excitation 264 and 296 nm for pyrene and BaP, respectively.

Results

Air monitoring data

The amount of extractable organic matter from indoor air and the mutagenicity of the extract were increased in all 5 living rooms after one week of wood combustion (Figure 3.1, Table 3.2). The indirect mutagenicity showed a stronger increase than the direct mutagenicity.

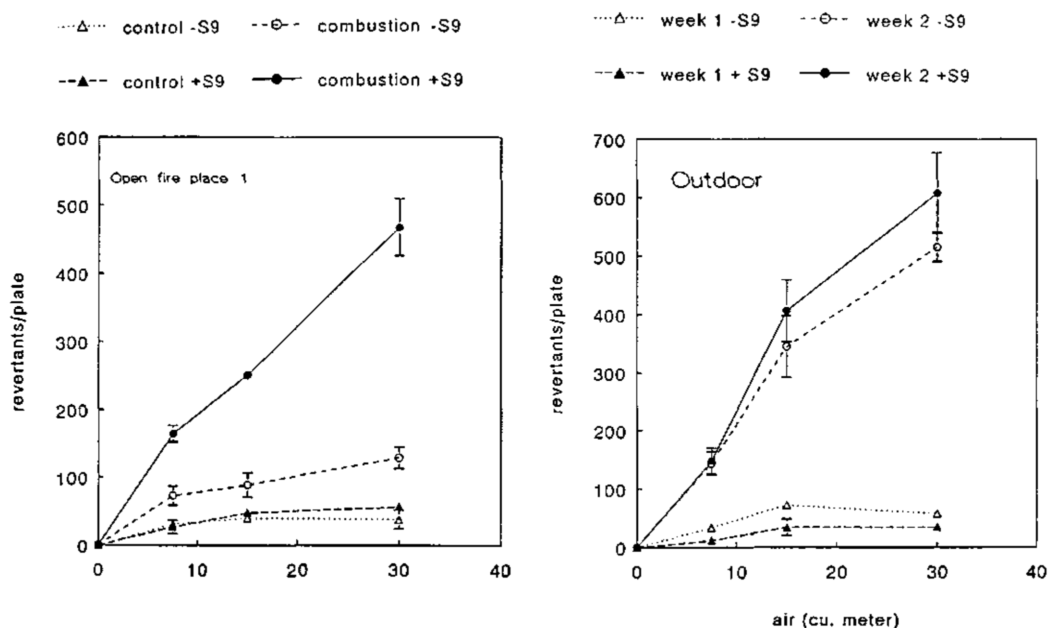

Figure 3.1 Mutagenicity of extracts of outdoor and indoor airborne particulate matter (APM) in TA 98 with or without rat liver Aroclor S9. For indoor APM a typical example of one livingroom is shown. Concentrations correspond to the volume of air sampled. Data are the mean $\pm$ SEM from 3-12 plates.

Table 3.2 Mutagenicity of extracts of airborne particulate matter in TA98 with or without rat liver Aroclor S9

Sample	$\mu g/m^{3,a}$	Revertants/30 $m^{3,b}$	
		-S9	+S9
1 Control	31.8	38	40
1 Combustion	39.3	128	468
2 Control	21.1	35	114
2 Combustion	38.8	136	365
3 Control	32.1	13	77
3 Combustion	47.6	176	383
4 Control	12.8	19	75
4 Combustion	38.9	217	364
5 Control	31.9	33	37
5 Combustion	34.5	110	264
Outdoor 1[c]	29.8	58	36
Outdoor 2	46.0	517	609

[a] Extractable organic matter/m^3 of sampled air.
[b] Number of revertants corresponding to 30 m^3 of sampled air.
[c] Outdoor 1 was sampled during the control week, while outdoor 2 was sampled during the combustion week.

Outdoor APM was collected simultaneously with indoor APM. The results of the mutagenicity assays showed that during the control week outdoor APM was hardly mutagenic (Figure 3.1, Table 3.2). In contrast, during the combustion week outdoor air mutagenicity was very high and also the amount of extractable organic matter in outdoor air was increased, although less than the mutagenicity. Meteorological data revealed that

in the control week westerly wind directions prevailed, while in the combustion week easterly wind directions prevailed (data not shown). Both indirect and direct mutagenicity of outdoor APM exceeded mutagenicity of indoor APM during the combustion week. Levels of pyrene and BaP in indoor air were 1.5-4.5 times increased after one week of wood combustion (Table 3.3). This increase was not directly correlated with the increase in indirect mutagenicity (Spearman rank correlation, $p > 0.10$).

Table 3.3 Concentrations of pyrene and benzo(a)pyrene in extracts of indoor airborne particulate matter

Sample	Pyrene (ng/m^3)[a]	Benzo(a)pyrene (ng/m^3)[a]
1 Control	0.6	0.2
1 Combustion	1.3	0.6
2 Control	0.6	0.2
2 Combustion	1.4	0.9
3 Control	n.d.	n.d.
3 Combustion	0.9	0.5
4 Control	0.5	0.2
4 Combustion	1.3	0.7
5 Control	0.8	0.2
5 Combustion	1.3	0.8

[a] Time-weighted average concentration over 1 week; n.d., not determined.

^{32}P-postlabelling analysis

In a first set of experiments the nuclease P1 treatment was applied to enrich adducted nucleotides from digested WBC DNA. The postlabelled mixtures were applied to 10-cm x 10-cm or 15-cm x 15-cm thin layer sheets. No consistent change in adduct levels due to

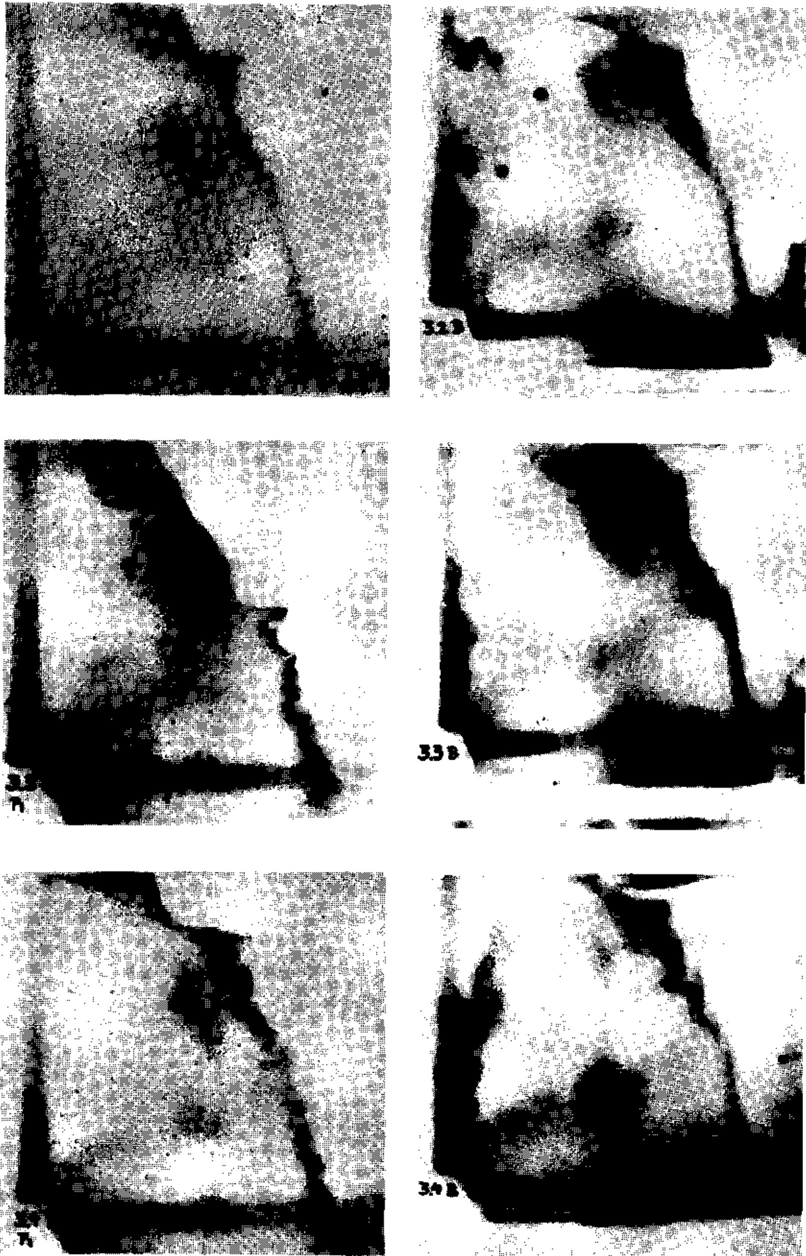

Figure 3.2 Adduct patterns of white blood cells of subject no. 3 exposed to wood combustion smoke. Enrichment of adducts took place by nuclease P1 digestion (left panel) or by butanol extraction (right panel). Sample no. 2 was taken when air sampling of the control week ended and wood combustion started. Sample no. 3 was taken directly after the end of the combustion week, and no. 4 one week later.

wood combustion was seen. Adduct levels of individual spots were low and total adduct levels ranged from 0.3-9.2 adducts/10^8 nucleotides. Each subject had his or her own typical adduct pattern. No additional spots appeared after exposure to RWC-derived APM. In fig. 3.2 the adduct pattern of one of the subjects is shown. In a second series of ^{32}P-postlabelling experiments the butanol extraction procedure was applied to enrich adducted nucleotides. Chromatography was carried out on 15-cm x 15-cm sheets. Comparison with the results after nuclease P_1 enrichment showed that butanol extraction resulted in a higher background level and in more intense spots. No additional spots appeared (fig. 3.2). Total adduct levels ranged from 3.9-40.7 adducts/10^8 nucleotides. As was the case for the experiments with nuclease P1 enrichment, in general no consistent change in exposure-related adduct levels was seen.

Discussion

Wood combustion in open fire places caused an increase in indoor air mutagenicity in all 5 living rooms studied. The indirect mutagenicity showed the strongest increase, which is in agreement with previous studies conducted by us and others (Alfheim and Ramdahl, 1984; Van Houdt et al., 1986). Infiltration of outdoor airborne particles may contribute to the mutagenicity of indoor APM (Van Houdt et al., 1986). The mutagenicity of outdoor APM was considerably higher during the week when open fire places were used as compared to the control week. This can be explained by the fact that in the control week westerly wind directions prevailed, while in the combustion week easterly wind directions were predominant. The latter conditions bring air masses over Wageningen that have passed industrial areas. Hence mutagenicity and other typical air pollution parameters are increased, while on days with westerly wind directions air masses come from the North Sea and levels of air pollution are low (Van Houdt et al., 1987). No significant difference was found between direct and indirect mutagenicity of outdoor APM, while for indoor APM the direct mutagenicity was lower. Because an intrinsic difference in infiltration capacity between direct and indirect outdoor airborne mutagens is very unlikely, we conclude that the observed increase in indirect mutagenicity of indoor APM in the

combustion week is caused by the use of open fire places and not by infiltration of outdoor airborne mutagens into the living rooms.

Several studies on indoor BaP levels due to wood combustion in dwellings in western societies have been reported. In the study of Moschandreas et al. (1980) indoor concentrations of BaP sampled during 24 h, were on average 4.7 ng/m^3 in residences where a presumably airtight (this was not specified by the authors) wood stove was used for residential heating. This was a 10-fold increase over the control value. Traynor et al. (1987) reported BaP indoor concentrations of 13 and 44 ng/m^3 during a 9-hour burning cycle when non-airtight wood-burning stoves were used. Control values were below 1 ng/m^3. In the study of Alfheim and Ramdahl (1984) RWC by airtight wood stoves and open fire places was compared. The use of wood stoves had a very moderate effect on indoor PAH levels, while the use of open fire places gave rise to BaP levels up to 18 ng/m^3. In the present study RWC caused a 2- to 4-fold increase in the levels of pyrene and BaP, respectively. Time-weighted average concentrations over 1 week were maximally 1.4 and 0.9 ng/m^3 of pyrene and BaP. If we take into account the burning time of the open fire places and calculate the increase in PAH levels, the maximum time-weighted average concentrations *over the burning time* of pyrene and BaP were 3.7 and 3.3 ng/m^3. These concentrations are lower than those found in the above-mentioned studies reported by others, probably because in our study the daily duration of the use of open fire places was shorter. In the above-mentioned reports wood stoves and open fire places were used for residential heating, while in our study open fire places were mainly used for social purposes, meaning that wood combustion started not earlier than in the late afternoon or beginning of the evening.

The increase in indirect mutagenicity after one week of wood combustion was not directly correlated with the increase in BaP concentrations. Because BaP itself cannot be responsible for the total mutagenic activity (the detection limit for BaP in an Ames test is much higher than the amount of BaP present in the APM samples), other mutagenic compounds are likely to contribute to the overall mutagenicity (Dasch, 1982; Asita et al., 1991). Concentrations of individual PAHs in wood smoke differ no more than about a factor 10 (Alfheim and Ramdahl, 1984; Zeedijk, 1986; Daisey et al., 1987; Boleij et al., 1989; Asita et al., 1991), which means that mutagenic PAHs other than BaP are probably

present in concentrations of ng/m^3 in our samples. In addition to PAHs several mutagenic hydroxylated, oxygenated and nitrated derivatives of PAHs have been detected in wood smoke (Alfheim and Ramdahl, 1984; Ramdahl, 1985; Löfroth et al., 1986). Because in the present studies no fractionation experiments were performed, the contribution of PAHs and PAH-derivatives to the overall mutagenicity cannot be quantified. Furthermore, the mutagenic activity derived from combustion sources cannot totally be ascribed to PAHs and derivatives (Schuetzle and Daisey, 1990).

Although our air monitoring studies showed that open fire places increase mutagenicity and levels of pyrene and benzo(a)pyrene in indoor air, no increase in DNA adducts (measured by ^{32}P-postlabelling) in WBC of exposed subjects was found. In fact adduct levels were low. None of the adducts found coeluted chromatographically with the BP-N^2-deoxyguanosine reference adduct. The level of adducts is of the same order of magnitude as that in WBC-DNA of non-smoking individuals not known to be occupationally exposed to carcinogens (Herbert et al., 1990; Hemminki et al., 1990a,b; Reddy et al., 1991). The origin of the adducts observed in our and the above-mentioned studies remains unknown. Our results correspond with those of Reddy et al. (1990), who found that RWC in wood stoves did also not increase DNA adducts of human WBC and placentas. However, no air monitoring was included in that study, so that a relationship between external exposure and internal body dose could not be investigated.

The lack of correlation between air monitoring data and WBC DNA adduct levels may have several causes. First, exposure might have been too low and/or too short. In three occupational studies roofers (Herbert et al., 1990), foundry workers (Reddy et al., 1991) and coke workers (Hemminki et al., 1990b) had higher levels of DNA adducts in their WBC measured by ^{32}P-postlabelling as compared to corresponding controls. These workers were exposed to airborne complex mixtures containing 0.05-90 μg BaP/m^3. Hemminki et al. (1990a) found an increase in DNA adducts of WBC in subjects living in a highly industrialized area in Poland as compared to countryside controls. Concentrations of BaP in this industrialized area were in the order of 0.015-0.057 μg/m^3. In these studies concentrations of BaP are 15-9000 times higher than the time-weighted average concentrations over 1 week in our study. Furthermore, subjects were exposed repeatedly

to such high levels of BaP. Subjects in our study were exposed for only one week to elevated, but still low concentrations of BaP.

Second, WBC might have a limited suitability as surrogate cells for the detection of exposure to airborne PAHs. Elevated levels of DNA adducts in WBC of roofers did not show a correlation with ambient air levels of PAHs, but did correlate with dermal exposure measured by means of skin wipes, suggesting the role of dermal absorption (Herbert et al., 1990). Also Hemminki et al. (1990a) suggested that oral or dermal exposure has a stronger effect on WBC DNA adduct levels than inhalation exposure. On the contrary Reddy et al. (1991) did find a correlation between adduct levels in WBC of foundry workers and airborne BaP concentrations.

Finally, presently unknown (non-)aromatic compounds might account for some of the mutagenicity of indoor APM, as already discussed. The ^{32}P-postlabelling methods used in the present study are very sensitive for the detection of PAH-DNA adducts. However, it should be recognized that other types of bulky eg. hydroxylated or oxygenated aromatic adducts and smaller adducts derived from exposure to non-aromatic compounds are probably not identified by the methods used. Thus the measured DNA adduct levels might be an underestimation of the actual genotoxic damage.

In conclusion, our study shows that although wood combustion in open fire places does increase indoor air mutagenicity and concentrations of PAHs, it does not give rise to increased levels of aromatic DNA adducts in WBC of exposed subjects, indicating that indoor pollution of this kind is probably not an important source of genotoxic damage to DNA.

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CHAPTER 4

STUDIES ON THE GENOTOXICITY OF AIRBORNE PARTICULATE MATTER IN THE DNA-REPAIR HOST-MEDIATED ASSAY

Summary

The genotoxic activity of benzo(a)pyrene (BaP), 2-nitrofluorene (NF) and airborne particulate matter (APM) was evaluated in the DNA-repair host-mediated assay after intraperitoneal or intratracheal administration. Dimethylnitrosamine (DMNA), used as a positive control, showed a genotoxic effect after both intraperitoneal and intratracheal administration, the strongest effect being found in liver, followed by lungs and kidneys, whereas a weak effect was observed in the spleen. In general no difference in genotoxicity was found between the two administration routes used. For BaP, although clearly positive in vitro, a moderate dose-dependent effect was found only in the liver after intraperitoneal administration. NF, which was positive in vitro both with and without a metabolizing system, produced no genotoxic effect in any of the organs tested after intraperitoneal administration. Extracts of APM which were genotoxic in vitro failed to cause a genotoxic effect in vivo by either route of administration. Possible explanations for the differences between the data obtained in vitro and in vivo are discussed.

This chapter is based on: Heussen GAH, Post JG, Alink GM (1990) Genotoxicity of benzo(a)pyrene, 2-nitrofluorene and airborne particulates in the DNA-repair host-mediated assay. Mutation Res 241:83-93.

Introduction

Several studies have shown the genotoxic activity of extracts of outdoor airborne particulate matter (APM) tested in vitro in microbial systems (Pitts et al., 1977; Dehnen et al., 1977; Tokiwa et al., 1977; Talcott and Wei, 1977; Van Houdt et al., 1987) or in mammalian cell systems using different genetic endpoints such as sister-chromatid exchanges (De Raat, 1983; Alink et al., 1983; Krishna et al., 1984; Hadnagy et al., 1989), chromosomal aberrations (Hadnagy and Seemayer, 1986; Hadnagy et al., 1989) and cell transformation (Seemayer et al., 1986). In addition extracts of indoor APM proved to be mutagenic in microbial systems (Alfheim and Ramdahl, 1984; Van Houdt et al., 1984, 1986).

However, little is known about the in vivo genotoxic potential of extacts of APM. Three studies have been conducted on the in vivo genotoxic activity of extracts of outdoor APM (Krishna et al., 1986; Crebelli et al., 1988; Motykiewicz et al., 1990). Only in the study of Motykiewicz et al. a positive genotoxic response could be detected. For a better understanding of the toxicological meaning of the genotoxicity data obtained in vitro so far more information is needed on the possible in vivo genotoxicity of extracts of APM.

In this study the DNA-repair host-mediated assay developed by Mohn (1984) was used to study genotoxic effects in vivo. In this assay mice are intrasanguineously injected with two *E. coli* strains which differ vastly in DNA repair capability. Mutagenic activity is determined as non-repairable DNA damage in *E. coli* cells present in various organs of mice resulting in differential killing of the two strains (Mohn, 1984). For the genotoxic activity of dialkylnitrosamines for example it was concluded that the results obtained in the host-mediated assay correlated better with carcinogenicity data than results obtained with in vitro assays (Kerklaan et al., 1981, 1983).

Previous studies conducted at our department have shown that mutagenicity of outdoor air in Wageningen is highest in the winter especially in periods with a wind direction between east and south. This was explained by the fact that with northerly wind directions airmasses come from the North Sea, while with southerly or easterly wind directions airmasses pass industrialized areas. The mutagenicity was positively correlated with other air pollution parametes such as SO_2, NO_2, NO and CO (Van Houdt et al., 1987). For indoor air smoking and to a smaller extent burning wood in an open fire place have been

shown to be important sources of mutagenicity (Van Houdt et al., 1984, 1986, 1987). Extracts derived from these sources which exhibit strong mutagenic activity in the Salmonella microsome assay were tested for their genotoxic potential in the DNA-repair host-mediated assay. Three reference compounds were also tested: dimethylnitrosamine (DMNA) because it has proved to be genotoxic in host-mediated assays (Mohn et al., 1983; Kerklaan et al., 1985), benzo(a)pyrene (BaP) (Sawicki et al., 1960; Sawicki, 1967; Bridbord et al., 1976; Phillips et al., 1983) and 2-nitrofluorene (NF) (for a review see Beije and Möller, 1988a) known to be present in ambient air particles.

Materials and methods

Chemicals

DMNA and dimethylsulfoxide (DMSO) were obtained from Merck (Amsterdam, The Netherlands). BaP was obtained from Sigma (St. Louis, USA), NF from Phaltz and Bauer inc. Stamford, Conn., streptomycin base from Pharmachemie (Haarlem, The Netherlands), and brietal sodium from Eli Lilly (Amsterdam, The Netherlands).

Animals

Male Balb/c mice with a bodyweight of about 25 g and an age of 10-13 weeks were used. For intraperitoneal administration (i.p.) DMNA was dissolved in phosphate buffered saline (PBS) and administered at a concentration of 50 or 500 μmol/kg body weight. BaP, NF and extracts of APM were dissolved in DMSO. BaP was administered at a concentration of 200 or 500 μmol/kg, NF at a concentration of 500 μmol/kg and extracts of particulates at a concentration corresponding to 2000 or 4000 m^3 of sampled air/kg. The maximum volume administered i.p. was 100 μl. For intratracheal instillation (i.t.) DMNA and extracts of particulate matter were dissolved or suspended in PBS containing 10% DMSO. DMNA was administered in a concentration of 500 μmol/kg and extracts of particulates in a concentration of 1052 or 1248 m^3/kg. For i.t. instillation animals were anesthetized with brietal sodium. Genotoxic agents were administered with the use of a blunt 22G canula, according to the procedure described by de Jong and Feron (1971). The maximum volume administered i.t. was 25 μl.

Sample collection

APM was collected in the winter periods of 1987/1988 and of 1988/1989 in Wageningen, as described in Chapter 2.

Extraction

Each filter was sonicated for 30 minutes in methanol with an ultrasonicator (50 Hz; Senior Instrument Corporation Copiage, NY, USA) as described previously (Van Houdt et al., 1989). Methanol extracts were filtered and evaporated to dryness. The residue was dissolved in DMSO for i.p. administration or suspended in a solution of PBS containing 10% DMSO.

Growth and suspension media

The media used were the same as described by Mohn (1984) with some minor modifications. PBS was purchased from Oxoid (Basingstoke, UK). The neutral red agar medium (NR-S-agar) for determining differential survival of lac^+ (red) versus lac^- (white) colonies contained per liter of medium: 20 g Bacto-peptone, 5 g Bacto-Tryptone, 10 g α-lactose, 5 g NaCl and 15 g Bacto-agar and was supplemented with neutral red (30 mg/l), streptomycine base (50 mg/l), thiamine (0.5 mg/ml), D-biotin (5 mg/ml), nicotinic acid (10 mg/ml) and 25 mg/ml L-histidine, L-arginine, L-proline and L-lysine. PEPS broth for preparing overnight cultures was as NR-S-agar, without Bacto-agar and neutral red, and with supplemented factors four times as much as in the NR-S-agar.

Bacterial strains

The strains used in this study were kindly provided by Prof. Mohn. Strain 343/753 is a uvrB, recA and Lac^+ derivative and strain 343/765 is uvr+, rec^+ and Lac^- For other genetic markers see Mohn (1984). Strains were kept in liquid nitrogen and every 2-3 weeks fresly thawed strains were plated on NR-S agar plates and kept at 4°C.

Genotoxicity testing

Liquid suspension assay

Overnight cultures of strain 765 and strain 753 were mixed at a ratio of 1:40, centrifuged, suspended in tris(hydroxymethyl)aminomethane buffer (pH 8.0; 0.12 M) and treated with ethylenediaminetetraacetate (EDTA, final concentration 0.5 mM) for 2 minutes. The reaction was stopped with $MgCl_2$ (final concentration 10 mM), cells were washed and suspended in PBS. Treatment with EDTA increases the surface permeability of the bacteria for large molecules such as actinomycin D (Leive, 1968). From preliminary experiments (data not shown) it was concluded that EDTA pretreatment of bacteria increased the sensitivity of the assay towards BaP and NF. Therefore this pretreatment was routinely used when testing BaP, NF and extracts of APM. For practical reasons the bacterial mix was diluted 1000 times in PBS prior to use. 0.1 ml of this diluted mix (about 10^6 bacteria) together with genotoxic agents were incubated at 37°C in a glass vial under gently shaking conditions for 120 minutes. 0.3 ml S9-mix or PBS were used for indirect or direct genotoxicity testing respectively. The volume was adjusted with PBS to 1 ml. S9-mix was prepared from Aroclor 1254 pretreated male Wistar rats according to Ames et al. (1975). After incubation the bacterial suspension was diluted in PBS and plated on NR-S-agar. Two days later colonies were counted and the survival of strain uvrB/recA as percentage of strain uvr^+/rec^+ was determined. The relative survival of the control was set at 100%. Each experiment was performed at least twice and carried out in triplicate, unless otherwise stated.

Host-mediated assay

Bacterial mix was prepared in the same way as described for the liquid suspension test, except that the bacterial mix was not diluted 1000 times prior to use. 0.1 ml of a mix of both strains with a viable cell titer of about 10^{10} cells/ml was injected intravenously into mice. Immediately afterwards genotoxic agents were administered i.p. or i.t. After 120 minutes animals were killed by cervical dislocation, organs were removed, washed in icecold 0.15M KCl, kept in PBS on ice and homogenized with a Polytron Homogenizer. Homogenates were diluted in PBS and 3 dilutions per homogenate were plated on NR-S-agar (the dilutions were for liver 5000, 10000 and 20000, for lungs 5, 10

and 20, for spleen 55, 110 and 275, and for kidneys 0, 2, 4). Determination of the relative survival of uvrB/recA was determined as described above. DMNA was used as a positive control. Each experiment was performed at least twice and per sample point 3 animals were used, unless otherwise stated.

Statistics

Data were tested for significance with Student's t-test. A significance level of $p<0.05$ was used.

Results

DMNA was tested as a positive control by i.p. and i.t. administration. Figure 4.1 and 4.2 show the results. It can be concluded that DMNA had a marked genotoxic effect in the liver, followed by the kidneys and lungs, whereas in the spleen a weak genotoxic effect was found. There was no significant difference in genotoxicity of DMNA between i.p. or i.t. administration, except in the kidneys where the effect after i.t. administration was less for the highest concentration.

The results of in vitro testing of BaP and NF are shown in figure 4.3. BaP as well as NF caused a genotoxic effect in the presence of S9. In addition NF caused an effect in the absence of S9. The difference between direct or indirect genotoxicity of NF was not significant ($p>0.05$). In a first series of experiments in vivo using i.p. administration in an equimolar dose as DMNA, BaP showed a dose-dependent effect in the liver ($p<0.005$), the lungs ($p<0.05$ at 200 μmol/kg and $p<0.025$ at 500 μmol/kg) and the spleen ($p<0.05$ at 200 μmol/kg and $p<0.025$ at 500 μmol/kg, Table 4.1). In later experiments these effects of BaP in lungs and spleen were less clear (Table 4.2). For NF no significant effect could be observed in any of the organs tested ($p>0.05$).

Extracts of APM from outdoor sources as well as from indoor sources (wood combustion) showed a genotoxic effect in vitro (figure 4.4). The outdoor extract caused a stronger effect than the indoor extract both for direct and indirect genotoxicity. In general the effect in the presence of S9 was smaller than the effect in the absence of S9.

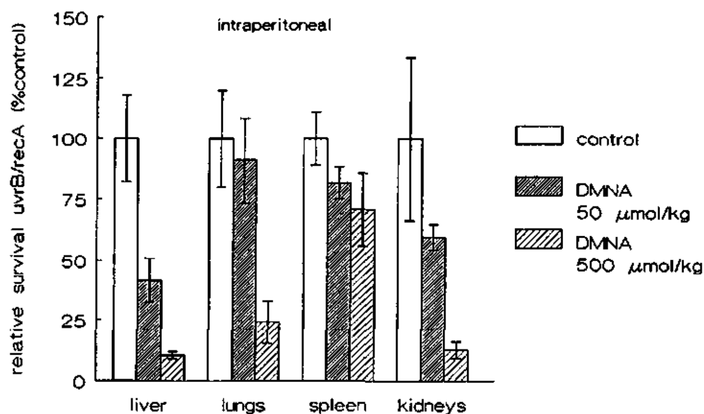

Figure 4.1 Relative survival of E. coli strains 343/753 (uvrB/recA) and 343/765 (uvr^+/rec^+) in different organs of mice after intraperitoneal administration of DMNA (50 and 500 μmol/kg bodyweight) or PBS (control, 4 ml/kg body weight). Results are expressed as mean ± s.d. (n=2).

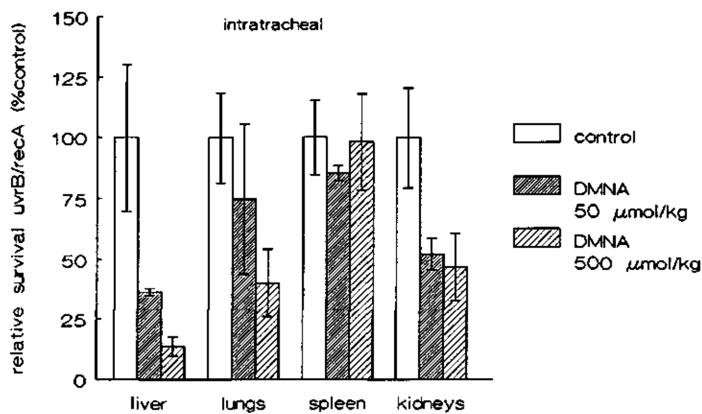

Figure 4.2 Relative survival of E. coli strains 343/753 (uvrB/recA) and 343/765 (uvr^+/rec^+) in different organs of mice after intratracheal administration of DMNA (50 and 500 μmol/kg bodyweight) or PBS (control, 4 ml/kg body weight). Results are expressed as mean ± s.d. (n=2, DMNA 50 μmol/kg or n=5, control and DMNA 500 μmol/kg).

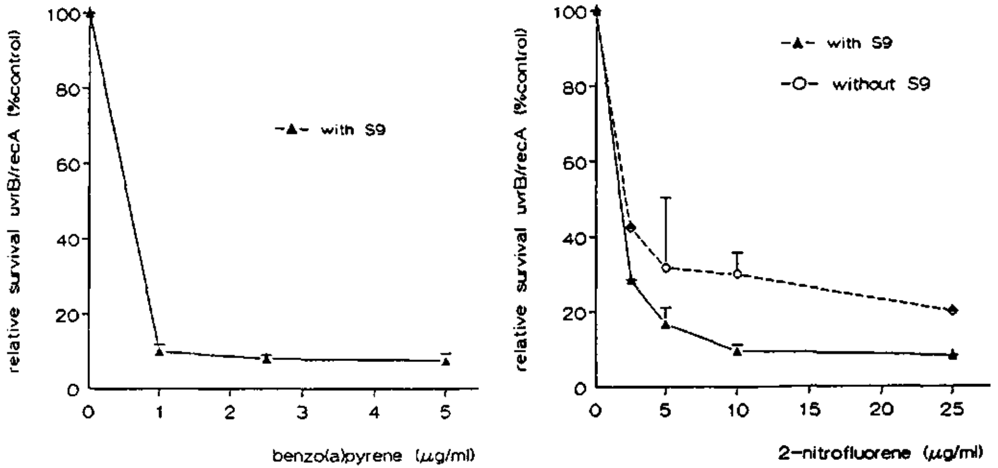

Figure 4.3 Relative survival of E. coli strains 343/753 (uvrB/recA) and 343/765 (uvr^+/rec^+) in suspension treated with BaP and NF in the presence or absence of rat liver Aroclor S9. Control was 2.5% DMSO. Results are expressed as mean ± s.d. (n=2, except NF 2.5 µg/ml and 25 µg/ml n=1).

Table 4.1 Genotoxicity of intraperitoneally administered BaP and NF in the DNA repair host-mediated assay

Treatment[a]	Relative survival uvrB/recA (% control)[b]			
	liver	lungs	spleen	kidneys
Control	100.0 ± 9.6	100.0 ± 17.4	100.0 ± 16.2	100.0 ± 9.5
BaP 200 µmol/kg	72.3 ± 5.2	70.3 ± 25.0	73.5 ± 15.5	89.3 ± 12.4
BaP 500 µmol/kg	59.9 ± 14.4	45.3 ± 24.7	64.0 ± 19.5	88.9 ± 12.5
Control	100.0 ± 17.5	100.0 ± 16.8	100.0 ± 7.5	100.0 ± 26.3
NF 500 µmol/kg	71.5 ± 20.0	72.5 ± 7.0	84.0 ± 4.0	45.0 ± 7.5

[a]BaP and NF were applied at a single intraperitoneal dose (200 or 500 µmol/kg body weight). Controls received solvent only (4 ml DMSO/kg body weight).
[b]Values are mean ± s.d. from 2 (NF) - 4 (BaP) experiments.

Table 4.2 Genotoxicity of intraperitoneally administered BaP in the DNA repair host-mediated assay when used as positive control

Treatment[a]	Relative survival uvrB/recA (% control)[b]			
	liver	lungs	spleen	kidneys
Control	100.0 ± 7.8	100.0 ± 18.5	100.0 ± 16.5	100.0 ± 18.8
BaP 500 μmol/kg	59.8 ± 13.4	87.3 ± 15.0	73.8 ± 19.4	88.8 ± 14.3

[a]BaP was applied at a single intraperitoneal dose (500 μmol/kg body weight). Controls received solvent only (4 ml DMSO/kg body weight).
[b]Values are mean ± s.d. from 7-8 experiments.

When these extracts were tested by i.p. administration in the DNA-repair host-mediated assay no genotoxic effect could be detected (Table 4.3).

For intratracheal administration extracts were suspended in PBS containing 10% DMSO. These extracts showed genotoxic activity in vitro (figure 4.5). Cigarette smoking proved to cause the strongest genotoxic activity. Also for these samples the direct genotoxicity was stronger than the indirect genotoxicity. In vivo these extracts failed to cause a genotoxic effect after i.t. administration (Table 4.4).

Discussion

The results obtained in this study after i.p. administration of DMNA in the DNA-repair host-mediated assay agree well with the data obtained by Mohn et al. (1983 and 1985) and Kerklaan et al. (1985). In general after i.t. administration of DMNA the results were comparable with those obtained after i.p. administration. Thus there appears to be a rapid absorption of i.t. instilled DMNA from the lungs into the bloodstream.

BaP showed a marked effect in vitro in the presence of S9. However, when tested in the host mediated assay only in the liver could a dose related effect be consistently detected. In some experiments an effect was seen in the lungs and in the spleen.

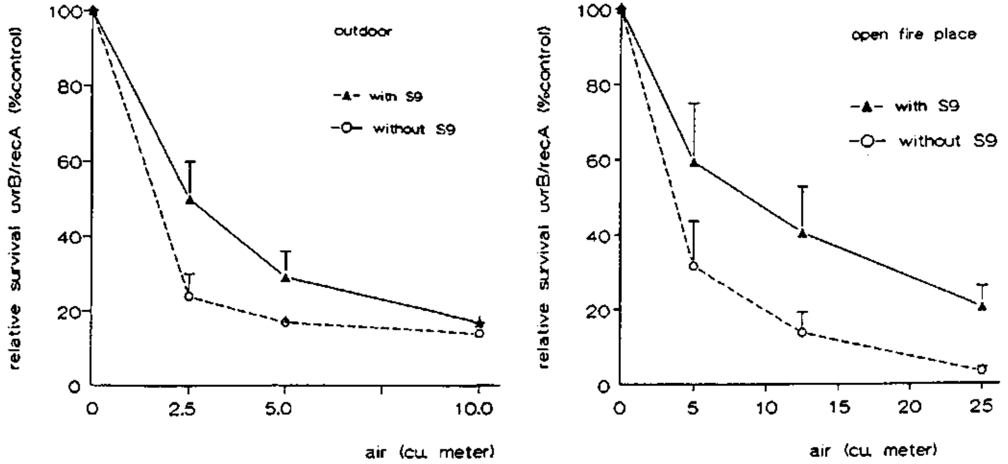

Figure 4.4 Relative survival of E. coli strains 343/753 (uvrB/recA) and 343/765 (uvr^+/rec^+) in suspension treated with extracts of outdoor airborne particles and of indoor airborne particles in the presence or absence of rat liver Aroclor S9. Control was 2.5% DMSO. Results are expressed as mean ± s.d. (n=2, except outdoor 10 m^3 +S9 and 5 m^3 -S9 n=1).

Table 4.3 Genotoxicity of intraperitoneally administered extracts of airborne particles in the DNA repair host mediated assay

Treatment[a]	Relative survival uvrB/recA (% control)[b]			
	liver	lungs	spleen	kidneys
Exp 1				
control	100.0 ± 5.1	100.0 ± 40.1*	100.0 ± 35.6	n.t.
open fire place	86.0 ± 14.2	113.6 ± 44.9	70.4 ± 14.0	n.t.
Exp 2				
control	100.0 ± 5.6	100.0 ± 16.1	100.0 ± 17.4	100.0 ± 28.3
open fire place	107.8 ± 24.3	76.5 ± 15.8	108.2 ± 14.5	100.0 ± 23.6
Exp 3				
control	100.0 ± 5.2	100.0 ± 17.6	100.0 ± 23.5	100.0 ± 8.1
outdoor	95.1 ± 29.5	92.4 ± 21.5	97.1 ± 12.7	109.3 ± 11.2
Exp 4				
Control	100.0 ± 5.0	100.0 ± 6.7	100.0 ± 9.7	100.0 ± 9.4
outdoor	93.1 ± 3.0	111.3 ± 27.6	111.4 ± 30.7	89.1 ± 14.7

[a]Extracts were applied at a single intraperitoneal dose (2000 m^3/kg body weight in Exp 1, 4000 m^3/kg body weight in the other experiments). Controls received solvent only (4 ml DMSO/kg body weight).
[b]Values are mean ± s.d. from 3 animals, except * mean ± s.d. from 2 animals; n.t. not tested.

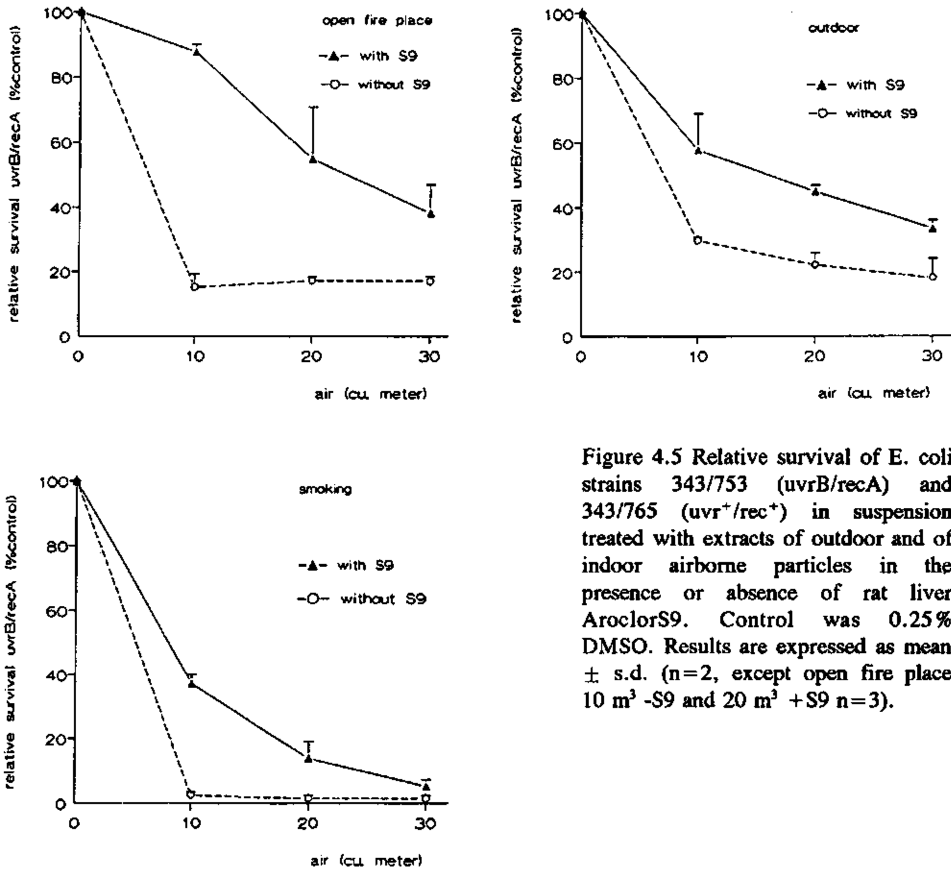

Figure 4.5 Relative survival of E. coli strains 343/753 (uvrB/recA) and 343/765 (uvr^+/rec^+) in suspension treated with extracts of outdoor and of indoor airborne particles in the presence or absence of rat liver AroclorS9. Control was 0.25% DMSO. Results are expressed as mean $\pm$ s.d. (n=2, except open fire place 10 m^3 -S9 and 20 m^3 +S9 n=3).

However, these results were not very reproducible. In other organs no significant dose-related effect could be detected. It appears that the dose level needed to detect genotoxic activity in vivo is much higher than might be expected from the in vitro results. Our results agree to some extent with the study of Knasmüller et al. (1989). In a host-mediated assay using the same bacterial strains they found a clear effect of BaP in the liver after i.p. administration, whereas in the lungs a moderate effect was detected. In the spleen, kidneys and stomach they could not find any effect. In contrast to our results Knasmüller et al. were able to detect a positive effect for BaP at a dose of 10 mg/kg

Table 4.4 Genotoxicity of intratracheally administered extracts of airborne particles in the DNA repair host-mediated assay

Treatment[a]	Relative survival uvrB/recA (% control)[b]			
	liver	lungs	spleen	kidneys
control	100.0 ± 14.6	100.0 ± 21.7	100.0 ± 18.5	100.0 ± 26.5
open fire place	69.5 ± 29.3	75.9 ± 29.2	120.2 ± 12.6	91.3 ± 29.8
control	100.0 ± 40.8	100.0 ± 27.4	100.0 ± 23.0	100.0 ± 32.4
smoking	n.t.	78.5 ± 36.0	138.3 ± 34.5	101.5 ± 21.0
control	100.0 ± 36.5	100.0 ± 29.1	100.0 ± 24.0	100.0 ± 26.8
outdoor	104.3 ± 8.0	85.3 ± 11.7	121.0 ± 11.1	124.4 ± 22.4

[a]Extracts were applied at a single intratracheal dose (open fire place 1052 m^3/kg body weight, outdoor and smoking 1248 m^3/kg body weight). Controls received solvent only (4 ml 10% DMSO in PBS/kg body weight).
[b]Values are mean ± s.d. from 2-4 experiments. n.t. not tested.

body weight, whereas we needed a dose of about 52 mg/kg bodyweight to obtain a positive effect. This difference may be due to the shorter period we used for the EDTA pretreatment. However, the period of 2 minutes we used was the maximum time, as this treatment caused a decrease in survival of the uvrB/recA strain of 50-60%. This was also observed by Zeilmaker (personal communication). In the above mentioned study of Knasmüller et al. no decrease in survival of the uvrB/recA strain was found after 3 minutes of EDTA treatment using the same EDTA concentration. The reason for this discrepancy remains unclear.

In host mediated assays different from the one described in this study no clear effect of BaP was observed. Batzinger et al. (1978) found a weak effect for BaP in an intraperitoneal host mediated assay using Salmonella strains TA 98 and TA 100 after 6-h incubation. In another intraperitoneal host mediated assay Simmon et al. (1979) could not find an effect of BaP to Saccharomyces cerevisiae D3 and Salmonella strains TA 1530,

TA 1353 and TA 1538. In an intrasanguineous host mediated assay using TA 98, TA 100 and TA 1538 Glatt et al. (1985) showed that neither BaP nor its metabolite BaP-4,5-oxide induced a positive effect. However, with the metabolite BaP-7,8-diol a positive effect in the liver was found.

The observed discrepancy between the concentrations needed in vivo to detect a genotoxic effect for BaP and the concentrations needed to do so in vitro could have several reasons. First, deactivation of metabolites of BaP as was suggested in the above mentioned study by Glatt et al. could be a reason. Second, the distance between the metabolizing cells and the cells in which the E. coli bacteria are trapped could be too great, so that most of the metabolites are deactivated before they reach the bacteria. Jenssen et al. (1979) found a mutagenic effect in a liver perfusion system as metabolizing system for DMNA only if the V79 cells used as indicator cells where placed in direct contact or in the near vicinity of the liver. They estimated the biological half-life of DMNA to be 1-5 seconds. For BaP no effect could be observed, even when the V79 cells where placed in direct contact with the liver. The authors concluded that metabolites of BaP are deactivated before they leave the liver. Other authors (Kuroki and Drevon, 1978; Reiners and Herlick, 1985; Jongen et al., 1986) have shown that BaP or its metabolite BaP-7,8-diol-9,10-epoxide (BPDE) were only mutagenic in vitro if there was direct or proximate contact between metabolizing cells and target cells.

Bacteria are trapped in the liver by macrophages, endothelial cells and Kupffer cells (Hauser and Matter, 1977). Although macrophages (Centrell et al., 1973; Harris et al., 1978) and endothelial cells (Omiecinski et al., 1978; Bond et al., 1981) have metabolic capacity, this capacity is less than in hepatocytes, so that the distance between metabolizing cells and cells in which the bacteria are trapped could be a determining factor. On the other hand Dock et al. (1989) estimated the biological half-live of BPDE to be about 1 minute. In view of the above-mentioned half-live of DMNA and the positive results obtained with DMNA obtained in the study of Jenssen et al., a half-live of 1 minute for BPDE would be enough for these metabolites to reach the E. coli cells and to give rise to a genotoxic effect. This is further supported by the study of Ginsberg and Atherholt (1989). 4 h after i.p. administration of BaP to mice they were able to isolate from the serum BPDE metabolites which were capable of forming DNA adducts. Serum

provided a protective effect towards hydrolysis of BaP metabolites. Lipoproteins, which have been suggested to be transporters of polycyclic aromatic hydrocarbons in the plasma (Revis et al., 1984), might cause the protective effect. In the study of Jenssen et al. a serum-free perfusion medium was used, and this could explain the negative result for BaP. It seems that mutagenic metabolites of BaP could be transported in vivo by the blood, and thus should be able to reach trapped bacteria in less or non-metabolizing cells in the DNA-repair host-mediated assay. That this indeed is the case was shown in our study and in the already mentioned study of Knasmüller et al. (1989).

2-Nitrofluorene showed a clear genotoxic effect in vitro. In vivo the strongest effect of NF was seen in the kidneys. However, this effect was not significant. Because the E. coli bacteria possess nitroreductase activity, as was shown by the direct acting activity of NF, an observed effect in vivo could be due to the metabolism of NF by the bacteria in stead of the metabolism of the host. Such an effect could thus be an artefact.

NF has been shown to be genotoxic in several in vivo systems (for a review see Beije and Möller, 1988a). However, the route of administration seems to be a major factor. Oral administration in general gave positive results, whereas i.p. administration in general gave negative results. Beije and Möller (1988b) were able to detect a positive unscheduled DNA synthesis response in liver hepatocytes after oral administration of NF. So the route applied in this study might explain the negative results obtained in the liver.

Extracts of outdoor as well as of indoor airborne particulate matter showed a genotoxic effect in vitro. The indirect action was less than the direct action. These results contrast with results obtained in the Ames test by van Houdt et al. (1984, 1986, 1987) in which the indirect acting mutagenicity is higher. This might be explained by the different genetic endpoints used in both systems.

In the host mediated assay no genotoxic activity in any of the organs tested could be found either after i.p. administration of a dose 20-40 times as high as the lowest dose which showed a positive effect in vitro (expressed as m^3/mouse versus m^3/ml) or after i.t. administration of a dose which showed a clear genotoxic effect in vitro. Due to the limited volume which could be tracheally instilled, no higher doses were tested. The negative results obtained in the host mediated assay, could perhaps be explained by the (relative) insensitivity of this assay for BaP and NF as already discussed. An additional

factor could be the low concentration of BaP and NF in airborne particles. These concentrations are in the order of ng/m³ of outdoor air (for BaP see de Raat et al., 1987; for references on NF see Beije and Möller, 1988a). However, it has to be kept in mind that the mutagenicity of outdoor airborne particles is not completely determined by the mutagenicity of polycyclic aromatic hydrocarbons (PAHs) and nitrated derivatives of PAHs present in airborne particles (De Raat, 1988), and that the compounds responsible for most of the mutagenic activity have not been identified yet (Schuetzle and Daisey, 1990).

Motykiewicz et al. (1990) found an increase of micronuclei in mouse bone marrow cells induced by an outdoor APM extract. In two other in vivo studies on the genotoxic activity of outdoor airborne particulate matter no genotoxic activity was detected. Although positive results were obtained in vitro by Krishna et al. (1986) based on sister chromatid exchanges (SCEs) as the genetic endpoint in bone marrow and spleen cells, no genotoxic activity was detected in vivo based on SCEs in the same cells. Crebelli et al. (1988) could not find a genotoxic effect in urine and faeces of rats treated with extracts of APM. In a micronucleus test they did not observe any effect either. These studies and our studies show that care has to be taken with the interpretation of positive in vitro results of genotoxicity of APM. Among other factors in vivo deactivation could play an important role in the negative results obtained. This deactivation is not well mimicked by in vitro systems. Therefore more information is needed on in vivo genotoxic activity of APM, to further evaluate a possible genotoxic risk to humans.

Note. About a year after our studies were published it was reported that for the poly(hetero)cyclic compounds aflatoxin B1 and actinomycin D, EDTA-permeabilisation of the cell wall of E. coli is of a transient nature when used in the host-mediated assay procedure, but not when used in the in vitro procedure (Zeilmaker et al., 1991). This could mean that permeabilised E. coli cells used in vitro are more sensitive to the genotoxic activity of BaP and NF then when used in the HMA. It is not known whether or not this difference in sensitivity is also applicable to APM (which might play a role in the absence of genotoxic activity of APM in the HMA), because the exact contribution in

genotoxicity of poly(hetero)cyclic compounds to the overall genotoxicity of APM is unknown.

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CHAPTER 5

INHIBITION OF GAP-JUNCTIONAL INTERCELLULAR COMMUNICATION BY OUTDOOR AND INDOOR AIRBORNE PARTICULATE MATTER

Summary

To investigate the inhibition of gap-junctional intercellular communication (GJIC) by extracts of airborne particulate matter (APM), V79 cells were incubated with extracts of APM and subsequently microinjected with the fluorescent dye Lucifer Yellow after which the number of fluorescent (=communicating) cells was determined. To compare inhibitory effects on GJIC with mutagenicity, APM was also tested in the Salmonella microsome assay. Six different extracts were tested for inhibition of GJIC in V79 cells and mutagenicity: two outdoor extracts representing a heavily polluted and a relatively clean sample, and four indoor extracts, taken either in livingrooms with or without wood combustion in an open fire place, or in a room with or without cigarette smoking. In addition three extracts were tested for inhibition of GJIC in primary cultures of rat alveolar type II cells, a possible target cell for inhaled particles. Non-cytotoxic doses of outdoor and indoor APM inhibited GJIC in V79 cells in a dose and time-dependent manner. Mutagenicity data and IC data were correlated. In addition to the effects on V79 cells, GJIC was also inhibited at non-cytotoxic doses in a concentration dependent manner in alveolar type II cells. Taken together, these results suggest that extracts of APM, in addition to the genotoxic activity that has been known for many years, also have a tumor-promoting activity.

This chapter is based on: Heussen GAH (1991) Inhibition of intercellular communication by airborne particulate matter. Arch Toxicol 65:252-256 and Heussen GAH, Alink GM (1992) Inhibition of gap-junctional intercellular communication by TPA and airborne particulate matter in primary cultures of rat alveolar type II cells. Carcinogenesis 13:719-722.

Introduction

A number of studies have shown that outdoor (Dehnen et al., 1977; Pitts et al., 1977; Tokiwa et al., 1977; Talcott and Wei 1977; Alink et al., 1983; Krishna et al., 1984; Seemayer et al., 1986; Van Houdt et al., 1987) and indoor (Alfheim and Ramdahl 1984; Van Houdt et al., 1984, 1986) airborne particulate matter (APM) is genotoxic. Because carcinogenesis is thought to be a multistep process, it is important that other parameters than those associated with initiation are studied.

Communication across gap junctions between cells in various tissues is considered as an important mechanism for control of cellular growth and differentiation (Loewenstein, 1979). Inhibition of gap-junctional intercellular communication (GJIC) was in the late 1970s postulated to be an important mechanism in tumor promotion (Yotti et al., 1979; Murray and Fitzgerald, 1979). More recently increasing evidence was obtained based on both in vitro and in vivo studies in favour of this hypothesis (Trosko et al., 1982; Trosko, 1987; Yamasaki, 1984a,b; for a review see Yamasaki, 1990).

To compare dose-effect relationships for tumor promoting activity of extracts of APM with initiating activity, mutagenicity studies were also performed. From previous studies conducted at our department it was shown that in the town of Wageningen extracts of APM showed the highest degree of mutagenicity if they were collected in the winter, especially when the wind was blowing from the east and south (Van Houdt et al., 1987). When the wind came from northerly directions, mutagenicity was lowest. These results were explained by the fact that with northerly winddirections airmasses come from the North Sea, while with southerly or easterly winddirections airmasses pass industrialized areas. For indoor air smoking and to a lesser extent wood combustion in an open fire place proved to be important sources of mutagenicity (Van Houdt et al., 1984, 1986).

In the present study the mutagenicity and inhibition of GJIC of 6 different APM extracts was tested. Two outdoor extracts representing a heavily polluted sample and a relatively clean sample were tested, and four indoor extracts differing in pollution sources were tested. In addition, the inhibitory potential of APM on GJIC in primary cultures of rat alveolar type II cells was studied. As far as we know these cells have not been used in studies on inhibition of GJIC. Alveolar type II cells were chosen because they are possible target cells for inhaled particles. Only recently it was shown by Bond et al.

(1990) that DNA adducts were formed in alveolar type II cells in rats after exposure by inhalation to diesel exhaust.

Materials and methods

Chemicals

Dimethylsulfoxide (DMSO) was obtained from Merck (Amsterdam, The Netherlands). 12-O-tetradecanoylphorbol-13-acetate (TPA), benzo(a)pyrene (BaP), 4-nitroquinoline-oxide and Percoll were obtained from Sigma (St. Louis, MO, USA). Phosphine R was obtained from ICN (NY, USA). Lucifer Yellow was purchased from Janssen Chemica (Beerse, Belgium).

Sample collection and extraction

APM was collected in the winter periods of 1988-1991 in Wageningen and extracted, as described in Chapter 2. Two indoor pollution sources were investigated: wood combustion in open fire places and cigarette smoking. Outdoor extracts representing heavily polluted samples (taken on days with southerly or easterly wind directions) and relatively clean samples (control, taken on days when the wind was blowing from the northwest) were also prepared.

Mutagenicity

Extracts were tested for mutagenicity in the Salmonella microsome assay, as described by Ames et al. (1975), with minor modifications (Van Houdt et al., 1986). Extracts were tested in strain TA 98 with and without Aroclor induced rat liver S9. BaP and 4-nitroquinoline-oxide were used as positive controls for indirect and direct mutagenicity respectively. Samples were tested in triplicate in two independent experiments. The number of spontaneous revertants was subtracted from all results.

Cytotoxicity

The cytotoxicity of the extracts was determined by the cloning efficiency of V79 cells or by measuring leakage of lactate dehydrogenase (LDH) in alveolar type II cells. For

cloning efficiency 200 cells/dish were plated, allowed to attach and APM extracts were added. DMSO concentration did not exceed 1%. After incubation, cells were washed once with Hank's Balanced Salt Solution (HBSS) and new medium was added (Ham's F10 [Flow Laboratories, UK] supplemented with 10% fetal calf serum [Gibco, Breda, The Netherlands] and 50 μg/ml gentamycin [Gibco]). Cells were cultured in a humidified atmosphere containing 5% CO_2. After 7 days the colonies were fixed, stained with Giemsa and scored. For LDH leakage, activity was measured in 100 μl supernatant. The maximum release of LDH was measured after scraping cells from the bottom after addition of 1 ml 0.5% Triton X-100 in 0.1M phosphate buffer (pH 7.4). This suspension was sonicated for 5 min and centrifuged after which LDH activity was measured in the supernatant (Mitchell et al., 1980).

Inhibition of gap-junctional intercellular communication

10^6 V79 cells were plated on 35 mm dishes (Greiner Labortechnik, Germany) and 24 h later confluent cultures were exposed to extracts of APM. Before microinjection cells were washed once with HBSS and new medium was added (Ham's F10 supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin and 20 mM HEPES pH 7.4). GJIC was determined after microinjection of a 10% Lucifer Yellow solution in 0.33 M lithium chloride in a single cell by means of a glass capillary tip (Clarke, Pangbourne, UK). The transfer of this dye to neigbouring cells is believed to occur via gap junctions, because it cannot diffuse through the membrane (Stewart, 1978; Lo and Gilula, 1979; Atkinson et al., 1981). At least 20 cells/dish were injected with the use of a vertical microinjection system coupled to a combined light and fluorescent phase contrast microscope (Olympus IMT-2, Japan). Glass capillary tips were prepared with an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of about 1 micron. Ten to fifteen min after the first injection the number of fluorescent cells was counted.

Alveolar type II cells were isolated from the lungs of female Wistar rats (age 4-6 months) as described earlier (Mason et al., 1977) using a Percoll gradient instead of albumin for the density gradient centrifugation. Approximately $4*10^5$ cells were plated in 0.2 ml medium in the middle of a 35 mm dish (Greiner). The medium used was Ham's F12 (Flow) containing 10 % fetal calf serum (Gibco), 50 μg/ml gentamycin (Gibco) and 1.25

μg/ml fungizone (Gibco). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C, and 18-20 h later 1.8 ml medium was added. Next day medium was changed. Again 24 h later (the third day after isolation) cells had formed a confluent monolayer in the middle of the dish containing about 70% type II cells (determined by staining with Phosphine R, as described earlier (Mason et al., 1977)). Per dish 15-20 cells were microinjected as described above. We also performed experiments with A549 cells, a human transformed alveolar type II cell line, but these cells had a very low intrinsic communication capacity (1-2 communicating cells per injection) and therefore can not be used in dye-coupling experiments.

Statistics

Data were tested at a significance level of $p<0.05$ with the Student t-test.

Results

Mutagenicity

As shown in Fig. 5.1, only the polluted outdoor sample was mutagenic, whereas the control outdoor sample was not mutagenic. The indirect mutagenicity was higher than the direct mutagenicity. Between the indoor pollution sources tested, extracts of APM produced by cigarette smoking caused a higher mutagenicity than did extracts of APM produced by wood combustion. For cigarette smoking the control sample was not mutagenic, whereas for wood combustion the control sample did show a mutagenic effect. However this effect was less than of the experimental sample. For the indoor samples also the indirect mutagenicity was higher than the direct mutagenicity.

Cytotoxicity

The outdoor sample was not cytotoxic towards V79 cells in any of the conditions tested (Table 5.1). The open fire place sample slightly reduced the cloning efficiency at the two highest doses tested after incubation for 2 h. Incubation for 24 h also reduced cloning efficiency. The cigarette sample was cytotoxic only at the highest dose tested. The

three samples tested in alveolar type II cells were not cytotoxic, as they did not increase LDH leakage at doses used for microinjection experiments (data not shown).

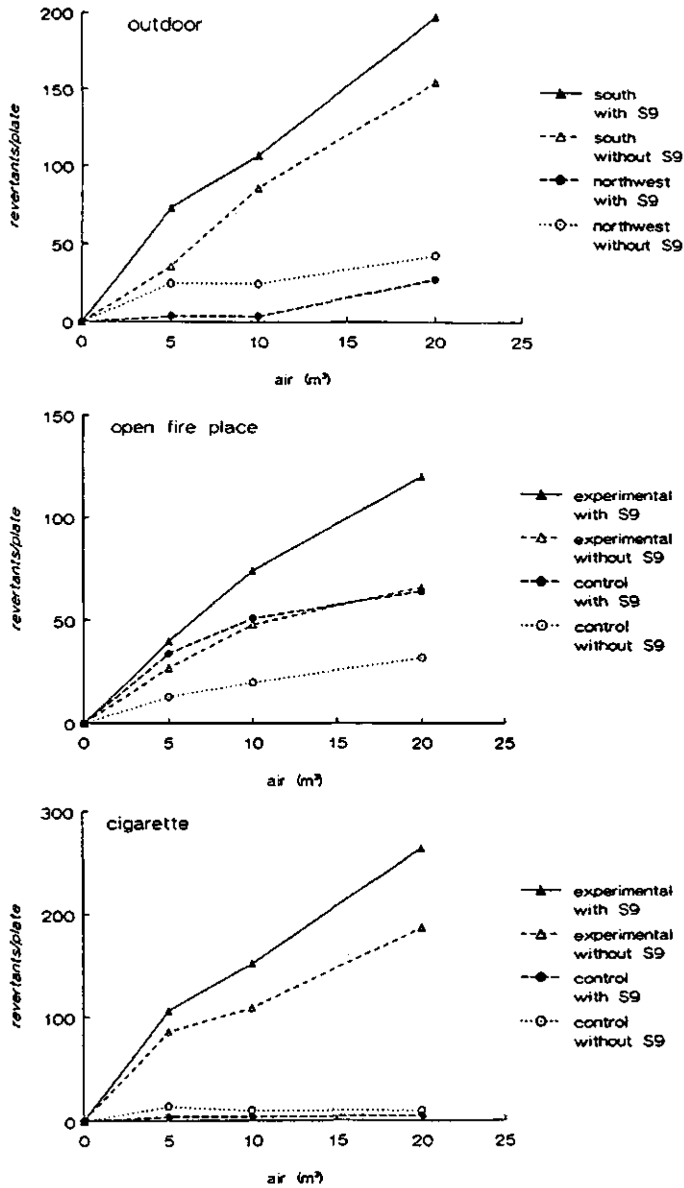

Figure 5.1 Mutagenicity of extracts of airborne particulate matter in TA 98 with or without rat liver Aroclor S9. Concentrations correspond to the volume of air sampled. Data are the mean ± SD from triplicate determinations.

Table 5.1 Cytotoxicity of extracts of airborne particulate matter towards V79 cells

Treatment	concentration[a] (m^3)	Incubation time (hours)	Relative cloning[b] efficiency
outdoor	0	2	100.0 ± 6.0 (3)
	5		n.t.
	7		n.t.
	10		n.t.
	15		86.7 ± 10.6 (2)
	20		86.5 ± 18.2 (2)
	0	7	100.0 ± 4.5 (2)
	4		107.2 ± 11.0 (2)
	0	24	100.0 ± 7.5 (2)
	4		98.3 ± 13.8 (2)
open fire place	0	2	100.0 ± 5.8 (5)
	5		n.t.
	7		92.6 ± 11.7 (2)
	10		94.5 ± 13.6 (2)
	15		75.7 ± 11.2 (2)
	20		73.4 ± 11.4 (2)
	0	7	100.0 ± 4.5 (2)
	4		82.8 ± 12.8 (2)
	0	24	100.0 ± 5.9 (2)
	4		75.2 ± 13.3 (2)
cigarette	0	2	100.0 ± 7.2 (3)
	5		95.9 ± 9.9 (3)
	7		105.2 ± 5.4 (2)
	10		91.8 ± 13.7 (3)
	15		70.0 ± 6.0 (2)
	20		n.t.

[a] Concentrations correspond to the volume of air sampled.
[b] Values are mean ± SD from n (number in parentheses) experiments;
n.t. not tested.

Inhibition of gap-junctional intercellular communication in V79 cells

Incubation of V79 cells for 1 h with 100 ng/ml 12-O-tetra-decanoylphorbol-13-acetate (TPA), used as a positive control, reduced the number of communicating cells from 7.60±1.86 to 1.57±0.43 (mean ± SD, 3 experiments). Next, different extracts of APM were tested. Extracts of APM caused a dose dependent inhibition of GJIC as can be seen in Fig. 5.2. The polluted outdoor sample inhibited GJIC, whereas the control outdoor sample did not. Extracts of APM produced by cigarette smoking inhibited GJIC, whereas the corresponding control sample did not show any effect. Extracts of APM produced by wood combustion also inhibited GJIC, while the corresponding control sample also showed an effect, although less than the experimental sample. Two extracts were tested for time dependent inhibition of GJIC. Both the polluted outdoor sample and the indoor sample taken in a room polluted by wood combustion, caused a marked time dependent decrease in communication, as is shown in Fig. 5.3. Mutagenicity data and data on inhibition of IC were correlated ($p < 0.005$, Spearman rank correlation).

Inhibition of gap-junctional intercellular communication in alveolar type II cells

Experiments showed an extensive dye-coupling between alveolar type II cells in control cultures (Figure 5.4A and B). Control cultures were exposed to dimethylsulfoxide (range 0.25-1%). The absolute number of communicating cells in control cultures was 148.3 ± 34.9 (mean ± SD, n=18 dishes). Exposure of cells to different concentrations of TPA resulted in a strong inhibition of IC (Figure 5.4C and D, Table 5.2). At a concentration of 1 ng/ml communication was inhibited by about 65%. Increasing the concentration to 10 ng/ml caused an almost complete inhibition of communication. Because cells were extensively dye coupled in control cultures and responded well to TPA, we concluded that these cells could serve as a model in dye-coupling experiments. Therefore three APM extracts were tested, and it was shown that all three extracts caused a strong inhibition of IC in a dose-dependent manner (Figure 5.5A-D, and 5.6).

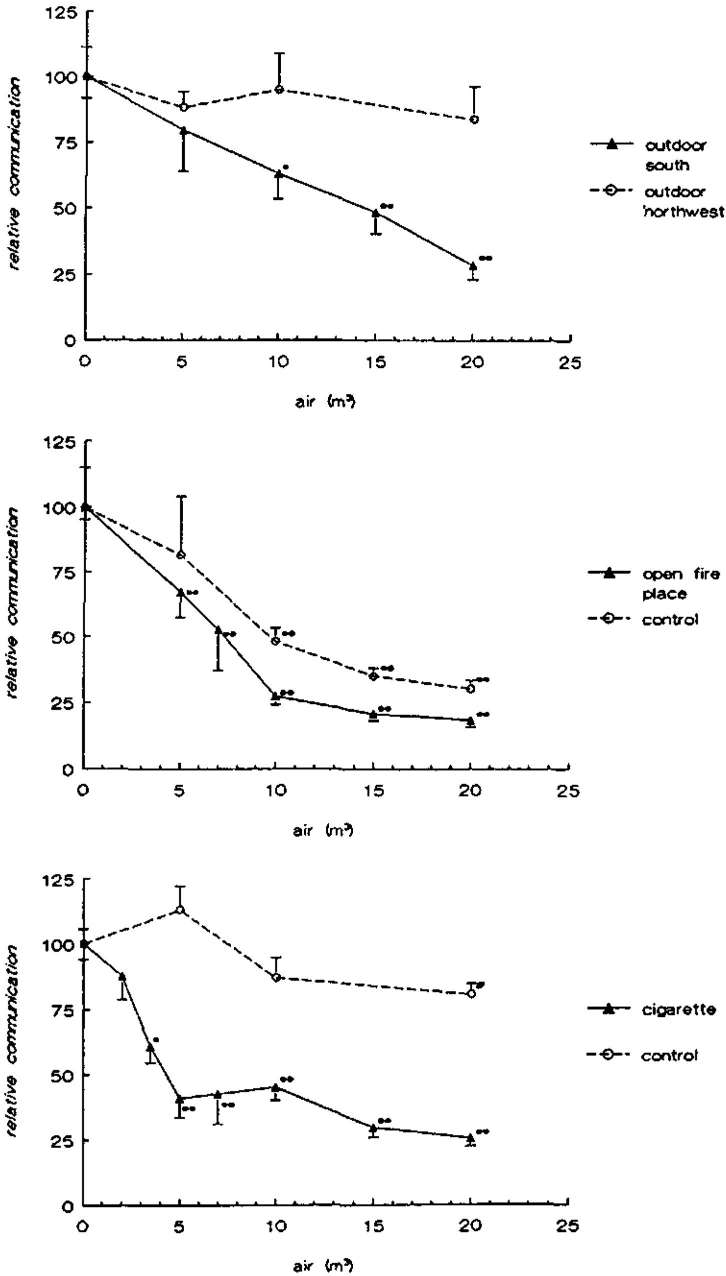

Figure 5.2 Dose-dependent inhibition of intercellular communication in V79 cells by extracts of airborne particulate matter. V79 cells were incubated for 2 hours with extracts of APM. Concentrations correspond to the volume of air sampled. Results are expressed as percentage of the control and are the mean ±SEM from 6-20 dishes. #, * and ** significant different from control, $p<0.05$, $p<0.01$ and $p<0.005$ respectively.

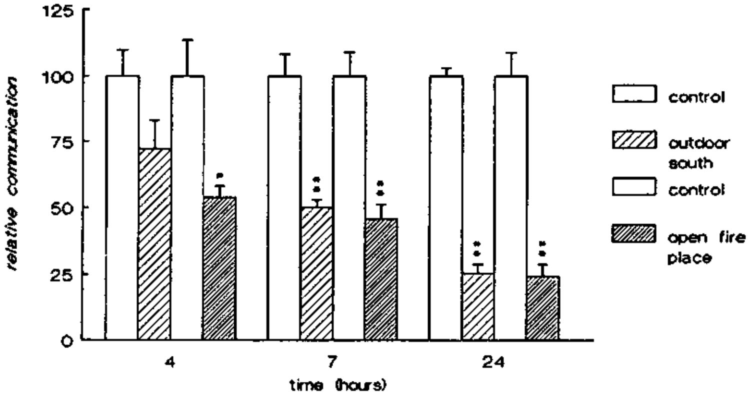

Figure 5.3 Time-dependent inhibition of intercellular communication in V79 cells by extracts of airborne particulate matter. V79 cells were incubated with extracts of APM corresponding to 4 m^3 of air. Control was 0.2% DMSO. Results are the mean±SEM from 6 dishes. * and ** significant different from control, $p<0.01$ and $p<0.005$ respectively.

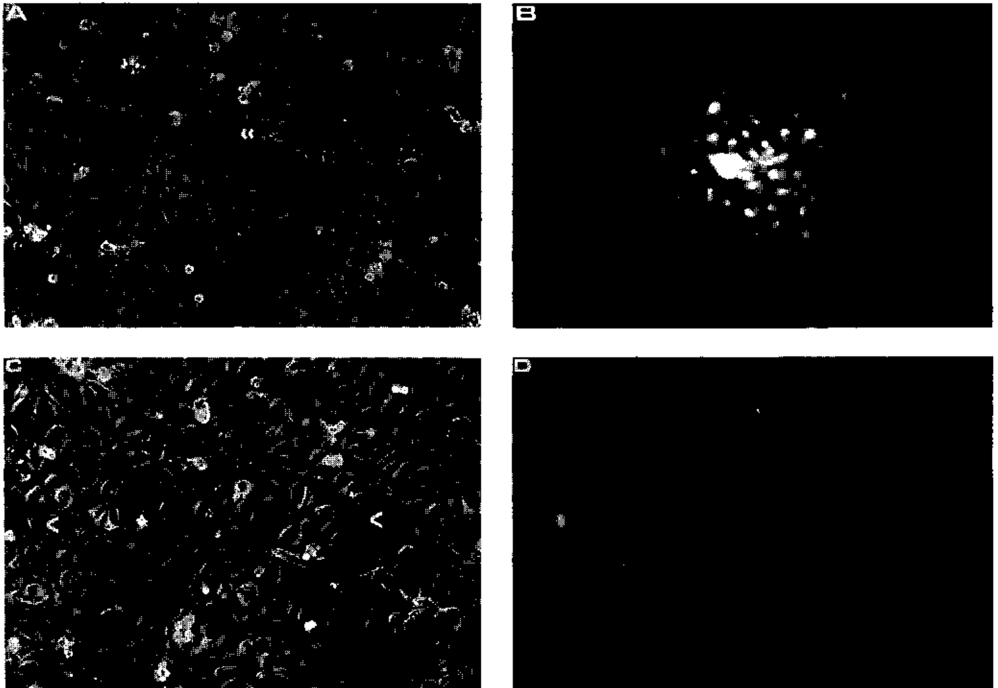

Figure 5.4 Dye-coupling between alveolar type II cells. The microinjected cells are marked with an arrow (original magnification X200). The left panel shows photographs taken under normal light, while the right panel shows photographs taken under UV light showing fluorescent cells. A and B represent control cultures. C and D are cultures exposed to a concentration of 100 ng/ml TPA.

Discussion

The mutagenicity data are in agreement with previous studies conducted at our department by Van Houdt et al. (1984, 1986, 1987). The indoor control samples for cigarette smoking and wood combustion differed in mutagenicity. This may be explained by differing infiltration of outdoor particles into these rooms. Infiltration of outdoor particles has been suggested by Van Houdt et al. (1986) to partially contribute to indoor air mutagenicity.

The results obtained in this study on the inhibition of GJIC by TPA in V79 cells agree well with the data obtained in the study of Zeilmaker and Yamasaki (1986). Extracts of APM were able to inhibit GJIC in V79 cells at non-cytotoxic doses. Dose-dependent as well as time-dependent inhibitions were found. Mutagenicity data and data on inhibition of GJIC were correlated, and the concentrations at which a positive effect was found, were in the same range.

Table 5.2 Inhibition of intercellular communication by TPA in primary cultures of rat alveolar type II cells

Treatment[a]	No. of communicating cells[b]	Relative communication
Control	135.9 ± 20.1	100.0 ± 14.7
TPA 1 ng/ml	45.8 ± 1.3	33.7 ± 1.0
Control	151.2 ± 18.2	100.0 ± 12.0
TPA 10 ng/ml	7.6 ± 1.9	5.0 ± 1.3
Control	126.8 ± 24.8	100.0 ± 19.6
TPA 100 ng/ml	9.8 ± 1.4	7.7 ± 1.1

[a] Cells were exposed for 1 h to solvent (0.25% DMSO) or TPA

[b] Values are mean ± SD from three dishes and are taken from representative experiments. Data from TPA treated cultures were significantly different from control cultures (Students t-test, $p < 0.005$)

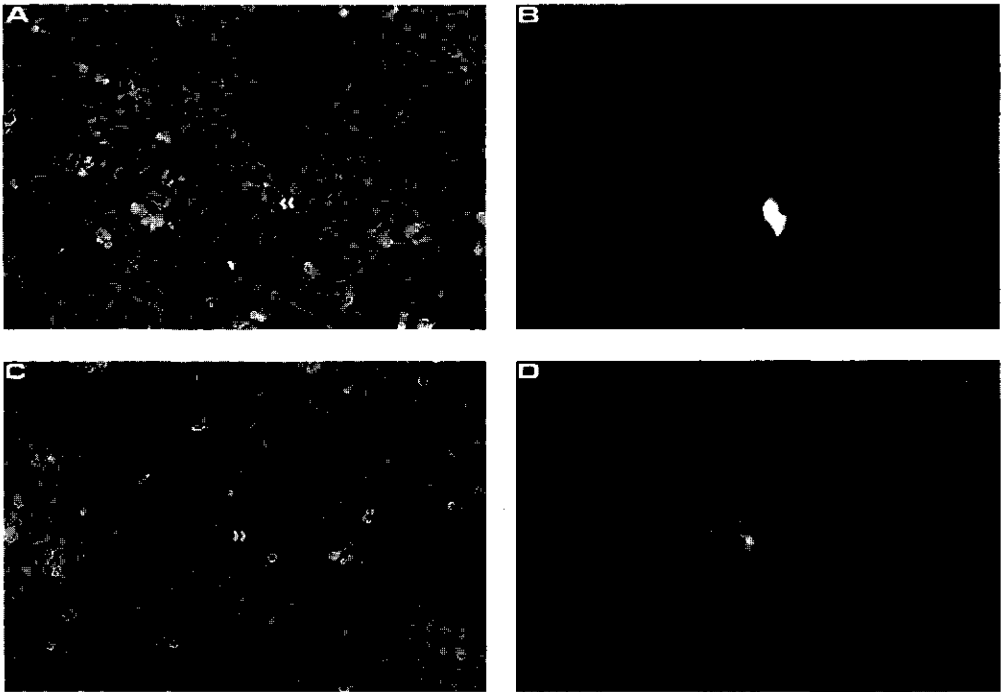

Figure 5.5 Legend as in fig. 5.4. A and B represent cultures exposed to cigarette smoking extract (obtained from 1.25 m^3 air), while C and D are cultures exposed to outdoor extract (obtained from 20 m^3 air).

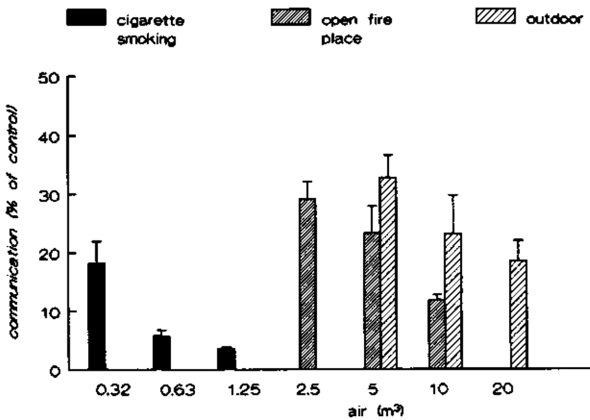

Figure 5.6 Inhibition of intercellular communication by extracts of airborne particulate matter in primary cultures of rat alveolar type II cells after a 2 hours exposure period. Cells were exposed to solvent (maximal 1% DMSO) or APM. Data are the mean $\pm$ SEM from 3-9 dishes. Control was set at 100%. Data from APM treated cultures were significantly different from control cultures (Students t-test, $p<0.005$).

The time-dependent inhibition of GJIC in V79 cells was irreversible up to 24 h. It is known that the inhibition of GJIC caused by TPA is transient dependent on the growth state of the cells (Enomoto and Yamasaki, 1985; Jongen et al., 1987; Mc.Gaffrey and Rosner, 1987). For TPA and related phorbol esters it is thought that they act via activation of protein kinase C (PKC) (Castagna et al., 1982; Nishizuka, 1986). Down regulation of PKC may also be related to the transient effect of TPA (Rodrigues-Pena and Rozengurt, 1984; Chida et al., 1986). The inhibitory effect on GJIC of another complex mixture, namely cigarette smoke condensate (CSC) is not transient (Jongen et al., 1987; Rutten et al., 1989; Van Der Zandt et al., 1990). In addition, TPA, but not CSC, has been shown to reduce the number of gap junctions in V79 cells (Jongen et al., 1987; Van Der Zandt et al., 1990) and it has recently been shown that these compounds induce different effects on gap junction morphology and quantity (Van Der Zandt et al., 1990). CSC does not seem to activate PKC (Van Der Zandt et al., 1990), and this suggests that these compounds act via different mechanisms. It might be possible that like cigarette smoke condensate, APM does not activate protein kinase C. However, studies have to be undertaken in order to prove this hypothesis.

Before experiments were started on inhibition of GJIC by APM in alveolar type II cells, we first had to investigate if these cells could be routinely used in such experiments. The dose levels of TPA, used as a positive control, at which an inhibition was found in alveolar type II cells were comparable to the doses used to inhibit IC in other studies (Heussen, 1991; Boreiko et al., 1989). Furthermore, these cells showed an intrinsic extensive dye-coupling. Therefore, primary cultures of alveolar type II cells can serve pre-eminently as a model in dye coupling experiments.

It is interesting to note that primary cultures of alveolar type II cells show more intercellular communication measured by dye-coupling than primary cultures of tracheal epithelial cells. In the study of Rutten et al. (1988) and in experiments performed by us (data not shown) maximally four tracheal epithelial cells were dye coupled. Because of this low communication capacity these cells can not be routinely used for the screening of airborne compounds with an inhibitory activity on IC. This difference in communication capacity between alveolar type II and tracheal epithelial cell cultures might be caused by the fact that cultures of type II cells were largely purified, whereas cultures of tracheal

epithelial cells were not purified and therefore consist of a mixed cell population. It is known that mixed cell populations sort out and form communication compartments (Pitts and Kam, 1985). As a consequence cells show a decreased communication capacity at the boundary layer with other cell types. In another study it was shown that cells in the dermal layer of the skin and cells in the epidermis were dye-coupled, while dermal-epidermal dye spread did not occur (Pitts et al., 1986).

Experiments showed that in addition to the inhibition in V79 cells, extracts of APM strongly inhibited GJIC at non-cytotoxic doses in a dose-dependent manner in alveolar type II cells. As there is increasing evidence that inhibition of GJIC is an important mechanism in tumor promotion (for a review see Yamasaki, 1990), this could mean that APM extracts are not only mutagenic, but are also possibly tumor promoting.

The compounds present in APM responsible for the inhibition of GJIC are unknown. Polycyclic aromatic hydrocarbons and nitrated derivatives are not likely to be responsible, because in experiments performed by us (data not shown) and others (Elmore et al., 1988; Budunova et al., 1990) several representatives of these compounds had no effect on GJIC. Studies on fractionated cigarette smoke showed that tumor promoters are found primarily in the weakly polar neutral and acidic fraction, which include certain unsaturated hydrocarbons and phenolic compounds (Hoffman et al., 1983). Fractionation of another complex mixture, namely asphalt fume condensate, revealed that alkylated ketones, alkylated naphtols and phenols inhibited metabolic cooperation most (Toraason et al., 1991). Of the above-mentioned compounds alkylated ketones and phenols were detected in APM (Cautreels and Van Cauwenberghe 1978). The same authors further detected several aliphatic acids in APM, some of which are known to be able to inhibit IC (Aylsworth et al., 1986).

In conclusion, the important finding of this study is that extracts of outdoor and indoor APM originating from different pollution sources, in addition to mutagenic activity, can inhibit GJIC, suggesting tumor promoting potency .

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CHAPTER 6

EFFECTS OF OUTDOOR AND INDOOR AIRBORNE PARTICULATE MATTER ON THE KINETICS OF VITAMIN A IN RATS

Summary

Vitamin A is an important regulator of normal epithelial differentiation and proliferation and might act in the promotion phase of carcinogenesis. Vitamin A and thyroid hormone metabolism are linked by a common plasma carrier protein transthyretin (TTR). Polychlorinated biphenyls (PCBs) and related organochlorine compounds deplete vitamin A and thyroxin (T4) by interaction with TTR and alteration of their metabolism in hepatic and other organs. In the present report, extracts of indoor and outdoor airborne particulate matter (APM), originating from different pollution sources, were tested for a possible interaction in vitro and in vivo with T4 and vitamin A. Furthermore, studies were performed to characterize compounds present in APM that interact with TTR. A third aim was to compare the interaction of APM extracts with TTR and Thyroxin Binding Globulin (TBG), the major carrier protein for T4 in humans. Results indicated that all APM extracts significantly interfered with T4 binding to TTR, but not with TBG. In all cases, the more polluted samples inhibited binding more than the corresponding control samples. Experiments showed that the neutral fraction of the APM extracts accounted for most of the inhibitory activity on T4-TTR binding. Polycyclic aromatic hydrocarbons (PAHs) and nitrated derivatives are not likely to be responsible for the activity of the neutral fraction, because several representatives of these compounds showed no or very little interaction with TTR. Pentachlorophenol, a compound with known inhibitory activity on T4-TTR binding, was detected in the organic acid fraction of both a cigarette smoke sample and an outdoor APM sample. A single treatment of rats with an outdoor or cigarette smoke APM extract depleted plasma T4 and

This chapter is based on: Heussen GAH, Hikspoors MLJ, Spenkelink A, Brouwer A, Koeman JH (1992) Inhibition of binding of thyroxin to transthyretin by outdoor and indoor airborne particulate matter and effects on thyroid hormone and vitamin A metabolism in rats. Arch Environ Contamin Toxicol 23:6-12 and Heussen GAH, Schefferlie GJ, Talsma MJG, van Til H, Dohmen MJW, Brouwer A, Alink GM (1993) Effects on thyroid hormone metabolism and depletion of lung vitamin A in rats by airborne particulate matter. J Toxicol Environ Health 38:419-434.

Abstract-continued

triiodothyronin levels and increased plasma retinol levels gradually over the time period studied,while liver retinol (in the highest dose group), lung retinol and retinyl palmitate levels were depleted. Taken together, these studies show that APM extracts have the potency to interfere with thyroid hormone metabolism both in vitro and in vivo and to deplete lung vitamin A in vivo. As vitamin A might have a protective effect in the process of lung carcinogenesis, APM might increase the susceptibility for the development of lung cancer.

Introduction

For more than a decade it is known that extracts of outdoor (Alink et al., 1983; Dehnen et al., 1977; Pitts et al., 1977; Talcott and Wei, 1977; Tokiwa et al., 1977) and indoor (Alfheim and Ramdahl, 1984; Van Houdt et al., 1984, 1986) APM are genotoxic. Because carcinogenesis is generally accepted to be a multistage process, it is important that parameters other than those associated with initiation are studied.

In the promotion phase, regarded as the phase following initiation, it is thought that an initiated cell may phenotypically alter into a transformed cell and clonally expand by means of a selective growth advantage. Deranged intercellular communication (IC) is considered as one of the most important mechanisms in tumor promotion (for a review see Yamasaki, 1990). Most cells have two different ways to communicate with other cells: a) by growth factor or hormone mediated IC or b) by cell contact-mediated IC. Among this latter form of IC, gap-junctional intercellular communication (GJIC) is believed to play a crucial role in the maintenance of tissue homeostasis. Therefore, in our previous studies we focused on the inhibition of GJIC by APM and showed that the communication was inhibited in V79 cells and in primary cultures of rat alveolar type II cells, a target cell for inhaled particles (Heussen, 1991; Heussen and Alink, 1992).

Interaction with growth and differentiation regulators has been postulated to be another mechanism of tumor promotion. Vitamin A is an important regulator of normal epithelial differentiation and proliferation (Sporn et al., 1984) and in several experimental studies (pro)vitamin A has been shown to prevent mutagenicity, malignant cell transformation, the formation of tumors in animal models or serves as an immunoenhancing agent (Krinsky, 1991, review). For

example, in the study of Beems (1984) with the Syrian golden hamster vitamin A deficiency increased the incidence of preneoplastic respiratory tract lesions induced by benzo(a)pyrene. However, no difference was seen in the number of respiratory tract tumors. Dogra et al. (1984, 1985) reported that the extent of binding of benzo(a)pyrene to lung DNA and the lung tumor incidence was higher in vitamin A deficient rats than in normal fed rats. Furthermore, vitamin A deficiency and cigarette smoke condensate treatment induce squamous metaplasia in cultured tracheal epithelium, which can be restored by vitamin A (Rutten et al., 1988a). Finally, physiological concentrations of vitamin A stimulate GJIC between hamster tracheal epithelial cells while pharmacological concentrations inhibit GJIC (Rutten et al., 1988b). Besides experimental studies, also epidemiological studies suggest that (pro)vitamin A may play a preventive role in the development of lung cancer (Ziegler, 1989, review; Van Poppel, 1992, review).

One of the mechanisms which may cause a depletion of vitamin A is by the interaction of chemicals with transthyretin (TTR). TTR is a plasma transport protein for thyroid hormones and by forming a complex with Retinol Binding Protein (RBP), also for vitamin A (Goodman, 1984). Chemicals compete with Thyroxin(T4)-TTR binding and might weaken the RBP-TTR complex, resulting in increased glomerular filtration of retinol-bound RBP and in depletion of vitamin A and thyroid hormone levels (Brouwer et al., 1988, 1990). An alternative route of depletion is by inducing enzymes involved in the metabolism of vitamin A and T4, such as UDP-glucuronyltransferases. Such a depletion might increase the susceptibility for the development of lung cancer, for example. In this study the T4-TTR binding test is used as a biomarker for measuring effects on the kinetics of vitamin A.

Several industrial chemicals or metabolites of these chemicals, like chlorinated phenols and benzenes, PCBs and dioxins strongly interact with T4 binding to TTR and decrease plasma T4 levels (Brouwer and Van den Berg, 1986a,b; Den Besten et al., 1991; Van den Berg et al., 1991; Van Raaij et al., 1991). Some structural features beneficial for TTR interaction have been identified, such as a hydroxy group on meta or para positions of an aromatic ring with one or two adjacent halogen atoms. Outdoor APM is a complex mixture of thousands of organic compounds distributed over many chemical classes (e.g. Cautreels and Van Cauwenberghe, 1976). The same holds true for APM derived from cigarette smoking (IARC, 1986). We argued that a complex mixture such as APM might be able to inhibit T4 binding to TTR because of the

possible presence of compounds which posses the above mentioned structural features beneficial for TTR interaction.

The first aim of the present study was to investigate the interaction of APM extracts with T4 binding. Four outdoor extracts representing two heavily polluted samples and two relatively clean samples, and four indoor extracts differing in pollution sources (wood combustion and cigarette smoking) were tested. In addition to the in vitro competition assays, the effects of two APM extracts were tested for interference with thyroid hormone and vitamin A kinetics in vivo.

The second aim was that, in case of a positive response, attention would be paid to the characterization of compounds present in APM which interfere with T4-TTR binding. An inhibitory effect on this binding has already been demonstrated for chlorinated phenols, PCBs and dioxins (Brouwer et al., 1990; Den Besten et al., 1991; Lans et al., 1991; Van den Berg, 1990). However, PCBs and dioxins can be excluded because these compounds require bioactivation and in our in vitro competition assay no metabolic system is added. Because pentachlorophenol (PCP) is the most active of the chlorinated phenols tested sofar, we focused on this compound. In addition, in this preliminary study six polycyclic aromatic hydrocarbons (PAHs) and nitrated derivatives (nitroPAHs) present in APM and nicotine, a major compound found in cigarettes, were tested in the same system. Considering the chemical structure of these compounds we did not expect them to interact with the thyroxin receptor at TTR. However, we wanted to test the possibility that these compounds could have an effect through aspecific binding on the protein.

The third aim of the present studies was to investigate the interaction of APM extracts with Thyroxin Binding Globulin (TBG). TTR is the major plasma transport protein of T4 in rats (Davis et al., 1970. Sutherland and Simpson-Morgan, 1975). In humans TBG is the major carrier, while TTR is of minor importance. Estimations of the percentages at which T4 is distributed over human plasma carrier proteins vary from 10-40% for TTR and from 50-80% for TBG (Petterson, 1989; Robbins and Bartalena, 1986). Information on a possible interaction of APM with TBG is important, in order to be able to extrapolate the effects on thyroid hormone metabolism from rats to humans.

Materials and Methods

Chemicals

Dimethylsulfoxide (DMSO) and dichloromethane (purity >99%) were obtained from Merck (Amsterdam, The Netherlands). Human TTR and TBG, L-thyroxin, benzo(a)pyrene (BaP) and 1,3-dinitropyrene were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Propylene glycol was obtained from Baker Chemicals (Deventer, The Netherlands). Pyrene, 1-nitropyrene and 2,7-dinitrofluorene were obtained from Aldrich (Bruxelles, Belgium). 2-nitrofluorene (NF) and L-nicotine were purchased from Phaltz and Bauer Inc. (Stanford CT, USA) and Riedel-de Haen (Seelze, Germany) respectively. Methanol (purity >99%) and retinol (purity 99%) were purchased from Janssen Chimica (Beerse, Belgium). Retinol acetate and palmitate were purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). L-[^{125}I]Thyroxine and Biogel P-6DG were obtained from Amersham International (PLC, UK) and Bio-Rad Laboratories (Richmond, CA) respectively.

Sample collection and extraction

APM was collected in the winter periods of 1989-1991 in Wageningen and extracted, as described in Chapter 2. Two indoor pollution sources were investigated: wood combustion in open fire places and cigarette smoking.

The outdoor sample tested in the animal experiment was part of a pooled sample composed of eleven samples taken on days with easterly wind directions and one sample taken on a day with northerly wind direction. This pooled extract inhibited T4 binding to TTR (data not shown). The outdoor sample which was fractionated and analyzed for the presence of PCP was part of a pooled sample composed of nine samples taken on days with easterly wind directions.

Because in the present study two samples had to be acid/base fractionated for subsequent PCP analysis, samples could not be methanol extracted. A comparison of methanol extraction with dichloromethane extraction showed dichloromethane to be a suitable solvent (data not shown).

Fractionation and pentachlorophenol analysis

Extracts were separated based on a fractionation scheme involving acid-base partitioning (Nishioka et al., 1985). First organic acids and phenolic compounds were separated from the extract by partitioning the extract 3 times with 0.1 M NaOH. In doing this, on top of the aqueous

phase a clear band of probably basic compounds was visible, suspended in and partitioned from the dichloromethane phase. This band was separated from the dichloromethane phase and collected after the third partitioning in the aqueous phase. The aqueous phases were combined and the acids and phenolic compounds were back extracted in dichloromethane after adjusting the pH to 1-2 with 10 M H_2SO_4. The aqueous phase (called first aqueous phase) was tested separately. The remaining dichloromethane phase of the extract was partitioned 3 times with 0.1 M H_2SO_4 in order to separate organic bases. Here again, on top of the aqueous phase a band (although smaller of size than the band described above) suspended in the dichloromethane phase was visible. After the third partitioning, this band was collected in the aqueous phase. After combining the aqueous phases the bases were back extracted in dichloromethane after adjusting the pH to 12-13 with 10 M NaOH. The aqueous phase (called second aqueous phase) was again tested separately. The remaining neutral fraction was not further separated.

For PCP analysis the acid-phenolic fractions were acetylated with acetic anhydride and acetyl-PCP was extracted in iso-octane. GC analysis was carried out on a Perkin Elmer 8700 gas chromatograph using a 30m x 0.2 mm i.d. CP Sil 8 CB colum (Chrompack) with helium as carrier gas. The GC temperature program conditions were as follows: 2 min at 100°C, 10°C/min for 100°C-240°C, and electron capture detector and inlet set at 300°C and 220°C respectively.

T4-TTR/TBG binding inhibition assay

A modification of the gel filtration procedure described by Somack et al. (1982) was developed. For the inhibition assay with APM extracts a chemoluminescence immunoassay was used in our first studies for the quantification of protein-bound T4, using standard Amerlite luminescence assay kits (Amersham, England). Therefore TTR (dissolved in Tris-HCl buffer (0.1 M, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA), final concentration 100 nM) was incubated overnight at 4°C with T4 (dissolved in 1.8 μl methanol, final concentration 180 nM) and with different concentrations of APM extracts (dissolved in 8.2 μl). The incubation took place in a total volume of 200 μl Tris buffer. Protein bound T4 was separated on a gel permeation chromatography column (Biogel-P6DG, bed volume 1 ml) which was centrifuged for 1 minute at 250 g. T4-bound to protein was quantified by chemoluminescence immunoassay. In later inhibition studies a somewhat different method using radioactivity was used. Therefore TTR or TBG (dissolved in Tris-HCl buffer (0.1 M, pH 8.0, containing 0.1 M NaCl and 1 mM EDTA, final concentration

30 nM) was incubated overnight at 4°C with 55 nM (70000 cpm) of ^{125}I-T4 with or without different doses of competitors. Unlabeled T4 was used as a positive control. The incubation took place in a total volume of 200 μl Tris buffer. PAHs, nitroPAHs, nicotine, APM extracts and fractions were dissolved in DMSO. T4 was dissolved in methanol/Tris 1:100. Protein bound T4 was separated as described for the chemoluminescence immunoassay. Eluent fractions containing protein-bound ^{125}I-T4 were quantified in a gamma counter. To compare the inhibitory potency of different samples, the IC_{50} (=concentration at which 50% inhibition is found) was calculated by linear regression from dose-response curves.

Animals and treatment

Female Wistar rats, age 3-4 months, were used. They had free access to food and water, and were housed under a 12 h light cycle. Animals were injected intraperitoneally with APM extracts or solvent. At different time points, animals were anesthetized with ether and blood was taken by means of an orbita punction. At the end of the experiments animals were sacrificed by anesthesia with ether and liver, lungs and blood (taken from the aorta) were collected. Blood was collected in heparinized tubes and centrifuged for 10 min at 8000 rpm in an Eppendorf centrifuge. Blood plasma and organs were stored at -20°C until analyses were performed.

Thyroid hormone assays

Total triiodothyronin (TT3), total thyroxin (TT4) and free thyroxin (FT4) were determined by means of chemoluminescence immunoassays using standard Amerlite luminescence assay kits (Amersham, England).

Vitamin A assays

Plasma retinol, hepatic and pulmonary retinol and retinyl esters were determined, after extraction of retinoids from plasma or organ homogenates in diisopropylether, by a reversed-phase HPLC method, as descibed earlier (Brouwer and Van den Berg, 1984). Retinyl acetate was used as an internal standard.

Statistics

Data were analyzed for statistical significance using Students t-test.

Results

Comparison of effect of APM extracts on T4 binding to TTR and TBG

Eight different extracts were tested for inhibition of T4 binding to TTR. The results showed that outdoor as well as indoor APM extracts were able to strongly interfere with T4 binding (Figure 6.1). In all cases the polluted sample inhibited T4 binding more than the corresponding control sample.

Four outdoor and two indoor samples were tested for both TTR and TBG interaction. All samples clearly inhibited T4-TTR binding. At the highest concentrations tested the most potent samples inhibited T4-TTR binding for almost 90%, while none of the samples tested in the same concentration range inhibited the binding of T4 to TBG (Table 6.1).

PCP analysis and effects of fractionation of APM extracts on T4-TTR binding

Fractionation of a cigarette smoke and outdoor APM sample resulted in mass recoveries of 98 and 90.9% respectively (Table 6.2). The aqueous phases represented about 1-10% of mass but, except for the first aqueous phase of the cigarette smoke APM sample, hardly inhibited T4 binding to TTR. When the mass distributions of these fractions were taken into consideration and a weighted activity was calculated, then these fractions accounted for maximally 4.9 and 0.6% of the activity of the cigarette smoke and outdoor APM sample respectively. The weighted activity of the basic fractions was neglectable. For both samples the neutral and acid fractions were the most active. Recovery of PCP throughout the fractionation scheme was >95%, as determined from studies with ^{14}C-PCP. PCP analysis of the acid fractions revealed that the cigarette and outdoor APM sample contained 6.6 and 0.23 ng PCP/m^3 of sampled air respectively (0.04 and 0.003 ng PCP/μg of extractable organic matter respectively). Because the neutral fraction contained most of the mass, this fraction accounted for most of the activity. In figure 6.2 dose-response curves of the complete extracts and of the two most active fractions are shown.

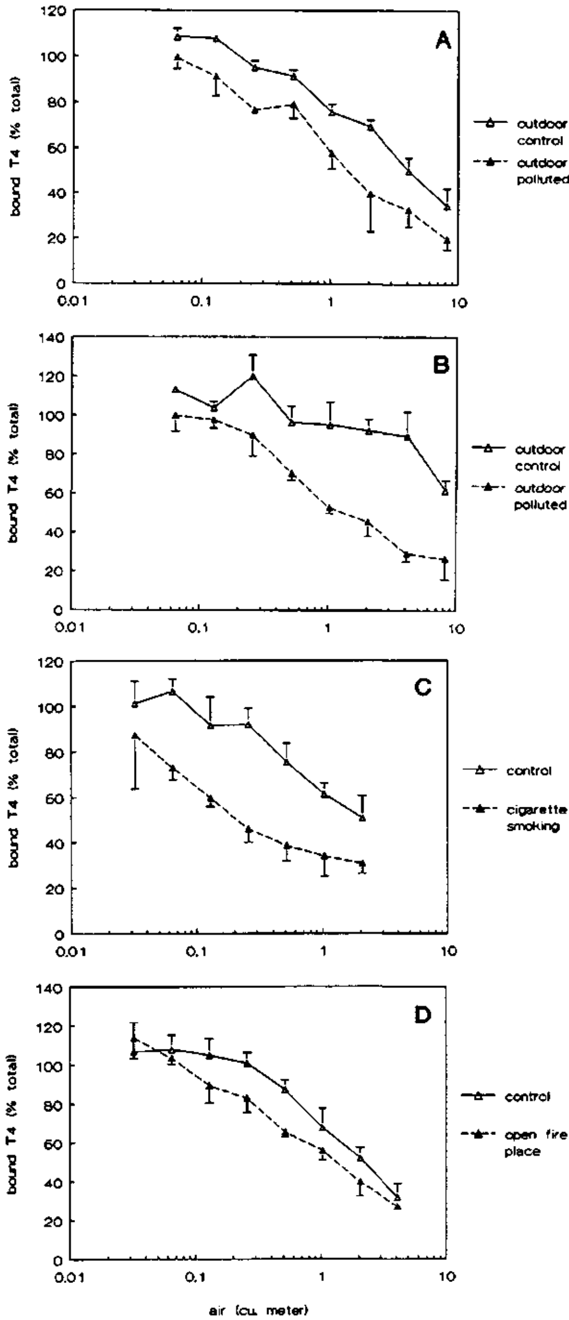

Figure 6.1 Inhibition of T4 binding to TTR by airborne particulate matter extracts. Data are the mean ± SD from two experiments. Concentrations correspond to the volume of sampled air. Outdoor samples were taken on different days: the control samples A and B on March 3 1990 and December 12 1990 respectively, the polluted samples A and B on January 27 1989 and November 25 1990 respectively.

Table 6.1 Effect of APM extracts on T4-TTR and T4-TBG binding

Sample	Concentration[b] (m^3)	% Bound T4[b] TTR	TBG
Outdoor (1)[a]	4.1	21.6	100[c]
	8.2	15.3	100
Outdoor (2)	4.1	45.7	100
	8.2	21.8	100
Outdoor[d](3)	0.06-8.2	IC_{50}=1.36 m^3	100
Outdoor[d](4)	0.06-8.2	IC_{50}=1.36 m^3	100
Cigarette	0.03-0.51	IC_{50}=0.09 m^3	100
Open fire place[d]	0.13-4.1	IC_{50}=1.28 m^3	100

[a] Outdoor no. 1 was taken at 23/11/90 when the wind direction was east; no. 2 was a pooled sample taken from 19-22/5/90 (east); no. 3 was taken at 25/11/90 (east); no. 4 was taken at 27/1/89 (south).
[b] Samples were tested in a concentration range -in which case the IC_{50} is given- or at the doses indicated in which case the corresponding values are given.
[c] Individual values ranged from 88.6-118.7.
[d] Values for T4-TTR inhibition were taken from figure 6.1.

Table 6.2 Effects of fractionation of APM extracts on T4 binding to TTR

Fraction	Extract Mass (mg)	Mass Distribution (%)	Activity IC_{50} (μg)	Distribution of Weighted Activity (%)[a]
Cigarette	139.5		28	
Aqueous I[b]	13.2	9.5	54	4.9
Base	5.3	3.8	45	2.4
Aqueous II	2.0	1.4	>>1000	<0.1
Acid	27.4	19.6	21	26.1
Neutral	88.8	63.7	29	61.5
Sum	136.7	98.0		94.9
Outdoor	100.8		20	
Aqueous I	10.0	9.9	311	0.6
Base	<1	<1	>>1000	<0.1
Aqueous II	5.5	5.5	>>1000	<0.1
Acid	3.2	3.2	10	6.4
Neutral	71.9	71.3	33	43.2
Sum	91.3	90.9		50.2

[a] Activity normalized to percent mass distribution of extract to total extractable mass. For example, the activity of the acid fraction of the cigarette sample (IC_{50}= 21 μg) is compared to the activity of the complete extract (IC_{50}= 28 μg), and then multiplied by the mass distribution of this fraction relative to the complete extract. Thus: 28 / 21 x 19.6% = 26.1%.

[b] For explanation, see Materials and Methods.

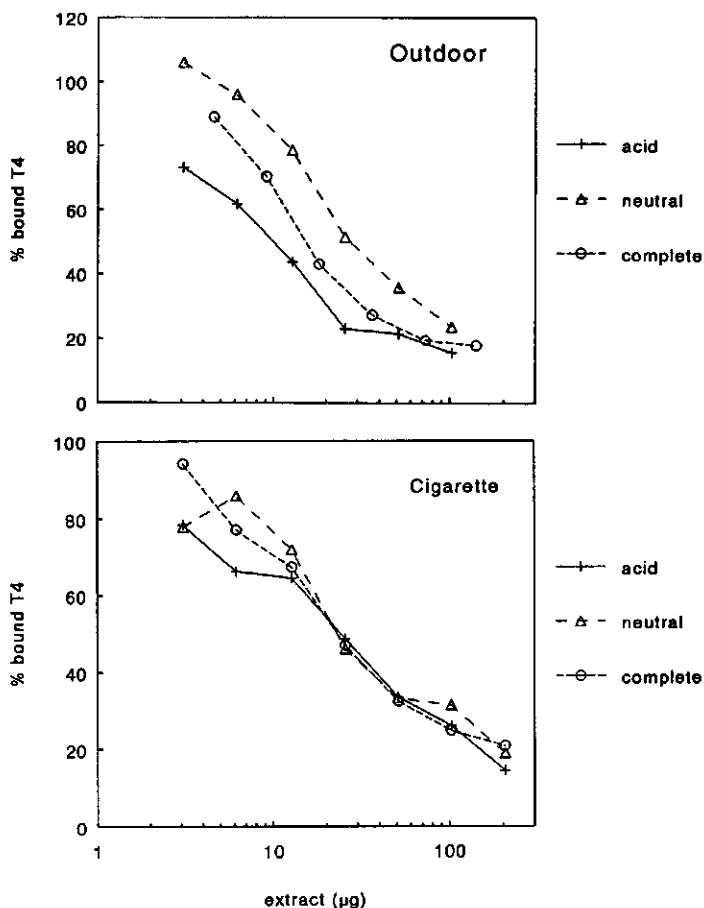

Figure 6.2 Inhibition of T4 binding to TTR by complete extracts, acid and neutral fractions of cigarette smoke and outdoor APM samples. Data are the mean from two independent experiments.

Effect of PAHs, nitroPAHs and nicotine on T4 binding to TTR

The two PAHs benzo(a)pyrene and pyrene did not interfere with T4 binding (Table 6.3). Of the nitroPAHs tested only 1-nitropyrene and 2,7-dinitrofluorene had an effect on T4 binding with an IC_{50} of 80.6 μM and $>>$ 532.9 μM respectively. Nicotine did also not inhibit T4 binding to TTR.

Table 6.3 Effects of PAHs, nitroPAHs and nicotine on TTR-T4 binding

Compound[a]	Concentration range (μM)			Inhibition[b]
Benzo(a)pyrene	16.0	-	1025.0	-
Pyrene	16.0	-	1025.0	-
1-Nitropyrene	16.0	-	512.5	±
1,3-Dinitropyrene	15.8	-	126.3	-
2-Nitrofluorene	24.2	-	775.6	-
2,7-Dinitrofluorene	133.2	-	532.9	±
Nicotine	2.0	-	252.6	-

[a] Compounds were tested in two independent experiments.
[b] - No inhibition of T4 binding, ± weak inhibition of T4 binding (IC_{50} of 1-nitropyrene and 2,7-dinitrofluorene were 80.6 μM and >> than 532.9 μM respectively).

Effect of cigarette smoke APM extract on thyroid hormone and vitamin A metabolism in rats

Two different doses of APM extract were tested. The low dose (43.2 mg extract/kg bodyweight) induced a significant reduction in plasma TT3 levels 6 and 24 h after animal treatment by 64 and 53%, respectively (Figure 6.3). At 6 h, TT3 levels were effected most. A comparable effect was seen for the high dose (81 mg extract/kg bodyweight) (Figure 6.4). Plasma TT4 levels were significantly reduced (by 39%) 6 h after treatment with the high dose. Seventy two h after treatment a significant increase by 46% was seen. The same trend was seen for the low dose. However, these effects were not significant. Significant reductions in FT4 levels by 47 and 25% were found in animals treated with the high dose 6 and 24 h after treatment respectively. The low dose induced a comparable non-significant effect. Because only the high dose caused significant effects on TT4 and FT4 levels, plasma and lung vitamin A levels were determined in animals treated with the high dose. Results showed a significant increase in plasma retinol levels 72 h after treatment (Figure 6.4). Lung retinol and retinyl palmitate levels were reduced in cigarette smoke extract exposed rats (Table 6.4). These reductions were however not statistically significant because of the large inter animal variations.

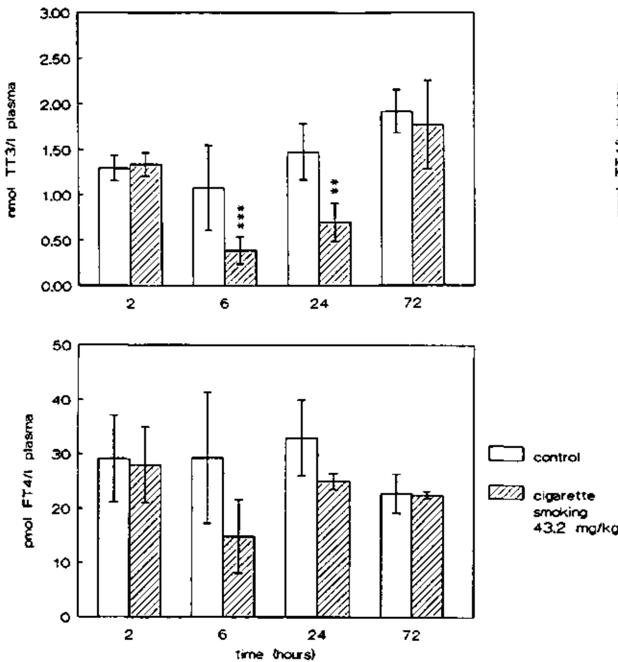

Figure 6.3 Effect of cigarette smoke APM extract (low dose) on plasma thyroid hormone levels in rats. Animals were intraperitoneally injected with 43.2 mg extract/kg body weight (corresponding to 272 m^3 of sampled air/kg) or solvent (0.546 ml DMSO/kg). Data are the mean $\pm$ SD from 3-5 animals. Asteriks indicate a statistical difference from control animals. ** $p<0.025$, *** $p<0.01$.

Effect of outdoor APM extract on thyroid hormone and vitamin A metabolism in rats

Because the cigarette smoke extracts induced the first noticeable effects on thyroid metabolism 6 hours after treatment, the effects 2 h after treatment were not studied for the outdoor APM extract. From the results in Figure 6.5 it can be observed that the low dose (81 mg/kg) significantly reduced TT3 plasma levels by 34% 6 h after treatment. Significant effects on TT4 and FT4 levels were not seen. However, the trend was the same as for the cigarette smoke sample and the high dose of the outdoor APM sample. This high dose (100 mg/kg) affected all three thyroid hormone parameters studied (Figure 6.6). The strongest effects were seen on plasma TT3 and FT4 levels. Six hours after treatment TT3, FT4 and TT4 levels were significantly reduced by 41, 39 and 28% respectively. Twenty four hours after treatment TT3 levels were still significantly reduced by 22%. At this time point no significant changes in TT4 and FT4 were found. At 72 h an increase was seen in TT4 and FT4 levels by 25 and 27% respectively. TT3 levels were not altered at this time point.

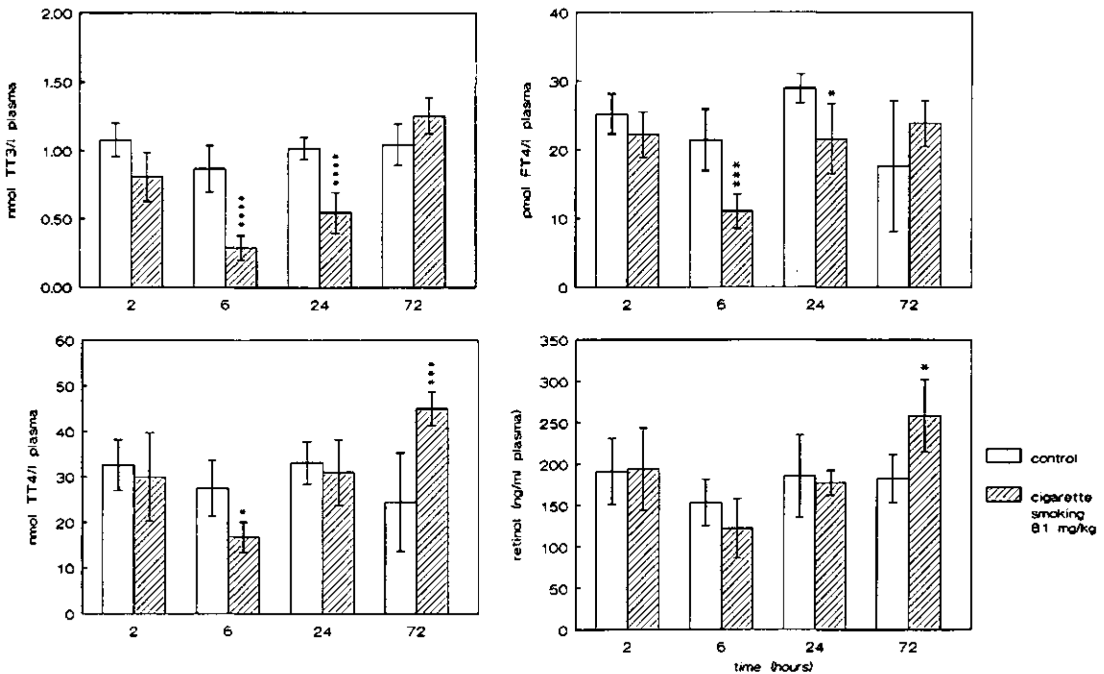

Figure 6.4 Effect of cigarette smoking APM extract (high dose) on plasma thyroid hormone and vitamin A levels in rats. Animals were intraperitoneally injected with 81 mg extract/kg body weight (corresponding to 502 m^3 of sampled air/kg) or solvent (1 ml DMSO/kg). Data are the mean $\pm$ SD from 4-5 animals. * $p<0.05$, *** $p<0.01$, **** $p<0.005$.

Significant changes in plasma vitamin A levels were not found after treatment of rats with the low dose (Figure 6.5). The high dose gradually increased plasma retinol levels over the time period studied, resulting in a significantly increase by 29% at 72 h after treatment (Figure 6.6). The low dose induced no significant changes in liver retinol and retinyl palmitate levels (Table 6.4). Lung retinol and retinyl palmitate levels were reduced by the low dose, but these reductions were not statistically significant because of the large inter animal variations. The high dose depleted lung retinol and retinyl palmitate levels significantly by 32 and 51% respectively (Table 6.4). Liver retinyl palmitate levels were not affected, but liver retinol levels were significantly reduced by 49%.

Table 6.4 Effect of APM extracts on rat liver and lung retinol and retinyl palmitate levels

Treatment[a]	organ (μg/g)[b]	retinol	retinyl palmitate (μg/g)[b]
Control	liver	ND	ND
Cigarette smoking 81 mg/kg	liver	ND	ND
Control	lung	0.13±0.10	2.50±3.76
Cigarette smoking 81 mg/kg	lung	0.07±0.07	0.80±0.43
Control	liver	25.9±17.5	205.8± 50.8
Outdoor 81 mg/kg	liver	50.4±42.1	235.9±104.5
Control	lung	0.41±0.23	2.00±2.41
Outdoor 81 mg/kg	lung	0.27±0.21	0.45±0.24
Control	liver	4.37±2.18	385.6±70.4
Outdoor 100 mg/kg	liver	2.24±0.88*	382.3±31.0
Control	lung	2.80±0.75	2.30±0.74
Outdoor 100 mg/kg	lung	1.90±0.92	1.11±0.45**

[a] Animals were intraperitoneally injected with APM extract.
[b] Data are the mean ± SD from 4-6 animals. ND, not determined. * and ** statistically significant from control, $p<0.05$ and 0.01 respectively.

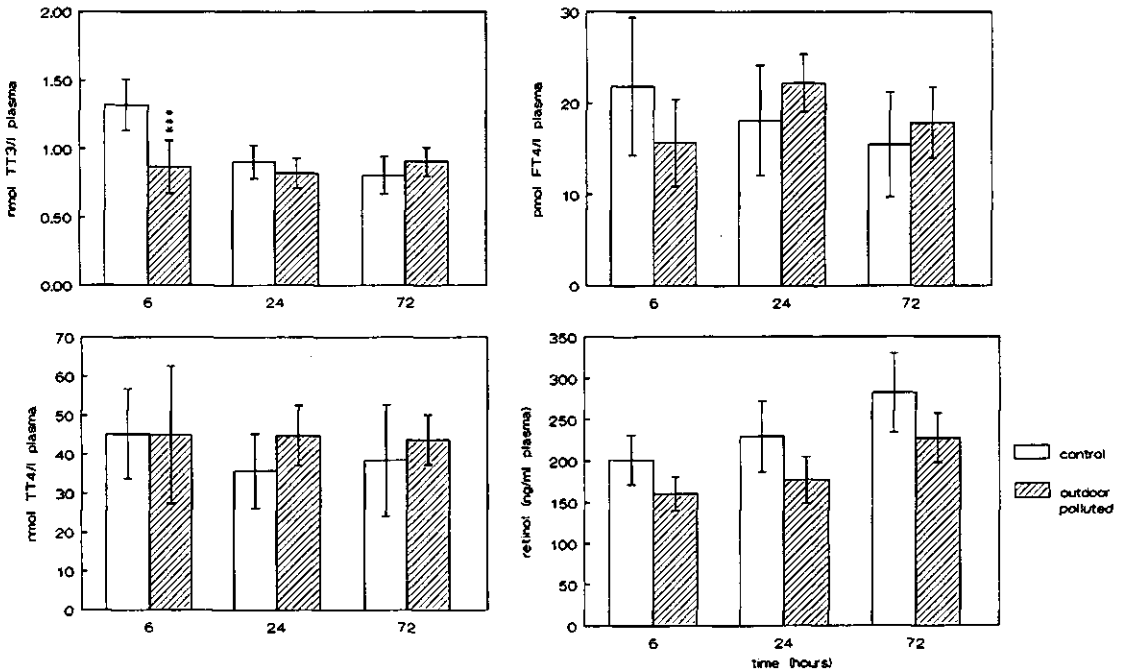

Figure 6.5 Effect of outdoor APM extract (low dose) on plasma thyroid hormone and vitamin A levels in rats. Animals were intraperitoneally injected with 81 mg extract/kg body weight (corresponding to 2222 m^3 of sampled air/kg) or solvent (1.27 ml propyleneglycol/kg). Data are the mean $\pm$ SD from 5 animals. *** $p<0.01$.

Discussion

The studies show that extracts of APM originating from different pollution sources inhibit T4 binding to TTR, indicating that the T4-TTR test may be used as a biomarker for studying effects on the kinetics of vitamin A. In all cases, the polluted sample had a more pronounced effect than the corresponding control sample. In order to identify the compounds present in APM which are responsible for the observed effect, several representatives of PAHs and nitroPAHs were tested for TTR interaction. Results showed that neither benzo(a)pyrene, nor pyrene, 2-nitrofluorene and 1,3-dinitropyrene inhibited T4 binding. On the contrary, 1-nitropyrene and 2,7-dinitrofluorene both did inhibit T4 binding. However, the concentrations at which these compounds had an effect

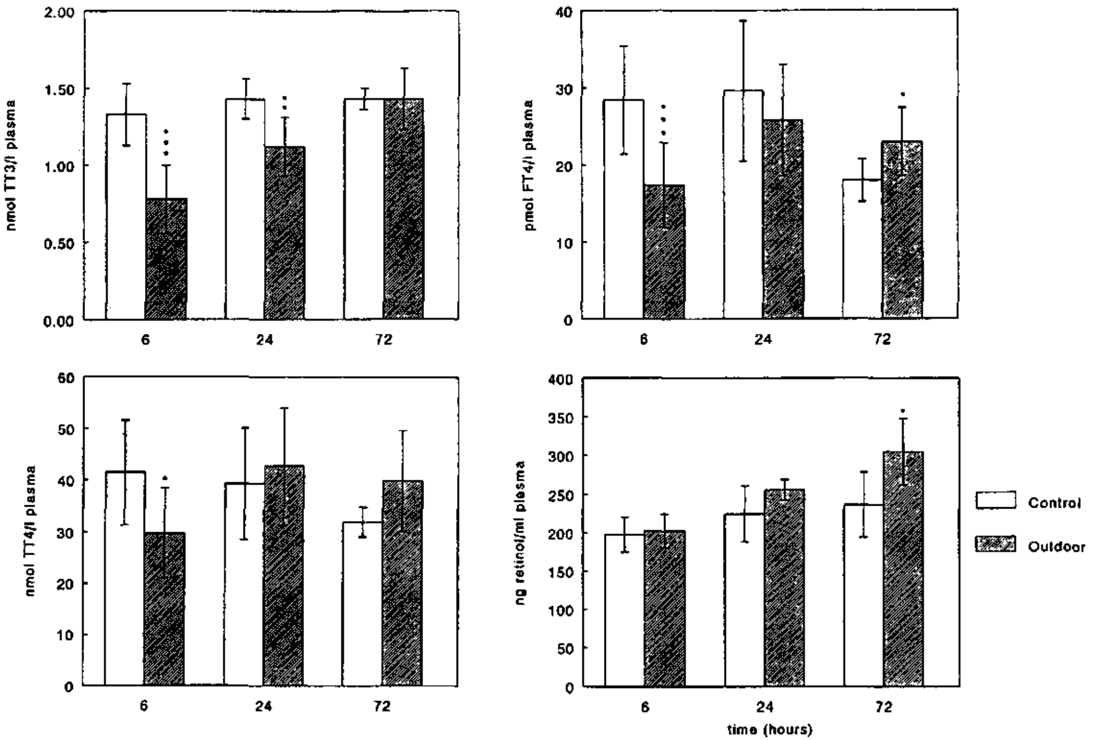

Figure 6.6 Effect of outdoor APM extract (high dose) on plasma thyroid hormone and vitamin A levels in rats. Animals were intraperitoneally injected with 100 mg extract/kg body weight (corresponding to 2743 m^3 of sampled air/kg) or solvent (1.57 ml propyleneglycol/kg). Data are the mean $\pm$ SD from 5-6 animals. * $p<0.05$, ** $p<0.025$, *** $p<0.01$.

were in the micromolar range. As levels of nitroPAHs in air are in the order of ng/m^3 (De Raat et al., 1987), this means that neither 1-nitropyrene nor 2,7-dinitrofluorene can be responsible, not even partly, for the effect caused by APM. Nicotine present in cigarette smoke APM also did not interfere with T4-TTR binding.

A compound that could be (partly) responsible for the interaction of APM with TTR is PCP. Indeed, PCP was detected in our cigarette smoke and outdoor APM sample. The amount of PCP detected in the outdoor APM sample is in agreement with literature data for rural air (IPCS, 1987). Taken into account a) the amount of PCP present in both the cigarette smoke and outdoor

APM sample, b) the IC_{50} of PCP of 2.3-4*10^{-8} M (Van den Berg, 1990; Den Besten et al., 1991) and c) the IC_{50} of both samples, the conclusion can be drawn that the amount of PCP in the samples is in itself not sufficient to account totally for the inhibition of T4 binding to TTR by these samples. The results clearly show that the activity is predominantly present in the neutral fraction. Despite the fact that 91% of the mass of the outdoor APM extract was recovered, the recovery of the weighted activity was only 50%. It seems unlikely that a loss of 8.7% of compounds accounts for 50% of the activity. A difference in activity of compounds in an unfractionated sample compared to its activity in subfractions might explain this phenomenon. It is noteworthy to mention that when fractionating APM samples, PAHs and nitroPAHs will partition in the neutral fraction. As our studies showed that several representatives of these compounds did not interact with TTR, other compounds present in the neutral fraction are likely to be responsible for the effect.

Although thyroid hormones play an important role in the growth and development of organs and in regulating basic metabolism (Henneman, 1986), there is no evidence at the present stage that these hormones are involved in the development of cancer. The only evidence obtained so far is that animal experiments indicate that there is a relationship between inhibition of thyroid homeostasis and the development of thyroid follicular cell neoplasia (for a review, see Hill et al. 1988). Nevertheless a disturbance in thyroid hormone metabolism may severely affect growth and differentiation. Both the cigarette smoke extract and the outdoor extract were able to induce changes in vivo in thyroid hormone metabolism. A time trend was visible for all measured thyroid hormone parameters: the reduction in thyroid hormones observed during the first 24 hours after animal treatment was followed by elevated levels of thyroid hormones after 3 days. This overcompensation may reflect a response of the thyroid gland in order to restore thyroid hormone homeostasis. As cigarette smoke and outdoor APM has been shown to inhibit T4-TTR binding in vitro, this suggests that the reduction in FT4 and TT4 levels in vivo is caused by the same phenomenon. It was recognized that TT3 levels were reduced most by APM. T3 is formed from T4 enzymatically by deiodination in the liver by 5'-deiodinase activity (Visser et al. 1984). Because T4 levels were reduced, this could mean that the amount of T4 available for deiodination was limited. In addition, it might be that outdoor APM is able to interfere in hepatic thyroid hormone metabolism by inhibiting 5'-deiodinase activity. As a consequence, the formation of T3 may be reduced, which may lead to decreased levels of TT3.

A decrease in liver vitamin A was found in rats and mice which were exposed for 5 weeks to 1 cigarette/day by Khanduja et al. (1984). However, no explanation was given for the mechanism of vitamin A depletion. Our hypothesis was that APM via interaction with TTR would deplete vitamin A. Both extracts tested significantly increased plasma retinol levels 72 hours after animal treatment. Although in general an inhibition of TTR-RBP binding results in decreased plasma retinol levels, this does not necessarily mean that the APM extract did not interfere with TTR-RBP binding. An effect on plasma retinol levels is in principle dependent upon two independent effects: a) increased mobilization of retinol from the liver and lung store into the circulation or b) inhibition of TTR-RBP binding. Lung and liver retinol and lung retinyl palmitate levels were reduced, suggesting an enhanced mobilization from these storage sites. Such a mobilization might be caused by an interaction of the APM extract with the enzymes retinyl palmitate hydrolase (responsible for the hydrolysis of retinyl esters into retinol) and acyl-CoA:retinol acyl transferase (responsible for the esterification of retinol into retinyl esters), shifting the balance in activities of these two enzymes towards an increased hydrolysis of retinyl esters and subsequent release of retinol into the circulation. A decrease in plasma retinol levels by interaction of APM with the TTR-RBP complex might therefore have been overcome by an enhanced mobilization from liver and lung retinol.

If its true that depletion of vitamin A increases the susceptibility for lung cancer, than APM exposure may contribute to an enhanced vulnerability for lung cancer. Further studies will be undertaken in order to test this hypothesis.

Another goal of our studies was to compare the effects of APM on T4-TTR and T4-TBG binding. Of the six extracts tested, none interfered with T4-TBG binding, while T4-TTR binding was clearly inhibited. The T4 binding sites on TBG are less well defined than the sites on TTR, which has been characterized extensively by X-ray diffraction (Blake and Oatley, 1977). In contrast, a hydroxy group in meta or para position on an aromatic ring with one or two adjacent halogen atoms has been identified as important structural features for TTR interaction. Data on interaction of environmental chemicals with TBG are scarce. PCP has hardly any affinity for TBG (about 1000x less than for TTR, Van den Berg, 1990), while tetra- and pentachlorobiphenyl hydroxy metabolites have no affinity at all (Willemsen et al., 1991). The afore-mentioned structural features for TTR interaction seem not to be beneficial for TBG interaction. We hypothesized that a complex mixture such as APM contains compounds that possess at present unknown struc-

tural features beneficial for TBG interaction. The fact that APM interacted with TTR but not with TBG was therefore surprising. This suggests that (some of) the compounds present in APM that interact with TTR might do so specifically because of the already mentioned structural features. In addition, because PAHs and nitroPAHs, compounds that do not possess these structural features, do not interact with TTR, this again suggests that the interaction of APM with TTR is specific.

The fact that APM interacted with TTR but not with TBG may have different consequences for the effects on thyroid hormone metabolism in rodents compared to humans. TBG is the major plasma carrier for thyroid hormones in man, and this carrier is lacking in rodents. This could mean that a displacement of T4 from TTR in humans might be counteracted by TBG, which may result in less severe effects on thyroid hormone status. However, such a displacement could still imply that the interaction with the RBP-retinol complex is weakened, resulting in a depletion of vitamin A.

In conclusion, the major findings of this study are that APM has the potency to interfere with thyroid hormone and vitamin A metabolism in vivo, leading to a depletion of liver and lung levels of retinol. The toxicological consequences of this phenomenon have been disscussed.

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CHAPTER 7

CARCINOGENICITY STUDY OF OUTDOOR AIRBORNE PARTICULATE MATTER IN NEWBORN MALE NMRI MICE

Summary

An organic extract of airborne particulate matter (APM) was tested for carcinogenicity at two dose levels in the newborn mouse bioassay. The samples used were taken under specific polluted conditions. The doses tested corresponded with 0.75 and 1.5 times the amount of air man inhales during lifetime. Benzo(a)pyrene which was used as a positive control significantly increased the lung tumor incidence. No evidence was found for a carcinogenic activity of the organic extract of APM. Considering the high dose of APM applied in this animal model and the much lower actual cumulative dose to which man is exposed to in many areas, the conclusion can be drawn that exposure to APM alone probably does not represent an important cancer risk for man.

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Introduction

For more than a decade outdoor airborne particulate matter (APM) has been sampled at various urban, industrial and rural locations to study mutagenicity. Extracts of APM derived from these locations induced mutations in vitro in microbial systems (Dehnen et al., 1977; Pitts et al., 1977; Talcott and Wei, 1977; Tokiwa et al., 1977) and sister-chromatid exchanges (Alink et al., 1983; De Raat, 1983; Krishna et al., 1984), chromosomal aberrations (Hadnagy et al., 1989; Hadnagy and Seemayer, 1986) and cell transformation in mammalian cell systems (Seemayer et al., 1986). In addition to this initiating activity, recent in vitro studies suggest that extracts of APM taken in a non-industrial area also may have a tumor promoting activity (Heussen, 1991; Heussen and Alink, 1992).

The studies mentioned above suggest that APM might pose a cancer risk to man. Outdoor APM is a complex mixture of thousands of organic compounds distributed over many chemical classes (e.g. Cautreels and Van Cauwenberghe, 1976; Schuetzle and Daisey, 1990) among which are many carcinogenic compounds. Considering the complexity of the aerosol mixture present in the environment, it is impossible to do carcinogenicity experiments with all the individual constituents and their combinations. The only feasible approach is to study the carcinogenic potential of the complete mixture. The present study was undertaken in order to investigate whether an average polluted outdoor APM sample taken in a non-industrial site (Wageningen, see Alink et al., 1983; Van Houdt et al., 1987) is able to induce lung tumors in the newborn mouse lung adenoma bioassay. This assay was chosen as an experimental animal model (Busby et al., 1985) because newborn mice compared to adult mice are highly sensitive to chemical carcinogens and produce lung tumors with relatively short latency periods. In addition, this bioassay is very sensitive for the measurement of carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) and nitroPAHs (Busby et al., 1988, 1989), compounds known to contribute to the mutagenicity of APM. The carcinogenic PAH benzo(a)pyrene (BaP) was used as a positive control.

Materials and Methods

Chemicals

Benzo(a)pyrene (BaP, purity approximately 98%) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Propylene glycol was obtained from Baker Chemicals (Deventer, The Netherlands).

Sample Collection and Extraction

APM was collected in the winter period of 1990-1991 in Wageningen and extracted as described in Chapter 2. The residue was dissolved in propylene glycol (PG). The outdoor sample was a pooled sample composed of twelve 24-h samples (Table 7.1). In Wageningen high mutagenic activity of outdoor APM is positively correlated with low temperatures and southerly/easterly wind directions. The latter conditions bring air masses over Wageningen that have passed industrial areas. Hence mutagenicity and other typical air pollution parameters are increased (Alink et al., 1983; Van Houdt et al., 1987). The BaP content and mutagenicity of the sample were determined as described in Chapter 3 and 5 respectively. The sample contained 22 ng BaP/mg extract (corresponding to 0.9 ng BaP/m^3 of sampled air) and induced 604 (without metabolic activation) and 687 (with metabolic activation) rev/mg extract.

Carcinogenicity assay

A total of 218 newborn male outbred NMRI mice (negative for Kilham's rat virus, Sendai virus, Reovirus type 3, Theiler (GD VII), PVM and Mycoplasma pulmonis) were used. The mice were divided into five groups and injected intraperitoneally with the vehicles propylene glycol (PG, 52 animals) and corn oil (18 animals), with the positive control BaP (dissolved in corn oil, 52 animals) or APM extract (dissolved in PG, 47 animals treated with the high dose, 49 animals with the low dose) in a total volume of 35 μl vehicle over a 2 week period. Treatment started on day 1 of life of mice with 5 μl vehicle, followed by a second injection of 10 μl vehicle on day 8 and a final injection on day 15 with 20 μl vehicle. The total dose for BaP was 0.40 μmol (=100 μg). The high APM dose (95 m^3 of sampled air/mouse) corresponds to 1.5 times the amount of air as has been inhaled by an adult person during lifetime (70 years, 70 kg). This dose

corresponded with the maximum amount of extract which was readily soluble in PG. The low APM dose corresponded to 0.75 times the amount of air inhaled during lifetime.

Mice were weaned at an age of 3 weeks and housed in macrolon cages type 3 with wood chip bedding under controlled conditions of temperature (21±1°C), relative humidity (55±5%) and light (12h light-12 dark cycle). They were fed water and RMH-B diet (Hope Farms, Woerden, The Netherlands) ad libitum. From weaning until week 12 individual mice weights were recorded weekly. From week 12-32 and from week 32-52 they were weighed every 4 and 2 weeks respectively.

After 1 year mice were killed by exsanguination under deep ether anaesthesia. At necropsy, mice were subjected to detailed post-mortem examination. Lungs and livers were weighed. Lungs were examined again under a dissection microscope with special attention paid to the possible occurrence, localization and multiplicity of lung masses. The lungs, livers and all macroscopic abnormalities were removed and fixed in a 4% buffered neutral formaldehyde solution. The lungs were trimmed, processed, embedded in paraffin wax and sectioned. Of each animal the five lung lobes were blocked together and three standardized sections were prepared. All tumor suspected lung masses noted upon macroscopy were sectioned separately. All gross abnormalities from all groups were sectioned. Sections were stained with haematoxylin and eosin and examined microscopically. Pulmonary tumors were classified as alveolar type II cell adenomas or carcinomas or as bronchiolar adenomas or carcinomas.

Statistics

Statistical analysis of tumor incidence was performed with a generalized linear model using log transformation (GLIM 3.77, Royal Statistical Society, London, UK). Body and organ weights were analysed by means of a Dunnet test.

Results

Figure 7.1 shows average weight increments of the 5 treatment groups. Terminal body weights were significantly increased in the high dose APM group ($p<0.01$). No statistically significant differences in lung and liver weights were recorded.

Table 7.1 Relevant meteorological conditions during collection of the samples which were pooled

Sample date	Mean temperature (°C)	Mean wind direction (grades)
17/12/90	0.2	60
14/01/91	0.2	60
15/01/91	0.7	90
16/01/91	-1.0	120
28/01/91	2.3	90
29/01/91	-1.2	90
30/01/91	-0.2	110
31/01/91	-1.8	130
01/02/91	-4.2	110
02/02/91	-5.5	0
03/02/91	-7.0	40
04/02/91	-4.4	60

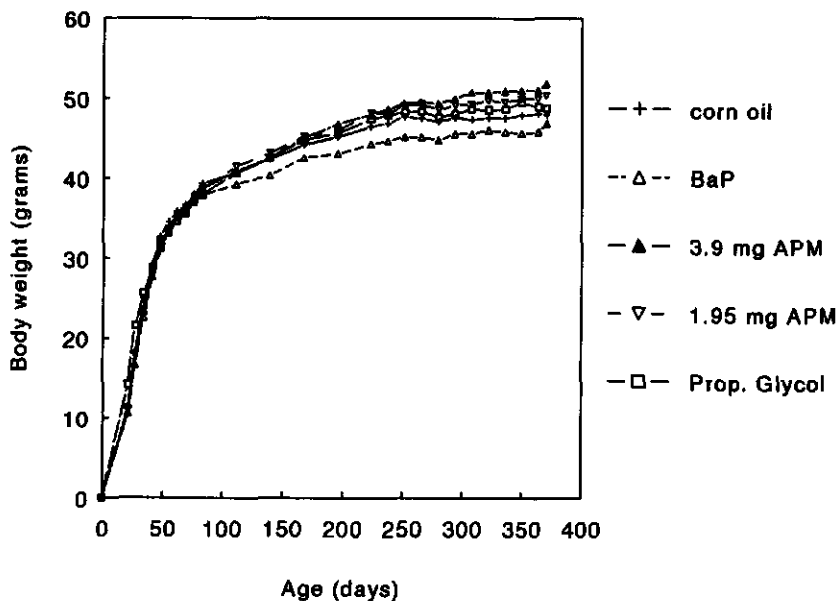

Figure 7.1 Average body weights of animals treated with vehicles, BaP or APM.

No mortality before weaning was observed. After weaning intercurrent mortality rarely occurred, i.e.: 3 animals, age 11 months, BaP group; 1 animal age 11 months, high dose APM group; 2 animals, age 7 and 8 months, low dose APM group.

Microscopical examination of non-pulmonary lesions revealed a sowewhat higher incidence of hepatocellular hyperplastic foci and adenomas in the BaP group in comparison with the other groups (data not shown), which showed very few hepatocellular lesions. Other non-pulmonary tumor incidences were low in all groups.

In the BaP group the mean number of pulmonary tumors per mouse was significantly increased as compared to the corn oil control group ($p = 0.036$; Table 7.2). This difference was mainly due to alveolar adenomas. The increase in alveolar adenomas was not significant ($p = 0.065$). No statistical significant changes were observed in the APM-dosed groups, although the high dose slightly increased the total number of tumors ($p = 0.17$), particularly alveolar adenomas, whereas the low dose decreased the total number of tumors compared to the control ($p = 0.15$). Considering the different tumor types, the number of bronchiolar adenomas was lower in the low-dose APM group than in the control group, but this decrease was not significant. Carcinomas were very seldom observed and there was no relation to any treatment.

The number of mice bearing more than one pulmonary tumor was increased in the BaP group. In the high-dose APM group, multiplicity was slightly higher than in the PG control group.

Discussion

The present study shows that the positive control group BaP was able to induce lung tumors in newborn male NMRI mice. The major tumor type found was classified as alveolar type II adenoma. Bronchiolar adenomas were less frequently observed and lung carcinomas were rare. Gunning et al. (1991) reported the same type of tumor distribution after a single treatment with BaP of 6 weeks old male A/J mice. Treatment of newborn Swiss-Webster BLU:Ha mice with BaP, in a dosage protocol identical to ours, induced only lung adenomas (Busby et al., 1988, 1989). In a comparable study with CD-1 mice again only lung adenomas were found (Wislocki et al., 1986). In these

Table 7.2 Summary of microscopic neoplastic pulmonary lesions.

Dose group	Control[a]	Control[b]	BaP	APM 3.9 mg	APM 1.95 mg
no. of animals	52	18	52	47	49
no. of tumor bearing mice	16	4	21	17	10
% of tumor bearing mice	30.8	22.2	40.4	36.2	20.4
total no. of tumors	16	4	30	20	10
% of alveolar adenomas	15.4	16.7	42.3	29.8	14.3
% of bronchiolar adenomas	13.5	5.6	11.5	10.6	4.1
% of alveolar carcinomas	0	0	1.9	0	0
% of bronchiolar carcinomas	1.9	0	1.9	2.1	2.0
mean no. of tumors/mouse	0.31	0.22	0.58[c]	0.43	0.20
multiplicity					
% of mice with 1 tumor	30.8	22.2	28.9	31.9	20.4
% of mice with 2 tumors	0	0	7.7	4.3	0
% of mice with 3 tumors	0	0	1.9	0	0

[a] Propylene glycol
[b] Corn oil
[c] Statistically different from corn oil group ($p = 0.036$)

studies lung adenomas were not further characterized as alveolar or bronchiolar adenomas. The fact that only adenomas and no carcinomas were found is in agreement with the other studies. Comparison of our data on lung tumor incidence with the already mentioned studies of Busby et al. and Wislocki et al. is somewhat difficult because of lack of dose-response curves, but it seems that the sensitivity for BaP of our newborn mouse model is somewhat less.

In the present study outdoor APM samples were taken when air pollution levels were increased. The pooled samples used appeared to be highly mutagenic. However, when tested in the newborn mouse assay no significant increase in carcinogenicity was observed. Only at the high dose there was a slight non-significant increase of the total number of lung tumors, while at the low dose there was a slight non-significant decrease of the total number of lung tumors. The overall conclusion is that the mutagenic activity does not appear to correspond with increased incidence of pulmonary tumors.

These results confirm earlier observations by showing that APM should not be considered as a potent initiator. Several studies with newborn mice have been reported in literature, but lung tumors in surviving mice were only found at lethal doses of APM (Asahina et al., 1972; Epstein et al., 1966, 1979; Leiter et al., 1942; Leiter and Shear, 1942; Sasaki et al., 1987). In the study of Epstein et al. (1979) subcutaneous injection of a non-toxic dose of urban APM in newborn mice gave rise to a small increase in pulmonary adenocarcinomas. The BaP content of their sample was 25 times higher than of our sample and because they treated the mice with 2.5 times as much extract as we did, the total dose of BaP was 62.5 times higher. Unfortunately, the amount of air corresponding with the BaP dose was not mentioned. Finally, in the study by Pott et al. (1980) in which non-toxic doses of urban outdoor APM were injected subcutaneously in adult mice, only local tumors were induced, which is generally not considered as a valid evidence for carcinogenicity (IARC, 1980, 1986).

In conclusion, in the present study no evidence for a carcinogenicity of APM was observed. As the APM samples were taken under specific conditions of high air pollution and man normally is exposed to much lower levels, the conclusion can be

drawn that exposure to APM alone probably does not pose an important cancer risk to man.

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CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

The mutagenicity of indoor and outdoor airborne particulate matter (APM) has been demonstrated by previous in vitro studies (Alink et al., 1983; Van Houdt et al., 1984, 1986, 1987). The aim of the present thesis was to contribute to a better understanding of the mode of action of APM in the pathogenesis of lung cancer by expanding the scope to the in vivo initiating (Chapter 3 and 4), tumor promoting (Chapter 5 and 6) and carcinogenic (Chapter 7) potential of APM.

The aromatic DNA adduct level in white blood cells was used as a biomarker for measuring the exposure of man to residential wood combustion particulate matter under conditions which most likely reflect the pattern of use of open fire places in The Netherlands (Chapter 3). Although indoor air mutagenicity and benzo(a)pyrene (BaP) and pyrene levels were increased, no combustion-related increase in the amount of adducts was observed. This indicates that these levels of aromatic compounds are probably not an important source of genotoxic damage to DNA.

In Chapter 4 experiments are described in which outdoor as well as indoor APM extracts were tested for in vivo genotoxicity in the DNA-repair host-mediated assay. Although the APM extracts were genotoxic in vitro, high doses of APM induced no genotoxic activity in mice. Because polycyclic aromatic hydrocarbons (PAHs) and nitroPAHs are known to contribute to the mutagenicity of APM, BaP and 2-nitrofluorene (NF) were used as positive controls. High concentrations of BaP caused a moderate effect in mice whereas NF was not genotoxic at all in vivo. It was concluded that the DNA-repair host-mediated assay is (relative) insensitive for PAHs and nitroPAHs. In addition, deactivation of APM in vivo might be responsible for the lack of genotoxicity observed.

Because it is generally accepted that carcinogenesis is a multi-step process we also studied the tumor promoting potential of APM. Inhibition of gap-junctional intercellular communication (GJIC) was used for measuring tumor promoting effects (Chapter 5). We showed that outdoor as well as indoor APM strongly inhibited GJIC in V79 Chinese hamster lung fibroblasts. Mutagenicity data, obtained with Salmonella typhimurium TA 98, and the data on inhibition of GJIC were correlated, and the concentrations at which a positive effect was found, were in the same range. V79 cells lack the capacity to metabolize foreign compounds. Thus the effects on communication

were caused by unmetabolized compounds. We also showed that communication was strongly inhibited in primary cultures of rat alveolar type II cells, a target cell for inhaled particles. Type II cells do have the capacity to metabolize foreign compounds, but it is unknown whether parent compounds and/or their metabolites inhibited IC.

Because vitamin A is an important regulator of normal epithelial differentiation and proliferation and a disturbance in its kinetics might influence the process of carcinogenesis, interactions of APM with the kinetics of vitamin A were studied (Chapter 6). A single high dose of APM extract increased plasma retinol levels in rats whereas lung vitamin A levels were depleted. Such a depletion might indicate an increased susceptibility for the development of lung tumors.

Finally, the carcinogenicity of outdoor APM in the newborn mouse bioassay was studied (Chapter 7). The APM samples were taken under conditions of increased air pollution. Because man is exposed to such high pollution levels only for a limited amount of time, the dose levels of APM applied in this animal model were an overestimation compared to the actual cumulative dose man is exposed to. BaP, used as a positive control, significantly increased the lung tumor incidence. On the contrary no evidence was found for a carcinogenic response to APM.

In a recent assessment of the state of the art in chemical carcinogenesis by Gori (1992) it is mentioned that initiation may not solely be of a mutagenic nature, but may more reflect a shift of the normal behavior of cells towards an adaptation to stressors adverse to cell and organism homeostasis, mediated by epigenetic events. It is further stated in that study that "initiation may be a trivial step in carcinogenesis, given that all cells in an organism are exposed since embryonic development to multiple initiator stresses, either physiologically generated of from the myriad of naturally ocurring external encounters." The idea of Gori implicates also that the initiating properties of chemicals might be of less importance then generally is assumed. All this would plead for a more important role of tumor promotion in the process of carcinogenesis. For cigarette smoking this indeed seems to be the case. For people who stop smoking the risk freezes at the time of quitting without further advantages, suggesting a promotional role of smoking. Upon smoking cessation the effects of nongenotoxic promoting agents abruptly stop. As a consequence preneoplastic lesions remain static or regress, whereas in the continuing smoker they progress (Doll and Peto, 1978; Weisburger, 1990). Furthermore, from epidemiological data of cigarette smoking and lung cancer it can be concluded that a threshold exists at measurable doses, again pleading for an important role of tumor promotion (Gori and Mantel, 1991).

As in the studies described in this thesis no evidence is found for an in vivo initiating or carcinogenic potential of APM, it might be that analogous with cigarette smoke also for APM the tumor promoting potential might be of more importance than the initiating potential. Therefore it is concluded that exposure to APM alone probably does not pose an important cancer risk to man. However, in combination with other factors a contribution of APM to the development of cancer can not be excluded.

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CHAPTER 9

SAMENVATTING EN SLOTBESCHOUWINGEN

Luchtverontreinigende componenten kunnen in de atmosfeer voorkomen als gassen of gebonden aan deeltjes, zogenaamde aerosolen. Aerosolen komen algemeen voor in buiten- en binnenlucht, waardoor de mens continu wordt blootgesteld. De concentraties kunnen echter aanzienlijk varieren in tijd en plaats. Volgens huidige wetenschappelijke inzichten kan blootstelling aan aerosolen mogelijk een gezondheidsrisico voor de mens betekenen, in het bijzonder voor het respiratoire systeem. In dit proefschrift ligt de nadruk op één van de meest gevreesde respiratoire ziekten, namelijk longkanker. Uit epidemiologische studies is bekend dat longkanker meer in stedelijke dan in landelijke gebieden voorkomt. Omdat in de jaren vijftig werd gevonden dat aerosolextracten afkomstig uit stedelijke gebieden tumoren in muizen konden veroorzaken, werd gedacht dat blootstelling aan aerosolen een risicofactor zou kunnen zijn voor het ontstaan van longkanker. Of aerosolen inderdaad een risicofactor vormen, is echter nog steeds onzeker. Deze onzekerheid wordt vooral veroorzaakt door het feit dat de huidige epidemiologische technieken en toxicologische modellen beiden hun beperkingen kennen. Bovendien is de chemische en fysische samenstelling van aerosolen zeer variabel, wat de onzekerheid nog verder vergroot.

Over de in vitro mutageniteit van binnen- en buitenluchtaerosol is reeds het nodige werk verricht op onze vakgroep (Alink et al., 1983; Van Houdt et al., 1984, 1986, 1987). Het doel van dit proefschrift was om bij te dragen aan een beter begrip van de werking van aerosolen in het proces van het onstaan van longkanker door de in vivo initiërende (Hoofdstuk 3 en 4), tumor promoverende (Hoofdstuk 5 en 6) en carcinogene potentie (Hoofdstuk 7) van aerosolen te onderzoeken.

In hoofdstuk 3 zijn experimenten beschreven waarin vrijwilligers werden blootgesteld aan binnenluchtaerosol door het gebruik van hun open haard. Het gebruik van de open haard was voor de Nederlandse situatie zo realistisch mogelijk. Dit betekent dat de haard niet zozeer voor verwarmingsdoeleinden werd gebruikt, maar meer voor de gezelligheid, en dus een beperkt aantal uren per dag brandde. Als biomarker voor de blootstelling aan aromatische stoffen werden in witte bloedcellen van blootgestelde personen DNA adducten bepaald. Hoewel de binnenlucht-mutageniteit en de concentraties aan benzo(a)pyreen (BaP) en pyreen verhoogd waren, werden er geen adducten gevonden die gerelateerd konden worden aan het gebruik van de open haard.

Dus de concentraties aan aromatische stoffen gemeten onder deze omstandigheden leiden waarschijnlijk niet tot een genotoxisch risico.

De in vivo genotoxiciteit van aerosolextracten werd getest met behulp van de DNA-repair host-mediated assay (Hoofdstuk 4). Hoewel de extracten in vitro genotoxisch waren, induceerden hoge doses aerosolextracten geen genotoxiciteit in muizen. Omdat polycyclische aromatische koolwaterstoffen (PAKs) en nitroPAKs bijdragen aan de mutageniteit van aerosolen, werden BaP en 2-nitrofluoreen (NF) als positieve controles getest. Hoge concentraties van BaP veroorzaakten een geringe genotoxiciteit in muizen, terwijl NF in het geheel niet genotoxisch was. Geconcludeerd werd dat de DNA-repair host-mediated assay (relatief) ongevoelig is voor PAKs en nitroPAKs. Bovendien kan deactivatie van aerosolen verantwoordelijk zijn voor de negatieve resultaten.

Omdat algemeen wordt aangenomen dat carcinogenese een meerstapsproces is, werd ook de tumor promoverende potentie van aerosolen bestudeerd. Remming van de intercellulaire communicatie door middel van gap-junctions (GJIC) werd gebruikt als een model voor tumor promotie (Hoofdstuk 5). Uit de studies bleek dat zowel buiten- als binnenluchtaerosol de GJIC sterk remde in V79 Chinese hamster long fibroblasten. De mutageniteit, gemeten met behulp van Salmonella typhimurium TA 98, en de remming van de GJIC waren sterk gecorreleerd. Bovendien lagen de concentraties waarbij een positieve respons werd gevonden in dezelfde range. V79 cellen missen de capaciteit om vreemde stoffen te metaboliseren. Daarom zijn de effecten op de GJIC veroorzaakt door ongemetaboliseerde stoffen. Uit de studies bleek verder dat de GJIC sterk geremd was in primaire cultures van ratten alveolaire type II cellen, een doelcel voor geïnhaleerde deeltjes. Type II cellen bezitten wel de capaciteit om vreemde stoffen te metaboliseren, maar het is onbekend of oorspronkelijke stoffen en/of metabolieten de GJIC remden.

Omdat vitamine A een belangrijke regulator is van de normale epitheliale differentiatie en proliferatie en een verstoring van de kinetiek het proces van de carcinogenese kan beïnvloeden, werden interacties van aerosolen met de kinetiek van vitamine A bestudeerd (Hoofdstuk 6). Toediening aan ratten van een hoge éénmalige dosis aerosolextract verhoogde het plasma retinol gehalte terwijl het vitamine A gehalte in de longen verlaagd werd. Een dergelijke verlaging kan betekenen dat de gevoeligheid voor het ontstaan van longtumoren toeneemt.

Tenslotte werd de carcinogeniteit van buitenluchtaerosol in de newborn mouse bioassay bestudeerd (Hoofdstuk 7). Buitenluchtaerosolen werden verzameld onder condities van hoge luchtverontreiniging. Mensen worden slechts gedurende een beperkte tijd aan zulke condities blootgesteld. Daarom

zijn de doses die in het diermodel gebruikt werden een overschatting van de reële cumulatieve dosis waaraan de mens gedurende zijn hele leven wordt blootgesteld. BaP, dat gebruikt werd als een positieve controle, verhoogde significant de longtumor incidentie. Daarentegen werden er geen aanwijzingen gevonden voor een carcinogene activiteit van het aerosolmonster.

In een recente beschouwing over de huidige stand van zaken in de chemische carcinogenese door Gori (1992) wordt gesteld dat mutaties niet de enige oorzaak zouden zijn van initiatie. Initiatie zou ook beschouwd kunnen worden als een verschuiving van het normale gedrag van cellen naar een adaptatie aan stressoren die bedreigend zijn voor de homeostase van cellen en weefsels. Een dergelijke adaptatie zou gemediëerd kunnen worden door epigenetische factoren. Bovendien wordt in bovenstaande studie gesteld dat "initiatie wel eens een onbelangrijke stap in de carcinogenese zou kunnen zijn, gegeven het feit dat alle cellen in een organisme sinds de embryonale ontwikkeling blootgesteld worden aan allerhande initiërende stressoren, die of van fysiologische oorsprong zijn of afkomstig van het grote aanbod vanuit de natuurlijke omgeving." De opvatting van Gori impliceert tevens dat de initiërende werking van stoffen wel eens minder belangrijk zou kunnen zijn dan tot nu toe werd gedacht. Dit alles zou pleiten voor een belangrijker rol van de tumor promotie in het proces van de carcinogenese. Voor sigarettenrook lijkt dit inderdaad het geval te zijn. Bij mensen die stoppen met roken wordt het risico op dat moment bevroren, het risico neemt dus niet verder toe, hetgeen een aanwijzing vormt voor een tumor promoverende werking. De effecten van niet-genotoxische tumor promoverende agentia houden na het stoppen met roken op. Het gevolg is dat preneoplastische lesies statisch blijven of in regressie gaan, terwijl bij mensen die blijven roken een verdere progressie van de lesies optreedt (Doll en Peto, Weisburger, 1990). Bovendien kan op grond van epidemiologische gegevens over het roken van sigaretten en het ontstaan van longkanker worden afgeleid dat er een drempeldosis bestaat, wat ook weer pleit voor een belangrijke rol van tumor promotie (Gori en Mantel, 1991).

Omdat in de studies in dit proefschrift geen aanwijzingen zijn gevonden dat aerosolen een in vivo initiërende of carcinogene potentie bezitten, zou het kunnen zijn dat net als bij sigarettenrook de tumor promoverende potentie van aerosolen belangrijker is dan de initiërende potentie. Daarom is blootstelling enkel en alleen aan aerosolen waarschijnlijk geen belangrijke risicofactor voor het ontstaan van kanker. Echter, in combinatie met andere factoren kan een bijdrage van aerosolen aan het ontstaan van kanker niet worden uitgesloten.

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