DNA-adduct kinetics, cell proliferation and precancerous changes in benzo[a]pyrene-treated hamster trachea in organ culture

Vorming en verwijdering van DNA-adducten, celvermeerdering en voorstadia van kanker in de luchtpijp van de hamster, na blootstelling aan benzo[a]pyreen in orgaankweek

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. J.A. van Ginkel
ingevolge het besluit van het College van Decanen
in het openbaar te verdedigen
op dinsdag 13 september 1994 des middags te 2.30 uur

door

Robert Roggeband

geboren op 11 december 1962, te Rotterdam

Promotoren: Prof. Dr VJ Feron
(Faculteit Diergeneeskunde; Universiteit Utrecht)

Prof. Dr WRF Notten
(Faculteit Geneeskunde; Erasmus Universiteit Rotterdam)

Co-promotor: Dr RA Baan
(Afdeling Genetische Toxicologie; TNO Voeding Rijswijk)

Het in dit proefschrift beschreven onderzoek werd uitgevoerd bij de afdeling Genetische Toxicologie van TNO Voeding.
Het drukken van dit proefschrift werd mede mogelijk gemaakt met financiële steun van TNO Voeding.

Lay-out en omslag: Marina Reudink

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Roggeband, Robert

DNA-adduct kinetics, cell proliferation and precancerous changes in benzo[a]pyrene-treated hamster trachea in organ culture / Robert Roggeband. - Utrecht : Universiteit Utrecht, Faculteit Diergeneeskunde
Proefschrift Universiteit Utrecht. - Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-393-0553-6
Trefw.: longkanker ; onderzoek.

Chapter 2

Improvements in the ^{32}P-postlabeling procedure to quantify bulky aromatic DNA adducts

IARC monographs, volume 124, pp 65-70

Chapter 3

Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* upon activation of benzo[a]pyrene by human, rabbit and rodent liver microsomes

Carcinogenesis, 14, 1945-1950

Chapter 4

DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture

Carcinogenesis, 15, 661-665

Chapter 5

Detection of DNA-adducts in basal and non-basal cells of the hamster trachea exposed to benzo[a]pyrene in organ culture

Journal of Histochemistry and Cytochemistry, in press

Chapter 6

Cell proliferation and DNA adducts in hamster tracheal epithelium exposed to benzo[a]pyrene in organ culture

Carcinogenesis, submitted

Chapter 7

DNA adducts and proliferation of basal and non-basal cells in relation to the development of precancerous lesions in the hamster trachea exposed to benzo[a]pyrene in organ culture

Cancer Research, submitted

AEC	3-amino-9-ethylcarbazole
Ah	aromatic hydrocarbon
ATP	adenosine 5'-triphosphate
B[a]P	benzo[a]pyrene
BPDE	7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene
(m)BSA	(methylated) bovine serum albumine
BrdU	bromodeoxyuridine
CCD	charge-coupled device
CD	circular dichroism
CYP	cytochrome P450
dA	deoxyadenosine
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DD	dihydrodiol dehydrogenase
dG	deoxyguanosine
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EH	epoxide hydrolase
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin *O*-deethylase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FLNS	fluorescence line-narrowing spectroscopy
FPLC	fast protein liquid chromatography
GA	glucuronic acid
GaM	goat-*anti*-mouse
GaR	goat-*anti*-rabbit
G6PD	glucose-6-phosphate dehydrogenase
GST	glutathione S-transferase
Gua	guanine
HPLC	high performance liquid chromatography
HTE	hamster tracheal epithelial
IARC	International Agency for Research on Cancer
IFM	immunofluorescence microscopy
i.p.	intraperitoneally
ISB	immuno-slot blot

LI	labeling index
LSM	laser-scanning microscopy
3-MC	3-methylcholanthrene
MN	micrococcal nuclease
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NER	nucleotide excision repair
NHS	normal hamster serum
NMR	nuclear magnetic resonance
NP1	nuclease P1
PAH	polycyclic aromatic hydrocarbon
PAP	prostate acid phosphatase
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEI	poly(ethylenimine)
PI	propidium iodide
PNK	polynucleotide kinase
PROD	pentoxyresorufin *O*-depentylation
RAL	relative adduct labeling
RIA	radio-immunoassay
SFS	synchronous fluorescence spectrophotometry
S/H	squamous metaplasia / hyperplasia
SPD	spleen phosphodiesterase
SVPD	snake venom phosphodiesterase
TBS	Tris-buffered saline
TE(N)	buffer containing Tris, EDTA, (NaCl)
TGF	transforming growth factor
TLC	thin-layer chromatography
TRITC	tetramethylrhodamine isothiocyanate
UDP	uridine diphosphate
UDS	unscheduled DNA synthesis
UGT	UDP-glucuronosyl transferase
USERIA	ultra-sensitive enzyme radio-immunoassay
UV	ultraviolet
WBC	white blood cells

Aan mijn ouders
Voor Patricia

Chapter

1 *Introduction*

Nowadays cancer is a major health problem in Western societies. It is one of the major causes of death, second only to circulatory failure. Lung cancer is the leading cause of cancer death in the US (Peto *et al* 1992, Mitsudomi *et al* 1993, Wattenberg 1993). With respect to the etiology of lung cancer, epidemiological research revealed that there is a link between the incidence of lung cancer and active smoking. About 30% of all cancer deaths and 85% of all lung cancer deaths in the US are associated with tobacco smoking (Loeb *et al* 1984, Fielding 1985). The contribution of environmental pollution and passive smoking to the etiology of lung cancer is undoubtedly present, but more difficult to assess (Kolonel 1993).

Tobacco smoke contains, amongst other compounds, a complex mixture of polycyclic aromatic hydrocarbons (PAHs) (IARC 1986). Apart from their presence in cigarette smoke, PAHs are ubiquitous in our environment as pollutants of air, soil, and water (IARC 1983). PAHs are released during various processes that involve incomplete combustion of fossil fuel and excessive broiling of meat. Occupationally PAH-exposed groups include coke-oven workers, roofers, chimney sweeps, asphalt-road builders. Non-occupational exposure to PAHs is found in smokers - active and passive - and in populations living in heavily industrialized areas. According to IARC, 11 different unsubstituted PAHs (see Figure 1 for their structures) have been proven to be carcinogenic in rodents (IARC 1973, 1983). In case of benzo[a]pyrene (B[a]P), a well-studied member of the class of PAH compounds, tumors are formed in various rodent tissues, depending on the route of administration. In humans, exposure to PAHs has been correlated with various types of cancer, *e.g.*, those of the lung (Redmond *et al* 1976, Hogstedt *et al* 1981) and the skin (Hammond *et al* 1976).

The idea that the genetic material plays a critical role in cancer initiation was first put forward by the Millers, who demonstrated that chemical carcinogens exert their biological effect by covalent interaction with DNA (Miller 1970, 1978). In tobacco-associated lung cancer the prevalent mutation in tumor DNA is the G:C to T:A transversion, which is in line with the preferential reaction of PAH metabolites with the G (guanine base) in DNA.

In 1968 an animal model for respiratory tract carcinogenesis was introduced, which proved to be useful to study the main characteristics of human lung cancer (Saffiotti 1968). In this model, the hamster trachea, neoplastic lesions induced by repeated instillation of B[a]P closely resembled the characteristics of human lung cancer. Later on,

it was found that the morphological characteristics that precede the development of neoplasia *in vivo*, *i.e.*, squamous metaplasia and hyperplasia, can also be induced in organ culture of the hamster trachea by exposure to cigarette smoke condensate (Rutten *et al* 1988a). Organ cultures offer the advantage to cell cultures of intact cell-to-cell interactions and allow the study of morphological and biochemical effects at different time points after exposure.

In this thesis, studies are described that were aimed at determining the interaction of B[a]P, the model compound for PAHs, with DNA in various cell types of the hamster trachea. To this purpose, hamster tracheas were exposed to B[a]P in organ culture. To detect B[a]P-DNA adducts, two methods were used. First, the ^{32}P-postlabeling technique, a biochemical assay that involves digestion of modified DNA to mononucleotides, followed by enrichment of adducted nucleotides, subsequent labeling and separation by use of thin-layer chromatography; this technique provides both quantitative and qualitative information with respect to overall DNA-adduct formation. The second approach comprises immunochemical assays, in particular *in situ* immuno-detection, which allows the determination of B[a]P-DNA adducts in specific cell types of the hamster tracheal epithelium by combining adduct-specific staining with staining of cell-specific components, such as cytokeratins. Data on B[a]P-DNA adduct kinetics in specific cell types, together with data on the proliferative status of these cell types and the determination of morphological effects that normally precede tracheal tumor formation *in vivo*, may give further insight into the process of respiratory tract carcinogenesis.

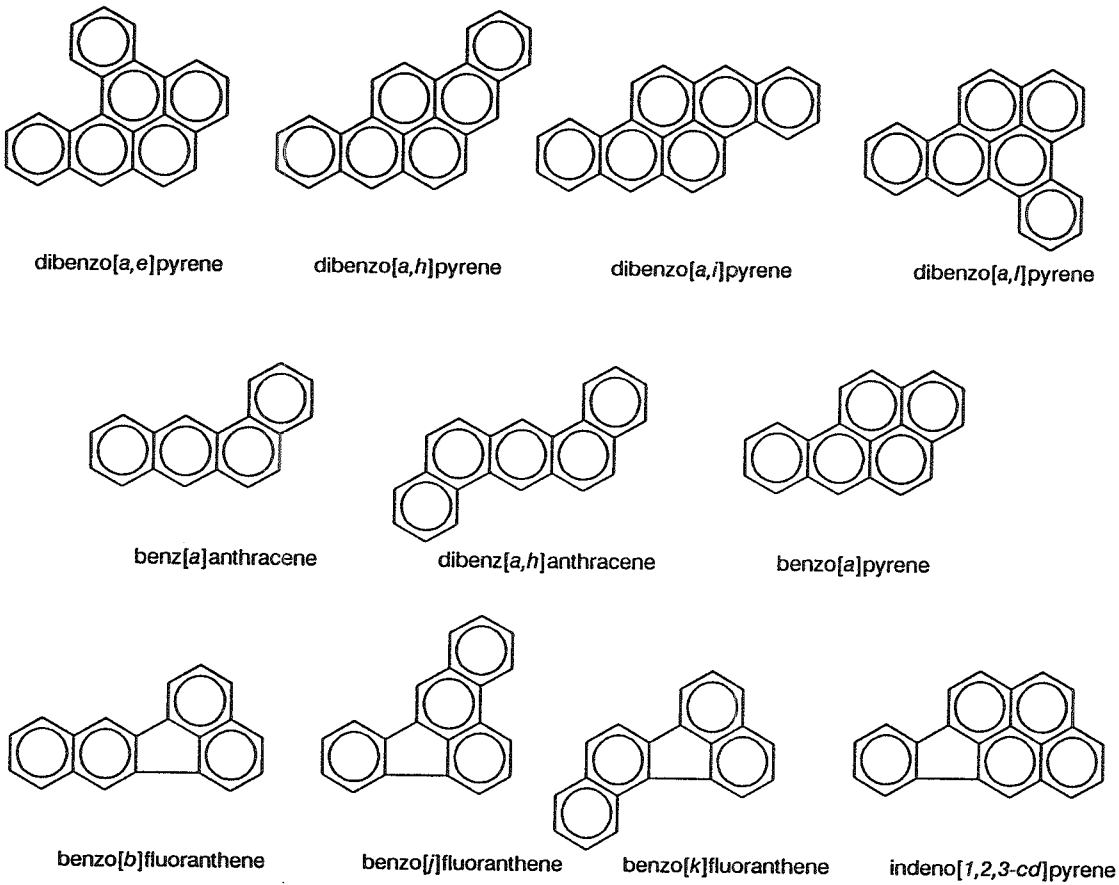

Figure 1 *Chemical structure of 11 PAH compounds, proven to be carcinogenic in rodents according to IARC (IARC 1973, 1983). Substituted PAH compounds, among which several are also carcinogenic in rodents, are not included in this Figure.*

1.2.1 Occurrence and sources of exposure

One of the most extensively studied members within the group of PAHs is B[a]P, which has been found carcinogenic in many rodent species: exposure to B[a]P can give rise to tumors in different organs, depending on the route of administration. The compound has been detected in the atmosphere, the soil, and food and water. Sources of exposure to B[a]P include both industrial activities and life-style factors. In tobacco smoke, B[a]P concentrations range from 5 to 80 ng per cigarette in mainstream smoke and 25 to 200 ng per cigarette in sidestream smoke. Cigarette smoking as such constitutes a major source of B[a]P exposure to humans (IARC 1986). A sad example of severe environmental pollution, especially in the soil, is found in the district of Katowice, Silesia, Poland, which is one of the most heavily polluted areas on earth. In that region, atmospheric concentrations of B[a]P presently are declining owing to the recent closure of PAH-emitting factories, but in the central part of Silesia, B[a]P was found to exceed many times the concentration of 10 $\mu g/m^3$ in ambient air. Taking into account that the daily intake of air is approximately 10 m^3, this would imply a relatively high daily respiratory burden of B[a]P in this particular area. In the surrounding area of Silesia, B[a]P concentrations in air were found to be as high as 57 ng/m^3 in January and 15 ng/m^3 in May (Hemminki *et al* 1990). The seasonal variation in B[a]P concentration is attributed to the extensive combustion of coal for residential heating during the winter months. In Western Europe, atmospheric PAH concentrations are several-fold lower, with B[a]P concentrations below 1 ng/m^3 in ambient air. In rural England, air concentrations of PAHs have decreased to a moderate extent between 1969 and 1989 (Jones *et al* 1992). However, it should be noted that concentrations of PAHs in soil are often still increasing, as deposition rates generally exceed removal through degradation, volatilization and leaching. Therefore, PAH contamination of soil remains a problem.

In the Total Human Environmental Exposure Study (THEES) with B[a]P, the relative contribution of inhalation and intake via food pathways were compared (Lioy *et al* 1988). In the winter, weekly inhaled doses of B[a]P varied between 80 and 400 ng while the weekly B[a]P dose ingested with the food varied between 10 and 4,000 ng. It was concluded that both entry routes were equally important. The dominance of one or the other pathway appeared to depend upon personal eating habits, on the type of residential heating used and on indoor smoking behaviour. Water consumption appeared

to be a minor source of B[a]P exposure in this study.

In the occupational setting, the atmospheric concentrations of PAHs may be several times higher as compared to environmental concentrations. In a Dutch study (van Schooten *et al* 1990) it was found that coke-oven workers from a steel-rolling factory were exposed to substantial concentrations of atmospheric PAHs (1-186 $\mu g/m^3$), including B[a]P (0.1-7.8 $\mu g/m^3$).

1.2.2 Structure, metabolism and reaction with DNA

B[a]P is a lipophilic, chemically rather inert compound. Cellular metabolism of B[a]P is directed to facilitate removal by enhancing the hydrophilicity of B[a]P, which is accomplished by the introduction of hydroxyl groups in the B[a]P-moiety. The enzyme system cytochrome P-450 is involved in this hydroxylation. B[a]P is metabolized to approximately 20 primary and secondary oxidized metabolites. Among the primary metabolites are three epoxides: the 4,5-epoxide, the 7,8-epoxide, and the 9,10-epoxide (see Figure 2 for numbering). In addition, several phenols have been isolated, among which are 3-OH-B[a]P and 9-OH-B[a]P. In the mid-70's the use of HPLC for isolation and separation of metabolite isomers resulted in significant progress in the elucidation of the metabolite profile of B[a]P (Selkirk *et al* 1976). The main three epoxides are converted by the action of epoxide hydrolase into 4,5-dihydro-dihydroxy-B[a]P, 7,8-dihydro-dihydroxy-B[a]P, and 9,10-dihydro-dihydroxy-B[a]P. These so-called dihydrodiols (or diols) can exist as *cis* and *trans* isomers. The 7,8-diol is of particular interest, as further metabolism of this specific diol is associated with the DNA-binding capacities and carcinogenic properties of B[a]P. The 7,8-diols formed enzymically are *trans* isomers and are (−)-enantiomers of high optical activity, which indicates that epoxidation of B[a]P followed by hydration is highly stereoselective (Thakker *et al* 1977, Conney 1982). The 7,8-diol can be metabolized to the 7,8-diol-9,10-epoxide, which is not degraded by epoxide hydrolase. The 9,10-epoxides of the 7,8-diol exist as a pair of diastereomers in which the 7-hydroxyl group is either *trans* (B[a]P-diolepoxide I or *anti*-B[a]P-diolepoxide) or *cis* (B[a]P-diolepoxide II or *syn*-B[a]P-diolepoxide) to the epoxide oxygen. The stereochemistry of these latter reactions is depicted in Figure 2.

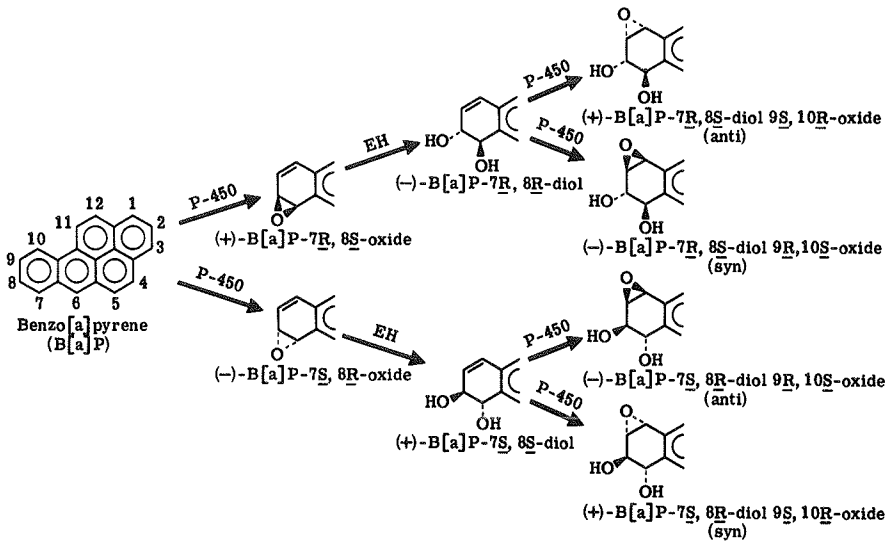

Figure 2 *Stereochemistry of the metabolic activation of B[a]P. P-450, cytochrome P-450; EH, epoxide hydrolase. Adapted from Hall and Grover 1990.*

The unique structural feature of this type of diolepoxide appears to be that the epoxide function is located on a saturated angular benzo ring and that it forms part of a bay-region of the B[a]P-molecule, *i.e.*, the region between C-atoms 10 and 11 (*cf* Figure 2). The bay-region diolepoxides show a high chemical reactivity, which can be attributed to the fact that they undergo ring opening to a carbonium ion much more easily than do non-bay region epoxides (Jerina *et al* 1980, Thakker *et al* 1980). The chemical nature of these diolepoxides sterically hinders enzymatic detoxification through hydrolysis of the epoxide function. Both *anti*- and *syn*-B[a]P-diolepoxide proved to be resistant to hydration by epoxide hydrolase. The high mutagenic potency of B[a]P-diolepoxide in bacterial and mammalian systems is in good accord with the postulate that the bay-region diolepoxides are likely to be good candidates for ultimate carcinogens, *i.e.*, metabolites responsible for binding to DNA, hence for initiating tumor formation (Phillips 1983).

In 1974, the first evidence was presented that a diolepoxide of B[a]P was the metabolite responsible for the observed binding to DNA (Sims *et al* 1974). It was

furthermore found that reaction of the (±)-*anti*-B[a]P-diolepoxide (*cf* Figures 2 and 3) with DNA *in vitro* predominantly resulted in binding to the exocyclic amino group of deoxyguanosine (Meehan *et al* 1977, Meehan and Straub 1979), while minor adducts were formed with deoxyadenosine and deoxycytidine. Evidence was also provided for the formation of adducts of (±)-*anti*-diolepoxide with DNA *in vivo* (Koreeda et al 1978). Harvey and Geacintov proposed a mechanism by which the diolepoxide reacts with the DNA base (Harvey and Geacintov 1988): rapid initial intercalation is followed by a rate-determining protonation to yield an intercalated carbonium ion which then reacts via one of two pathways. The major route involves the formation of an intercalated tetrahydroxy-B[a]P (tetraol) complex while only about 8% of the carbonium ion reacts to form a covalent adduct.

Recently, detailed NMR and CD spectral data were published for all the possible adducts formed upon alkylation of the exocyclic amino groups of deoxyguanosine and deoxyadenosine by *anti*- and *syn*-B[a]P-diolepoxide (Cheng *et al* 1989). A re-evaluation (Sayer *et al* 1991) of the covalent DNA adducts of the diolepoxides revealed that the major adducts of the enantiomers of *syn*-diolepoxide to polyguanylic acid resulted from *cis* opening of the epoxide by the exocyclic amino group of guanine, rather than *trans* opening as had been reported before. In case of the *anti*-diolepoxide, *trans* ring opening was the major route by which the exocyclic amino group opened the epoxide. Apart from the reported DNA-binding capacities of *anti*-B[a]P-diolepoxide, other metabolites of B[a]P may, to a lesser extent, also be capable of reacting with DNA.

For mechanistic studies on the carcinogenic potential of B[a]P and other PAHs, the availability of methods for the detection of adducts formed with DNA are an absolute necessity. Under physiological conditions, only few such adducts are induced in relation to the total amount of DNA. Therefore, sensitive methods to detect small amounts of DNA adducts are of great importance. With the advent of the ^{32}P-postlabeling procedure (see 1.3.1) it has become possible to detect small quantities of DNA adducts, in particular those induced by polyaromatic compounds. Following administration of B[a]P and various B[a]P metabolites, adduct formation was studied by use of ^{32}P-postlabeling in different organs of the rat (Ross *et al* 1991). By comparison of adduct patterns obtained after treatment with B[a]P metabolites with those found after treatment with the parent B[a]P molecule, the relative importance of various metabolic pathways could be estimated. Among all possible monohydroxy derivatives, only 2-, 9- and 12-OH B[a]P produced detectable levels of DNA adducts. Of the 4,5-, the 7,8-, and the 9,10-diol, only the 7,8-diol resulted in adduct formation. Exposure to B[a]P resulted in adducts that were also detected after exposure to the metabolites 9-OH-B[a]P and B[a]P-7,8-diol.

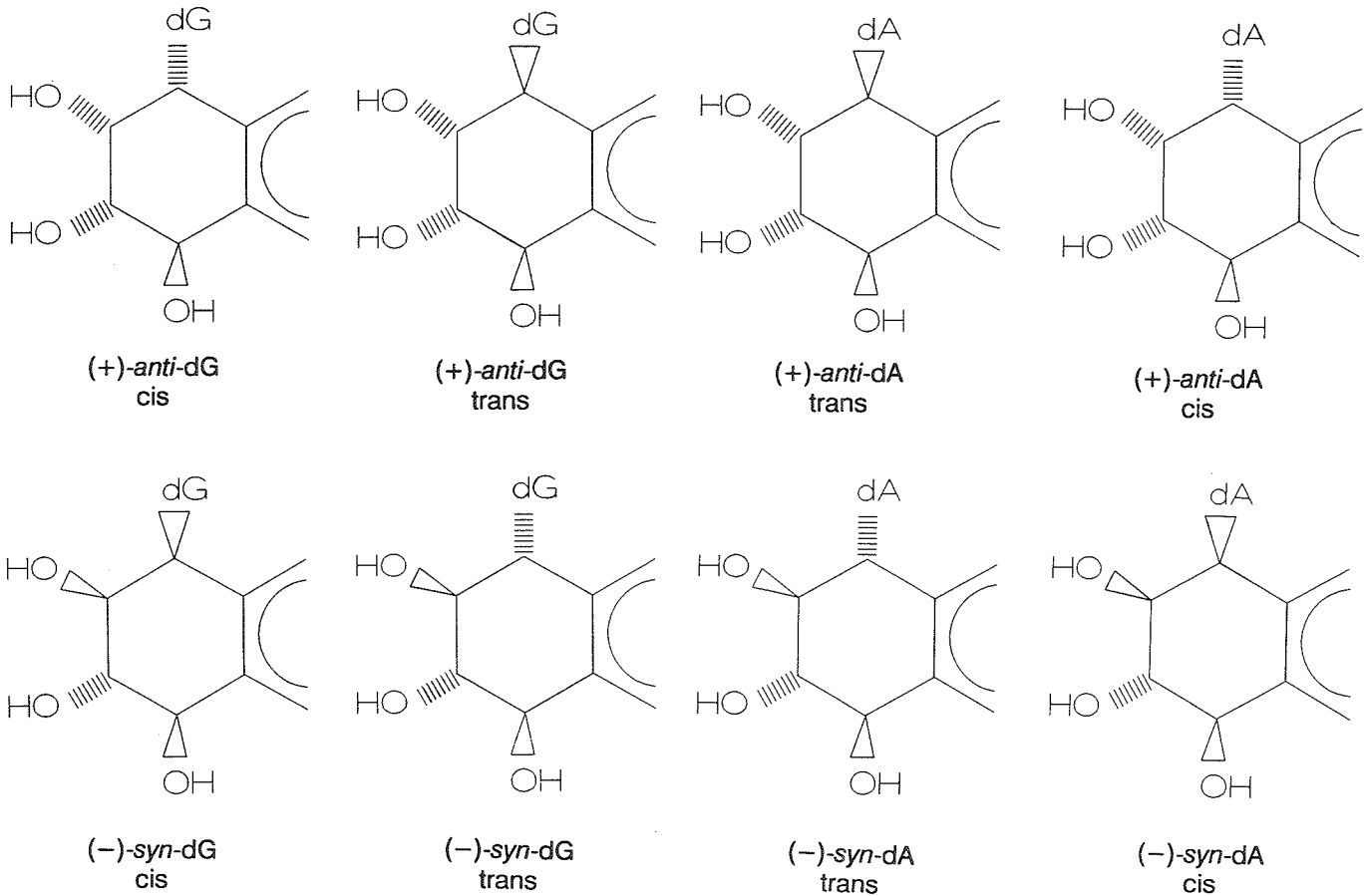

Figure 3 *Possible adducts of (+)-anti or (−)-syn B[a]P-diolepoxide to deoxyguanosine (dG) and deoxyadenosine (dA).*

The metabolism of B[a]P and the subsequent DNA-binding capacities of B[a]P-metabolites can be studied in various model systems. One approach would be the direct incubation of individual B[a]P-metabolites with DNA. Chemical synthesis of metabolites, however, is time-consuming and requires rather complex organic chemistry. A more practical approach to generate B[a]P metabolites is through the use of the enzyme systems present in microsomes, *i.e.*, fractions of smooth endoplasmic reticulum containing cytochrome P-450, which can be prepared by differential centrifugation. Most often, liver microsomes are used from rodents that have been pretreated with specific enzyme inducers, such as 3-methylcholanthrene (3-MC), a known inducer of a cytochrome P450 isozyme that is active in the metabolism of planar substrates such as PAHs. Comparative studies, in which 3-MC-induced liver microsomes were co-incubated with [^{3}H]-B[a]P, revealed major differences in metabolism between species (Selkirk and Wiebel 1979). Furthermore, it became apparent that major differences exist in B[a]P-DNA adduct formation when *in vitro* activation with cell cultures is compared with activation by use of microsomes. Using the latter way of activation with rat liver microsomes, the so-called K-region epoxide of B[a]P (B[a]P-4,5-epoxide) appears as a major DNA-reactive metabolite (Ashurst and Cohen 1982), whereas in cell cultures activation of B[a]P predominantly results in the formation of bay-region diolepoxides. In view of these results, in cell cultures an important role was attributed to the enzyme epoxide hydrolase which could destroy the K-region epoxides (Alexandrov *et al* 1980).

In vitro activation systems with cell cultures have been widely applied to study species differences in B[a]P metabolism and adduct formation. In early-passage embryo cell-cultures from BALB/c and Sencar mice, Wistar and Fischer 344 rats, and Syrian golden hamsters, B[a]P-DNA adduct formation was studied after exposure of the cells to B[a]P (2 μM). In both rat cell strains no significant amounts of (+)-*anti*-B[a]P-diolepoxide adducts were found after 5 or 96 hours of exposure, while in both the mouse and the hamster cell lines the proportion of (+)-*anti*-B[a]P-diolepoxide adducts increased with the length of exposure (Sebti *et al* 1985). When rat (Sprague Dawley) and human mammary epithelial cells were exposed to B[a]P (2 μM) for 24 hours, the major adduct in the human cells was that of the (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine (B[a]P-diolepoxide-N^2-dG), whereas in the rat cells a large proportion of *syn*-B[a]P-diolepoxide-derived adducts was observed (Moore *et al* 1987). This preference for the formation of the latter type of adducts was also found in rat (Wistar) dermal fibroblasts (Alexandrov *et al* 1988). In that study the use of rabbit (New Zealand White) dermal fibroblasts resulted mainly in the formation of adducts derived from (+)-*anti*-B[a]P-diolepoxide.

In vitro organ cultures may provide a better substitute than cell cultures for *in vivo* metabolism and activation. In organ cultures, morphology and functionality of cell types

can be preserved over a prolonged period of time (Van Scott *et al* 1986). With regard to the applicability of skin organ culture to imitate *in vivo* skin metabolism of B[a]P, however, some contradictory results have been published (Weston *et al* 1982, Watson *et al* 1989).

In vivo, B[a]P metabolism may vary between species and organs. Although tissue-specific adducts have been described, there are indications that the liver plays a central role in the metabolism of B[a]P (Wall *et al* 1991). In that respect it is interesting to note that B[a]P metabolites, such as the highly reactive diolepoxide, can be transported in their active form in serum to various target tissues (Ginsberg and Atherholt 1989, 1990, Kwei *et al* 1992). Further evidence for a crucial role of the liver in B[a]P metabolism is given by the fact that with respect to the tissue distribution of covalent DNA damage the route of exposure is less important than the nature of the carcinogen itself (Schurdak and Randerath 1989).

1.2.3 Other routes of B[a]P activation

In general, PAHs with relatively high ionization potentials are activated by the diolepoxide pathway, whereas the ones with relatively low ionization potentials, which represent the most potent carcinogenic PAHs, are activated by a combination of the one-electron oxidation and the diolepoxide pathway (Cavalieri *et al* 1993; see Figure 4 for metabolic routes). One-electron oxidation of PAH compounds generates radical cations, which form predominantly labile adducts, which are lost from DNA by depurination. By comparison of retention times on HPLC in different solvent systems and by comparison of fluorescence spectra with those of authentic adducts, B[a]P-6-N7Guanine, B[a]P-6-C8Guanine and B[a]P-6-N7Adenine were characterized (Devanesan *et al* 1992). Reaction of DNA with B[a]P in the presence of 3-MC-induced rat liver microsomes resulted in 81% depurination adducts and 19% stable adducts, while in DNA reacted with (+)-*anti*-B[a]P-diolepoxide the amount of depurination adduct B[a]P-diolepoxide-10-N7Adenine was only 1.8% of the total.

In an *in vivo* experiment mouse skin was treated with B[a]P for 4 hours, whereafter both stable and labile DNA adducts were analyzed. It appeared that 74% of the adducts had been released by depurination, while the stable B[a]P-diolepoxide-N^2dG adduct accounted for 22% of the adducts (Rogan *et al* 1993). The extensive depurination leaves numerous apurinic sites in DNA. In the absence of appropriate repair, these sites can lead to the induction of mutations through mispairing (Schaaper *et al* 1983).

Postlabeling analysis revealed that also stable adducts derived from the one-electron oxidation route, could be found in mouse skin treated with B[a]P (Bodell *et al*

1989). The identity of these adducts could not be confirmed yet, although the presence of the stable one-electron oxidation adduct B[a]P-6-C8deoxyguanosine has been confirmed *in vitro* (Rogan *et al* 1988).

Another metabolic route for activation of B[a]P involves the formation of benzylic esters (see Figure 4 for metabolic route). This pathway has been proposed to consist of three reactions: (i) bioalkylation of the unsubstituted B[a]P at a centre of high-electron density (leading to methyl substitution), (ii) oxidation of this side-chain methyl group to form a hydroxymethyl metabolite, (iii) esterification of the hydroxyalkyl side chain. DNA adducts derived from metabolites generated by this pathway, have been detected by means of postlabeling analysis in subcutaneous rat tissue, after subcutaneous injection of B[a]P or postulated intermediates of the benzylic ester pathway. However, the level of adducts derived from the pathway leading to the formation of diolepoxides was 15 to 50 times higher (Stansbury *et al* 1994).

Epoxidation of the B[a]P-7,8-diol is also accomplished by non-cytochrome P-450 catalysts (Reed *et al* 1984, Hughes *et al* 1989). Enzyme reactions that generate peroxyl free-radicals are efficiently capable of inserting oxygen atoms into the 9,10-double bond of the B[a]P-7,8-diol. Parent PAH compounds, however, are no substrate for these enzyme reactions. B[a]P-7,8-diol is also epoxidized *in vitro* by activated granulocytes in a myeloperoxidase-mediated reaction (Mallet *et al* 1991, Petruska *et al* 1992). Peroxyl free radical-dependent epoxidation of (+)-B[a]P-7,8-diol primarily gives (−)-*anti*-B[a]P-diolepoxide, while the cytochrome P450-mediated epoxidation of (+)-B[a]P-7,8-diol primarily results in the formation of (+)-*syn*-B[a]P-diolepoxide. Despite the fact that both B[a]P-diolepoxide diastereomers are about equally reactive with DNA, no (−)-*anti*-B[a]P-diolepoxide adducts were found in mice that were treated topically with (+)-B[a]P-7,8-diol, which indicates that in mice skin peroxyl free radical-dependent epoxidation plays a minor role *in vivo* (Reddy *et al* 1992a, b).

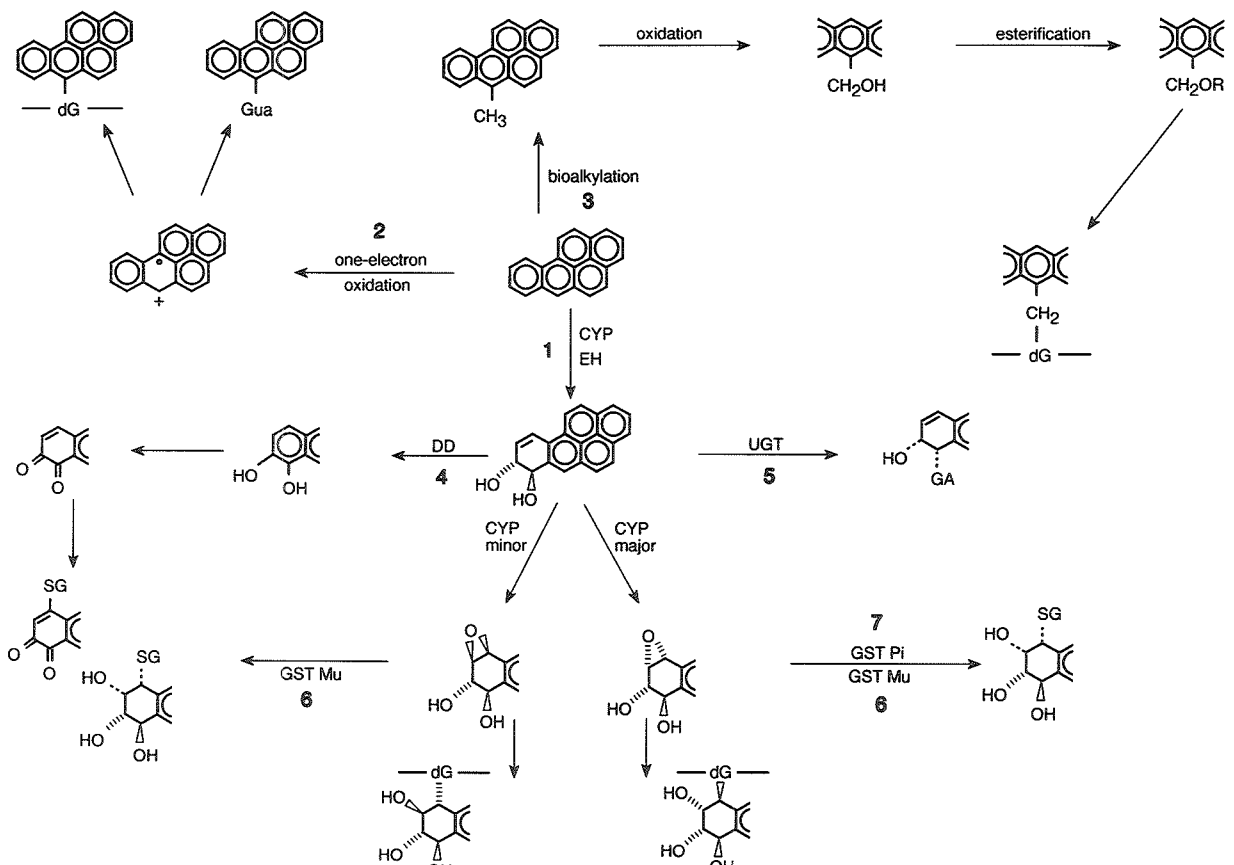

Figure 4 *Diagram of major metabolic enzymes involved in B[a]P metabolism. CYP, cytochrome P450; DD, dihydrodiol dehydrogenase; dG, deoxyguanosine; EH, epoxide hydrolase; GA, glucuronic acid; GST Mu, glutathione S-transferase class Mu; GST Pi, glutathione S-transferase class Pi; Gua, guanine; SG, glutathione; UGT, UDP glucuronosyl transferase. References: 1, Hall and Grover 1990; 2, Cavalieri et al 1993; 3, Stansbury et al 1994; 4, Penning 1993; 5, Fahl et al 1978; 6, Mannervik et al 1985; 7, Robertson et al 1986a,b.*

1.2.4 *Cytochrome P450*

Cytochrome P450 (CYP) is a monooxygenase, *i.e.*, an enzyme that inserts one atom of oxygen into its substrate. The activity of this class of enzymes requires the integrity of an electron flow between the cofactor NADPH (in some cases NADH) and the oxygenated form of CYP (Nebert *et al* 1981). The reduced form of CYP then activates an oxygen molecule, of which one atom goes into the substrate, whereas the other one is reduced to water. Monooxygenases, generally considered as activating or phase I enzymes, are now known to be a vast superfamily of more than one hundred distinct gene products, each with its characteristic substrate and specificities. An update on recommended nomenclature is published biennially (Nebert *et al* 1989, 1991, Nelson *et al* 1993).

CYP is located in the endoplasmic reticulum of liver, kidney, and other tissues of mammals (Parke *et al* 1991). Probably the most extensively studied members of the cytochrome P450 class are those of the CYP1A subfamily, which is characterized by (i) its affinity for planar substrates and its ability to oxygenate molecules in conformationally hindered (bay region) positions, and (ii) the mode by which the enzyme is induced, which involves the interaction of inducers with the aromatic hydrocarbon (Ah) receptor (Nebert 1989). The CYP1A-inducing agents interact with this steroid-like regulatory receptor, whereupon the receptor-inducer complex binds to specific regions of the DNA, which then results in an increased transcription of the *CYP1A* gene (Parke *et al* 1991) and *de novo* synthesis of a specific enzyme. There are, however, indications that CYP content and activity are also regulated at the translational or post-translational level. The CYP1A subfamily comprises two enzymes, namely CYP1A1, which reacts with aromatic hydrocarbon substrates such as B[a]P, and CYP1A2, which reacts with aromatic amines.

The response of enzyme activity to specific inducers may vary from one species to another. The degree of induction can be quantified enzymically by use of specific substrates for each isoform studied, *e.g.*, CYP1A1 induction can be determined on the basis of enzymatic O-deethylation of 7-ethoxyresorufin. B[a]P was found to be capable of inducing cytochrome CYP1A in *in vitro* cell cultures (Smolarek *et al* 1987, Eberhart *et al* 1992) as well as *in vivo* (Ayrton *et al* 1990). Antibodies directed against specific cytochrome P450 enzymes made it possible to confirm their presence in specific cell types (Plopper *et al* 1987, Voigt *et al* 1989, Guo *et al* 1990). Based on studies with human liver microsomes, B[a]P and antibodies against different cytochrome P450 isozymes, a major role for an isozyme from the CYP3A family in human liver B[a]P metabolism was proposed (Shimada *et al* 1989, McManus *et al* 1990). A substantial role for CYP1A1/2 in extrahepatic human tissue can, however, not be ruled out.

1.2.5 *Glutathione S-transferases*

The glutathione S-transferases (GSTs; phase II enzymes) are an important group of enzymes involved in the detoxification of electrophilic arylating agents, although in some specific cases conjugates were found to be more harmful than the non-conjugated compounds (van Bladeren 1988, Monks *et al* 1990). Several isozymes exist, which are mainly present in the cytosol. They all catalyze the nucleophilic addition of the tripeptide glutathione to substrates that have electrophilic functional groups (Armstrong 1991).

The transferases from rat, man, and mouse have been divided into three classes, Alpha, Mu and Pi, based on similar structural and catalytic properties (Boyer 1989, Vos and van Bladeren 1990). An interesting aspect of the GSTs is their variable expression in different tissues. Furthermore, in humans the types of isozymes present in different individuals also are quite variable. Indeed, about 30-40% of the population lacks the Mu type of GST. This particular isozyme has a high affinity towards epoxides (Mannervik *et al* 1985) and may thus play an important role in protection against reactive B[a]P diolepoxides. However, it has been shown that GST isozymes of the Pi class also are quite efficient in this respect: notable activity towards the (+)-*anti*-B[a]P-diolepoxide was found for both rat and human GST Pi isozymes (Robertson *et al* 1986a, b).

Many papers have been published in which a correlation is described between the absence or presence of specific GST isozymes and the occurrence of neoplasia in specific tissues (Howie *et al* 1990, Moorghen *et al* 1991, Ranganathan and Tew 1991, Bell *et al* 1993). There are indications that expression of GST Mu is a determinant of genetic susceptibility to lung cancer among smokers (Zhong *et al* 1991, Hayashi *et al* 1992). Among smokers with lung cancer significantly more individuals lacked GST Mu isozyme than among smokers without cancer (Seidegård *et al* 1986, 1990). This might be understood as the result of the disease process itself. However, van Poppel *et al* demonstrated that in heavy smokers who lacked the GST Mu isozyme there was a significant increase in cytogenetic damage, as measured by the extent of sister-chromatid exchanges in lymphocytes, compared to heavy smokers who were GST Mu-positive (van Poppel *et al* 1992). In another study the absence of the GST Mu isozyme in the human lung was associated with relatively high PAH-DNA adduct levels in the same sample, which indicates an important role of GST Mu in preventing the formation of PAH-DNA adducts *in vivo* by scavenging the reactive metabolite (Shields *et al* 1993a).

1.2.6 *UDP-glucuronosyltransferases and dihydrodiol dehydrogenases*

Other deactivation enzymes involved in B[a]P metabolism include the UDP-glucuronosyl transferases (UGTs). UGTs are membrane-bound enzymes of the endoplasmic reticulum and are found primarily in the liver. They catalyze the transfer of glucuronic acid from UDP-glucuronic acid to compounds possessing -OH, -COOH, $-NH_2$, or -SH functions (Tephly 1990). UGTs are a family of isozymes, with marked substrate specificity (Jin *et al* 1993). Addition of UDP-glucuronic acid to incubation mixtures containing microsomes and B[a]P caused a dose-dependent conjugation of mainly quinone and phenol metabolites (Fahl *et al* 1978), although affinity for dihydrodiols has been observed for some UGT isozymes.

Dihydrodiol dehydrogenases (DDs) have also been implicated in the detoxification of proximate carcinogenic metabolites (trans-dihydrodiol) of PAHs (Penning 1993). Human liver contains relatively high levels of this particular enzyme, while the levels in the lung are low. This suggests that DD plays an important role in PAH detoxification in the liver, while the low levels of DD in the lung may contribute to the susceptibility of this tissue to PAH-induced carcinogenesis (Penning and Sharp 1990).

1.2.7 *Persistence and repair of B[a]P-DNA adducts*

DNA repair is a cellular response that is associated with the restoration of the normal nucleotide sequence following induction of damage to the genome. Residual DNA damage, as a consequence of inadequate DNA repair, may have a serious impact on the fate of the cell (McMillan 1992). Persistent DNA adducts, but also labile DNA adducts leading to apurinic sites, are important with regard to the process of tumor initiation in various tissues or cell types. Differences in DNA-repair capacity have been described at several levels. At the interindividual level, differences in repair rates may reflect both acquired and host factors (Harris 1989). Differences in repair rate among species, *e.g.*, in rat and hamster tracheas, have been correlated with differences in tumor susceptibility (Ishikawa *et al* 1980, Kuper and Benford 1991). Within one species, repair capacities may vary from one strain to the other (Slaga 1988).

B[a]P-DNA adducts form a block for DNA polymerase (Reardon *et al* 1990). This block may allow DNA repair to take place in the meantime (Buratowski 1993); this so-called transcription-coupled repair has been described for UV-induced thymidine dimers in DNA (Sachsenmaier *et al* 1994). The cell possesses various repair mechanisms to restore the integrity of its DNA. Bulky lesions, such as adducts derived from B[a]P and other PAH, are removed via the nucleotide excision repair pathway (NER). NER can be

divided into three successive stages, *i.e.*, (i) the incision, which involves the recognition of the adduct and the subsequent cleavage of the DNA strand, (ii) the excision and repair synthesis, during which about 30 to 100 nucleotides are incorporated, and (iii) the ligation, which restores the integrity of the DNA strands. The replacement of a relatively large number of excised nucleotides during NER makes it possible to determine the extent of repair either *in vivo* or *in vitro*, by measurement of the incorporation of radiolabeled nucleotides during the ligation phase. This so-called unscheduled DNA synthesis (UDS) assay has been widely applied (Lonati-Galligani *et al* 1983, Brambilla and Martelli 1992).

Biphasic disappearance of adducts has been described for B[a]P-DNA adducts in various rodent organs (Kulkarni and Anderson 1984, Brauze *et al* 1991, Lu *et al* 1993). The first, relatively short phase of rapid disappearance of adducts is characterized by active DNA repair, cell death and cell turnover. The second phase is characterized by a relatively slow decline in adduct level, which may be indicative of the fact that part of the adducts were present in a small subpopulation of the cells that cycled slowly and had a low rate of DNA repair. There is evidence that upon administration of B[a]P a substantial storage of the compound in fatty tissues takes place (Uziel and Haglund 1988). Slow release of B[a]P or B[a]P metabolites from storage sites and subsequent increase of the adduct level must therefore be taken into consideration when determining the persistence of adducts *in vivo*.

Repair of B[a]P-induced DNA lesions has been described at various levels. There are indications that DNA repair is adduct specific; a difference in repair rate was found between the adduct derived from *anti*- and *syn*-B[a]P-diolepoxide (Celotti *et al* 1993). Recently, it has been shown that B[a]P-diolepoxide-derived adducts were preferentially repaired in active genes as compared to silent genes (Chen *et al* 1992). In this study it was found that adducts are removed faster from the transcribed strand than from the non-transcribed strand in the active gene. The loss of adducts from the silent gene was even lower than that from the overall genome. As more than 90% of the genome is not actively transcribed, it was expected that the loss of adducts from the silent gene more or less reflected that in the overall genome. It was therefore postulated that also the chromatin structure is of importance in determining repair rate.

It may well be that the three levels of repair, *i.e.*, (i) slow repair of silent genes, (ii) fast repair of active genes and (iii) accelerated repair of the transcribed strand of active genes, as proposed for UV-induced cyclobutane pyrimidine dimers (Mullenders *et al* 1991) are also valid for B[a]P-induced adducts.

1.2.8 *B[a]P-induced mutations*

The presence of unrepaired or incorrectly repaired DNA adducts during the S-phase of the cell cycle may give rise to mutations in the DNA after the completion of the cell cycle. Mutations in critical genes may lead to the initiation of carcinogenesis. After *in vitro* modification of plasmids containing the human proto-oncogene H-*ras1*, with *anti*-B[a]P-diolepoxide, the modified plasmid appeared to have transforming activities when transfected into NIH 3T3 cells. The mutations induced by *anti*-B[a]P-diolepoxide were predominantly G:C to T:A and A:T to T:A transversions (Vousden *et al* 1986). In lung adenocarcinomas the activation of the *ras* oncogene by mutation in codon 12 was related with smoking status (Rodenhuis *et al* 1988). The mutation profile of the activated *ras* genes revealed that G:C to T:A transversions are the most frequently detected mutations in adenocarcinomas and large cell carcinomas of the lung (Reynolds *et al* 1991). In human lung and liver cancers, G:C to T:A transversions in specific regions of the p53 tumor-suppressor gene also are frequently observed (Puisieux *et al* 1991, Ruggeri *et al* 1993). It may be that the PAH fraction of cigarette smoke is responsible for the activation of *ras* genes in human large cell carcinoma and adenocarcinomas of the lung, because G:C to T:A transversions were also the most frequently detected mutations in activated *ras* genes in B[a]P-induced mouse lung tumors (You *et al* 1989). *In vitro* studies with plasmids containing the sequence of the rat or human *ras* gene, revealed preferential targeting of specific codons in the *ras* gene by the diolepoxide of B[a]P (Reardon *et al* 1989, Dittrich and Krugh 1991).

The one-electron oxidation pathway may give rise to the formation of adducts that are labile. The subsequent release of these B[a]P-base adducts during depurination leaves apurinic sites in DNA, which can lead to the induction of mutations through mispairing if improperly repaired (Schaaper *et al* 1983). In studies on germ-cell mutagenesis in male mice, evidence was presented that incorrect repair of apurinic sites or mispairing during replication may occur after fertilization, which may give rise to mutations at an early stage of embryonic development (van Zeeland *et al* 1990). It is interesting to speculate on a possible causal role of apurinic sites in the formation of G:C to T:A transversions, often seen in human lung cancers. In prokaryotic systems, preferential insertion of an adenine, opposite to the apurinic site, occurs (the A-rule). However, evidence was recently provided that this does not appear to be a general rule in mammalian systems (Cabral-Neto *et al* 1992, Gentil *et al* 1992). This would imply a minor role of apurinic sites in the formation of G:C to T:A transversions in human lung cancer. We can, however, not rule out the possibility that B[a]P-DNA adducts that are released through depurination may be significant inducers of genetic effects.

1.3.1 The ^{32}P-postlabeling procedure

The postlabeling assay, initially developed by Randerath and coworkers (Randerath *et al* 1981), is most noted for its sensitivity (1 adduct per 10^{10} nucleotides) and applicability to structurally diverse classes of chemicals, although it is especially suited for PAH-DNA adducts. The extensive use of the postlabeling technique has helped in the elucidation of PAH-metabolism pathways *in vivo* (Singletary *et al* 1990, Ross *et al* 1990). Also, postlabeling of DNA adducts derived from the incubation of liver microsomes or S9 preparations with parent PAH compounds (Gallagher *et al* 1991, Lecoq *et al* 1991) or of adducts derived from the binding of chemically synthesized reactive PAH metabolites with DNA (Reddy *et al* 1992b, Canella *et al* 1991) can be of use to identify the chemical nature of adducts formed *in vivo*.

The classical assay involves the following sequential steps: (i) digestion of DNA to deoxynucleoside 3'-monophosphates; (ii) enrichment of modified nucleotides for which several methods are available; (iii) attachment of a ^{32}P label to the 5'-hydroxyl function of modified nucleotides thereby creating a 3',5'-biphosphate; (iv) separation and detection of modified ^{32}P-labeled nucleotides by thin-layer chromatography (TLC) and autoradiography, and (v) quantification of modified nucleotides by determination of radioactivity. Instead of TLC, more modern assays involve adduct separation by HPLC and on-line counting of radioactivity (see Figure 5 for general outline of ^{32}P-postlabeling).

A relatively new approach in postlabeling is the so-called dinucleotide method. It involves DNA hydrolysis with nuclease P1 and prostate acid phosphatase, to yield adducts in the form of dinucleotides, while unmodified nucleotides are obtained as nucleosides. The major advantage in this procedure is that adducts are enriched as a consequence of this DNA digestion and thus no additional steps are required. Furthermore, recoveries of polyaromatic adducts tend to be somewhat higher as compared with the biphosphate procedure (Randerath *et al* 1989b).

It is evident that the purity of the DNA is of crucial importance for the reliability of the assay. DNA should be free of RNA and protein (Beach and Gupta 1992): postlabeled RNA adducts may also show up and interfere with DNA-adduct quantification. Protein may interfere during the entire procedure. Optimal conditions to digest the DNA are dependent on the chemical nature of the adducts. In case of B[a]P-modified DNA, digestion to mononucleotides was optimized (Reddy and Randerath

1986), but for DNA containing other PAH-adducts, optimal digestion conditions need to be established. Incomplete digestion may yield adduct-containing oligonucleotides, which would be missed in the ^{32}P-postlabeling. On the other hand, it should be realized that too lengthy digestion incubations may result in significant adduct loss, probably by adsorption onto the walls of plastic tubes (Perin-Roussel *et al* 1990).

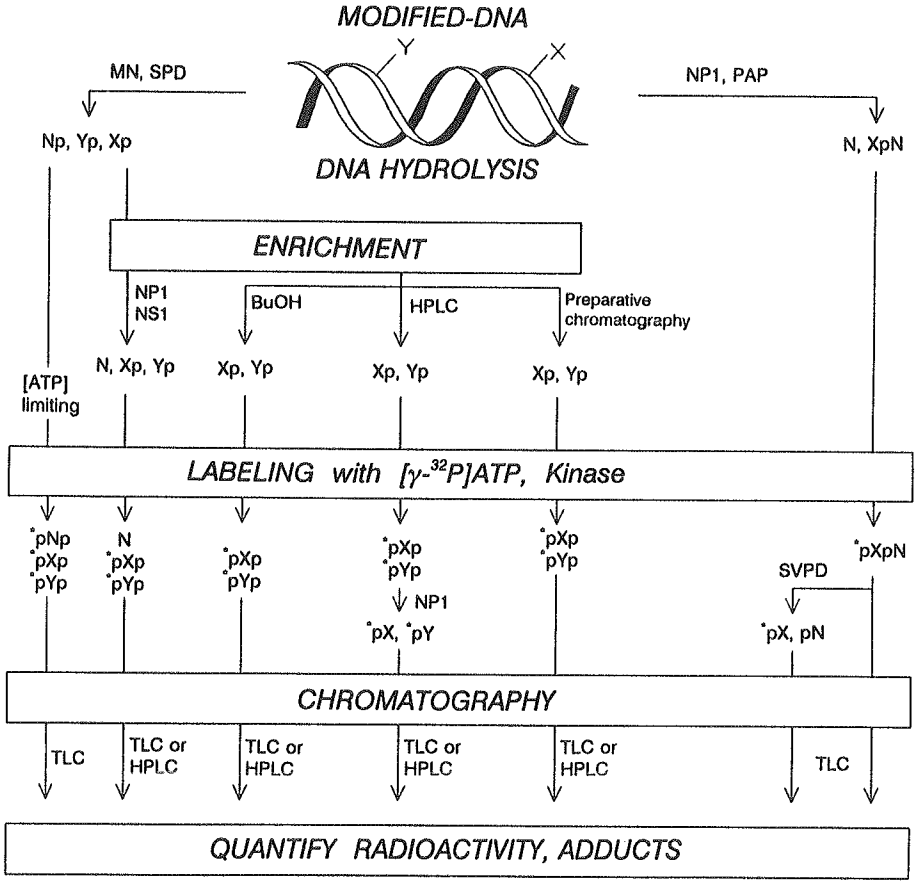

Figure 5 *Diagram of the steps in the ^{32}P-postlabeling procedure. BuOH, n-butanol; MN, micrococcal nuclease; N, normal nucleoside; Np, normal nucleoside 3'-monophosphate; pN, normal nucleoside 5'-monophosphate; pNp, normal nucleoside 3',5'-di[5'-^{32}P]phosphate; NP1, nuclease P1; NS1, nuclease S1; PAP, prostate acid phosphatase; SPD, spleen phophodiesterase; SVPD, snake venom phosphodiesterase; Xp and Yp, nucleoside 3'-monophosphate adduct; pXp and pYp, nucleoside 3',5'-di[5'-^{32}P]phosphate adduct; pX and pY, nucleoside 5'-[^{32}P]monophosphate adduct; XpN, 5'-adducted dinucleotide monophosphate; pXpN, 5'-[^{32}P]phosphomono-ester of XpN. From Gorelick 1993.*

With respect to the adduct enrichment, several methods have been developed. These include (i) butanol extraction (Gupta 1985); (ii) treatment with nuclease P1 or S1 (Reddy and Randerath 1986, Reddy 1991); and (iii) HPLC pre-separation (Dunn and San 1988). These enrichment steps greatly improved the sensitivity of the assay compared to the initial standard procedure (Randerath *et al* 1981). They also yield better results than the so-called intensification approach (Randerath *et al* 1985), in which adducts are preferentially labeled under conditions of ATP deficiency. Butanol enrichment involves the preferential extraction of aromatic and/or hydrophobic adducts into this organic solvent, while normal nucleotides will remain in the aqueous phase. Nuclease P1 and S1 readily hydrolyse 3'-nucleoside monophosphates (dNp) to their corresponding nucleosides. By virtue of their chemical structure, many modified nucleotides are resistant to the action of these enzymes. Nucleosides are not substrate for subsequent labeling with kinase, and thus only the modified nucleotides will become labeled during the subsequent incubation with polynucleotide kinase (PNK) and γ-[^{32}P]-ATP. The recovery of specific adducts may vary significantly from one enrichment procedure to the other. In general, the more polar adducts show a lower recovery in the butanol procedure as compared to the nuclease P1 enrichment (Gupta and Earley 1988). The use of these two enrichment procedures in parallel may serve to probe the structural characterization of adducts of unknown carcinogens (Gallagher *et al* 1991). Radiolabeling following enrichment involves PNK-catalyzed enzymatic transfer of ^{32}P from γ-[^{32}P]-ATP to the 5'-position of modified nucleotides to create 3',5'-biphosphate nucleosides. Crucial to this step is the presence of an adequate amount of PNK and a molar excess of γ-[^{32}P]-ATP. The labeling efficiency is negatively influenced by relatively high levels of unmodified nucleotides, which may remain in the mixture after less successful enrichment (Vaca *et al* 1992). Despite optimal conditions of labeling, the overall absolute recoveries of PAH-DNA adducts in postlabeling are rather low, ranging from 4 to 60% (Segerback and Vodicka 1993).

Subsequent to adduct labeling, aliquots of samples are applied on PEI-cellulose sheets for separation by multidirectional TLC (see Figure 6). In the first step (D1) the excess γ-[^{32}P]-ATP and labeled normal nucleotides are eluted onto a paper wick attached to the PEI sheet. In both D3 and D4 the modified nucleotides migrate and form a specific adduct pattern. The D5 step is included as a final 'clean-up' step. Spots on the TLC can be visualized by means of screen-enhanced autoradiography, which is the method most commonly used. Recently, also the use of the storage phosphor-imaging technique has been described for detection and quantification of DNA adducts (Reichert *et al* 1992). An additional advantage of this method, apart from its reported increased sensitivity compared to autoradiography, is that it allows retrospective analysis of samples

without the requirement of reanalysis of a replicate sample.

Adduct quantification is in most cases based on relative adduct labeling (RAL), in which the labeling of modified nucleotides is compared to the labeling of an external standard, namely deoxyadenosine 3'-monophosphate. However, another way of quantification has been described (Shields *et al* 1993b). This method is based on the concomitant labeling of a known amount of deoxyguanosine 3'-monophosphate (dGMP), added to each sample after enrichment just prior to labeling. Each sample to be analyzed thus contains an internal standard that can be quantified by applying the appropriate separation conditions on TLC. This way of adduct quantification may provide a better estimate of the adduct level because the standard is actually present in the same labeling mixture as the unknown samples. However, adduct recoveries are not taken into account using this procedure. Adduct recoveries may depend to a large extent upon the modification level of the DNA (Gorelick and Wogan 1989, Shields *et al* 1993a, b). In that case, it would be highly desirable for accurate quantification to include standard-DNA samples that contain known amounts of specific adducts. Preferably, the adduct levels in these standards should be determined by use of an independent method. Furthermore, they should be within the same modification range as is expected for the samples to be analyzed. Part of this thesis deals with development and application of such absolute standard-DNA samples to quantify B[a]P-DNA adducts.

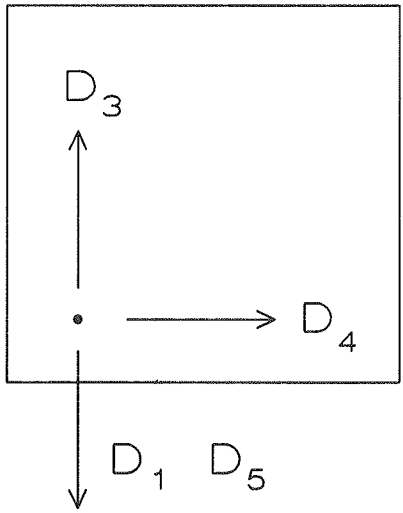

Figure 6 *Diagram of TLC, indicating the directions of the sequential chromatographic steps. The dot indicates the spot where the DNA digest is applied.*

Inherent in any highly sensitive assay is the presence of confounding factors. The release of unidentified chemicals from test tubes was found to result in the formation of adducts to guanine, adenine, and cytosine (Beach and Gupta 1992). Non-adduct compounds, such as tetraols, are also substrate for polynucleotide kinase labeling. However, the labeling efficiency of, *e.g.*, the B[a]P-tetraol was 2000-fold less compared to B[a]P-DNA adducts, which makes it unlikely for residual amounts of tetraols to interfere with labeling of DNA adducts (Masento *et al* 1989).

Another aspect that is related to the high sensitivity of the assay, is the detection of the so-called I-compounds, which is a collective term for the wide array of chromatographically separable indigenous adducts found in a variety of animal tissues. These adducts apparently arise in the absence of exogenous treatment and are tissue, species, sex, diet and age specific (Randerath *et al* 1989a, Li and Randerath 1990, Li *et al* 1990). More recently, I-compounds were also detected in the human brain (Randerath *et al* 1993).

1.3.2 Adduct detection by use of fluorescence spectroscopy

As PAHs generally are good fluorophores, spectroscopic techniques can be used for PAH-DNA adduct detection. Two methods of this kind have been applied and will be briefly discussed here, namely synchronous fluorescence spectrophotometry (SFS) and fluorescence line-narrowing spectroscopy (FLNS).

In conventional fluorescence spectroscopy the spectra of PAHs are usually complex and often show many broad peaks. In synchronous spectra, where emission and excitation are scanned at the same time with a constant wavelength difference, only one peak emerges, provided that the 0-0 band difference of the molecule of interest (so-called Stokes' shift) is used as the wavelength difference. SFS was applied to determine B[a]P-DNA adducts in *in vitro* modified DNA (Vähäkangas *et al* 1985). Typically, isolated DNA is heated at 90°C for several hours in the presence of HCl. In this way, covalently bound B[a]P will be released from the DNA as tetrahydroxy-B[a]P (tetraol). These tetraols can be extracted from the DNA solution with organic solvents. Collected tetraol fractions are then evaporated to dryness and the residue is redissolved in an aqueous solution. This solution can then be analyzed by scanning excitation and emission synchronously, with a fixed wavelength difference of 34 nm, characteristic for the pyrene moiety. In SFS, tetraols of B[a]P produce a fingerprint signal with an excitation maximum of 344 nm. By use of the appropriate standards, the amount of tetraols liberated from the DNA can be calculated. This is indicative of the amount of B[a]P-DNA adducts initially present. SFS does not provide information with respect to the DNA base the B[a]P-moiety was initially

attached to.

The SFS technique has been applied to detect B[a]P-DNA adducts in lung from smokers (Weston and Bowman 1991) and in placenta from women who had been smoking during pregnancy (Manchester *et al* 1990). The lower limit of detection was around one adduct in 10^8 nucleotides, but in order to reach this sensitivity about 500 μg of DNA was required. SFS turned out to be > 10-fold less sensitive for detection of other PAH-tetraols tested so far, compared to the detection of B[a]P-tetraol (Bowman *et al* 1990). SFS has also been applied to analyze B[a]P metabolites in human and animal urine (Uziel *et al* 1987). Part of this thesis deals with the use of SFS in establishing a series of B[a]P-DNA standards for adduct quantification by ^{32}P-postlabeling.

Fluorescence line-narrowing spectroscopy (FLNS) is suitable to obtain high-resolution optical spectra of molecules embedded in amorphous solids for which conventional absorption spectra yield broad vibronic bands (Jankowiak and Small 1989). In FLNS a narrow-banded laser selectively excites molecules whose individual absorption spectra coincide with the energy of the laser. The low temperature (liquid helium; 4.2 K) reduces the mobility of the molecules, thereby maintaining the excitation selection, which results in very sharp signals in the emission spectra. FLNS can be applied for detection of adducts in high molecular-weight DNA as well as of nucleoside adducts. For the B[a]P-DNA adduct, a lower detection limit of around 3 adducts in 10^8 nucleotides was determined with only 20 μg of DNA (Jankowiak *et al* 1988). In addition to being a selective and sensitive method, FLNS is also a practical technique. For a laser-based method the technology is relatively simple (see Discussion 8.2.2).

1.3.3 Immunoassays

For immunochemical detection of DNA adducts, polyclonal antisera or monoclonal antibodies are used that have been raised in mice, rats or rabbits against specific DNA modifications. To elicit an immune response, modified DNA or modified nucleotides (DNA adducts) must be coupled to an immunogenic carrier-protein before immunization of the animal. Whereas each polyclonal antiserum is unique and, therefore, can never be replaced with an identical serum, the supply of a particular monoclonal antibody is unlimited, provided that the producing clone is stable in time. Although antibodies are usually raised against one specific type of adduct, cross-reactivity of antisera and even monoclonal antibodies with other types of structurally related adducts has been reported (Newman *et al* 1990).

With respect to the immunochemical detection of DNA adducts, several methods have been developed. In the radio-immunoassay (RIA), one of the first techniques used,

radioactively labeled hapten, *e.g.*, a [^{3}H]-labeled adduct, competes in solution with unlabeled adduct from digested DNA for the antibody-binding sites (Rajewsky *et al* 1980). For detection of PAH-DNA adducts, solid-phase enzyme immunoassays appeared more sensitive than RIA (reviewed in Phillips 1990, dell'Omo and Lauwerys 1993). In this type of assays, the amount of antibody bound to unlabeled antigen is determined by use of an enzyme-linked second antibody. A signal is obtained by enzymatic cleavage of a chromogenic or fluorogenic substrate (enzyme-linked immunosorbent assay or ELISA), or by conversion of a radiolabeled substrate into a labeled product (ultra-sensitive enzyme RIA or USERIA). Thirdly, provided that the DNA modifications are heat- or alkali-stable, they can be quantified by use of the immuno-slot blot (ISB) assay, following the blotting of DNA on filters (Nehls *et al* 1984). For both competitive ELISA and USERIA, the lower detection limit for PAH-DNA adducts is around one adduct in 10^7 to 10^8 nucleotides (Santella *et al* 1987, Weston *et al* 1987). In solid-phase enzyme immunoassays, serial dilutions of highly modified B[a]P-DNA are often used to construct a calibration curve to determine adduct levels. This may, however, lead to an underestimation of the actual adduct level, as the affinity of the antiserum may depend on the level of DNA modification (van Schooten *et al* 1987).

These *in vitro* tests mentioned above, all start with isolated DNA and therefore do not allow the study of adducts in specific cell types. The application of antibodies in *in situ* assays makes it possible to detect DNA adducts in single cells. Some aspects of this assay will be discussed in more detail as some of these also apply to the above-mentioned techniques. In *in situ* assays cells are fixed and put onto slides. To obtain an optimal accessibility of the adducts, DNA is denatured *in situ*, whereupon the cells are treated with a proteinase and RNAse. Subsequently, the cells are incubated with the adduct-specific antibody, followed by an incubation with a second antibody.

The most commonly used second antibodies are the fluorochrome-containing conjugates and the immunoperoxidases. In case of the former, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are widely used fluorochromes. However, FITC fades more rapidly upon irradiation compared to TRITC (Larsson 1988). With the appropriate light source, in combination with emission and excitation filters and a device for signal detection and data acquisition, it is possible to quantify the amount of fluorescence per cell, which is indicative of the number of adducts present. In case of B[a]P, monoclonal antibodies that were specifically raised against the B[a]P-diolepoxide-N^{2}dG adduct have been described (Baan *et al* 1988b). These antibodies were successfully applied for B[a]P-DNA adduct quantification by means of immunofluorescence microscopy both in *in vitro* and *in vivo* studies (Baan *et al* 1988a, 1990).

A factor that limits the sensitivity of the use of fluorescence in adduct quantification is the autofluorescence of cells, *i.e.*, fluorescence derived from endogenous compounds and as such not related to the presence of adducts. In this respect, the use of rhodamine conjugates is to be preferred, as much of the autofluorescence is detected at the excitation-wavelength optimum of FITC. To overcome the problem of autofluorescence in a more general way, the use of immunoperoxidases in *in situ* detection can be useful. Immunoperoxidases form a group of enzymes that convert specific substrates into dark-coloured precipitates in the presence of H_2O_2. In this respect diaminobenzidine (DAB) appears to be a quite suitable substrate: staining results in an intensely dark-brown precipitate, the light absorbance of which is relatively stable in time. The intensity of DAB staining is an indirect measure of the number of adducts that was present initially. By use of the DAB staining, B[a]P-DNA adducts have been localized immunocytochemically in animal and human tissues (Shamsuddin and Gan 1988, van Schooten *et al* 1991). In order to quantify the number of adducts at the level of the single cell, the intensity of DAB staining can be determined with a conventional light microscope in combination with an image-analysis programme (Scherer *et al* 1988).

When antibodies are used for adduct quantification, one should realize that only relative adduct levels are obtained. Dependent upon the accessibility of the adducts and the intrinsic properties of the antibody, only a certain fraction of the adducts will be recognized. DNA loss during *in situ* procedures may contribute to an underestimation of the actual adduct level. Problems with quantification may also arise when a single compound leads to formation of more than one type of adduct, as the affinity of the antibody for these various adducts may differ. Ideally, affinity for each type of adduct formed by a single compound should be determined under conditions that are the same as those in the assay to be used. In case of compounds that mainly give one type of adduct, calibration of immunochemical adduct determination may be feasible by a combination of antibody staining with the results of an independent adduct-detection assay, *e.g.*, the ^{32}P-postlabeling. This can be done by treatment of, *e.g.*, white blood cells (WBC) with (±)-*anti*-B[a]P-diolepoxide, which gives rise to formation of mainly one type of adduct, and processing of these WBC, partly for DNA isolation and subsequent postlabeling and partly for *in situ* assays (see also Chapter 5).

1.4.1 *Structure and function of the trachea*

The main function of the trachea is to condition the incoming air: the remaining suspended matter is trapped and removed, while the temperature and the humidity of the air are adjusted to physiological conditions. As depicted in Figure 7, three main structures can be discerned. These are the pseudostratified epithelium, the lamina propria, and the C-shaped rings of cartilage (Rhodin 1974). The cartilage rings give support to the tissue and allow dilatation and constriction of the trachea. The lamina propria provides nutrition for the epithelium. Several blood vessels, mast cells, fibroblasts, smooth muscle cells and serous glands can be found in this layer (Rhodin 1974). The tracheal epithelial lining is composed of four major cell types: basal cells, ciliated cells, small mucous granule cells, and mucous goblet cells. Less frequently occurring cell types generally include intermediate cells, brush cells, serous cells, and neuroendocrine cells (McDowell 1982). All tracheal epithelial cells are connected to the basal lamina by hemidesmosomes (McDowell 1982). The secretory cell types produce a viscous glycoprotein mixture which is deposited onto the luminal surface. Ciliated cells transport the secreted mucous layer together with trapped particles towards the upper parts of the respiratory tract.

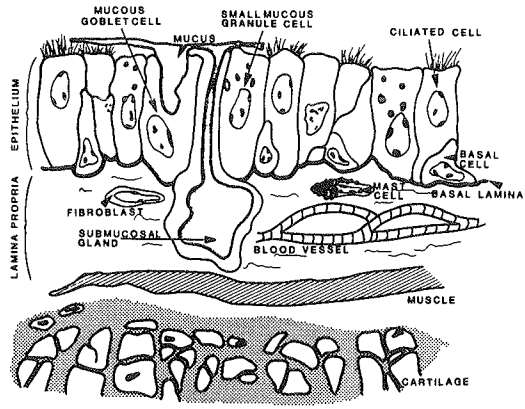

Figure 7 *Basic organization of the tracheal epithelium (Rhodin 1974).*

The hamster trachea model to study the induction of respiratory tract tumors *in vivo*, described originally by Saffiotti in 1968, involves repeated intratracheal instillations of B[a]P (Saffioti *et al* 1968, Harris *et al* 1971, 1972, Feron *et al* 1972). Syrian golden hamsters proved to be a useful species because they are relatively resistant to chronic respiratory disease, while spontaneous lung tumors rarely occur. Furthermore, the histological features of the respiratory tract of the hamster resemble those of the human (McDowell *et al* 1978a, Becci *et al* 1978a). It appeared that the morphology of the precancerous lesions in the hamster trachea induced by exposure to B[a]P has close resemblance with the morphological characteristics of human bronchial cancer. Since the Saffiotti publication, several studies have also provided evidence that the normal hamster tracheobronchial and the human bronchial epithelium share many similarities (Becci *et al* 1978b, c, McDowell *et al* 1978b, Trump *et al* 1978, McDowell and Trump 1983). These observations support the concept that the hamster tracheal tumor represents a valid model for studies and interpretations on the etiology of human lung carcinoma.

The introduction of the *in vitro* organ culture of the trachea (Lane and Miller 1976, Lane *et al* 1976) made it possible to study chemically-induced effects in the respiratory tract in a more extensive and dose-controlled way than in whole-animal experiments. Organ cultures allow complete control of the environment, easy manipulation of the model, and continuous monitoring of effects. The use of serum-free, hormone-supplemented media in organ cultures allowed tissue preservation for at least several weeks (Lane and Miller 1976, Van Scott *et al* 1986). With respect to the relative amounts of the various cell types within the tracheal epithelium, freshly isolated hamster epithelium contains about 40% ciliated cells, while in *in vitro* culturing this was found to be significantly lower, ranging from 20 to 30%, depending on the culture medium used (Sigler *et al* 1987). This may be related to the fact that ciliated cells in general are more susceptible to changes in the environment, such as the transition from air (*in vivo*) to culture medium (*in vitro*). However, the relative amount of ciliated cells in organ culture may have been underestimated due to internalization of cilia, which renders it difficult to discriminate between ciliated and secretory cells.

Hamster tracheas, kept in organ culture and exposed to cigarette smoke condensate - a mixture of compounds of which some are carcinogenic - showed a dose-dependent induction of pathological effects that precede tumor development *in vivo*, such as nodular hyperplasia and metaplasia (Chopra and Cooney 1985, Rutten *et al* 1988a). The *in vitro* hamster trachea model thus provides opportunities to study the formation and repair of DNA adducts in relation to the development of precancerous lesions.

1.5.1 The cell cycle

The division of a cell is a highly regulated process, which comprises several phases. After the mitotic phase, which encompasses nuclear and cytoplasmic division, the cell either enters the G_1 phase or becomes quiescent in a nonproliferative state (G_0 phase). The time spent in the G_0 phase is not only cell type dependent but is also influenced by other factors, such as hormonal stimulation. A cell in G_1 phase, after some time, enters the S-phase, which begins with the synthesis of DNA and ends when the DNA content of the nucleus has doubled. The cell then enters the G_2 phase, which continues until mitosis starts. The G_1, S, and G_2 phases together comprise about 90% of the total cell-cycle time.

1.5.2 Cell proliferation in the hamster tracheal epithelium

Proliferation of cells is a necessary step for the clonal expansion of cells that leads to carcinogenesis. It is generally accepted that the small mucous granule cell and the basal cell are the cell types that are responsible for cell renewal in the hamster tracheal epithelium (Nettesheim *et al* 1990). However, it has been shown that under certain conditions the ciliated cell also is capable of dividing to a limited extent (Rutten *et al* 1988b). The basal cell is the cell type from which all major cell types in the tracheal epithelium originate (Inayama *et al* 1988, Nettesheim *et al* 1990) and may therefore be seen as the major stem cell. The normal tracheal epithelium shows a rather slow turnover and it is likely that at a given moment most cells are in the G_0 phase of the cell cycle (Jetten 1991). A strict balance exists between cellular proliferation and differentiation, which is controlled by the action of several factors. *In vitro* studies have shown that proliferation is stimulated by EGF/TGF-α and insulin-like growth factors and is negatively regulated by TGF-β (reviewed in Jetten 1989, 1990). It is likely that these factors also play an important role *in vivo*.

Under certain conditions, quiescent cells may be recruited from G_0 to reenter the cell cycle. Mutations, translocations, deletions, and amplifications in genes that control proliferation of tracheal epithelial cells may lead to a disturbance of the delicate equilibrium between proliferation and differentiation (reviewed in Jetten 1991). Certain oncogene products are known to be specific substitutes for growth factors. Normally, the

sustained presence of certain growth factors is needed to allow a cell to proceed through the G_1 phase. Oncogenic proteins may compensate for this necessity, thereby stimulating cells to proliferate (Aaronson 1991).

1.5.3 Methods to determine the proliferative state

There are several ways to determine the percentage of cells that are in S-phase in both *in vivo* and *in vitro* experiments. The most commonly used method is to monitor the incorporation of the DNA precursor thymidine. After incubation with [^{3}H]thymidine, sections of the organ are placed on a microscope slide, overlaid with a photographic emulsion, and prepared for autoradiography. The incorporation of radiolabeled thymidine can be scored as black grains over the nucleus. The thymidine analogue bromodeoxyuridine (BrdU) has also been used instead of [^{3}H]thymidine. BrdU-labeled DNA can be visualized with immunohistochemical techniques. The BrdU and the [^{3}H]thymidine labeling produced essentially the same results (Goldsworthy *et al* 1991). Double-labeling procedures, in which a continuous labeling with either [^{3}H]thymidine or BrdU is followed or preceded by a pulse-labeling with either BrdU or [^{3}H]thymidine have provided more detailed information on the exact time point of changes in cell proliferation (Hume and Thompson 1990, Hyatt and Beebe 1992).

Another marker for cell proliferation that has recently gained much attention is the proliferating cell nuclear antigen (PCNA), which is intricately involved in cell replication processes (Dietrich 1993). The expression of PCNA starts in late G_1, reaching its maximum during the S-phase. The major advantage of the use of PCNA over that of BrdU or [^{3}H]thymidine is that retrospective assessment of the proliferative rates in archival tissues is still possible due to the conservation of this marker in fixed tissues. Besides its involvement in DNA replication, PCNA also plays a role in DNA excision repair (Stivala *et al* 1993).

The more general aim of the work described in this thesis was to study the genotoxic effects of B[a]P exposure in specific cell types in hamster tracheal epithelium, maintained in *in vitro* organ culture. Experiments were focused on determining the relationship between DNA-adduct formation and repair in specific cell types, proliferation of specific cell types and the development of precancerous lesions in tracheal epithelium. Several studies have already provided evidence that the hamster is well-suited to study respiratory tract carcinogenesis. Morphologically, the tumors that develop in hamster trachea after repeated intratracheal instillation of B[a]P resemble those found in the human respiratory tract (see 1.4.2). Histological features that precede tumor formation *in vivo*, such as squamous metaplasia and hyperplasia, could also be induced *in vitro* (in organ culture) after exposure of hamster tracheas to cigarette-smoke condensate. The interaction of carcinogenic compounds with DNA is closely associated with the initiation of carcinogenesis. The model compound B[a]P is metabolized to various reactive metabolites that are capable of reacting with DNA. Animal studies have shown a good correlation between tumor induction and the formation of one specific adduct, *viz.* that between (+)-*anti*-B[a]P-7,8-dihydroxy-9,10-epoxide and deoxyguanosine (B[a]PdG).

Various techniques to detect DNA adducts are available (see 1.3). Two of these techniques were applied in this thesis, namely the ^{32}P-postlabeling analysis and the *in situ* immunofluorescence microscopy technique. The first assay allows sensitive adduct quantification; furthermore, by simultaneous analysis of the appropriate adduct standards, some structural information can be obtained. Optimization of the ^{32}P-postlabeling procedure, especially with respect to adduct quantification, was achieved. The second assay, in which antibodies against specific DNA adducts were used, allows the study of adduct formation at the level of the individual cell. Antibodies directed against the B[a]PdG adduct were generated, characterized and found to be suitable for use in our *in vitro* hamster trachea model.

Hamster tracheas in organ culture were exposed to B[a]P according to different exposure regimes. A procedure, developed for use in the hamster trachea model, allowed us to simultaneously and discriminatingly detect B[a]P-DNA adducts in basal and non-basal cells *in situ*. In this way, cell-type-specific adduct formation and removal could be studied. The relative amount of proliferating cells was also determined in these preparations for both basal and non-basal cells. By parallel investigation of the

development of precancerous lesions in the tracheal epithelium, more insight in the process of carcinogenesis and the specific cell types involved could be obtained.

In chapter 2 some methodological aspects of the ^{32}P-postlabeling procedure are discussed. With respect to DNA-adduct quantification, a series of B[a]P-modified DNA samples was prepared to serve as calibration standards. The respective adduct levels of these B[a]P-modified DNA standards were established in an independently assay, namely by use of synchronous fluorescence spectrophotometry. Two of these DNA samples were routinely included as external standards in each postlabeling experiment, which provided a more reliable adduct quantification compared to the more commonly used relative adduct labeling.

An application of the postlabeling procedure is described in chapter 3. In a series of *in vitro* studies microsomes from various species, including man, were incubated with B[a]P and either untreated DNA or white blood cells. These microsomes comprise subcellular fractions containing endoplasmic reticulum with several types of the bioactivating cytochrome P450 enzymes. The data obtained provide insight into the formation and stability of reactive B[a]P-metabolites that bind to DNA and give rise to different adduct patterns.

In chapter 4 a study is presented on the pattern of B[a]P-induced DNA-adduct formation and removal in tracheas from Syrian golden hamsters and Wistar rats in organ culture. This comparison is of particular interest, in view of the striking differences between these species with respect to susceptibility to development of tracheal tumors.

The use of antibodies in adduct quantification *in situ* allows the study of the induction of adducts in specific cell types. Chapter 5 describes the generation of an antibody that appeared suitable to recognize B[a]P-DNA adducts in epithelial cells of the hamster trachea. By combining the adduct-specific staining with the staining of a basal cell-specific cytokeratin we were able to selectively determine adduct levels in the basal cell. This specific technique was applied in an induction-repair experiment with hamster tracheas that had been exposed to B[a]P for 2 days, whereafter the tracheas were transferred to a B[a]P-free medium and cultured for another 3 days.

Cell proliferation is a prerequisite for the clonal expansion of cells that leads to tumor development. Under normal conditions, the process of cell proliferation is strictly regulated; recently, the role of tumor-suppressor genes in the negative regulation of cell proliferation has gained much attention. In chapter 6, the effect of different concentrations of B[a]P on cell proliferation and DNA-adduct level in hamster tracheal epithelium was studied in more detail.

Chapter 7 describes the relation between cell-specific adduct formation in hamster tracheas exposed to B[a]P in organ culture, along with cell-specific proliferation and the

development of precancerous lesions that precede tumor development *in vivo*, such as squamous metaplasia and hyperplasia.

A general discussion and concluding remarks are presented in chapter 8.

Aaronson SAL (1991) Growth factors and cancer. *Science*, **254**, 1146-1153.

Alexandrov K, Dansette PM, Frayssinet C (1980) Effect of purified epoxide hydrolase on metabolic activation and binding of benzo(a)pyrene to exogenous DNA. Shift of the activation pathway. *Bioch Biophys Res Com*, **93**, 611-616.

Alexandrov K, Sala M, Rojas M (1988) Differences in the DNA adducts formed in cultured rabbit and rat dermal fibroblasts by benzo(a)pyrene and (−)-benzo(a)pyrene-7,8-diol. *Cancer Res*, **48**, 7132-7139.

Armstrong RN (1991) Glutathione S-transferases: reaction mechanism, structure, and function. *Chem Res Toxicol*, **4**, 131-140.

Ashurst SW, Cohen GM (1982) The formation of benzo(a)pyrene-deoxyribonucleoside adducts *in vivo* and *in vitro*. *Carcinogenesis*, **3**, 267-273.

Ayrton AD, McFarlane M, Walker R, Neville S, Coombs MM, Ioannides C (1990) Induction of the P-450 I family of proteins by polycyclic aromatic hydrocarbons: possible relationship to their carcinogenicity. *Toxicol*, **60**, 173-186.

Baan RA, van den Berg PTM, Steenwinkel M-JST, van der Wulp CJM (1988a) Detection of benzo(a)pyrene-DNA adducts in cultured cells treated with benzo(a)pyrene diol-epoxide by quantitative immunofluorescence microscopy and ^{32}P-postlabelling; immunofluorescence analysis of benzo(a)pyrene-DNA adducts in bronchial cells from smoking individuals. In Bartsch H Hemminki K, O'Neill IK, eds. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention (IARC Scientific Publications No.89), IARC, Lyon, 146-154.

Baan RA, van den Berg PTM, Watson WP, Smith RJ (1988b) *In situ* detection of DNA adducts formed in cultured cells by benzo(a)pyrene diolepoxide (BPDE), with monoclonal antibodies specific for the BP-deoxyguanosine adduct. *Toxicol Environm Chem*, **16**, 325-339.

Baan RA, van den Berg PTM, van Delft JHM, Roza L, van der Meer EG, van der Wulp CJM (1990) *In situ* detection of DNA damage in single cells or tissue sections by quantitative immunofluorescence microscopy. In Mendelsohn ML, Albertini RJ, eds. Mutation and the environment, part A: Basic mechanisms, progress in clinical and biological research, vol 340A, Wiley-Liss, New York, 101-112.

Beach AC, Gupta RC (1992) Human biomonitoring and the ^{32}P-postlabeling assay. *Carcinogenesis*, **13**, 1053-1074.

Becci PJ, McDowell EM, Trump BF (1978a) The respiratory epithelium. II. Hamster trachea, bronchus, and bronchioles. *J Natl Cancer Inst*, **61**, 551-561.

Becci PJ, McDowell EM, Trump BF (1978b) The respiratory epithelium. IV. Histogenesis of epidermoid metaplasia and carcinoma *in situ* in the hamster. *J Natl Cancer Inst*, **61**, 577-586.

Becci PJ, McDowell EM, Trump BF (1978c) The respiratory epithelium. VI. Histogenesis of lung tumors induced by benzo[a]pyrene-ferric oxide in the hamster. *J Natl Cancer Inst*, **61**, 607-618.

Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW (1993) Genetic risk and carcinogenic exposure: a common inherited defect of the carcinogen-metabolism gene glutathione S-transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J Natl Cancer Inst*, **85**, 1159-1164.

van Bladeren PJ (1988) Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *TIPS*, **9**, 295-299.

Bodell WJ, Devanesan PD, Rogan EG, Cavalieri EL (1989) ^{32}P-Postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* and *in vivo*. *Chem Res Toxicol*, **2**, 312-315.

Boyer TD (1989) The glutathione S-transferases: an update. *Hepatology*, **9**, 486-496.

Bowman ED, Shields PG, Poirier MC, Santella RM, Manchester DK, Farmer PB, Harris CC, Weston A (1990) Determination of complete fluorescence excitation-emission matrices for polycyclic aromatic hydrocarbon-DNA adducts found in human lung and placenta. *Proc Am Assoc Cancer Res*, **31**, 89 (abstract).

Brambilla G, Martelli A (1992) Grain counting in the *vitro* hepatocyte DNA-repair assay. *Mutat Res*, **272**, 9-15.

Brauze D, Mikstacka R, Baer-Dubowska W (1991) Formation and persistence of benzo(a)pyrene-DNA adducts in different tissues of C57BL/10 and DBA/2 mice. *Carcinogenesis*, **12**, 1607-1611.

Buratowski S (1993) DNA repair and transcription: the helicase connection. *Science*, **260**, 37-38.

Cabral-Neto JB, Gentil A, Caseira-Cabral RE, Sarasin A (1992) Mutation spectrum of heat-induced abasic sites on a single-stranded shuttle vector replicated in mammalian cells. *J Biol Chem*, **267**, 19718-19723.

Canella K, Peltonen K, Dipple A (1991) Indentification of (+) and (−)*anti* benzo[a]pyrene dihydrodiol epoxide-nucleic acid adducts by the ^{32}P-postlabeling assay. *Carcinogenesis*, **12**, 1109-1114.

Cavalieri EL, Rogan EG, RamaKrishna NVS, Devanesan PD (1993) Mechanisms of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene activation: qualitative aspects of the stable and depurination DNA adducts obtained from radical cations and diol epoxides. In Garrigues P, Lamotte M, eds. Polycyclic aromatic compounds. Synthesis, properties, analytical measurements, occurrence and biological effects, Gordon and Breach Science Publishers, Langhorne, 725-732.

Celotti L, Ferraro P, Furlan D, Zanesi N, Pavanello S (1993) DNA repair in human lymphocytes treated *in vitro* with (±)-*anti* and (±)-*syn*-benzo[a]pyrene diolepoxide. *Mutat Res*, **294**, 117-126.

Chen R-H, Maher VM, Brouwer J, van de Putte P, McCormick JJ (1992) Preferential repair and strand-specific repair of benzo[a]pyrene diol epoxide adducts in the *HPRT* gene of diploid human fibroblasts. *Proc Natl Acad Sci*, **89**, 5413-5417.

Cheng SC, Hilton BD, Roman JM, Dipple A (1989) DNA adducts from carcinogenic and noncarcinogenic enantiomers of benzo(a)pyrene dihydrodiol epoxide. *Chem Res Toxicol*, **2**, 334-340.

Chopra DP, Cooney RA (1985) Histogenesis of benzo(a)pyrene-induced lesions in tracheal explants. *Virch Arch B Cell Pathol*, **48**, 299-315.

Conney AH (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: GHA Clowes memorial lecture. *Cancer Res*, **42**, 4875-4917.

Devanesan PD, RamaKrishna NVS, Todorovic R, Rogan EG, Cavalieri EL, Jeong H, Jankowiak R, Small GJ (1992) Identification and quantitation of benzo(a)pyrene-DNA adducts formed by rat liver microsomes *in vitro*. *Chem Res Toxicol*, **5**, 302-309.

Dietrich DR (1993) Toxicological and pathological applications of proliferating cell nuclear antigen (PCNA), a novel endogenous marker for cell proliferation. *Crit Rev Toxicol*, **23**, 77-109.

Dittrich KA, Krugh TR (1991) Mapping of (±)-*anti*-benzo[a]pyrene diol epoxide adducts to human c-Ha-ras1 protooncogene. *Chem Res Toxicol*, **4**, 277-281.

Dunn BP, San RHC (1988) HPLC enrichment of hydrophobic DNA-carcinogen adducts for enhanced sensitivity of ^{32}P-postlabeling analysis. *Carcinogenesis*, **9**, 1055-1060.

Eberhart J, Coffing SL, Anderson JN, Marcus C, Kalogeris TJ, Baird WM, Park SS, Gelboin HV (1992) The time-dependent increase in the binding of benzo(a)pyrene to DNA through (+)-*anti*-benzo(a)pyrene-7,8-diol-9,10-epoxide in primary rat hepatocyte cultures results from induction of cytochrome P450Ia1 by benzo(a)pyrene treatment. *Carcinogenesis*, **13**, 297-301.

Fahl WE, Shen AL, Jefcoate CR (1978) UDP-glucuronosyl transferase and the conjugation of benzo(a)pyrene metabolites to DNA. *Bioch Biophys Res Com*, **85**, 891-899.

Feron VJ (1972) Respiratory tract tumours in hamsters after intratracheal instillations of benzo(a)pyrene alone and with furfural. *Cancer Res*, **32**, 28-36.

Fielding JE (1985) Smoking: heath effects and control. *N Eng J Med*, **313**, 491-498.

Gallagher JE, Kohan MJ, George MH, Lewtas J (1991) Improvement in the diagnostic potential of ^{32}P-postlabeling analysis demonstrated by the selective formation and comparative analysis of nitrated-PAH-derived adducts arising from diesel particle extracts. *Carcinogenesis*, **12**, 1685-1691.

Gentil A, Cabral-Neto JB, Mariage-Samson R, Margot A, Imbach JL, Rayner B, Sarasin A (1992) Mutagenicity of a unique apurinic/apyrimidinic site in mammalian cells. *J Mol Biol*, **227**, 981-984.

Ginsberg GL, Atherholt TB (1989) Transport of DNA-adducting metabolites in mouse serum following benzo(a)pyrene administration. *Carcinogenesis*, **10**, 673-679.

Ginsberg GL, Atherholt TB (1990) DNA adduct formation in mouse tissues in relation to serum levels of benzo(a)pyrene-diol-epoxide after injection of benzo(a)pyrene or the diol-epoxide. *Cancer Res*, **50**, 1189-1194.

Goldsworthy TL, Morgan KT, Popp JA, Butterworth BE (1991) Guidelines for measuring chemically induced cell proliferation in specific rodent target organs. In Chemically induced cell proliferation: implications for risk assessment, Wiley-Liss, New York, 253-284.

Gorelick NJ (1993) Application of HPLC in the ^{32}P-postlabeling assay. *Mutat Res*, **288**, 5-18.

Gorelick NJ, Wogan GN (1989) Fluoranthene-DNA adducts: identification and quantification by an HPLC-^{32}P-postlabeling method. *Carcinogenesis*, **10**, 1567-1577.

Guo J-F, Brown R, Rothwell CE, Bernstein IA (1990) Levels of cytochrome P-450-mediated aryl hydrocarbon hydroxylase (AHH) are higher in differentiated than in germinative cutaneous keratinocytes. *J Invest Dermatol*, **94**, 86-93.

Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen-DNA adducts. *Cancer Res*, **45**, 5656-5662.

Gupta RC, Earley K (1988) ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis*, **9**, 1687-1693.

Hall M, Grover PL (1990) Polycyclic aromatic hydrocarbons: metabolism, activation and tumour initiation. In Cooper CS, Grover PL, eds. Chemical carcinogenesis and mutagenesis, Cooper CS, Grover PL, eds, Springer Verlag, Berlin, 327-372.

Hammond EC, Selikoff IJ, Lawther PL, Seidman H (1976) Inhalation of benzpyrene and cancer in man. *Ann NY Acad Sc*, **271**, 116-124.

Harris CC, Sporn MB, Kaufman DG, Smith JM, Baker MS, Saffiotti U (1971) Acute ultrastructural effects of benzo(a)pyrene and ferric oxide on the hamster tracheobronchial epithelium. *Cancer Res*, **31**, 1977-1989.

Harris CC, Sporn MB, Kaufman DG, Smith JM, Jackson FE, Saffioti U (1972) Histogenesis of squamous metaplasia in the hamster tracheal epithelium caused by vitamin A deficiency of benzo[a]pyrene-ferric oxide. *J Natl Cancer Inst*, **48**, 743-761.

Harris CC (1989) Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. *Carcinogenesis*, **10**, 1563-1566.

Harvey RG, Geacintov NE (1988) Intercalation and binding of carcinogenic hydrocarbon metabolites to nucleic acids. *Acc Chem Res*, **21**, 66-73.

Hayashi S-I, Watanabe J, Kawajiri K (1992) High susceptibility to lung cancer analyzed in terms of combined genotypes of P450IA1 and Mu-class glutathione S-transferase genes. *Jpn J Cancer Res*, **83**, 866-870.

Hemminki K, Grzybowska E, Chorazy M, Twardowska-Saucha K, Sroczynski JW, Putman KL, Randerath K, Phillips DH, Hewer A, Santella RM, Young TL, Perera FP (1990) DNA adducts in humans environmentally exposed to aromatic compounds in an industrial area of Poland. *Carcinogenesis*, **11**, 1229-1231.

Hogstedt C, Andersson K, Frenning B, Gustavsson A (1981) A cohort on mortality among long time employed Swedish chimney sweeps. *Scand J Work Environ Health Suppl*, **1**, 72-78.

Howie AF, Bell D, Hayes PC, Hayes JD, Beckett GJ (1990) Glutathione S-transferase isoenzymes in human bronchoalveolar lavage: a possible early marker for the detection of lung cancer. *Carcinogenesis*, **11**, 295-300.

Hughes MF, Chamulitrat W, Mason RP, Eling TE (1989) Epoxidation of 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene via a hydroperoxide-dependent mechanism catalyzed by lipoxygenases. *Carcinogenesis*, **10**, 2075-2080.

Hume WJ, Thompson J (1990) Double labelling of cells with tritiated thymidine and bromodeoxyuridine reveals a circadian rhytm-dependent variation in duration of DNA synthesis and S phase flux rates in rodent oral epithelium. *Cell Tissue Kinet*, **23**, 313-323.

Hyatt GA, Beebe DC (1992) Use of a double-label method to detect rapid changes in the rate of cell proliferation. *J Histochem Cytochem*, **40**, 619-627.

IARC (1973) Monographs on the evaluation of carcinogenic risk of the chemical to man. Certain polycyclic aromatic hydrocarbons and heterocyclic compounds. Volume 3, IARC, Lyon.

IARC (1983) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Polynuclear aromatic compounds, part 1, chemical, environmental and experimental data. Volume 32, IARC, Lyon.

IARC (1986) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Tobacco smoking. Volume 38, IARC, Lyon.

Inayama Y, Hook GER, Brody AR, Cameron GS, Jetten AM, Gilmore LB, Gray T, Nettesheim P (1988) The differentiation potential of tracheal basal cells. *Lab Invest*, **58**, 706-717.

Ishikawa T, Takayama S, Ide F (1980) Autoradiographic demontration of DNA repair synthesis in rat tracheal epithelium treated with chemical carcinogens *in vitro*. *Cancer Res*, **40**, 2898-2903.

Jankowiak R, Cooper RS, Zamzow D, Small GJ (1988) Fluorescence line narrowing-nonphotochemical hole burning spectrometry: femtomole detection and high selectivity for intact DNA-PAH adducts. *Chem Res Toxicol*, **1**, 60-68.

Jankowiak R, Small GJ (1989) Fluorescence line-narrowing spectroscopy in the study of chemical carcinogenesis. *Anal Chem*, **61**, 1023A-1032A.

Jerina DM, Sayer JM, Thakker DR, Yagi H (1980) Carcinogenicity of polycyclic aromatic hydrocarbons: the bay-region theory. In Pullman B, Ts'O POP, Gelboin HV, eds. Carcinogenesis: fundamental mechanisms and environmental effects. D Reidel, Dordrecht, 1-12.

Jetten AM (1989) Multistep process of squamous differentiation in tracheobronchial epithelial cells *in vitro*: analogy with epidermal differentiation. *Env Health Persp*, **80**, 149-160.

Jetten AM, Vollberg TM, Nervi C, George MD (1990) Positive and negative regulation of proliferation and differentiation in tracheobronchial epithelial cells. *Am Rev Respir Dis*, **142**, S36-S39.

Jetten AM (1991) Growth and differentiation factors in tracheobronchial epithelium. *Am J Physiol*, **260**, L361-L373.

Jin C-J, Miners JO, Burchell B, Mackenzie PI (1993) The glucuronidation of hydroxylated metabolites of benzo(a)pyrene and 2-acetylaminofluorene by cDNA-expressed human UDP-glucuronosyltransferases. *Carcinogenesis*, **14**, 2637-2639.

Jones KC, Sanders G, Wild SR, Burnett V, Johnston AE (1992) Evidence for a decline of PCBs and PAHs in rural vegetation and air in the United Kingdom. *Nature*, **356**, 137-140.

Kolonel LN (1993) Lung cancer: another consequence of a high-fat diet? *J Natl Cancer Inst*, **85**, 1886-1887.

Koreeda M, Moore PD, Wislocki PG, Levin W, Conney AH, Yagi H, Jerina DM (1978) Binding of benzo(a)pyrene 7,8-diol-9,10-epoxides to DNA, RNA, and protein of mouse skin occurs with high stereoselectivity. *Science*, **199**, 778-781.

Kulkarni MS, Anderson MW (1984) Persistence of benzo(a)pyrene metabolite: DNA adducts in lung and liver of mice. *Cancer Res*, **44**, 97-101.

Kuper A, Benford DJ (1991) Unscheduled DNA synthesis in tracheal epithelial cell cultures. *Toxicol in Vitro*, **5**, 511-513.

Kwei GY, Zaleski J, Irwin SE, Thurman RG, Kauffman FC (1992) Conjugation of benzo(a)pyrene 7,8-dihydrodiol-9,10-epoxide in infant Swiss-Webster mice. *Cancer Res*, **52**, 1639-1642.

Lane BP, Miller SL (1976) Carcinogen-induced changes in tracheal epithelium cultured in serum-free, chemically defined medium. *J Natl Cancer Inst*, **56**, 991-996.

Lane BP, Miller SL, Drummond EJ (1976) Use of tracheal organ cultures in toxicity testing. *Env Health Persp*, **16**, 89-98.

Larsson L-I (1988) Immunocytochemistry: theory and practice. CRC Press, Boca Raton, Florida, 272 p.

Lecoq S, Ni She M, Grover PL, Platt KL, Oesch F, Phillips DH (1991) The *in vitro* metabolic activation of dibenz[a,h]anthracene, catalyzed by rat liver microsomes and examined by ^{32}P-postlabelling. *Cancer Lett*, **57**, 261-269.

Li D, Randerath K (1990) Association between diet and age-related DNA modifications (I-Compounds) in rat liver and kidney. *Cancer Res*, **50**, 3991-3996.

Li D, Xu D, Randerath K (1990) Species and tissue specificities of I-compounds as contrasted with carcinogen adducts in liver, kidney and skin DNA of Sprangue-Dawley rats, ICR mice and Syrian hamsters. *Carcinogenesis*, **11**, 2227-2232.

Lioy PL, Waldman JM, Greenberg A, Harkov R, Pietarinen C (1988) The total human environmental exposure study (THEES) to benzo(a)pyrene: comparison of the inhalation and food pathways. *Arch Env Health*, **43**, 304-312.

Loeb LA, Ernster VL, Warner KE (1984) Smoking and lung cancer: an overview. *Cancer Res*, **44**, 5940-5958.

Lonati-Galligani M, Lohman PHM, Berends F (1983) The validity of the autoradiographic method for detecting DNA repair synthesis in rat hepatocytes in primary culture. *Mutat Res*, **113**, 145-160.

Lu L-JW, Anderson LM, Jones AB, Moskal TJ, Salazar JJ, Hokanson JA, Rice JM (1993) Persistence, gestation stage-dependent formation and interrelationsship of benzo(a)pyrene-induced DNA adducts in mothers, placentae and fetuses of *Erythrocebus patas* monkeys. *Carcinogenesis*, **14**, 1805-1813.

Mallet WG, Mosebrook DR, Trush MA (1991) Activation of (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene to diolepoxides by human polymorphonuclear leukocytes or myeloperoxidase. *Carcinogenesis*, **12**, 521-524.

Manchester DK, Wilson VL, Hsu IC, Choi JS, Parker NB, Mann DL, Weston A, Harris CC (1990) Synchronous fluorescence spectroscopic, immunoaffinity chromatographic and ^{32}P-postlabeling analysis of human placental DNA known to contain benzo[a]pyrene diol epoxide adducts. *Carcinogenesis*, **11**, 553-559.

Mannervik B, Alin P, Guthenberg C, Jensson H, Tahir K, Warholm M, Jörnvall H (1985) Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc Natl Acad Sci*, **82**, 7202-7206.

Masento MS, Hewer A, Grover PL, Phillips DH (1989) Enzyme-mediated phosphorylation of polycyclic hydrocarbon metabolites: detection of non-adduct compounds in the ^{32}P-postlabelling assay. *Carcinogenesis*, **10**, 1557-1559.

McDowell EM, Barrett LA, Glavin F, Harris CC, Trump BF (1978a) The respiratory epithelium. I. Human bronchus. *J Natl Cancer Inst*, **61**, 539-549.

McDowell EM, McLaughlin JS, Merenyi DK, Kieffer RF, Harris CC, Trump BF (1978b) The respiratory epithelium. V. Histogenesis of lung carcinomas in the human. *J Natl Cancer Inst*, **61**, 587-606.

McDowell EM (1982) Histogenesis and morphogenesis of bronchial neoplasms. In Shimosato Y, Melamed MR, Nettesheim P, eds. Morphogenesis of lung cancer, vol 1, CRC Press, Boca Raton, Florida, 1-36.

McDowell EM, Trump BF (1983) Histogenesis of preneoplastic and neoplastic lesions in tracheobronchial epithelium. *Surv Synth Path Res*, **2**, 235-279.

McManus ME, Burgess WM, Veronese ME, Huggett A, Quattrochi LC, Tukey RH (1990) Metabolism of 2-acetylaminofluorene and benzo(a)pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. *Cancer Res*, **50**, 3367-3376.

McMillan TJ (1992) Residual DNA damage: what is left over and how does this determine cell fate? *Eur J Cancer*, **28**, 267-269.

Meehan T, Straub K, Calvin M (1977) Benzo(a)pyrene diol epoxide covalently binds to deoxyguanosine and deoxyadenosine in DNA. *Nature*, **269**, 725-727.

Meehan T, Straub K (1979) Double-stranded DNA stereoselectively binds benzo(a)pyrene diol epoxides. *Nature*, **277**, 410-412.

Miller EC (1978) Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. *Cancer Res*, **38**, 1479-1496.

Miller JA (1970) Carcinogenesis by chemicals: an overview. GHA Clowes memorial lecture. *Cancer Res*, **30**, 559-576.

Mitsudomi T, Oyama T, Kusano T, Osaki T, Nakanishi R, Shirakusa T (1993) Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J Natl Cancer Inst*, **85**, 2018-2023.

Monks TJ, Anders MW, Dekant W, Stevens JL, Lau SS, van Bladeren PJ (1990) Glutathione conjugate mediated toxicities. *Toxicol Appl Pharmacol*, **106**, 1-19.

Moore CJ, Pruess-Schwartz D, Mauthe RJ, Gould MN, Baird WM (1987) Interspecies differences in the major DNA adducts formed from benzo(a)pyrene but not 7,12-dimethylbenz(a)anthracene in rat and human mammary cell cultures. *Cancer Res*, **47**, 4402-4406.

Moorghen M, Cairns J, Forrester LM, Hayes JD, Hall A, Catan AR, Wolf CR, Harris AL (1991) Enhanced expression of glutathione S-transferases in colorectal carcinoma compared to non-neoplastic mucosa. *Carcinogenesis*, **12**, 13-17.

Mullenders LHF, Vrieling H, Venema J, van Zeeland AA (1991) Hierarchies of DNA repair in mammalian cells: biological consequences. *Mutat Res*, **250**, 223-228.

Nebert DW, Eisen HJ, Negishi M, Lang MA, Hjelmeland LM (1981) Genetic mechanisms controlling the induction of polysubstrate monooxygenase (P-450) activities. *Ann Rev Pharmacol Toxicol*, **21**, 431-462.

Nebert DW (1989) The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol*, **20**, 153-174.

Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R, Waterman MR (1989) The P450

superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA Cell Biol*, **8**, 1-13.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ (1991) The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol*, **10**, 1-14.

Nehls P, Adamkiewicz J, Rajewsky MF (1984) Immuno-slot-blot: a highly sensitive immunoassay for the quantitation of carcinogen-modified nucleosides in DNA. *J Cancer Res Clin Oncol*, **108**, 23-29.

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ , Gunsalus IC, Gotoh O, Okuda K, Nebert DW (1993) The P450 superfamily: updated on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol*, **12**, 1-51.

Nettesheim P, Jetten AM, Inayama Y, Brody AR, George MA, Gilmore LB, Gray T, Hook GER (1990) Pathways of differentiation of airway epithelial cells. *Env Health Persp*, **85**, 317-329.

Newman MJ, Weston A, Carver DC, Mann DL, Harris CC (1990) Serological characterization of polycyclic aromatic hydrocarbon diolepoxide-DNA adducts using monoclonal antibodies. *Carcinogenesis*, **11**, 1903-1907.

dell'Omo M, Lauwerys RR (1993) Adducts to macromolecules in the biological monitoring of workers exposed to polycyclic aromatic hydrocarbons. *Crit Rev Toxicol*, **23**, 111-126.

Parke DV, Ioannides C, Lewis DFV (1991) The role of the cytochromes P450 in the detoxication and activation of drugs and other chemicals. *Can J Physiol Pharmacol*, **69**, 537-549.

Penning TM, Sharp RB (1990) Characterization of dihydrodiol dehydrogenase in human liver and lung. *Carcinogenesis*, **11**, 1203-1208.

Penning TM (1993) Dihydrodiol dehydrogenase and its role in polycyclic aromatic hydrocarbon metabolism. *Chem Biol Int*, **89**, 1-34.

Perin-Roussel O, Perin F, Zajdela F (1990) ^{32}P-Post-labeling analysis of DNA adducts in mouse embryo fibroblasts treated with dibenzo[a,e]fluoranthene and its major metabolites. *Carcinogenesis*, **11**, 301-306.

Peto R, Lopez AD, Boreham J, Thun M, Heath Jr C (1992) Mortality from tobacco in developed countries: indirect estimation from national vital statistics. *Lancet*, **339**, 1268-1278.

Petruska JM, Mosebrook DR, Jakab GJ, Trush MA (1992) Myeloperoxidase-enhanced formation of (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene-DNA adducts in lung tissue in vitro: a role of pulmonary inflammation in the bioactivation of a procarcinogen. *Carcinogenesis*, **13**, 1075-1081.

Phillips DH (1983) Fifty years of benzo(a)pyrene. *Nature*, **303**, 468-472.

Phillips DH (1990) Modern methods of DNA adduct determination. In Cooper CS, Grover PL, eds. Chemical carcinogenesis and mutagenesis, Springer Verlag, Berlin, 503-546.

Plopper CG, Cranz DL, Kemp L, Serabjit-Singh CJ, Philpot RM (1987) Immunohistochemical demonstration of cytochrome P-450 monooxygenase in clara cells throughout the tracheobronchial airways of the rabbit. *Exp Lung Res*, **13**, 59-68.

van Poppel G, de Vogel N, van Bladeren PJ, Kok FJ (1992) Increased cytogenetic damage in smokers deficient in glutathione S-transferase isozyme μ. *Carcinogenesis*, **13**, 303-305.

Puisieux A, Lim S, Groopman J, Ozturk M (1991) Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. *Cancer Res*, **51**, 6185-6189.

Rajewsky MF, Müller R, Adamkiewicz J, Drosdziok W (1980) In Pullman B, Ts'O POP, Gelboin HV, eds. Carcinogenesis: fundamental mechanisms and environmental effects, D Reidel, Dordrecht, 207-218.

Randerath E, Agrawal HP, Weaver JA, Bordelon CB, Randerath K (1985) ^{32}P-Postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with, 7,12-dimethylbenz[a]anthracene. *Carcinogenesis*, **6**, 1117-1126.

Randerath K, Reddy MV, Gupta RC (1981) ^{32}P-Labeling test for DNA damage. *Proc Natl Acad Sci*, **78**, 6126-6129.

Randerath K, Liehr JG, Gladek A, Randerath E (1989a) Age-dependent covalent DNA alterations (I-compounds) in rodent tissues: species, tissue and sex specificities. *Mutat Res*, **219**, 121-133.

Randerath K, Randerath E, Danna TF, van Golen KL, Putman KL (1989b) A new sensitive ^{32}P-postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to

radiolabeled dinucleotides and nucleoside 5'-monophosphates. *Carcinogenesis*, **10**, 1231-1239.
Randerath K, Putman KL, Osterburg HH, Johnson SA, Morgan DG, Finch CE (1993) Age-dependent increases of DNA adducts (I-compounds) in human and rat brain. *Mutat Res*, **295**, 11-18.
Ranganathan S, Tew KD (1991) Immunohistochemical localization of glutathione S-transferases α, μ and π in normal tissue and carcinomas from human colon. *Carcinogenesis*, **12**, 2383-2387.
Reardon DB, Bigger CAH, Strandberg J, Yagi H, Jerina DM, Dipple A (1989) Sequence selectivity in the reaction of optically active hydrocarbon dihydrodiol epoxides with rat H-*ras* DNA. *Chem Res Toxicol*, **2**, 12-14.
Reardon DB, Bigger CAH, Dipple A (1990) DNA polymerase action on bulky deoxyguanosine and deoxyadenosine adducts. *Carcinogenesis*, **11**, 165-168.
Reddy AP, Pruess-Schwartz D, Ji C, Gorycki P, Marnett LJ (1992a) ^{32}P-postlabeling analysis of DNA adduction in mouse skin following topical administration of (+)-7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene. *Chem Res Toxicol*, **5**, 26-33.
Reddy AP, Pruess-Schwartz D, Marnett LJ (1992b) Separation of (+)-*syn*- and (−)-*anti*-benzo[a]pyrene dihydrodiol epoxide-DNA adducts in ^{32}P-postlabeling analysis. *Chem Res Toxicol*, **5**, 19-25.
Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543-1551.
Reddy MV (1991) Nuclease S1-mediated enhancement of the ^{32}P-postlabeling assay for aromatic carcinogen-DNA adducts. *Carcinogenesis*, **12**, 1745-1748.
Redmond CK, Strobono BR, Cypress RH (1976) Cancer experience among coke by-product workers. *Ann NY Acad Sci*, **271**, 102-115.
Reed GA, Grafstrom RC, Krauss RS, Autrup H, Eling TE (1984) Prostaglandin H synthase-dependent co-oxygenation of (±)-7,8-dihydroxy-7,8-dihydro benzo(a)pyrene in hamster trachea and human bronchus explants. *Carcinogenesis*, **5**, 955-960.
Reichert WL, Stein JE, French B, Goodwin P, Varanasi U (1992) Storage phosphor imaging technique for detection and quantitation of DNA adducts measured by the ^{32}P-postlabeling assay. *Carcinogenesis*, **13**, 1475-1479.
Reynolds SH, Anna CK, Brown KC, Wiest JS, Beattie EJ, Pero RW, Iglehart JD, Anderson MW (1991) Activated protooncogenes in human lung tumors from smokers. *Proc Natl Acad Sci*, **88**, 1085-1089.
Rhodin JAG (1974) Respiratory system. In Rhodin JAG, ed. Histology, a text and atlas, Oxford University Press, London, pp 618-624.
Robertson IGC, Guthenberg C, Mannervik B, Jernström B (1986a) Differences in stereoselectivity and catalytic efficiency of three human glutathione transferases in the conjugation of glutathione with 7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Cancer Res*, **46**, 2220-2224.
Robertson IGC, Jensson H, Mannervik B, Jernström B (1986b) Glutathione transferases in rat lung: the presence of transferase 7-7, highly efficient in the conjugation of glutathione with the carcinogenic (+)-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene. *Carcinogenesis*, **7**, 295-299.
Rodenhuis S, Slebos RJC, Boot AJM, Evers SG, Mooi WJ, Wagenaar SS, van Bodegom PC, Bos JL (1988) Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. *Cancer Res*, **48**, 5738-5741.
Rogan EG, Cavalieri EL, Tibbels S, Cremonesi P, Warner C, Nagel D, Tomer K, Cerny RL, Gross ML (1988) Synthesis and identification of benzo[a]pyrene-guanine nucleoside adducts formed by electrochemical oxidation and by horseradish peroxidase catalyzed reaction of benzo[a]pyrene with DNA. *J Am Chem Soc*, **110**, 4023-4029.
Rogan EG, Devanesan PD, RamaKrishna NVS, Higginbotham S, Padmavathi NS, Chapman K, Cavalieri EL, Jeong H, Jankowiak R, Small GJ (1993) Identification and quantitation of benzo[a]pyrene-DNA adducts formed in mouse skin. *Chem Res Toxicol*, **6**, 356-363.
Ross J, Nelson G, Kligerman A, Erexson G, Bryant M, Earley K, Gupta RC, Nesnow S (1990) Formation and persistence of novel benzo(a)pyrene adducts in rat lung, liver, and peripheral blood lymphocyte DNA. *Cancer Res*, **50**, 5088-5094.
Ross J, Nelson G, Erexson G, Kligerman A, Earley K, Gupta RC, Nesnow S (1991) DNA adducts in rat lung, liver and peripheral blood lymphocytes produced by i.p. administration of benzo(a)pyrene metabolites and derivatives. *Carcinogenesis*, **12**, 1953-1955.

Ruggeri B, DiRado M, Zhang SY, Bauer B, Goodrow T, Klein-Szanto AJP (1993) Benzo[a]pyrene-induced murine skin tumors exhibit frequent and characterstic G to T mutations in the p53 gene. *Proc Natl Acad Sci*, **90**, 1013-1017.

Rutten AAJJL, Beems RB, Wilmer JWGM (1988a) Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture II. A histomorphological study. *Virch Archiv B Cell Pathol*, **55**, 177-186.

Rutten AAJJL, Beems RB, Wilmer JWGM, Feron VJ (1988b) Ciliated cells in vitamin A-deprived cultured hamster tracheal epithelium do divide. *In Vitro Cell & Develop Biol*, **24**, 931-935.

Sachsenmaier C, Radler-Pohl A, Müller A, Herrlich P, Rahmsdorf HJ (1994) Damage to DNA by UV light and activation of transcription factors. *Bioch Pharmacol*, **47**, 129-136.

Saffiotti U, Cefis F, Kolb LH (1968) A method for the experimental induction of bronchogenic carcinoma. *Cancer Res*, **28**, 104-124.

Santella RM, Gasparo F, Hsieh L-L (1987) Quantitation of carcinogen-DNA adducts with monoclonal antibodies. *Prog Exp Tumor Res*, **31**, 63-75.

Sayer JM, Chadna A, Agarwal SK, Yeh HJC, Yagi H, Jerina DM (1991) Covalent nucleoside adducts of benzo(a)pyrene 7,8-diol 9,10-epoxides: structural reinvestigation and characterization of a novel adenosine adduct on the ribose moiety. *J Org Chem*, **56**, 20-29.

Schaaper RM, Kunkel TA, Loeb LA (1983) Infidelity of DNA synthesis associated with bypass of apurinic sites. *Proc Natl Acad Sci*, **80**, 487-491.

Scherer E, van Benthem J, Terheggen PMAB, Vermeulen E, Winterwerp HHK, den Engelse L (1988) Immunocytochemical analysis of DNA adducts in single cells: a new tool for experimental carcinogenesis, chemotherapy and molecular epidemiology. In Bartsch H Hemminki K, O'Neill IK, eds. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention (IARC Scientific Publications No.89), IARC, Lyon, 286-291.

van Schooten FJ, Kriek E, Steenwinkel M-JST, Noteborn HPJM, Hillebrand MJX, van Leeuwen FE (1987) The binding efficiency of polyclonal and monoclonal antibodies to DNA modified with benzo[a]pyrene diol epoxide is dependent on the level of modification. Implications for quantitation of benzo[a]pyrene-DNA adducts *in vivo*. *Carcinogenesis*, **8**, 1263-1269.

van Schooten FJ, Hillebrand MJX, van Leeuwen FE, Lutgerink JT, van Zandwijk N, Jansen HM, Kriek E (1990) Polycyclic aromatic hydrocarbon-DNA adducts in lung tissue from lung cancer patients. *Carcinogenesis*, **11**, 1677-1681.

van Schooten FJ, Hillebrand MJX, Scherer E, den Engelse L, Kriek E (1991) Immunocytochemical visualization of DNA adducts in mouse tissues and human white blood cells following treatment with benzo(a)pyrene or its diol epoxide. A quantitative approach. *Carcinogenesis*, **12**, 427-433.

Schurdak ME, Randerath K (1989) Effects of route of administration on tissue distribution of DNA adducts in mice: comparison of 7H-dibenzo(c,g)carbazole, benzo(a)pyrene, and 2-acetylaminofluorene. *Cancer Res*, **49**, 2633-2638.

Sebti SM, Pruess-Schwartz D, Baird WM (1985) Species- and length of exposure-dependent differences in the benzo(a)pyrene: DNA adducts formed in embryo cell cultures from mice, rats and hamsters. *Cancer Res*, **45**, 1594-1600.

Segerbäck D, Vodicka P (1993) Recoveries of DNA adducts of polycyclic aromatic hydrocarbons in the ^{32}P-postlabelling assay. *Carcinogenesis*, **14**, 2463-2469.

Seidegård J, Pero RW, Miller DG, Beattie EJ (1986) A glutathione transferase in human leukocytes as a marker for the susceptibility to lung cancer. *Carcinogenesis*, **7**, 751-753.

Seidegård J, Pero RW, Markowitz MM, Roush G, Miller DG, Beattie EJ (1990) Isoenzyme(s) of glutathione transferase (class Mu) as a marker for the susceptibility to lung cancer: a follow up study. *Carcinogenesis*, **11**, 33-36.

Selkirk JK, Croy RG, Wiebel FJ, Gelboin HV (1976) Differences in benzo(a)pyrene metabolism between rodent liver microsomes and embryonic cells. *Cancer Res*, **36**, 4476-4479.

Selkirk JK, Wiebel FJ (1979) Comparative metabolism of benzo(a)pyrene in rodent liver and embryonic cells in tissue culture. *Prog Exp Tumor Res*, **24**, 61-72.

Shamsuddin AKM, Gan R (1988) Immunocytochemical localization of benzo(a)pyrene-DNA adducts in human tissue. *Hum Pathol*, **19**, 309-315.

Shields PG, Bowman ED, Harrington AM, Doan VT, Weston A (1993a) Polycyclic aromatic hydrocarbon-DNA adducts in human lung and cancer susceptibility genes. *Cancer Res*, **53**, 3486-3492.

Shields PG, Harris CC, Petruzelli S, Bowman ED, Weston A (1993b) Standardization of the ^{32}P-postlabeling assay for polycyclic aromatic hydrocarbon-DNA adducts. *Mutagenesis*, **8**, 121-126.

Shimada T, Martin MV, Pruess-Schwartz D, Marnett LJ, Guengerich FP (1989) Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res*, **49**, 6304-6312.

Sigler RE, Jones RT, Hebel JR, McDowell EM (1987) Hamster tracheal organ culture in serum-free media: a quantitative comparison of *in vitro* epithelial morphology with that of *in vivo* controls. *In Vitro Cell & Develop Biol*, **23**, 100-110.

Sims P, Grover PL, Swaisland A, Pal K, Hewer AJ (1974) Metabolic activation of benzo(a)pyrene proceeds by a diol-epoxide. *Nature*, **252**, 326-328.

Singletary KW, Parker HM, Milner JA (1990) Indentification and *in vivo* formation of ^{32}P-postlabeled rat mammary DMBA-DNA adducts. *Carcinogenesis*, **11**, 1959-1963.

Slaga TJ (1988) Interspecies comparisons of tissue DNA damage, repair, fixation, and replication. *Env Health Persp*, **77**, 73-82.

Smolarek TA, Morgan SL, Kelley J, Baird WM (1987) Effects of pretreatment with benzo(a)pyrene on the stereochemical selectivity of metabolic activation of benzo(a)pyrene to DNA-binding metabolites in hamster embryo cell cultures. *Chem Biol Int*, **64**, 71-82.

Stansbury KH, Flesher JW, Gupta RC (1994) Mechanism of aralkyl-DNA adduct formation from benzo[a]pyrene *in vivo*. *Chem Res Toxicol*, **7**, 254-259.

Stivala LA, Prosperi E, Rossi R, Bianchi L (1993) Involvement of proliferating cell nuclear antigen in DNA repair after damage induced by genotoxic agents in human fibroblasts. *Carcinogenesis*, **14**, 2569-2573.

Tephly TR (1990) Isolation and purification of UDP-glucuronosyltransferases. *Chem Res Toxicol*, **3**, 509-516.

Thakker DR, Yagi H, Akagi H, Koreeda M, Lu AYH, Levin W, Wood AW, Conney AH, Jerina DM (1977) Metabolism of benzo(a)pyrene VI. Stereoselective metabolism of benzo(a)pyrene and benzo(a)pyrene 7,8-dihydrodiol to diol epoxides. *Chem Biol Int*, **16**, 281-300.

Thakker DR, Yagi H, Levin W, Wood AW, Conney AH, Jerina DM (1980) Metabolic activation of polycyclic aromatic hydrocarbons at the bay-region. In Breimer DD, ed. Towards better safety of drugs and pharmaceutical products, Elsevier, North-Holland, 207-226.

Trump BF, McDowell EM, Glavin F, Barret LA, Becci PJ, Schürch W, Kaiser HE, Harris CC (1978) The respiratory epithelium. III. Histogenesis of epidermoid metaplasia and carcinoma *in situ* in the human. *J Natl Cancer Inst*, **61**, 563-575.

Uziel M, Ward RJ, Vo-Dinh T (1987) Synchronous fluorescence measurement of BaP metabolites in human and animal urine. *Anal Lett*, **20**, 761-776.

Uziel M, Haglund R (1988) Persistence of benzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene in Fischer 344 rats: time distribution of total metabolites in blood, urine and feces. *Carcinogenesis*, **9**, 233-238.

Vaca CE, Löfgren M, Hemminki K (1992) Some quantitative considerations about DNA adduct enrichment procedures for ^{32}P-postlabelling. *Carcinogenesis*, **13**, 2463-2466.

Vähäkangas K, Haugen A, Harris CC (1985) An applied synchronous fluorescence spectrophotometric assay to study benzo[a]pyrene-diolepoxide-DNA adducts. *Carcinogenesis*, **6**, 1109-1116.

Van Scott MR, Yankaskas JR, Boucher RC (1986) Culture of airway epithelial cells: research techniques. *Exp Lung Res*, **11**, 75-94.

Voigt JM, Kawabata TT, Burke JP, Martin MV, Guengerich FP, Baron J (1990) *In situ* localization and distribution of xenobiotic-activating enzymes and aryl hydrocarbon hydroxylase activity in lungs of untreated rats. *Mol Pharmacol*, **37**, 182-191.

Vos RME, van Bladeren PJ (1990) Glutathione S-transferases in relation to their role in the biotransformation of xenobiotics. *Chem Biol Int*, **75**, 241-265.

Vousden KH, Bos JL, Marshall CJ, Phillips DH (1986) Mutations activating human c-Ha-*ras1* protooncogene (*HRAS1*) induced by chemical carcinogens and depurination. *Proc Natl Acad Sci*, **83**, 1222-1226.

Wall KL, Gao W, te Koppele JM, Kwei GY, Kauffman FC, Thurman RG (1991) The liver plays a central role in the mechanism of chemical carcinogenesis due to polycyclic aromatic hydrocarbons. *Carcinogenesis*, **12**, 783-786.

Watson WP, Smith RJ, Huckle KR, Wright AS (1989) Use of organ cultures in human risk assessment: comparisons of benzo(a)pyrene-DNA adducts in mouse and human skin. *Toxicol in Vitro*, **3**, 69-76.

Wattenberg LW (1993) Prevention-therapy-basic science and the resolution of the cancer problem: presidential address. *Cancer Res*, **53**, 5890-5896.

Weston A, Grover PL, Sims P (1982) Metabolism and activation of benzo(a)pyrene by mouse and rat skin in short-term organ culture and *in vivo*. *Chem Biol Int*, **42**, 233-250.

Weston A, Trivers G, Vähäkangas K, Newman M, Rowe M, Mann D, Harris CC (1987) Detection of carcinogen-DNA adducts in human cells and antibodies to these adducts in human sera. *Prog Exp Tumor Res*, **31**, 76-85.

Weston A, Bowman ED (1991) Fluorescence detection of benzo[a]pyrene -DNA adduct in human lung. *Carcinogenesis*, **12**, 1445-1449.

You M, Candrian U, Maronpot RR, Stoner GD, Anderson MW (1989) Activation of the K-*ras* protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. *Proc Natl Acad Sci*, **86**, 3070-3074.

van Zeeland AA, de Groot A, Neuhäuser-Klaus A (1990) DNA adduct formation in mouse testis by ethylating agents: a comparison with germ-cell mutagenesis. *Mutat Res*, **231**, 55-62.

Zhong S, Howie AF, Ketterer B, Taylor J, Hayes JD, Beckett GJ, Wathen CG, Wolf CR, Spurr NK (1991) Glutathione S-transferase mu locus: use of genotyping and phenotyping to assess association with lung cancer susceptibility. *Carcinogenesis*, **12**, 1533-1537.

Chapter
2

Improvements in the ^{32}P-postlabeling procedure to quantify bulky aromatic DNA adducts

M-JST Steenwinkel, R Roggeband, JHM van Delft and RA Baan

Department of Genetic Toxicology, TNO Medical Biological Laboratory, PO Box 45, 2280 AA Rijswijk, The Netherlands

IARC monographs, 124, 65-70, 1993

Abstract

This contribution describes methodological modifications and improvements that may contribute to inter-assay reproducibility and more accurate adduct quantification for ^{32}P-postlabeling. Firstly, an anion-exchange chromatography procedure was developed to determine the amount of DNA used per assay and to check its purity, in particular to verify the absence of contaminating RNA. Secondly, calibration standards were prepared, in order to correct for differences in recovery. The modification levels of these standards were determined by synchronous fluorescence spectrophotometric analysis. Thirdly, the effect on adduct levels of exposure to light during postlabeling was investigated. Exposure of polyaromatic DNA adducts on a PEI-cellulose plate reduced the amounts of adducts detected considerably.

Introduction

There is a growing need to evaluate the carcinogenic risk in groups of workers exposed to polycyclic aromatic hydrocarbons (PAHs). Among these are roofers and workers in coal mines, cokeries, wood-conservation plants, etc (1). The detection and quantification of PAH-DNA adducts in blood samples is a widely used approach to determine exposure to PAHs, aimed at providing a basis for risk assessment (2).

The ^{32}P-postlabeling assay (3-5) is especially suitable for the analysis of adduct formation resulting from exposure to complex mixtures of PAHs of unknown composition. In the procedure most commonly used, DNA is enzymically digested to 3'-monophosphates of normal and adducted nucleosides. Following selective removal of the unmodified nucleotides by so-called enhancement steps, *e.g.*, treatment with nuclease P1 (6) or extraction with n-butanol (7), the modified nucleotides are labelled at the 5'-position with ^{32}P and detected by autoradiography after multidirectional chromatography. Under optimal conditions, detection limits of 1 adduct/10^9 - 10^{10} nucleotides have been reported for the ^{32}P-postlabeling assay. Because many factors determine the efficiencies of digestion, dephosphorylation (nuclease P1 enhancement) and phosphorylation, the reproducibility of the ^{32}P-postlabeling is often not satisfactory. In this contribution, some modifications and improvements of the technique are described.

Contamination with RNA

Contamination of DNA samples with RNA is a disturbing factor in the ^{32}P-postlabeling assay for two reasons: firstly, spectrophotometric determination of the DNA content in a sample contaminated with RNA gives an overestimation of the actual amount of DNA. Secondly, the RNA may also contain adducts that can be ^{32}P-postlabeled, and their presence may influence the DNA-adduct labeling. In our laboratory, ^{32}P-postlabeling experiments with nuclease P1 enhancement were carried out with DNA modified both *in vivo* and *in vitro* with benzo[a]pyrene (B[a]P), with and without RNA contamination. When the results were compared, however, no additional adduct spots were detected on the chromatograms of the RNA-contaminated samples. Although the amount of adducts was generally the same in both cases, occasionally it seemed to be higher when RNA contamination had been removed (results not shown). This could be explained by a less efficient nuclease P1-mediated dephosphorylation of unmodified ribonucleotides, which would affect the subsequent ^{32}P-labeling of modified DNA nucleotides. With regard to the effects of contaminating RNA on postlabeling of DNA adducts, it should be realized that RNA adducts can also be formed when

microsomes, which often contain RNA, are used for the metabolic activation of chemicals before their reaction with DNA.

A procedure was therefore developed to determine the amount of RNA contamination in DNA. An aliquot of the digested DNA was analyzed by anion-exchange column chromatography in a fast protein liquid chromatography system (Mono Q, Pharmacia). The four deoxyribonucleoside-3'-monophosphates (3'dNMPs) are separated by elution with an NaCl gradient. Furthermore, 3'dGMP can be distinguished from 3'GMP, which is derived from contaminating RNA. The detection limit of dGMP and GMP in this system is 0.01 nmol. Elution patterns of digested DNA, with and without contaminating RNA, are shown in Figure 1.

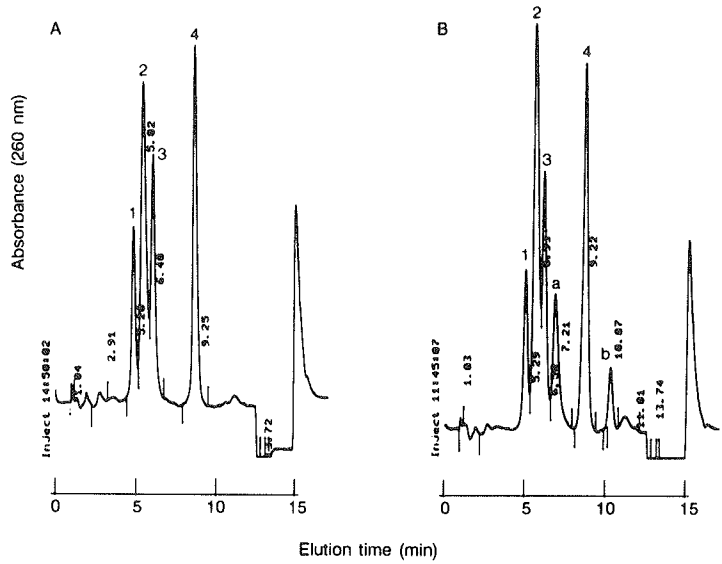

Figure 1 *Fast protein liquid chromatogram of digested DNA without (A) and with (B) RNA contamination. The numbers 1, 2, 3 and 4 indicate the normal deoxynucleotides 3'dCMP, 3'dTMP, 3'dAMP and 3'dGMP, respectively; a and b indicate the nucleotides derived from RNA (3'UMP and 3'GMP).*

The precise amount of input-DNA and of contaminating RNA (lower detection limit 0.1% w/w) can thus be calculated from the same elution pattern by use of peak-areas and appropriate calibration curves.

Adduct quantification

To convert the radioactivity counted on the chromatogram after ^{32}P-postlabeling into the amount of adducts, the relative adduct labeling (RAL) method is commonly used. However, the labeling efficiency of a given DNA adduct may differ from that of deoxyadenosine-3'-monophosphate (3'-AMP), with which it is compared. An alternative method for adduct quantification has been developed using reference DNA samples containing known amounts of B[a]P-N^2-dG adduct. This adduct was selected as a reference adduct because its recovery in the nuclease P1 method as well as in the butanol-extraction procedure is approximately 100% (8). A series of standards was prepared by *in vitro* modification of DNA with B[a]P diol-epoxide (BPDE). Figure 2 shows a chromatogram with the reference adduct B[a]P-N^2-dG originating from one of these standards after ^{32}P-postlabeling.

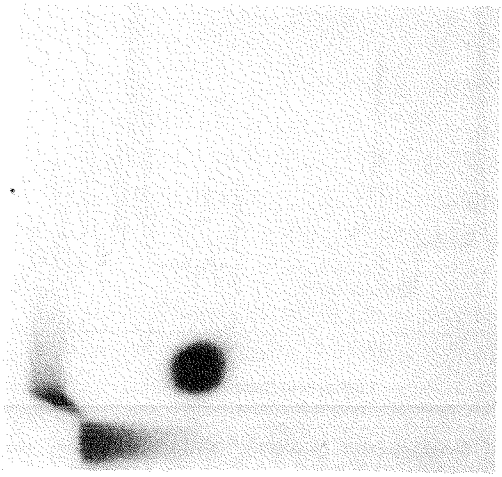

Figure 2 *Autoradiogram of the ^{32}P-postlabeled B[a]P-N^2-dG reference adduct, as present in DNA treated with BPDE in vitro (8.4 adducts/10^8 nucleotides; 0.28 fmol/µg DNA).*

The adduct levels were determined by release from the DNA of the B[a]P moieties as tetraols (tetrahydroxy-tetrahydro-B[a]P) through treatment with 0.05 N HCl for 6 h at 90 °C, followed by quantification of the tetraols by use of synchronous fluorescence spectrophotometry (SFS) with a fixed difference of 34 nm between excitation and emission wavelengths (9). The concentration of the B[a]P-tetraol standard, used for

calibration of the SFS, was determined by UV spectrophotometry (ϵ_{344} = 45,700). The B[a]P modification levels in this calibration series ranged from 0.03 to 20.5 adducts/10^6 nucleotides (0.1 to 62.3 fmol adduct/μg DNA) (Figure 3). Two of these standards, with modification levels in the same range as expected for the test samples, are routinely included in each postlabeling experiment. Because these samples serve as internal standards, the quantification of the adducts in the test samples is not influenced by differences between experiments in the specific activities of the γ-[^{32}P]ATP or in the efficiencies of dephosphorylation (P1 nuclease) or phosphorylation (T4 kinase). It should be realized, however, that intrinsic influences between different types of adducts in their susceptibility to dephosphorylation or phosphorylation are not taken into account.

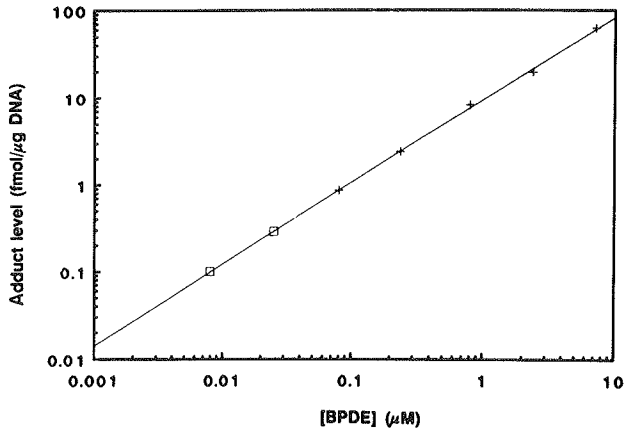

Figure 3 *Modification level of B[a]P-DNA as measured by SFS as a function of the treatment concentration of BPDE (the adduct levels indicated by □ were obtained by extrapolation, as they were below the detection limit of SFS).*

Influence of light

When a ^{32}P-postlabeling experiment is carried out in bright daylight, a loss of up to 40% of polyaromatic adducts is sometimes observed on the chromatogram, when compared with the results obtained under subdued light. There is evidence that this effect is increased when the chromatograms are dry (10). During a postlabeling experiment, chromatograms are routinely washed and dried between developments. When the light conditions are not controlled, the variations in the periods during which the dry chromatograms are exposed to daylight will affect the results of adduct quantification. The UV-A (wavelength range 330-400 nm) present in daylight appeared of special

interest because it passes through glass and is absorbed by (poly)aromatic compounds. Indeed, when chromatograms were exposed to high dosages of UV-A and violet light, a rapid decrease of more than 60% of the amount of adducts was observed (not shown). Therefore, the effect of daylight exposure on PAH adduct levels is probably due to photochemical decomposition by UV-A.

To investigate the effect of the light under normal experimental conditions, a postlabeling experiment was carried out with B[a]P-modified DNA samples. After chromatography in D1, the chromatograms were dried and exposed to daylight behind a glass window for 1 to 8 h. Subsequent chromatographic steps were carried out in subdued light conditions (yellow fluorescent light, Phillips TLD 36W/16). Because the light intensity varied considerably from one hour to the other, the total amount of daylight to which the chromatograms were exposed was recorded with a thermopile (Hewlett-Packard 8330A). The results of these experiments, shown in Figure 4, demonstrate a light-dependent reduction of the amount of adducts detected. The adduct level was compared with that obtained for a sample that was worked up under conditions of subdued light. After an 8-h daylight exposure, as described above, the reduction of the amount of adducts was similar to that observed for samples handled in normal daylight conditions throughout the postlabeling procedure.

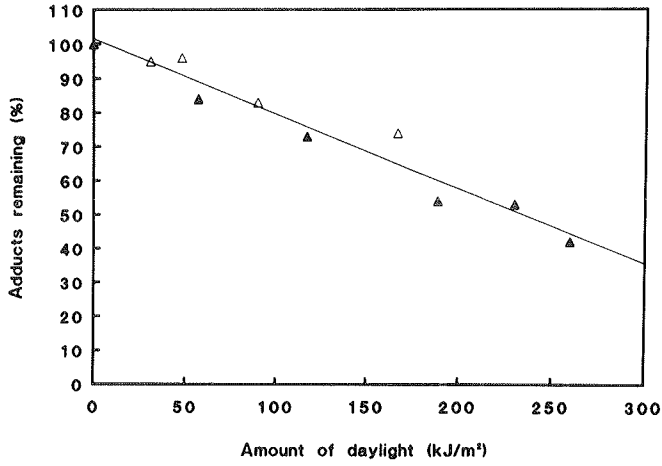

Figure 4 *The reduction of the amount of ^{32}P-postlabeled adducts as a function of the amount of daylight the dry chromatograms were exposed to after chromatography in D1. The symbols △ and ▲ represent data from two independent experiments.*

Conclusions

Three general conclusions can be drawn: (1) Because of the rather complex and partly unknown effects of RNA contamination in DNA samples that are assayed by ^{32}P-postlabeling it is important to check that all RNA has been removed. The FPLC procedure developed for this purpose also allows precise quantification of the amount of DNA. (2) Inclusion of internal standards in each postlabeling experiment allows corrections to be made for experimental variations. The use of standards with known levels of adducts allows a more accurate calculation of the amount of adducts. This calculation is independent of the exact specific activity of the γ-[^{32}P]ATP or of the comparative postlabeling of dAMP (RAL method). (3) Photochemical decomposition of PAH-DNA adducts under the influence of light, particularly UV-A, may reduce the amount of adducts on the chromatogram to 30-40%. Therefore, exposure of chromatograms, especially when dry, to daylight should be avoided.

Acknowledgements

The technical assistance of Mr MJ Latuhihin is gratefully acknowledged. We thank Dr JW Hofstraat for valuable discussions. We also thank Dr L Roza for his help with the UV-A irradiation experiments and for critical reading of the manuscript.

References

1. IARC Monographs on the evaluation of carcinogenic risks to humans (1984-1985), Vols 32-35: Polynuclear Aromatic Hydrocarbons, IARC, Lyon, France.
2. Baan RA, Fichtinger-Schepman AMJ, van Loon AAWM, van Delft JHM (1991) Methods for detection of DNA lesions induced by genotoxic agents; developments, and applications in monitoring human exposure. In: New Horizons in Biological Dosimetry, Gledhill BL and Mauro F, Eds, Progress in clinical and biological research, vol 372, Wiley-Liss, New York, pp 229-241.
3. Randerath K, Reddy MV, Gupta RC (1981) ^{32}P-labeling test for DNA damage. *Proc Natl Acad Sci USA*, **78**, 6126-6129.
4. Watson WP (1987) Post-radiolabelling for detecting DNA damage. *Mutagenesis*, **2**, 319-331.
5. Phillips DH (1990) Modern methods of DNA adduct determination. In: Chemical carcinogenesis and mutagenesis, vol I, Cooper CS and Grover PL, Eds, Springer Verlag, Heidelberg, pp 503-546.
6. Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543-1551.
7. Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabelling analysis of aromatic carcinogen:DNA adducts. *Cancer Research*, **45**, 5656-5662.
8. Gupta RC, Early K (1988) ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis*, **9**, 1687-1693.
9. Vähäkangas K, Haugen A, Harris CC (1985) An applied synchronous fluorescence spectrophotometric assay to study benzo(a)pyrene-diolepoxide-DNA adducts. *Carcinogenesis*, **6**, 1109-1116.

10. Hofstraat JW, Engelsma M, van de Nesse RJ, Brinkman UATh, Gooijer C, Velthorst NH (1987) Coupling of narrow-bore liquid chromatography to thin-layer chromatography, Part 2. Application of fluorescence-based spectroscopic techniques for off-line detection. *Anal Chim Acta*, **193**, 193-207.

Chapter

3 *Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed in vitro upon activation of benzo[a]pyrene by human, rabbit, and rodent liver microsomes**

R Roggeband, APM Wolterbeek[1], AAJJL Rutten[1], and RA Baan

TNO Medical Biological Laboratory, Department of Genetic Toxicology, PO Box 5815, 2280 HV, Rijswijk and [1] TNO Toxicology and Nutrition Institute, Department of Biological Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands.

*This paper is dedicated with great appreciation to Dr Frits Berends on the occasion of his retirement from the TNO Medical Biological Laboratory.

Carcinogenesis, 14, 1945-1950, 1993

Abstract

An interspecies comparison was made of the DNA-adducts formed *in vitro* upon incubation of rat liver DNA (RL-DNA) with benzo[a]pyrene (B[a]P) in the presence of liver microsomes. Incubations were carried out with RL-DNA, B[a]P (100 μM), and liver microsomes from hamsters, mice, rabbits, rats, 3-methylcholanthrene (3MC) pretreated rats, and from humans. To analyze the adduct profiles, the ^{32}P-postlabeling technique with the nuclease P1-enhancement procedure was used. The total amount of adduct formed varied greatly with the species; also the number of adduct spots detected was different, ranging from 1 to 5. In all incubations the B[a]P-N^2-deoxyguanosine adduct was formed. Relative to the total adduct level, the level of this adduct varied from 26% with rat, 54% with hamster, 56% with 3MC-pretreated rat, 58% with mouse, and 75% with rabbit, to 100% with human liver microsomes. In human liver microsomes both the total amount of cytochrome P-450 per mg microsomal protein and the ethoxyresorufin *O*-deethylation (EROD) activity were low compared to that in animal liver microsomes. In microsomes from 3MC-pretreated rats the EROD activity was strongly induced. There was no correlation between EROD activity in non-induced microsomes and total adduct level. To compare B[a]P-DNA adduct formation in human white blood cells (WBC) with that in RL-DNA, WBC were incubated with B[a]P and 3MC-pretreated rat microsomes. The adduct profile in WBC-DNA differed from that observed after incubation of RL-DNA: the B[a]P-N^2-deoxyguanosine adduct in WBC-DNA accounted for 97% of the total adduct level. It is concluded that the ^{32}P-postlabeling method is a suitable technique to

investigate both qualitative and quantitative differences in B[a]P-DNA adduct formation between species. Furthermore, the incubation of microsomes from the liver (or other sources) with a genotoxic agent and isolated DNA or cells can be a useful approach to study the formation and stability of reactive intermediates that are able to bind to DNA, also with respect to differences between species or tissue.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemical carcinogens that are ubiquitous environmental pollutants (1). Exposure to PAHs occurs via the respiratory tract, the digestive tract, or the skin. To become carcinogenic, PAHs have to be activated through metabolic conversion. PAHs are metabolized mainly by the enzymes of the cytochrome P-450I family, present in specific cell types in various tissues. Metabolism of PAHs has been elucidated in great detail through studies of benzo[a]pyrene (B[a]P), the widely used model compound for PAHs (2,3). The formation of epoxides is the initial step in the metabolism of unsubstituted PAHs (4). The so-called 'bay-region' epoxides which contain a sterically hindered area, *e.g.*, the region between C-10 and C-11 in B[a]P, show the highest chemical reactivity (5). Epoxides can be hydrated to yield diols or they can isomerize to phenols. The majority of the PAH metabolites are detoxicated and either excreted as conjugates of glutathione via the urinary tract or as conjugates of glucuronic acid via the digestive and/or urinary tract (4). A small proportion of the reactive metabolites binds covalently to cellular macromolecules, such as DNA, and forms adducts (6-8). In the case of B[a]P, the (+)-*anti*-B[a]P-7,8-diol-9,10-epoxide ((+)-*anti*-BPDE) is considered to be the ultimate carcinogen. In metabolism studies, liver microsomes are often used to achieve activation of PAHs (9-12). With respect to the metabolism of PAHs, it is known that both quantitative and qualitative differences exist between different rodent species (13).

The objective of the work presented here was to study the B[a]P-DNA adduct formation *in vitro* after incubations of DNA with B[a]P and rabbit, rodent, or human liver microsomes, by use of the ^{32}P-postlabeling method (14-17). The sensitivity of this method and the specific adduct patterns obtained upon multidirectional thin-layer-chromatography render it possible to study both quantitative and qualitative aspects of adduct formation. Species-specific biotransformation was studied by use of microsomes, obtained from hamster, rat, mouse, rabbit, and human livers. Liver microsomes were chosen because the liver plays a central role in the mechanism of PAH carcinogenicity (18). Also, liver microsomes were isolated from rats that had been treated with 3-methylcholanthrene (3MC), a known P-450IA inducer in rats (19). Both the

ethoxyresorufin *O*-deethylation (EROD, indicative of activity of P-450IA1 and P-450IA2, (20)) and the pentoxyresorufin *O*-dependentylation (PROD, indicative of P-450IIB, (21)) activity of all microsome preparations were determined.

Furthermore, a comparison was made between the adducts formed upon incubation of DNA with B[a]P and liver microsomes from 3MC-treated rats (3MC-microsomes), and those found upon incubation of human white blood cells (WBC) with B[a]P and these microsomes.

Materials and methods

Chemicals and enzymes

Glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase (G6PD), nuclease P1, and RNAse T1 were obtained from Boehringer, Mannheim, FRG. RNAse T1 was heated at 80°C for 5 min to remove DNAse activity. Micrococcal endonuclease, spleen phosphodiesterase, RNAse A, benzo[a]pyrene, and 3-methylcholanthrene were obtained from Sigma, St. Louis, MO, USA. T4 polynucleotide kinase was purchased from Biolabs, Beverly, MA, USA and proteinase K from Merck, Darmstadt, FRG. Racemic *anti*-BPDE was obtained from Chemsyn, Lenexa, KS, USA. γ-[^{32}P]-ATP was purchased from Amersham, Buckinghamshire, UK.

Preparation of microsomes

Livers (from young male animals that served as untreated controls in other experiments) were isolated from rats (Wag/MBL and Wistar), mice (BALB/c), rabbits (New Zealand White), and hamsters (Syrian Golden) and homogenized with a Potter Elvehjem homogenizer in 3 volumes of 0.1 M potassium phosphate buffer, pH 7.4 (1 g of liver in 3 ml of buffer). The liver suspension was centrifuged at 9,000 g for 30 min in a cooled (4°C) Beckman centrifuge. Thereafter, the supernatant (S9) was centrifuged at 100,000 g for 90 min. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Procedures for preparation of microsomes were identical for livers of all species.

To obtain 3MC-induced microsomes, male young adult Wistar rats were injected i.p. with 3MC in corn oil (40 mg 3MC/kg body weight) on three consecutive days. One day after the third injection, liver microsomes were prepared according to the protocol described above.

Human microsomes were obtained from livers of four individuals (all males). The

liver tissue (recovered from two persons who died in a traffic accident and two persons who died of cerebral haemorrhage) had been kept frozen (-80°C) for 6 months to 7 years.

The cytochrome P-450 and protein content of the microsomes as well as the EROD and PROD activity were determined in triplicate according to the protocol described by Rutten *et al* (22,23).

Isolation of RL-DNA

Livers from untreated male rats were cut into pieces and homogenized in buffer A (250 mM sucrose, 100 mM EDTA, pH 7.4); per gram 9 ml was used (all volumes are expressed per g liver as starting material). The homogenate was centrifuged (4,000 g, 1 min, 4°C) and the pellet was resuspended in 9 ml buffer A. Subsequently, the solution was centrifuged (1,000 g, 10 min, 4°C). The pellet was resuspended in 5 ml buffer B (250 mM sucrose, 25 mM EDTA, and 1% Triton X-100, pH 7.4) and kept at 4°C for 30 min. Thereafter the chromatin was isolated by centrifugation (1,000 g, 10 min, 4°C). Triton X-100 was removed by resuspending the chromatin in 5 ml buffer C (10 mM Tris-HCl pH 7.6, 25 mM EDTA), followed by centrifugation (1,000 g, 10 min, 4°C). This last step was repeated once and finally the chromatin pellet was resuspended in 2.5 ml TEN buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, and 100 mM NaCl) and incubated overnight at 37°C with proteinase K (100 μg/ml) and 1% SDS. Extractions of DNA were carried out with equal volumes of phenol, phenol-chloroform-isoamylalcohol (25:24:1, v/v/v), and chloroform-isoamylalcohol (24:1, v/v). The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 6.0, and 3 volumes of ethanol at -20°C, washed with 70 % ethanol at -20°C and dried *in vacuo*. Subsequently, the DNA was dissolved in 2.5 ml TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and treated serially with a mixture of RNAses A (50 μg/ml) and T1 (50 U/ml) for 90 min and proteinase K (100 μg/ml) for 60 min at 37°C. DNA was extracted, precipitated, washed and dried as described above. It was dissolved in MQ water and the concentration was estimated spectrophotometrically (1 mg of DNA per ml = 20 absorbance units at 260 nm). The $A^{260}/_{280}$ was 1.78.

Modification of RL-DNA

RL-DNA dissolved in Millipore-filtered (MQ) water (300 μl, 0.85 mg/ml) was mixed with 200 μl medium (100 mM Hepes pH 7.1, 1 mM $MgCl_2.6H_2O$, 10 mM glucose-6-phosphate, 1.6 mM NADP, 1 unit G6PD/ml) and 100 μl of a microsome suspension (protein content varied from 15.2 to 26.7 mg/ml in the different microsome

preparations, see also Table 1). B[a]P, dissolved in DMSO, was added to a final concentration of 100 μM and the mixture was incubated for 90 min in a humidified incubator (37°C, 5% CO_2) according to the procedure described by Gorelick and Wogan (10). The final concentration of DMSO was 0.5% (v/v). After the incubation, DNA was extracted and purified as described above. It should be emphasized that despite the fact there is no RNA present in RL-DNA, incubations with microsomes can introduce RNA contamination which may influence the postlabeling assay. Contaminating RNA is, therefore, removed afterwards by a supplementary treatment of the modified RL-DNA with RNAses (see DNA isolation).

Isolation and B[a]P treatment of human WBC

Blood was obtained from a healthy non-smoking female and collected in tubes containing EDTA. WBC were isolated at 4°C within 1 h after sampling. Red blood cells were lysed with a buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4 (3 ml of buffer per ml of blood). The white blood cells were then collected by centrifugation at 125 g and resuspended in RPMI medium without serum. The number of cells was determined in a haemocytometer (Burker, FRG) and adjusted to 5 x 10^6 cells per ml. The cell suspension was supplemented with glucose-6-phosphate (final concentration 5 mM), NADP (0.8 mM), and G6PD (0.4 units/ml). The microsome suspension was added (5% v/v). B[a]P was dissolved in DMSO and added to a final concentration of 100 μM; the final concentration of DMSO was 0.5 % (v/v).

Subsequently, the mixture containing cells, microsomes, and B[a]P was transferred onto petridishes. After 5 h of incubation in a humidified incubator (37°C, 5% CO_2) the cells were scraped off the petridishes and washed with RPMI medium. Cells were collected by centrifugation at 125 g and frozen (-24°C). DNA from WBC was isolated by means of phenolic extractions. Typically, WBC (1 x 10^7) were suspended in 1 ml TEN buffer and treated further as described under Isolation of RL-DNA.

^{32}P-postlabeling analysis of B[a]P-DNA adducts

The postlabeling assay was performed as described by Reddy and Randerath (15). DNA (5-15 μg) was digested with micrococcal nuclease (0.6 units) and spleen phosphodiesterase (0.012 units), and subsequently with nuclease P1 (3 units). The modified nucleotides were labelled by incubation with 37 MBq γ-[^{32}P]-ATP (sp.act. > 5,000 Ci/mmol) and 50 units T4-polynucleotide kinase for 30 min at 37°C.

The postlabeled mixtures were applied to 20-cm x 20-cm poly(ethylenimine)(PEI)-

cellulose sheets (JT Baker, Phillipsburg, NJ, USA). A paper wick was attached to the top of each TLC sheet; the sheets were developed overnight in 1 M sodium phosphate, pH 6.0 (D1). After two washes with water the sheets were developed in 8.5 M urea, 3 M lithium formate, pH 3.5 (D3) for 6½ h and in 8.5 M urea, 0.8 M lithium chloride, 0.5 M Tris, pH 8.0 (D4), also for 6½ h. To remove any remaining impurities, the sheets were developed overnight in 1.7 M sodium phosphate, pH 6.0 (D5, in the direction of D1).

The adducts were localized by autoradiography on Kodak XAR-5 film with an intensifying screen. The spots on the PEI-cellulose sheets detected by autoradiography were cut out and the radioactivity was determined by liquid scintillation counting. The level of DNA adducts was calculated on the basis of the concurrent analysis of standard samples carrying known amounts of [^{3}H]B[a]P-adducts. The exact amount of input DNA and the absence of RNA were determined by chromatography of an aliquot of the DNA digest on an FPLC-column (24).

Results

Cytochrome P-450 analysis of the microsomes

Table 1 summarizes the characteristics of the microsome preparations used. In human liver microsomes the amount of cytochrome P-450 per mg microsomal protein was significantly lower ($p=0.0003$, Student's t-test) than in animal liver microsomes. Also the EROD activity was lower in human liver compared to animal liver microsomes.

Table 1 *Characteristics of microsome preparations from various species.*

Species	Protein[a]	Cytochrome P-450[b]	EROD[c]	PROD[c]
hamster	15.2 ± 0.3	0.63 ± 0.04	41.0 ± 1.3	2.9 ± 0.1
mouse	22.6 ± 0.4	0.73 ± 0.13	37.8 ± 0.6	5.5 ± 0.5
rat (Wag/MBL)	23.7 ± 2.7	0.74 ± 0.16	NA	3.6 ± 0.1
rat (Wistar)	22.5 ± 2.1	0.71 ± 0.02	34.2 ± 1.6	3.8 ± 0.3
rat-3MC (Wistar)	18.8 ± 1.0	0.59 ± 0.04	120 ± 6.5	10.0 ± 0.0
rabbit	17.5 ± 0.8	0.38 ± 0.12	23.5 ± 0.5	4.4 ± 0.7
human (061285)	22.3 ± 1.6	0.13 ± 0.03	6.9 ± 0.8	0.2 ± 0.0
human (071087)	16.7 ± 0.2	0.15 ± 0.00	10.1 ± 3.3	ND
human (250589)	26.7 ± 3.4	0.15 ± 0.02	5.9 ± 0.4	ND
human (130192)	20.0 ± 1.5	0.13 ± 0.01	7.3 ± 0.9	ND

[a]: microsomal protein content in mg per ml ± SD (n=3)
[b]: total amount of cytochrome P-450 in nmol per mg protein ± SD (n=3)
[c]: activity in pmol resorufin formed per min per mg protein ± SD (n=3)
NA: not analyzed
ND: not detectable

Strikingly, induction of the metabolic activity with 3MC in Wistar rats did not result in a higher amount of total cytochrome P-450 per mg microsomal protein. However, EROD and PROD were higher in the rat 3MC-microsomes compared to the untreated control.

^{32}P-postlabeling analysis of B[a]P-DNA adducts

Adducts formed in RL-DNA

Microsomes obtained from livers of various species were used to activate B[a]P *in vitro*. RL-DNA was added as a target molecule. During the various B[a]P experiments 5 different adduct spots were detected, of which the relative positions are indicated schematically in Figure 1.

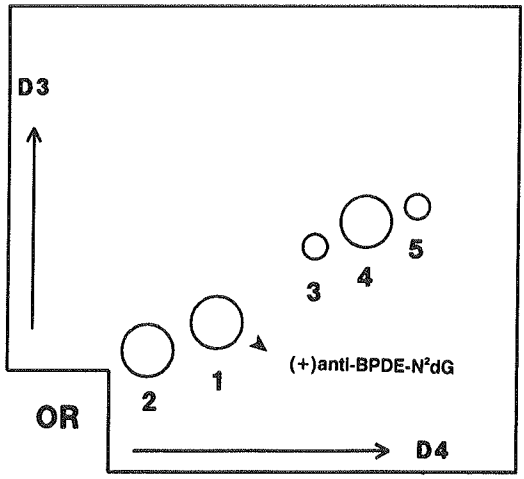

Figure 1 *Diagram of adduct profile, obtained upon postlabeling of B[a]P-modified DNA. The position of adduct 1 corresponds with that of the adduct formed in the reaction between (+)-anti-BPDE and dG.*

With liver microsomes from both the Wag/MBL rat (Figure 2a) and the Wistar rat (not shown) two adduct spots were observed. Adduct 1 co-chromatographed with the 3'5'-biphosphate of N^2-B[a]P-deoxyguanosine (Figure 3a), the synthetic adduct obtained after incubation of (±)-*anti*-BPDE with RL-DNA (*trans* addition product of dG and (+)-*anti*-BPDE). The identity of adduct 2 and of other minor adducts observed in further experiments (see below), is not known.

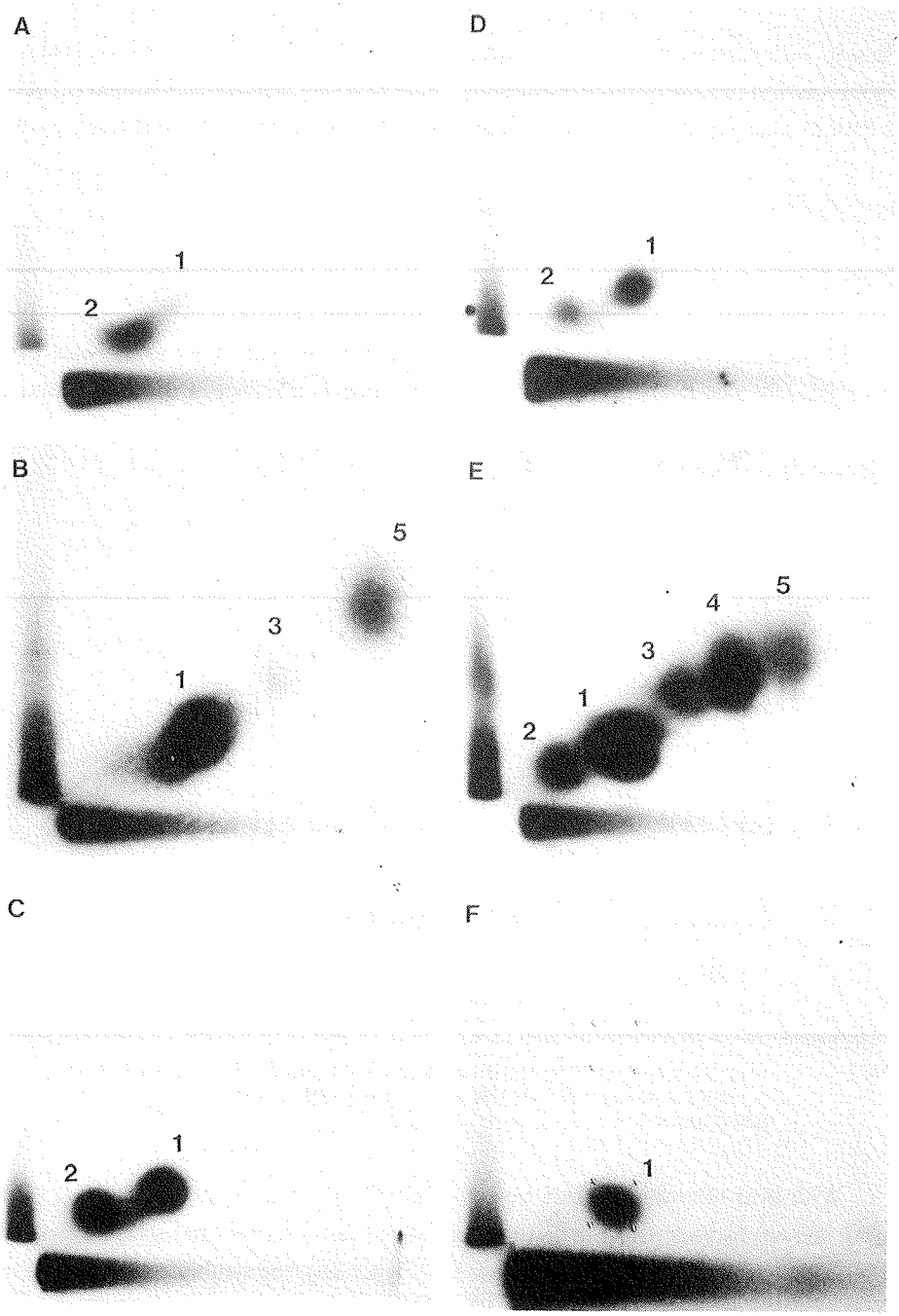

Figure 2 *Autoradiograms of B[a]P-modified RL-DNA, obtained after incubation with both Wistar and Wag/MBL rat(A), rabbit(B), hamster(C), mouse(D), rat-3MC(E), and human(F) liver microsomes. Films (A)-(D) were exposed for 44 h, film (E) was exposed for 22 h, and film (F) was exposed for 64 h at -70°C.*

Table 2 *B[a]P-DNA adduct levels in add/10^8n after in vitro incubations of RL-DNA with B[a]P in the presence of rodent, rabbit, or human liver microsomes. Values are given as adduct levels/1.5 mg microsomal protein[a].*

Species	Adduct 1[b]	Adduct 2	Adduct 3	Adduct 4	Adduct 5
hamster	98.0 ± 0.3 (54%)[c]	82.6 ± 1.0 (46%)	ND	ND	ND
hamster[d]	71.1 ± 19.2 (53%)	64.3 ± 16.0 (48%)	ND	ND	ND
mouse	12.0 ± 5.2 (57%)	9.0 ± 4.7 (43%)	ND	ND	ND
mouse[d]	14.0 ± 0.9 (61%)	9.0 ± 4.7 (39%)	ND	ND	ND
rat (Wag/MBL)	7.4 ± 2.9 (26%)	21.2 ± 10.1 (74%)	ND	ND	ND
rat (Wag/MBL)[d]	5.3 ± 0.2 (26%)	14.6 ± 0.6 (74%)	ND	ND	ND
rat (Wistar)	2.6 ± 0.2 (32%)	5.4 ± 0.0 (68%)	ND	ND	ND
rat (Wistar)[d]	2.2 ± 0.3 (30%)	5.0 ± 0.6 (70%)	ND	ND	ND
rat-3MC (Wistar)	365 ± 97 (54%)	68.7 ± 6.5 (10%)	60.9 ± 3.4 (9%)	140 ± 24 (21%)	42.9 ± 0.8 (6%)
rat-3MC (Wistar)[d]	433 ± 157 (58%)	83.6 ± 26.8 (11%)	59.8 ± 8.6 (8%)	126 ± 20 (17%)	42.4 ± 4.7 (6%)
rabbit	105 ± 7 (75%)	ND	12.8 ± 0.3 (9%)	ND	22.8 ± 8.9 (16%)
rabbit[d]	54.2 ± 6.4 (76%)	ND	8.1 ± 2.8 (11%)	ND	9.2 ± 1.1 (13%)
human 061285	2.9 ± 0.2	ND	ND	ND	ND
human 071087	2.9[e]	ND	ND	ND	ND
human 250589	4.6 ± 0.7	ND	ND	ND	ND
human 130192	2.2 ± 0.2	ND	ND	ND	ND

[a]: mean of two independent postlabeling assays with range of values
[b]: see Figure 1 for adduct numbering
[c]: relative percentage of one adduct
[d]: independent second experiment with the same batch of microsomes
[e]: only one postlabeling assay
ND: not detectable

Rat-liver microsomes yielded a total adduct level per 1.5 mg of microsomal protein of 24.3 ± 10.2 adducts/10^8 nucleotides (add/10^8n) in the case of the Wag/MBL rat and 7.6 ± 0.6 add/10^8n for the Wistar rat. For both rat strains, adduct 2 was predominant (74%), compared with adduct 1 (26%). With rabbit-liver microsomes (Figure 2b) the total adduct level was 106 ± 37 add/10^8n, distributed over adduct 1 (75%), adduct 2 (15%), and adduct 5 (10%). There were no qualitative differences between the results of *in vitro* incubations with hamster liver microsomes (Figure 2c), and mouse liver microsomes (Figure 2d). In both cases the relative amounts of adducts 1 and 2 were comparable (54-

59% of adduct 1 and 46-41% of adduct 2). With hamster liver microsomes the total adduct level was 158 ± 34 add/10^8n, compared to 22.0 ± 7.2 add/10^8n with mouse liver microsomes. For human liver microsomes, incubations with B[a]P and RL-DNA (Figure 2f) resulted in only one spot, *viz.* adduct 1. The total adduct levels determined after incubation of the four different human liver-microsome preparations with B[a]P and RL-DNA, ranged from 2.9 to 4.6 add/10^8n. When corrected for the amount of cytochrome P-450 present during the microsomal incubations, the total B[a]P-DNA adduct levels with human liver microsomes were in the same range as those found with rat and mouse liver microsomes.

When liver microsomes isolated from 3MC-pretreated rats were used in *in vitro* incubations with B[a]P and RL-DNA (Figure 2e) about a 100-fold overall increase in B[a]P-DNA adduct level was observed, compared to that found with microsomes from non-induced rats (711 ± 183 vs 7.6 ± 0.8 add/10^8n). Furthermore, the adduct profile showed some major differences compared to that obtained with non-induced rat-liver microsomes. The level of adduct 1 (~56%) was 399 ± 135 add/10^8n; the level of adduct 2, which was the major adduct observed in incubations with non-induced rat liver microsomes, was 76.2 ± 20.9 add/10^8n, *i.e.* only about 10% of the total adducts. A fairly large additional adduct spot appeared in the incubations with 3MC rat liver microsomes, *viz.* adduct 4 (133 ± 23 add/10^8n) which accounted for approximately 19%. Two minor spots, adducts 3 (60.4 ± 6.6 add/10^8n, 9%) and 5 (42.7 ± 3.4 add/10^8n, 6%) could also be detected. Adducts 3 and 4 may be derived, at least in part, from 3MC carried over during microsome preparation. The chemical may still be present in rat liver at 24 h after the third dose of 3MC. In a control experiment in which 3MC-induced rat-liver microsomes were incubated with RL-DNA in the absence of B[a]P these two spots in positions 3 and 4 could be detected, although the levels of adducts 3 and 4 were about 3-fold lower in incubations without B[a]P compared to incubations with B[a]P (results not shown).

When untreated RL-DNA was analyzed in the postlabeling assay, several spots could be detected (Figure 3b). The intensity of the individual spots would correspond to adduct levels in the range of 0.2 to 0.9 add/10^8n. In the postlabeling assay none of these adducts co-migrated with any of the B[a]P-DNA adducts observed in other experiments.

Comparison of the EROD activity of non-induced microsomes (Table 1) and the total B[a]P-DNA adduct levels obtained with the same preparation (Table 2) showed no correlation. Apparently, in livers the EROD activity is not predictive of the amount of B[a]P-DNA adducts formed. However, 3MC-induced microsomes show an increased level of EROD and PROD activity and give rise to a considerable increase in DNA adduct induction.

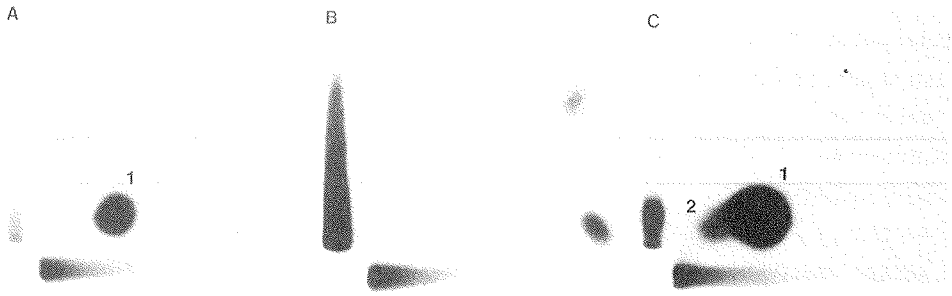

Figure 3 *Autoradiogram of (A) [^{3}H]B[a]P-modified DNA at a level of 43 add/10^{8}n, (B) RL-DNA incubated with B[a]P in the absence of microsomes, and (C) B[a]P-modified WBC-DNA, obtained after incubation of WBC with 3MC-induced rat-liver microsomes and B[a]P. Films (A), (B), and (C) were exposed for 22, 64, and 4 h, respectively, at -70°C.*

Adducts formed in human WBC

When human WBC were incubated with B[a]P (100 μM) in the presence of 3MC rat-liver microsomes (Figure 3c) adduct 1 was formed almost exclusively (97%); the DNA modification level was 903 ± 400 add/10^{8}n. The level of adduct 2 was 25.6 ± 12.8 add/10^{8}n (3%). The adducts 3, 4, and 5 could not be detected in incubations with human WBC and B[a]P.

Adduct 1 could also be detected following incubations of WBC with B[a]P (100 μM) in the absence of microsomes. However, the modification level was only 1.3 add/10^{8}n. The level of adduct 2, if formed, was too low to be detected.

Discussion

A comparative study was carried out on the *in vitro* activation of B[a]P by liver microsomal preparations from various species. Purified rat-liver DNA was used as a target and the formation of B[a]P-DNA adducts was analyzed by use of the ^{32}P-postlabeling method. It should be noted that microsomes often contain RNA. The presence of RNA may interfere with the adduct quantification in the postlabeling assay (24). Therefore, even though the initial DNA had been purified from RNA, after the incubation with microsomes DNA should again be treated with RNAses.

The EROD activity is correlated with the ability of microsomes to metabolize planar PAHs. Despite the fact that both the EROD activity and the total DNA-adduct level were lower with human liver microsomes compared to rabbit and rodent liver microsomes, there was no correlation between the EROD activity in non-induced microsomes and the total DNA-adduct level. Apparently, B[a]P is metabolized by other P450 isozymes, besides P450IA1 and IA2. This is supported by the work of Nebert (19). Pretreatment of rats with 3MC resulted in a clear induction of the EROD activity along with an enhanced DNA-adduct formation. However, the total amount of cytochrome P-450 per mg of microsomal protein was not elevated. In a study of Wortelboer *et al* (25) a similar observation was made: in male Wistar rats that were treated i.p. with β-naphthoflavone (an inducer of the EROD activity in rats) the EROD activity was increased 24-fold, compared with the control animals while in the same animals the total amount of P-450 was increased only 2.5-fold compared to the controls.

One of the adducts observed in the present study, adduct 2, has a low mobility in D3 and D4. Several studies with cultured cells from the rat, provide evidence for the formation of DNA adducts derived from (±)-*syn*-BPDE (26-28). In order to find out whether adduct 2 is derived from (±)-*syn*-BPDE, RL-DNA was reacted with (±)-*syn*-BPDE (a generous gift of Dr A Dipple). After purification, the (±)-*syn*-BPDE modified-DNA was analyzed by postlabeling. None of the (±)-*syn*-BPDE DNA adducts co-chromatographed with adduct 2 (not shown), so it can be concluded that this adduct is not derived from (±)-*syn*-BPDE. Another possibility may be that adduct 2 is derived from a metabolite of 9-OH-B[a]P. By using 3MC-induced rat-liver microsomes Ashurst and Cohen (29) found that the major adduct formed in incubations with B[a]P and DNA co-chromatographed with an adduct derived from a metabolite of 9-OH-B[a]P. Ross *et al* (30) exposed rats to 9-OH-B[a]P intraperitoneally; the major adduct formed in liver, lung, and peripheral blood lymphocytes had a low mobility in both D3 and D4 compared to the reference (+)-*anti*-BPDE-dG adduct. The authors suggest that the major adduct is derived from the 4,5-epoxide of 9-OH-B[a]P. The chromatographic behaviour of this adduct in their experiments is comparable to that of our adduct 2. Therefore, adduct 2 may be derived from the 9-OH-4,5-epoxide of B[a]P. Experiments are ongoing to confirm the identity of this adduct. The reason that in our incubations of 3MC-induced rat-liver microsomes with B[a]P and DNA adduct 2 was not a major adduct may be related to the level of epoxide hydrolase activity, which can shift metabolism towards diolepoxide formation. Bodell *et al* (9), who also used 3MC-induced rat-liver microsomes in *in vitro* incubations with B[a]P and DNA, detected two major spots, of which one (55%) was identified as (+)-*anti*-BPDE-dG.

Incubations of human liver microsomes with B[a]P and RL-DNA resulted in relatively low adduct levels, compared to the incubations with animal liver microsomes. This can be explained by a rather low content of cytochrome P-450 per mg microsomal protein in human liver microsomes. However, the total B[a]P-DNA adduct levels were in the same range as those observed with rat and mouse liver microsomes when adduct levels were corrected for the amount of cytochrome P-450 present during the incubations. The biotransformation capacity of the human microsomes may have been reduced upon storage of the human liver tissue (32,33). However, in the relatively fresh human liver microsomes (stored for 6 months) the adduct level was in the same range as found for the microsomes that had been stored for several years. Furthermore, there were no qualitative differences in adduct patterns between the different human liver microsome preparations. A more reliable comparison, however, can only be made when a fresh human liver specimen becomes available. In contrast to the animal liver microsomes, the use of human liver microsomes resulted in only one adduct spot, *viz.* adduct 1. In the study by Moore *et al* (28), in which human mammary epithelial cells were exposed to B[a]P (2 μM), also mainly the (+)-*anti*-BPDE-deoxyguanosine adduct was formed.

Human WBC are able to metabolize PAHs to a relatively low extent (34). In our experiments, exposure of WBC to B[a]P (100 μM) without an additional metabolic activation system yielded a total adduct level of approx. 1.3 add/10^8n. The study reported by Gupta *et al* (34) showed several adduct spots in incubations of WBC with 30 μM B[a]P, whereas we observed just one adduct, *viz.* the (+)-*anti*-BPDE-dG (adduct 1). The main adduct (62%) in their study showed the same characteristics as our adduct 1. Generally, the total adduct level in their experiments was also higher (2.6-48.0 add/10^8n; 12 individuals) compared to our result. It should be pointed out that we determined the capacity to metabolize B[a]P of WBC from only one individual. Also, interlaboratory variation in the postlabeling technique makes it rather difficult to make a quantitative comparison between results of different studies (35).

The use of 3MC-induced rat-liver microsomes in the incubations of WBC with B[a]P gave rise to a considerable increase in WBC-DNA adduct formation. Two adduct spots were observed, of which adduct 1 accounted for 97%. In RL-DNA, 3MC-microsomes induced 5 different adducts. Adducts 3 and 4, which are probably derived from 3MC itself, at least in part, were not found in DNA of WBC. This might be related to the stability of the 3MC-metabolites. Furthermore, in the WBC incubations the ratio between adduct 1 and 2 changed in favour of adduct 1, compared to incubations with RL-DNA. This change in adduct profile may reflect the stability of reactive metabolites, the attainability of DNA, and/or the presence of cellular proteins and enzymes.

The results presented in this paper demonstrate that the postlabeling assay is a very suitable method to study and compare various biological systems with respect to their capability to bio-activate PAHs into products that can react with DNA. This approach not only allows the study of the overall capacity of such systems to convert PAHs into DNA-adduct-forming compounds, it also provides insight into the differences in the specificity with which the systems perform these conversions. Moreover, a useful indication can be obtained as to the stability of the products of the bio-activation, by comparing the adducts formed during an incubation of the system with the substance under investigation together with purified DNA, with those resulting from an identical incubation with DNA inside cells.

Acknowledgements

We thank professor V.J. Feron for critically reading the manuscript. Furthermore we thank Dr E. Kriek for the [^{3}H]B[a]P-DNA and Dr A. Dipple for the (±)-*syn*-BPDE.

References

1. IARC (1973) Certain polycyclic aromatic hydrocarbons and heterocyclic compounds. IARC monographs on the evaluation of carcinogenic risk of the chemical to man. IARC, Lyon, Vol 3, pp 91-136.
2. Conney, A.H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogens by polycyclic aromatic hydrocarbons : GHA Clowes Memorial Lecture. *Cancer Res*, **42**, 4875-4917.
3. Phillips DH (1983) Fifty years of benzo(a)pyrene. *Nature*, **303**, 468-472.
4. Hall M, Grover PL (1990) Polycyclic aromatic hydrocarbons: metabolism, activation and tumour initiation. In: Chemical Carcinogenesis and Mutagenesis, Cooper CS, Grover PL, Eds, pp 327-372, Springer Verlag, Heidelberg, FRG.
5. Thakker DR, Yagi H, Levin W, Wood AW, Conney AH, Jerina DM (1980) Metabolic activation of polycyclic aromatic hydrocarbons at the bay-region. In: Towards better safety of drugs and pharmaceutical products, Elsevier/North-Holland Biomedical Press, Breimer DD, Ed., pp 207-226.
6. Wogan GN, Gorelick NJ (1985) Chemical and biochemical dosimetry of exposure to genotoxic chemicals. *Env Health Persp*, **62**, 5-18.
7. Baan RA, van den Berg PTM, Steenwinkel M-JST, van der Wulp CJM (1988) Detection of benzo(a)pyrene-DNA adducts in cultured cells treated with benzo(a)pyrene diol-epoxide by quantitative immunofluorescence microscopy and ^{32}P-postlabelling; immunofluorescence analysis of benzo(a)pyrene-DNA adducts in bronchial cells from smoking individuals. In: Methods for detecting DNA damaging agents in humans: Applications in Cancer Epidemiology and Prevention, Bartsch H, Hemminki K, O'Neill IK, Eds, pp 146-154, IARC Scientific Publications No.89, Lyon, France.
8. Stowers SJ, Anderson MW (1985) Formation and persistance of benzo(a)pyrene metabolite-DNA adducts. *Env Health Persp*, **62**, 31-39.
9. Bodell WJ, Devanesan PD, Rogan EG, Cavalieri EL (1984) ^{32}P-postlabeling analysis of benzo(a)pyrene-DNA adducts formed *in vitro* and *in vivo*. *Chem Res Toxicol*, **2**, 312-315.
10. Gorelick NJ, Wogan GN (1989) Fluoranthene-DNA adducts: identification and quantification by an HPLC-^{32}P-postlabeling method. *Carcinogenesis*, **10**, 1567-1577.

11. Lecoq S, Ní Shé M, Grover PL, Platt KL, Oesch F, Phillips DH (1991) The *in vitro* metabolic activation of dibenz[a,h]anthracene, catalyzed by rat liver microsomes and examined by ^{32}P-postlabelling. *Cancer Lett*, **57**, 261-269.
12. Kawabata TT, White KLJr (1989) Benzo(a)pyrene metabolism by murine spleen microsomes. *Cancer Res*, **49**, 5816-5822.
13. Mass MJ, Kaufman DG (1983) A comparison between the activation of benzo(a)pyrene in organ cultures and microsomes from the tracheal epithelium of rats and hamsters. *Carcinogenesis*, **4**, 297-303.
14. Gupta RC, Earley K (1988) ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. *Carcinogenesis*, **9**, 1687-1693.
15. Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **9**, 1543-1551.
16. Randerath K, Reddy MV, Gupta RC (1981) ^{32}P-Labeling test for DNA damage. *Proc Natl Acad Sci USA*, **78**, 6126-6129.
17. Gupta RC, Reddy MV, Randerath K (1982) ^{32}P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis*, **3**, 1081-1092.
18. Wall KL, Gao W, te Koppele JM, Kwei GY, Kauffman FC, Thurman RG (1991) The liver plays a central role in the mechanism of chemical carcinogenesis due to polycyclic aromatic hydrocarbons. *Carcinogenesis*, **12**, 783-786.
19. Nebert DW (1989) The Ah locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol*, **20**, 153-174.
20. Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos*, **2**, 583-588.
21. Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, Guengerich FP (1985) Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring the induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys*, **238**, 43-48.
22. Rutten AAJJL, Falke HE, Catsburg JF, Topp R, Blaauboer BJ, van Holsteijn I, Doorn L, van Leeuwen FXR (1987) Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. *Arch Toxicol*, **61**, 27-33.
23. Rutten AAJJL, Falke HE, Catsburg JF, Wortelboer HM, Blaauboer BJ, Doorn L, van Leeuwen, FXR, Theelen R, Rietjens IMCM (1992) Interlaboratory comparison of microsomal ethoxyresorufin and pentoxyresorufin *O*-dealkylation determinations: standardization of assay conditions. *Arch Toxicol*, **66**, 237-244.
24. Steenwinkel M-JST, Roggeband R, van Delft JHM, Baan RA (1993) Improvements in the ^{32}P-postlabelling procedure to quantify bulky aromatic adducts, in: Postlabelling methods for detection of DNA adducts, Phillips DH, Castegnaro M, Bartsch H, Eds, IARC Scientific Publications No 124, Lyon, France, pp 65-70.
25. Wortelboer HM, de Kruif CA, van Iersel AAJ, Falke HE, Noordhoek J, Blaauboer BJ (1991) Comparison of cytochrome P-450 isoenzyme profiles in rat liver and hepatocyte cultures. *Bioch Pharmacol*, **42**, 381-390.
26. Alexandrov K, Sala M, Rojas M (1988) Differences in the DNA adducts formed in cultured rabbit and rat dermal fibroblasts by benzo(a)pyrene and benzo(a)pyrene-7,8-diol. *Cancer Res*, **48**, 7132-7139.
27. Sebti SM, Pruess-Schwartz D, Baird WM (1985) Species- and length of exposure-dependent differences in the benzo(a)pyrene:DNA adducts formed in embryo cell cultures from mice, rats, and hamsters. *Cancer Res*, **45**, 1594-1600.
28. Moore CJ, Pruess-Schwartz D, Mauthe RJ, Gould MN, Baird WM (1987) Interspecies differences in the major DNA adducts formed from benzo(a)pyrene but not 7,12-dimethylbenz(a)anthracene in rat and human mammary cell cultures. *Cancer Res*, **47**, 4402-4406.
29. Ashurst SW, Cohen GM (1982) The formation of benzo(a)pyrene-deoxyribonucleoside adducts *in vivo* and *in vitro*. *Carcinogenesis*, **3**, 267-273.

30. Ross J, Nelson G, Erexson G, Kligerman A, Earley K, Gupta RC, Nesnow S (1991) DNA adducts in rat lung, liver and peripheral blood lymphocytes produced by i.p. administration of benzo(a)pyrene metabolites and derivatives. *Carcinogenesis*, **12**, 1953-1955.
31. King HWS, Thompson MH, Brookes P (1975) The benzo(a)pyrene deoxyribonucleoside products isolated from DNA after metabolism of benzo(a)pyrene by rat liver microsomes in the presence of DNA. *Cancer Res*, **34**, 1263-1269.
32. Frazier JM, Tyson CA, McCarthy C, McCormick JJ, Meyer D, Powis G, Ducat L (1989) Potential use of human tissues for toxicity research and testing. *Toxicol Appl Pharmacol*, **97**, 387-397.
33. Powis G, Jardine I, VanDyke R, Weinshilboum R, Moore D, Wilke T, Rhodes W, Nelson R, Benson L, Szumlanski C (1988) Foreign compound metabolism studies with human liver obtained as surgical waste, relation to donor characteristics and effects of tissue storage. *Drug Metab Dispos*, **16**, 582-589.
34. Gupta RC, Earley K, Sharma S (1988) Use of human peripheral blood lymphocytes to measure DNA binding capacity of chemical carcinogens. *Proc Natl Acad Sci USA*, **85**, 3513-3517.
35. Savela K, Hemminki K, Hewer A, Phillips DH, Putman KL, Randerath K (1989) Interlaboratory comparison of the ^{32}P-postlabelling assay for aromatic DNA adducts in white blood cells of iron foundry workers. *Mutat Res*, **224**, 485-492.

Chapter

4

DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture

R Roggeband, APM Wolterbeek[1], OWM Melis[1], ME Wittekoek[1], AAJJL Rutten[1], VJ Feron[1] and RA Baan

TNO Nutrition, Department of Genetic Toxicology, PO Box 5815, 2280 HV Rijswijk, and [1]Department of Biological Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands

Carcinogenesis, 15, 661-665, 1994

Abstract

Syrian golden hamsters are much more susceptible than Wistar rats to the induction of tracheal tumors by benzo[a]pyrene (B[a]P). To investigate whether this difference is reflected in the pattern of DNA-adduct induction and removal, tracheas from either species were isolated and exposed to B[a]P (5 μg/ml) in organ culture. At various time-points B[a]P-DNA adducts were quantified by ^{32}P-postlabeling; unscheduled DNA synthesis (UDS) and cell proliferation were determined by [^{3}H]-thymidine incorporation during 18 h before sampling. In an induction-repair experiment tracheas were exposed to B[a]P for two days, and cultured for another for four days without B[a]P. After two days of exposure total B[a]P-DNA adduct levels were 10 times higher in hamster compared to rat tracheas. In hamster tracheas one major adduct was formed (95%), *viz.* the adduct between (+)-*anti*-B[a]P-diolepoxide and deoxyguanosine (BPDE-N^{2}dG). In rat tracheas BPDE-N^{2}dG comprised about 60% of the total B[a]P-DNA adduct level. The other major adduct found in rat tracheas is probably derived from interaction of *syn*-BPDE and deoxyadenosine. During exposure to B[a]P in hamsters the adduct level increased to 36 ± 19 adducts/10^6 nucleotides (add/10^{6}n) on day 2. Two days after removal of B[a]P the B[a]P-DNA adduct level had decreased to 60% of that on day 2; there was no further decrease in the B[a]P-DNA adduct level, despite considerable cell proliferation at the end of the six-day culture period. UDS increased during exposure to B[a]P and decreased after removal of B[a]P. In rats removal of B[a]P did not lead to a decrease in the B[a]P-DNA adduct level, which agreed with the observed absence of UDS. In a second experiment tracheas were exposed to B[a]P continuously for 15 days. In hamster tracheas the total B[a]P-DNA adduct level increased from 11 ± 0.7 add/10^{6}n after 1 day of exposure to 105 ± 2 add/10^{6}n after 15 days; also UDS increased with increasing exposure until day 11. Cell proliferation was low at the end of the culture

period. In rat tracheas no progressive increase in the B[a]P-DNA adduct level was seen, UDS was not increased, and cell proliferation had increased significantly at the end of the exposure period. The extent of adduct induction in the trachea of the two species corresponded with the different susceptibilities to B[a]P-induced tumor formation.

Introduction

The lung is a major site of tumor formation in humans. Epidemiological studies show a higher relative risk of lung cancer for the smoking population compared to non-smoking controls (1). With the ^{32}P-postlabeling method tobacco-smoke specific DNA adducts could be detected in lung tissue from cigarette smokers (2-5). Morphologically, the type of tumors observed in the lungs of humans shows a close similarity to benzo[a]pyrene (B[a]P)-induced respiratory tract tumors in hamsters (6-8).

In vitro exposure of hamster tracheas to B[a]P or cigarette-smoke condensate (9) resulted in hyperplasia and squamous metaplasia, which are generally considered to be precursors of respiratory-tract tumors (7,10). The Syrian golden hamster is susceptible to tumor formation in the trachea after exposure to B[a]P (11). After multiple intratracheal instillations of B[a]P epithelial hyperplasia and squamous metaplasia with various degrees of atypia, and tumors were observed in the trachea of hamsters. In contrast, similarly-treated rats did not show any epithelial alterations including tumors in the trachea, but developed lung tumors. This difference in B[a]P-susceptibility of the trachea between hamsters and rats was attributed to differences in the mucosal barrier of the trachea, preventing B[a]P to penetrate the tracheal epithelial cells of rats. However, the results of a study by Autrup *et al* (12) indicated that B[a]P-DNA adducts could be detected in rat trachea epithelial cells after *in vitro* exposure to B[a]P.

Several groups (12-14) already reported a difference in total B[a]P-DNA adduct levels between hamster and rat tracheas, exposed to B[a]P *in vitro* during 24 h. The objective of the experiments described in the present paper was to study differences in DNA-adduct formation and DNA-repair between B[a]P-exposed hamster and rat tracheas in organ culture. In an induction-repair experiment B[a]P (5 μg/ml) was present for two consecutive days, whereafter B[a]P was removed while the tracheas were kept in culture for another four days. In an induction-accumulation experiment B[a]P was present continuously during 15 days. At various time-points in both experiments tracheas were prepared for analysis of UDS and cell proliferation by incorporation of [^{3}H]-thymidine. DNA-adducts were analyzed by ^{32}P-postlabeling. In the latter assay, the reaction product of deoxyguanosine with the reactive *anti*-B[a]P-diolepoxide (*anti*-BPDE) was used as a calibration standard. In an attempt to identify the unknown adducts

observed in the postlabeling chromatograms, control experiments were carried out with the *syn*-isomer of the reactive B[a]P-diolepoxide.

Materials and methods

Chemicals and enzymes

Racemic *anti*-BPDE was obtained from Chemsyn, Lenexa, KS. Racemic *syn*-BPDE was a kind gift of Dr A Dipple. γ-[^{32}P]-ATP was purchased from Amersham, Buckinghamshire, UK. B[a]P, micrococcal endonuclease, spleen phosphodiesterase, and RNAse A were obtained from Sigma Chemicals, St. Louis, MO. Nuclease P1 and RNAse T1 were obtained from Boehringer Mannheim, Mannheim, Germany. RNAse T1 was heated at 80°C for 5 min to remove DNAse activity. T4 polynucleotide kinase was purchased from New England Biolabs, Beverly, MA and proteinase K was purchased from Merck, Darmstadt, Germany.

Culturing of tracheal rings

Ten-week old male Syrian golden hamsters and Wistar rats (obtained from Harlan/CPB, Zeist, The Netherlands) were sacrificed by an *ip* overdose of Nembutal (Ceva, Paris, France). Tracheas were isolated aseptically, and adherent connective tissue was removed. Thereafter, hamster and rat tracheas were cut into three to four and eight to nine rings, respectively. Tracheal rings were randomly allocated to the wells of 24-well plates (Costar, Cambridge, MA) and cultured in serum-free, hormone-supplemented Ham's F12 medium containing 2 mM L-glutamine (Flow Laboratories, Herts, UK), 1 μM hydrocortisone (Sigma), 5 μg/ml bovine pancreatic insulin (Sigma), 5 μg/ml human transferrin (Sigma), 25 ng/ml epidermal growth factor (Sigma), and 50 μg/ml gentamycin (Flow). Each well contained four tracheal rings derived from four different hamsters or rats. Plates were placed on a rocker (8 to 9 cycles per minute) in an incubator in a humidified atmosphere of 40% O_2, 55% N_2, and 5% CO_2 at 37°C.

Induction-repair experiment

On the day before treatment (day −1) hamster (n=40) and rat (n=16) tracheas were isolated, cleaned and placed in culture. Starting on day 0, the tracheal rings were exposed to B[a]P (5 μg/ml) for 48 h. B[a]P was dissolved in DMSO; the final concentration of DMSO in the medium was 0.1% (v/v). Samples were taken at day 0

(control), day 1 and day 2. At the end of day 2, just after sampling, all remaining tracheal rings were washed, transferred to a medium without B[a]P, and cultured for another four days during which samples were taken at 24-h intervals (see Figure 1). Sampling comprised scraping off epithelial cells for DNA isolation and adduct analysis (^{32}P-postlabeling), and preparation of sections for the assays of UDS and cell proliferation (see below).

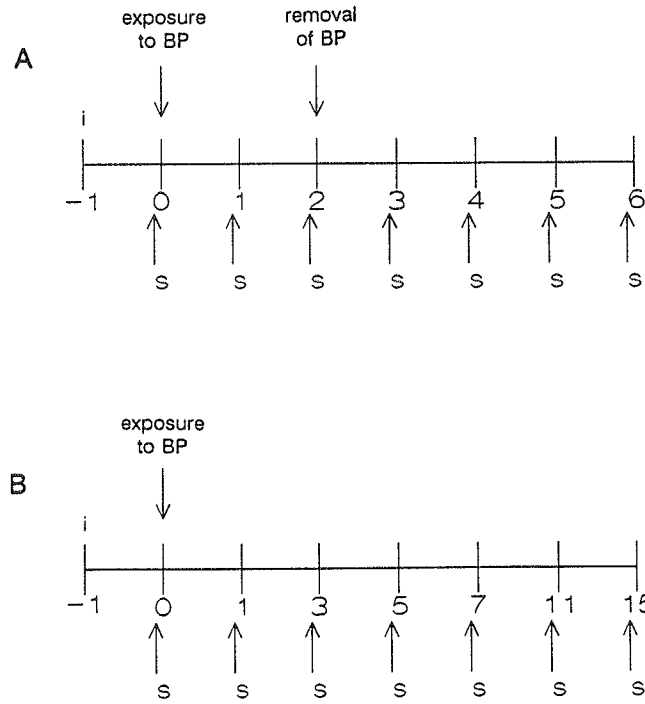

Figure 1 *Diagram of the induction-repair (A) and the induction-accumulation (B) experiment. The "i" indicates the isolation of the tracheas. The "s" indicates the sampling-points on the respective days.*

Induction-accumulation experiment

Hamster (n=30) and rat (n=12) tracheas were isolated, cleaned and placed in culture on day −1. On day 0 trachea rings were exposed to B[a]P (5 μg/ml) which continued for 15 days. Every day medium was removed and replaced with fresh medium containing freshly dissolved B[a]P. Samples were taken on days 1, 3, 5, 7, 11, and 15 (after 24, 72, 120, 168, 264, and 360 h of exposure, respectively).

DNA isolation

At each selected time-point during both the induction-repair and the induction-accumulation experiment the epithelial cells were scraped off the sampled tracheal rings (12 per time-point). Cells were collected in PBS and centrifuged at 125 g. The cell pellet was suspended in 250 μl TEN buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA.Na_2, and 100 mM NaCl). Thereafter, 250 μl of TEN containing 1% SDS and proteinase K (100 μg/ml) was added and the mixture was incubated for 18h at 37°C. DNA was isolated by phenolic extractions with equal volumes of phenol, phenol-chloroform-isoamylalcohol (25:24:1, v/v/v), and chloroform-isoamylalcohol (24:1, v/v). The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 6.0 and 3 volumes of ethanol at -20°C. DNA was washed with 70 % ethanol in water at -20°C and dried *in vacuo*. Subsequently, DNA was dissolved in 250 μl TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA.Na_2) and treated serially with a mixture of RNAses A (50 μg/ml) and T1 (50 U/ml) for 90 min and proteinase K (100 μg/ml) for 60 min at 37°C. Phenolic extractions were repeated and finally DNA was precipitated, washed, and dried as described above. DNA was dissolved in Millipore-filtered (MQ) water and the concentration was determined spectrophotometrically (1 mg of DNA per ml = 20 absorbance units at 260 nm).

^{32}P-postlabeling analysis of B[a]P-DNA adducts

The ^{32}P-postlabeling assay was performed as described by Reddy and Randerath (15) with some modifications. DNA (2-3 μg in 18 μl) was digested with micrococcal nuclease (0.4 U) and spleen phosphodiesterase (0.008 U) for 3 h at 37°C, and subsequently treated with nuclease P1 (2 U) for 40 min at 37°C. The modified nucleotides were labelled with 37 MBq γ-[^{32}P]-ATP (sp.act. > 185 TBq/mmol) by incubation with 50 U T4-polynucleotide kinase for 30 min at 37°C.

The postlabeled mixtures were applied to 20-cm x 20-cm poly(ethylenimine)(PEI)-cellulose sheets (JT Baker, Phillipsburg, NJ, USA). A paper wick was attached to the top of each TLC sheet; the sheets were developed overnight in 1 M sodium phosphate, pH 6.0 (D1). After two washes with water the plates were developed in 8.5 M urea, 3 M lithium formate, pH 3.5 (D3) for 6.5 h and in 8.5 M urea, 0.8 M lithium chloride, 0.5 M Tris, pH 8.0 (D4), also during 6.5 h. In order to remove any remaining impurities, the plates were developed overnight in 1.7 M sodium phosphate, pH 6.0 (D5, in the direction of D1). The adducts were localized by autoradiography at -70°C on Kodak XAR-5 film with intensifying screens.

The spots on the PEI-cellulose sheets detected by autoradiography were cut out and the radioactivity was determined by liquid scintillation counting. DNA-adduct levels were calculated on the basis of the results of concurrent postlabeling analysis of standard DNA samples carrying known amounts of B[a]P-adducts prepared by *in vitro* reaction with *anti*-BPDE (16). The exact amount of input DNA and the absence of RNA were determined by chromatography of the DNA digest on FPLC, as described previously (16).

In the induction-repair experiment the B[a]P-DNA-adduct levels in the first postlabeling experiment were twice as high as the levels in the second postlabeling experiment; this was due to differences in labeling of the standard samples. However, kinetics of adduct-formation and removal were comparable between the two experiments.

Reaction of (±)-syn-BPDE with DNA, dAMP and dGMP

In an attempt to identify the unknown adducts observed after postlabeling of B[a]P-modified DNA from rat and hamster, various reference adducts were prepared, by incubation of DNA or nucleotides with the *syn*-isomer of the reactive BPDE. One volume of purified rat-liver DNA (1 mg/ml in 0.1 M Tris-HCl, pH 7) was incubated with 0.1 volume of (±)-*syn*-BPDE (1 mg/ml in $MgSO_4$-dried acetone) for 12 h at 37°C. The reaction mixture was extracted three times with an equal volume of water-saturated 1-butanol, and twice with an equal volume of water-saturated ether. The DNA was then precipitated from the aqueous phase with alcohol as described above (see DNA isolation).

Solutions of dAMP (40 μM in MQ water) and of dGMP (75 μM in MQ water) were each mixed with an equal volume (±)-*syn*-BPDE (1 mg/ml in dry acetone) for 12 h at 37°C. The B[a]P-modified dNMPs were purified by Sephadex-LH20 chromatography. Both the (±)-*syn*-BPDE modified DNA and the (±)-*syn*-BPDE modified dNMPs were subjected to ^{32}P-postlabeling analysis, run in parallel with treated DNA samples.

UDS and cell proliferation

Slides were prepared for UDS as described by Wolterbeek *et al* (17). Briefly, tracheal rings were cultured for 18 h in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (Amersham, sp. act. 1.81 TBq/mmol). Semi-thin sections (1 μm) of the tracheal rings were dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York). Autoradiograms were exposed for 4-6 weeks at -30°C. UDS was expressed as net grains per nucleus which was calculated as the number of silver grains over the nucleus minus the number of silver grains over a nuclear-sized area in the cytoplasm. At each time-point a total of four

sections (on two different slides) were scored. Per section 50 nuclei were scored.

Cell proliferation was determined in the sections that were also used for UDS (18). In the epithelium the total number of labeled cells (basal and non-basal cells) was counted. Furthermore, the total number of epithelial cells per section was counted. At each time-point cells were counted around the whole circumference (600-1200 cells) of eight different sections (on four slides). The labeling index (LI) was defined as the percentage of labeled cells amongst the total number of cells counted.

Results

Induction-repair experiment

Hamster and rat tracheas were exposed to B[a]P (5 μg/ml, ~20 μM) for two consecutive days, whereafter B[a]P was removed and tracheas were kept in culture for another four days. In DNA isolated from hamster trachea epithelial cells an increase in the total B[a]P-DNA adduct level, as measured with the ^{32}P-postlabeling method, from day 0 (start of exposure to B[a]P) to day 2 (end of exposure to B[a]P) was observed (Figure 2).

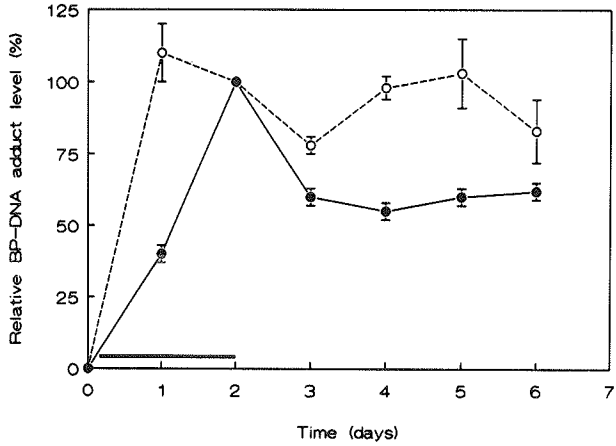

Figure 2 *Relative B[a]P-DNA adduct level in hamster (-●-) and rat (-o-) tracheas exposed to B[a]P (5 μg/ml) for two days. The 100% level at 48 h corresponds to 36 ± 19 add/10^{6}n in case of hamster tracheas and 3.5 ± 1.7 add/10^{6}n in case of rat tracheas. The horizontal bar indicates the period of B[a]P-exposure. For each of two postlabeling experiments, the results obtained with the sample collected on day 2 was set at 100%. Error bars represent range of values.*

The adduct level on day 2 corresponded to 36 ± 19 adducts per 10^6 nucleotides (add/10^6n). One day after washing out B[a]P the adduct level had decreased to 60% ± 2% of the level on day 2. There was no further decrease in adduct level during culture. A typical adduct pattern obtained with DNA from the hamster trachea is depicted in Figure 3. At all time-points the TLC results showed one predominant adduct in the hamster trachea (adduct 1), which co-migrated with the adduct formed between (+)-*anti*-BPDE and deoxyguanosine. It accounted for 95% of the total adduct level.

In the DNA of rat trachea epithelial cells the B[a]P-DNA adduct level was 3.7 ± 1.5 add/10^6n after one day exposure to B[a]P. However, contrary to the hamster, there was no further increase in the B[a]P-DNA adduct level after two days of exposure. Also in the rat trachea B[a]P-DNA adducts could still be detected four days after removal of B[a]P (Figure 2). Actually, this was almost 90 % of the level found at day 2. After 48 h of exposure to B[a]P the total B[a]P-DNA adduct level in the rat trachea was 10-fold lower than in the hamster trachea. As depicted in Figure 3, the adduct pattern on TLC of rat trachea epithelial cells differed considerably from that of hamster; while adduct 1 was almost the only adduct in the hamster, the relative amount in rat trachea was about 60 %. The other substantial adduct in the rat trachea, *viz.* adduct 3, comprised about 30 % of total adducts.

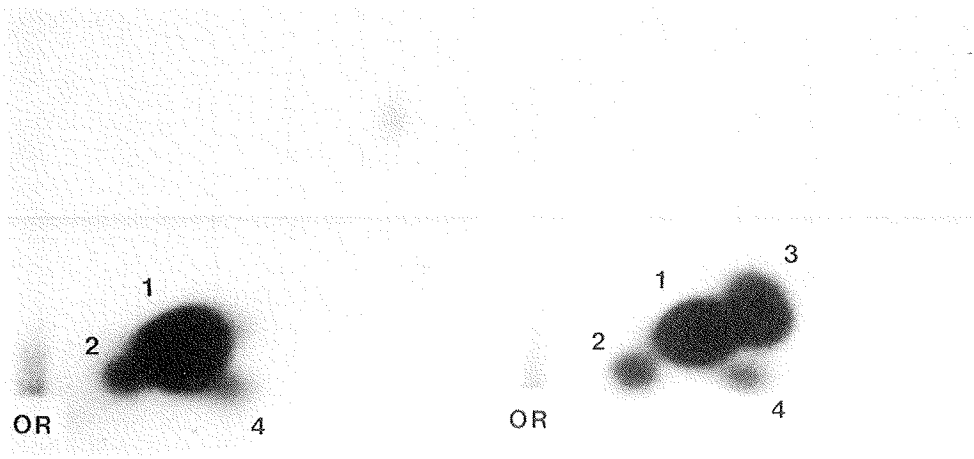

Figure 3 *Typical adduct pattern on TLC of postlabeled DNA from hamster (left) and rat (right) tracheas. The films were exposed for 6 h (hamster) and 26 h (rat) at -70°C. Adduct 1 was identified by co-chromatography as the interaction product of (+)-anti-BPDE and dG.*

The chromatographic behaviour of adduct 3 closely resembles that of one of the two adducts derived from incubation of (±)-*syn*-BPDE with dAMP (see Figure 4). This suggests that adduct 3 is derived from interaction of (±)-*syn*-BPDE and deoxyadenosine. Adduct 2 was a minor adduct (10% of the total adduct level).

In order to determine DNA-repair activity, the unscheduled DNA synthesis (UDS) was scored in trachea sections. In hamster trachea epithelial cells the exposure to B[a]P led to a well measurable induction of UDS (Figure 5). The control level on day 0 of 0.22 ± 0.09 increased to 0.54 ± 0.03 on day 1 (statistically significant; Student's t-test; p=0.0056). On day 2 (48 h exposure to B[a]P) and day 3 (one day after removal of B[a]P) the UDS was also significantly higher than on day 0 (Student's t-test; p=0.0003 and p=0.0017, respectively). UDS at days 4, 5, and 6 was not increased compared to that on day 0. In contrast, in rat trachea epithelial cells UDS was not increased after exposure to B[a]P at any time-point (see Figure 5).

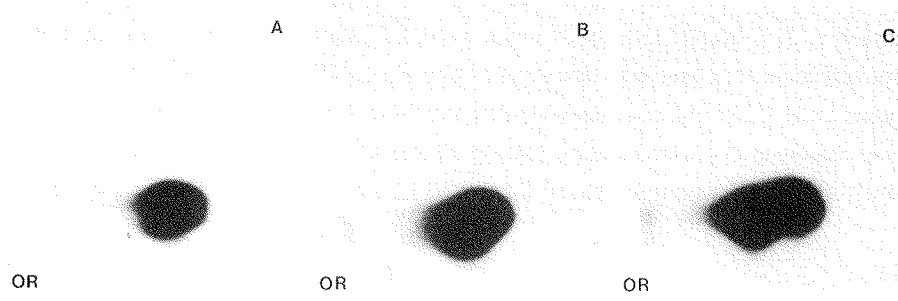

Figure 4 *Adduct pattern on TLC of postlabeled (±)-syn-BPDE-modified DNA (A), dGMP reacted with (±)-syn-BPDE (B), and dAMP with (±)-syn-BPDE (C). Film (A) was exposed for 30 min and films (B) and (C) were exposed for 4 h at -70°C.*

During exposure to B[a]P, cell proliferation was low in hamster tracheas (LI < 2.5%). On days 4, 5, and 6 cell proliferation had recovered (LI varied from 15 to 20%). Despite this rather extensive cell proliferation there was no further decrease in the total B[a]P-DNA adduct level on days 4, 5, and 6 compared to day 2. In general, cell proliferation in hamster tracheas was higher in non-basal cells than in basal cells. In rat tracheas the LI varied from 6 to 12% during exposure to B[a]P. On days 4, 5, and 6 the LI decreased from 12% to <1%. No clear difference was found between non-basal and basal cells with respect to cell proliferation in rat tracheas.

Induction-accumulation experiment

Figure 6 shows the postlabeling data of DNA from tracheas that had been exposed to B[a]P continuously during 15 days. In hamster trachea epithelial cells, a steady increase in the total B[a]P-DNA adduct level from 11 ± 0.7 add/10^6n on day 1 to 105 ± 2 add/10^6n on day 15 was observed. Adduct 1 comprised about 95% of the total adducts, comparable with the results of the induction-repair experiment.

In rat trachea epithelial cells the total adduct level on day 1 was 6.1 ± 1.5 add/10^6n. Contrary to the hamster, in the rat trachea no steady increase in adduct level was found; on day 15 the total adduct level was 8.1 ± 0.3 add/10^6n. The adduct patterns of both the induction-repair and the induction-accumulation experiment were comparable, with the exception of adduct 4, a minor adduct (<10%), which was not observed in the induction-repair experiment. The difference in total adduct level between the hamster and rat trachea increased from about 2-fold at day 1 to about 13-fold on day 15.

In the hamster trachea epithelial cells UDS (Figure 7) was significantly increased on days 1, 3, 5, 7, and 11 (Student's t-test; $p<0.025$ at all time-points) compared to the unexposed control in the induction-repair experiment (Figure 5, day 0). In rat trachea epithelial cells UDS did not increase with exposure time. Cell proliferation activity in hamster tracheas decreased during culture to less than 1% at day 15. In rat tracheas, however, cell proliferation increased from a LI of 5% at day 1 to 20% at day 15.

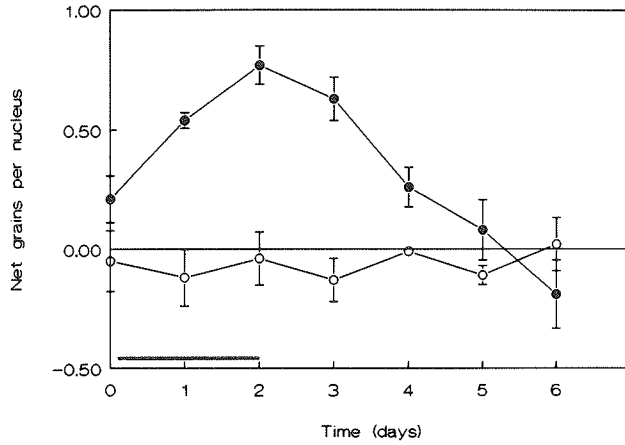

Figure 5 *UDS in hamster (-●-) and rat (-o-) tracheas exposed to B[a]P (5 µg/ml) for two days. The horizontal bar indicates the period of B[a]P-exposure. Error bars represent SD (n=8).*

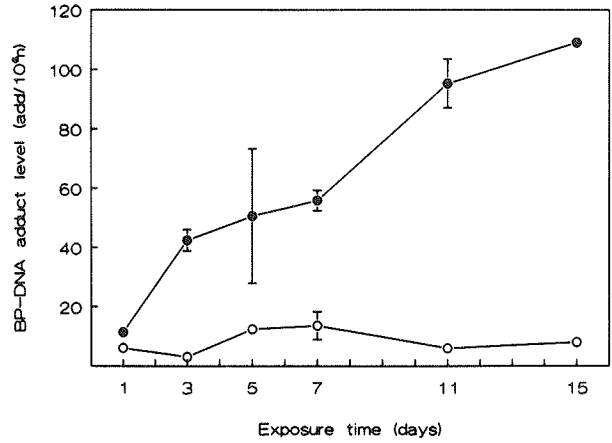

Figure 6 *B[a]P-DNA adduct level in hamster (-●-) and rat (-o-) tracheas exposed to B[a]P (5 µg/ml) continuously. Error bars represent range of values of two postlabeling assays (error bars sometimes within the size of the symbol).*

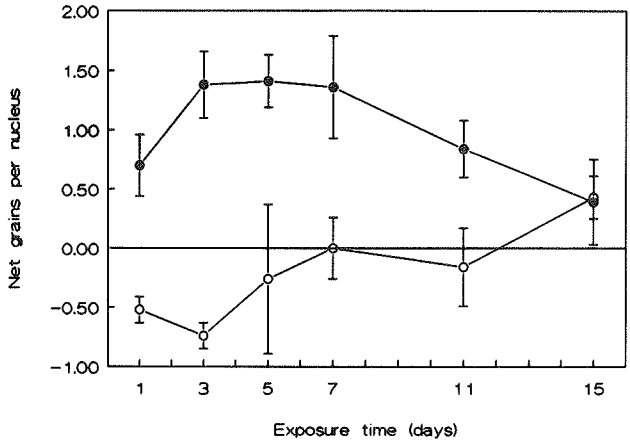

Figure 7 *UDS in hamster (-●-) and rat (-o-) tracheas exposed to B[a]P (5 µg/ml) continuously. Error bars represent SD (n=8).*

Discussion

In view of the remarkable difference between the hamster and the rat with respect to the susceptibility to B[a]P-induced tumor formation in the trachea, it is of interest to establish whether a similar difference can be observed during the early stages of tumorigenesis, *viz.* with respect to DNA-adduct formation. In this paper the results are presented of experiments in which tracheas from Syrian golden hamsters and Wistar rats were exposed to B[a]P *in vitro* during various periods of time. This *in vitro* organ culture has proven to be an attractive model to study the initial stages of tumor development in the respiratory tract (9). The amount of DNA adducts in the epithelial cells of the tracheas was determined at different time points by use of the ^{32}P-postlabeling assay, which also yielded the adduct pattern as revealed by TLC. Furthermore, DNA-repair synthesis (UDS) and the extent of cell proliferation were determined in preparations sampled at the same time points.

In an induction-repair experiment tracheas from the two species were exposed to B[a]P for two days, followed by post-treatment incubation for four days to allow repair to proceed. The kinetics of adduct formation in epithelial cells during B[a]P exposure and adduct removal during the post-treatment incubation were different: in the hamster trachea the amount of DNA adducts increased during B[a]P treatment, reached a maximum on day 2, then dropped to a level at ≈60% of the maximum value, which

remained constant on days 4, 5 and 6. In the rat trachea the adduct level reached a plateau value already on day 1 and remained more or less the same during days 2-6. There was a striking difference between the two species as to the total B[a]P-DNA adduct level, which was about 8 times as high in hamster than in rat trachea. In an induction-accumulation experiment tracheas were exposed continuously to B[a]P for 15 days. A steady increase in the DNA-adduct level was observed in the hamster, and a constant, low level in the rat trachea epithelial cells. At the end of the incubation period there was a 13-fold difference between the adduct levels in hamster and rat. A few other studies have been published in which B[a]P-DNA-adduct formation in rat and hamster tracheas was compared, after exposure for 24 h to 1 or 1.5 μM [^{3}H]-B[a]P (12-14); the differences in total adduct levels varied from 2- to 17-fold between the species, which is similar to our observations. This difference in B[a]P-DNA-adduct induction in hamster and rat trachea is in line with the difference in susceptibility of the two species to B[a]P-induced tracheal tumor formation.

Besides quantitative data on adduct levels, the ^{32}P-postlabeling assay also provides information on adduct patterns. The TLC chromatogram of postlabeled hamster tracheal DNA showed that one adduct (adduct 1) was formed almost exclusively (95% of the total amount), whereas another adduct (adduct 2) comprised only about 2%. Adduct 1 was tentatively identified by co-migration as the reaction product of (+)-*anti*-BPDE and deoxyguanosine (BPDE-N^{2}dG). Adduct 2 was not identified; it might originate from the reaction of 9-hydroxy-B[a]P-4,5-epoxide with DNA (19). In rat tracheal DNA, adducts 1, 2 and a third adduct (adduct 3) were observed in a 60:10:30 ratio. The chromatographic behaviour of adduct 3, when compared with that of appropriate standards, suggested that this adduct results from interaction of the *syn*-isomer of the B[a]P-diolepoxide ((±)*syn*-BPDE) and deoxyadenosine. Autrup *et al* (12) reported that in rat tracheas BPDE-deoxyadenosine adducts accounted for about 20% of the total modification. Adduct 4 was not identified. However, its low recovery in the butanol extraction procedure (results not shown) suggests that the adduct is more polar than adduct 1, possibly as a consequence of more than two hydroxyl groups on the B[a]P-moiety.

From the qualitative assessment of the adduct patterns on TLC it appears that epithelial cells in both hamster and rat trachea are capable of metabolic conversion of B[a]P into reactive metabolites, under the experimental conditions used. In tracheal DNA from both species the *anti*-BPDE-N^{2}dG adduct (adduct 1) was detected, albeit in different proportions. This adduct is considered to play a role in B[a]P-induced carcinogenesis. In addition, in rat DNA the *syn*-BPDE-dA adduct was detected. Although there are qualitative differences in the adduct patterns of hamster and rat tracheal DNA, it can be concluded that the different susceptibilities of these species to B[a]P-induced

tracheal tumor formation cannot be attributed to a specific adduct that is present in the hamster and absent in the rat. This conclusion appears to be a valid one since there are indications that B[a]P-metabolism and B[a]P-DNA adduct formation during *in vitro* organ culture are not too different from those *in vivo*. This follows from the observation that adduct patterns of postlabeled DNA from the trachea of *in vivo* B[a]P-treated hamsters (intratracheal intubation, ref. 17) were qualitatively comparable with those from the present *in vitro* study.

Bulky lesions, such as those induced by B[a]P, are repaired through the excision-repair pathway, which can be visualized by incorporation of [^{3}H]-thymidine during resynthesis of DNA (UDS). In parallel to adduct analysis by ^{32}P-postlabeling, the extent of UDS was determined in samples collected at the same time points. In the induction-repair experiment a significant increase in UDS was detected in hamster trachea cells. The time interval of maximum UDS coincided with the time point at which the highest B[a]P-adduct level was observed. After removal of B[a]P, UDS gradually decreased to control levels on days 5 and 6, despite the fact that ≈60% of the adducts were still present. One explanation could be that part of the adducts is not susceptible to repair, and appears persistent. However, this is less likely because the adduct patterns observed in the TLC chromatograms at the various time points were similar. Other explanations could be the absence of active DNA repair in certain cell types, *e.g.*, non-dividing cells (see below), or gradual loss of overall repair activity under the conditions of the *in vitro* organ culture. A similar UDS pattern in the hamster trachea was observed in the induction-accumulation experiment, albeit at a somewhat higher level. In this case, an increased UDS level was observed up to day 7, and a gradual decrease to control values on day 15, despite the fact that a steady accumulation of DNA adducts was seen. This may be explained by a deficit of the repair system due to the large amount of DNA lesions by that time. In both experiments, rat tracheal cells did not show significant levels of UDS. Apparently, the low level of DNA damage does not evoke a measurable UDS response.

Reduction of DNA-adduct levels, which are expressed per unit amount of DNA, may be the result not only of active repair but also of ongoing cell proliferation. Along with the analysis of repair, the incorporation of [^{3}H]-thymidine was also used to determine the relative number of cells in S-phase (expressed as the labeling index), as an indicator of cell proliferation. In the epithelial cells of the hamster trachea, a relatively high labeling index was observed on days 4, 5 and 6 of the induction-repair experiment, while the total amount of DNA isolated was approximately the same at all time points. Furthermore, tissue sections of the trachea did not show an increase of the thickness of the epithelial wall during the course of the experiment. This may indicate that cell

proliferation in the epithelium is counterbalanced by cell loss at the epithelial surface. Remarkably, the total amount of B[a]P-DNA adducts per μg DNA did not change during that time period, despite the rather high labeling index. This may be explained by a preferential occurrence of proliferation of those cells which contained only few adducts or which had their adducts removed before entering into S-phase owing to rapid DNA-repair, and/or by an uneven distribution of B[a]P-DNA adducts over the various cell types. Also, the time needed to proceed through the S-phase in B[a]P-exposed tracheal epithelial cells may be important in this respect; since indications point out that the cell-cycle in this *in vitro* system amounts to 3-4 days, the S-phase may very well take a few days, in which case we may not be able to detect any substantial change in the B[a]P-DNA level per μg DNA over such a period. The use of adduct-specific antibodies will allow the study of adduct formation and repair *in situ*, in different epithelial cell types within the trachea. Experiments along this line are ongoing.

Acknowledgements

We thank Dr A Dipple for his generous gift of *syn*-BPDE. Furthermore we thank Mrs M-JST Steenwinkel for her advice on the postlabeling experiments and Dr F Berends for his helpful discussions. This work was sponsored in part by the Directorate-General of Labour, Dutch Ministry of Social Affairs and Employment.

References

1. International Agency for Research on Cancer (1986) IARC Monographs on the evaluation of the carcinogenic risks of chemicals to humans. Vol 38. Tobacco smoking. IARC, Lyon, France.
2. Phillips DH, Hewer A, Martin CN, Garner RC, King MM (1988) Correlation of DNA adduct levels in human lung with cigarette smoking. *Nature*, **336**, 790-792.
3. Randerath E, Miller RH, Mittal D, Avitts TA, Dunsford HA, Randerath K (1989) Covalent DNA damage in tissues of cigarette smokers as determined by ^{32}P-postlabeling assay. *J Natl Cancer Inst*, **81**, 341-347.
4. van Schooten FJ, Hillebrand MJX, van Leeuwen FE, Lutgerink JT, van Zandwijk N, Jansen HM, Kriek E (1990) Polycyclic aromatic hydrocarbon-DNA adducts in lung tissue from lung cancer patients. *Carcinogenesis*, **11**, 1677-1681.
5. Dunn BP, Vedal S, San RHC, Kwan W-F, Nelems B, Enarson DA, Stich HF (1991) DNA adducts in bronchial biopsies. *Int J Cancer*, **48**, 485-492.
6. Feron VJ, van den Heuvel PD, Koeter HBMW (1980) Significance of particle size of benzo(a)pyrene for the induction of respiratory tract tumors in hamsters. *Int J Cancer*, **25**, 301-307.
7. McDowell EM (1982) Histogenesis and morphogenesis of bronchial neoplasms. In Shimosato Y, Melamed MR, Nettesheim P (eds), Morphogenesis of lung cancer. Vol 1, CRC Press, Boca Raton, FL, pp 1-36.
8. McDowell EM, Trump BF (1983) Histogenesis of preneoplastic and neoplastic lesions in tracheobronchial epithelium. *Surv Synth Pathol Res*, **2**, 235-279.

9. Rutten AAJJL, Beems RB, Wilmer JWGM (1988) Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture II. A histomorphological study. *Virch Arch B Cell Pathol*, **55**, 177-186.
10. Becci PJ, McDowell EM, Trump BF (1978) The respiratory epithelium. IV. Histogenesis of epidermoid metaplasia and carcinoma *in situ* in the Hamster. *J Natl Cancer Inst*, **61**, 577-586.
11. Schreiber H, Martin DH, Pazmiño N (1975) Species differences in the effect of benzo(a)pyrene-ferric oxide on the respiratory tract of rats and hamsters. *Cancer Res*, **35**, 1654-1661.
12. Autrup H, Wefald FC, Jeffrey AM, Tate H, Schwartz RD, Trump BF, Harris CC (1980) Metabolism of benzo(a)pyrene by cultured tracheobronchial tissues from mice, rats, hamsters, bovines and humans. *Int J Cancer*, **25**, 293-300.
13. Daniel FB, Schut HAJ, Sandwisch DW, Schenck KM, Hoffmann CO, Patrick JR, Stoner GD (1983) Interspecies comparisons of benzo(a)pyrene metabolism and DNA-adduct formation in cultured human and animal bladder and tracheobronchial tissues. *Cancer Res*, 43, 4723-4729.
14. Mass MJ, Kaufman DG (1983) A comparison between the activation of benzo(a)pyrene in organ cultures and microsomes from the tracheal epithelium of rats and hamsters. *Carcinogenesis*, **4**, 297-303.
15. Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **9**, 1543-1551.
16. Steenwinkel M-JST, Roggeband R, van Delft JHM, Baan RA (1993) Improvements in the ^{32}P-postlabelling procedure to quantify bulky aromatic adducts, in: Postlabelling methods for detection of DNA adducts, Phillips DH, Castegnaro M, Bartsch H, Eds, IARC Scientific Publications No 124, Lyon, pp 65-70.
17. Wolterbeek APM, Roggeband R, Steenwinkel M-JST, Baan RA, Rutten AAJJL (1993) Formation and repair of benzo(a)pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis. *Carcinogenesis*, **14**, 463-467.
18. Rutten AAJJL, Wilmer JWGM, Beems RB (1988) Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture I. A cell proliferation study. *Virch Arch B Cell Pathol*, **55**, 167-175.
19. Roggeband R, Wolterbeek APM, Rutten AAJJL, Baan RA (1993) Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* upon activation of benzo[a]pyrene by human, rabbit, and rodent liver microsomes. *Carcinogenesis*, **14**, 1945-1950.

Chapter

5 *Detection of DNA-adducts in basal and non-basal cells of the hamster trachea exposed to benzo[a]pyrene in organ culture*

R Roggeband, PTM van den Berg, CJM van der Wulp and RA Baan

TNO Nutrition and Food Research Institute, Division of Toxicology, Department of Genetic Toxicology, PO Box 5815, 2280 HV Rijswijk, The Netherlands

Journal of Histochemistry and Cytochemistry, in press

Abstract

DNA adducts were quantified in hamster tracheas exposed to benzo[a]pyrene (B[a]P) in organ culture, in basal as well as in non-basal cells, by means of *in situ* detection with an adduct-specific rabbit antiserum (W2/01), and with a mouse monoclonal antibody against human cytokeratins 5 and 8 (RCK102) to identify hamster trachea basal cells. Recognition by W2/01 of the adduct of (+)-*anti*-7,8-dihydroxy-9,10-epoxide of B[a]P (B[a]P-diolepoxide; BPDE) to deoxyguanosine (dG) was checked on human white blood cells (WBC) exposed to B[a]P together with 3-methylcholanthrene (3MC)-induced rat-liver microsomes. By comparison with the adduct levels determined by ^{32}P-postlabeling, a lower detection limit of about 1 adduct per 10^6 nucleotides could be deduced. Next, tracheal rings were exposed to B[a]P (40 μM) in organ culture for 2 days, then washed and cultured without B[a]P for another 3 days. At different time points epithelial cells were isolated and cytospin preparations made. Staining of B[a]P-DNA adducts combined with that of cytokeratin (both visualized with fluorescence), allowed the detection of adducts in both basal and non-basal cells in the same preparation. B[a]P-DNA adduct formation in basal and non-basal cells after 2 days of exposure to B[a]P was not different. However, upon removal of B[a]P the adducts disappeared significantly faster from basal cells than from non-basal cells. The combination of the two antibodies mentioned above thus allows selective determination of B[a]P-DNA adduct levels in different cell types. This could be of importance with regard to the involvement of specific cell types in the process of tumor initiation.

Polycyclic aromatic hydrocarbons (PAHs) form a group of wide-spread environmental pollutants (1). One major biological effect of these compounds is the formation of the so-called PAH-DNA adducts, which are formed after covalent interaction of reactive PAH-metabolites and nucleotides. Several methods aimed at the determination of the level of PAH-DNA adducts have been developed. These include both biochemical (2-9) and immunochemical (10-17) techniques. A widely used biochemical technique is the ^{32}P-postlabeling assay, which is renowned for its sensitivity (1 adduct in 10^{10} nucleotides) and requires only μg amounts of DNA. This method, however, does not give information on DNA-adduct levels in different cell types in mixed populations. The immunochemical techniques are about three orders of magnitude less sensitive than the postlabeling method. However, the use of antibodies against DNA-adducts offers the possibility to study DNA-adduct formation at the level of the single cell. Several groups (13,14,16,17) reported the use of antibodies in *in situ* detection of benzo[a]pyrene (B[a]P)-DNA adducts. At our Institute, a mouse-monoclonal antibody (II.E4) has been developed that recognizes B[a]P-DNA adducts (14). It was applied both in *in vitro* studies, in which human fibroblasts (14) or white blood cells (WBC) (16) were treated with the diolepoxide of B[a]P, and *in vivo* studies (15), in which bronchial cells were obtained from smoking individuals.

In the study presented in this paper, we aimed at DNA-adduct detection in specific cell types, *i.e.*, the basal and the non-basal cell of the hamster trachea. With respect to the role of specific cell types in the process of respiratory tract carcinogenesis, it is important to be able to measure adducts in these cell types. To this end we combined immunocytochemical detection of adducts with the identification of the cells of interest by use of another antiserum. For this purpose the monoclonal mouse antibody RCK102 was available, which had been raised against human cytokeratins 5 and 8, but was shown to be specific also for the basal cell of the hamster trachea (18). To facilitate the combination of both detection methods, the availability of non-mouse anti-adduct antibodies appeared desirable. For that reason the rabbit antiserum W2/01 was generated. Then, a procedure was developed that enabled us to both determine the amount of B[a]P-DNA adducts per cell and discriminate between basal and non-basal cells in one preparation, by use of immunofluorescence microscopy (IFM). The use of only fluorescence markers in double-staining procedures instead of the combination of fluorescence and colourigenic substrates such as 3-amino-9-ethylcarbazole (AEC) and 3,3'-diaminobenzidine (DAB) has specific advantages. The use of colourigenic substrates may result in precipitated products that are relatively unstable in alcoholic solutions

(AEC) or are too intense, such that they obscure the fluorescence (DAB).

Interactive image-processing of both adduct-specific and cell type-specific fluorescence images allowed the determination of the B[a]P-DNA adduct level in basal as well as non-basal cells of the hamster trachea. In an induction-repair experiment hamster trachea rings were exposed to B[a]P (40 μM) for two days. Thereafter, the tracheal rings were cultured for three more days in B[a]P-free medium. Induction and disappearance of B[a]P-DNA adducts were determined in basal and non-basal cells of the hamster tracheal epithelium.

Materials and methods

Chemicals, antibodies and enzymes

Benzo[a]pyrene (B[a]P), propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), BSA and RNAse A were obtained from Sigma, St Louis, MO. γ-[^{32}P]-ATP (~110 TBq/mmol) was purchased from Amersham, Buckinghamshire, England. DMSO and proteinase K were from Merck, Darmstadt, Germany. GaM-FITC was purchased from Southern Biotechnology Associates, Birmingham, AL. GaR-FITC and GaR-TRITC were purchased from Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD. RCK102 was purchased from Organon Teknika, Eindhoven, The Netherlands.

Treatment of WBC with B[a]P and subsequent postlabeling

Human WBC were collected and exposed to B[a]P (concentration ranging from 0 to 100 μM; exposures in triplicate) for 5 h in the presence of 3MC-induced rat-liver microsomes as described before (19). After exposure the WBC were divided into two portions. One was processed for analysis by immunofluorescence microscopy (IFM) and from the other portion DNA was isolated for ^{32}P-postlabeling. WBC-DNA was subjected to the postlabeling assay as described by Gupta *et al* (8). Briefly, 7.5 μg of DNA was digested with micrococcal endonuclease (Sigma, 33 mU/μl) and spleen phosphodiesterase (Sigma, 0.67 mU/μl) in a total volume of 22.5 μl. After digestion, ammonium formate (final concentration 10 mM) and tetrabutyl ammoniumchloride (Sigma, final concentration 1 mM) were added and extractions were carried out twice with equal volumes of water-saturated n-butanol (Aldrich Chemie, Bornem, Belgium). Pooled butanol-fractions were extracted with an equal volume of water. Next, 2 μl 200 mM Tris pH 9.5 was added to the butanol fraction which was then dried *in vacuo* in a Speed Vac. The residue was resuspended in 25 μl of butanol and dried again. Finally, the digest was

resuspended in 3 μl of Millipore-filtered water and labeled with γ-[^{32}P]-ATP (3,000 Ci/mmol; 1.7 MBq per sample) in the presence of polynucleotide kinase (New England Biolabs, Beverly, MA; 10U/μl; 3.9 U per sample) for 30 min at 37°C. The labeled nucleotides were separated by multi-directional chromatography on polyethyleneimine thin-layer sheets (JT Baker, Phillipsburg, NJ). Adducts were quantified with the use of B[a]P-DNA standards as described by Steenwinkel *et al* (20).

Polyclonal antiserum W2/01

As a hapten to generate the polyclonal antibody W2/01, untreated rat-liver DNA was reacted with (±)-*anti*-B[a]P-diolepoxide (Chemsyn, Lenexa, KS). Next, the B[a]P-modified DNA (modification level about 3%) was made single-stranded and complexed with methylated-BSA (mBSA). A Flemish Giant rabbit (7 kg and 15 months of age) was immunized three times with 200 μg of B[a]P-DNA-mBSA intradermally at four places on the back. The first and second immunization were given on day 0 and day 28 together with Freunds complete adjuvant, the third was given on day 52, with Freunds incomplete adjuvant. The animal was bled three weeks after the final immunization, serum was isolated and used without further purification.

Processing of WBC for IFM

WBC were fixed in methanol:acetic acid (9:1) at room temperature and stored at -20°C. Before analysis, WBC were put on multiwell-slides (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD) by use of a pipet and processed as described by Lesko *et al* (21), with slight modifications. Slides with WBC were rehydrated in 0.1 N HCl at 4°C for 3 min, treated with RNAse A (0.1 mg/ml) for 1 h at 37°C, and dehydrated in an ethanol series. To denature the DNA *in situ*, the slides were incubated in 0.15 N NaOH in 70% ethanol for 2 min at room temperature. To prevent rewinding of DNA, the slides were incubated in 3.5% formaldehyde (Riedel-de Haën, Seelze, Germany) in 70% ethanol for 30 sec, and washed in 70% ethanol for 30 sec. Subsequent treatment with proteinase K (2 μg/ml) in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA.Na_2 was for 10 min at 37°C. After washing, the preparations were pre-incubated with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 1% BSA (w/v), for 30 min at room temperature. Then, the preparations were incubated with adduct-specific antibody (W2/01; 1:2,000) for 18 h at 4°C and with either FITC- or TRITC-labeled GaR-IgG (1:75) for 1 h at 37°C. All antibody dilutions were made in PBS, containing 1% BSA (w/v). The cells were then counterstained in PI (50

ng/ml) in case of FITC-labeled GaR-IgG or DAPI (20 ng/ml) in case of TRITC-labeled GaR-IgG, mounted in glycerol/Tris-buffered saline (9:1), coverslipped and examined by means of IFM.

Exposure of hamster tracheas to B[a]P and subsequent processing for IFM

Tracheas were isolated from eight Syrian golden hamsters and cultured as tracheal rings (four rings per trachea) as described by Wolterbeek *et al* (22). Starting on day 0 (one day after isolation of the tracheas) tracheal rings were exposed to B[a]P (40 μM) for two days. At the end of day 2 the tracheal rings were transferred to B[a]P-free medium and cultured for another three days. The medium was refreshed each day. At day 2 (48 h exposure to B[a]P), day 3 (24 h after washing out B[a]P), day 4 and day 5 eight rings were removed. The epithelial cells were liberated from the cartilage by treatment with 0.05% trypsin in PBS containing 0.025% EDTA.Na_2 for 24 h at 4°C. After trypsinization epithelial cells were suspended in FCS and cytospin preparations were made on aminosilane-precoated microscope slides. Next, the cells were fixed in methanol for 10 min at room temperature. Cells were rehydrated in 2*SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0) at 37°C for 1 h, treated with RNAse A (0.1 mg/ml) for 1 h at 37°C, and dehydrated in an ethanol series. DNA denaturation and proteinase K treatment were as described above. After washing, the cytospin preparations were pre-incubated in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 5% normal hamster serum (NHS), for 1 h at room temperature. Then, the preparations were incubated with W2/01 (1:2,000) for 18 h at 4°C. Finally, the cells were washed and incubated successively with RCK102 (undiluted) for 1 h at 37°C, GaR-TRITC (1:75) for 1 h at 37°C, and GaM-FITC (1:75) for 1 h at 37°C. All antibody dilutions were made in TBS, containing 5% NHS. The cells were counterstained with DAPI (20 ng/ml), mounted in glycerol/TBS (9:1), coverslipped and examined by means of IFM.

Image recording and processing

For the recording of fluorescence images two set-ups were used, *viz.* a non-confocal scanning laser microscope (LSM) and a CCD (charge coupled device) camera on top of a fluorescence microscope. Below both systems are described in more detail.

The LSM system

In the scanning laser microscope (Zeiss LSM 41, Germany) a single-line 488nm Ar^+ laser (Siemens) scans the specimen once in 2 s. A dichroic mirror FT 510 (Zeiss) separates the faint fluorescence emission light, emerging from FITC and PI, from the abundant reflected light (488 nm). The FITC-fluorescence is selected by means of a bandpass barrier filter (BP 515-565), that of PI by a longpass emission filter (LP 590). While the specimen is scanned line by line (512 lines/image), a photomultiplier-tube converts the selected component of the fluorescence into a continuous electrical signal. Each line is sampled (A/D conversion) 512 times. Thus, a complete image is represented by 262,144 picture elements (pixels); it is stored in the local LSM memory. A custom-developed, mouse-driven recording program, with a graphical interface, is applied to store the images in the memory of a recording computer (Silicon Graphics Personal Iris 4D/25), which is connected to the LSM via an IEEE-488 bus. From the computer memory the images are directly and transparently written over the LAN (Ethernet) onto a remote disk of a workstation (Silicon Graphics 4D/35). On this machine, image processing, using SCIL-Image as a basic toolbox, can be done automatically (in batch) or in an interactive way.

The CCD system

This consists of a liquid-nitrogen cooled CCD camera (LN_2 Astromed Ltd., Cambridge, England) placed on top of a Leitz Orthoplan fluorescence microscope. The specimen is excited by light of a 100 watt dc mercury-arc lamp, filtered with a bandpass filter, adapted to the fluorochrome used. For TRITC it is a BP 515-560 filter, for FITC BP 485/20 and for DAPI BP 340-380. The respective dichroic mirrors are DM 580 (TRITC), DM 510 (FITC), and DM 400 (DAPI). The lamp illuminates the total specimen, resulting in a fluorescence image selected by means of the emission filters LP 590 (TRITC), BP 515-560 (FITC) and BP 430-500 (DAPI). This image is projected onto the CCD chip of the camera using a 63x oil-immersion objective. The camera is controlled by special image pre-processing hardware (Astromed Ltd), placed in a personal computer (Unix). On this PC runs a custom-written recording program, essentially similar to the one in the LSM set-up, that allows recording of large sequences of images. These are directly transported over the LAN to the same disk of the workstation as in the case of the LSM set-up. The user-chosen type of image processing can be done in the same way as with the LSM.

This paper describes the use of a procedure which allows detection of B[a]P-DNA adducts in basal as well as non-basal cells of the hamster trachea. A polyclonal rabbit antibody, directed against B[a]P-modified DNA, was used together with a mouse monoclonal antibody, specific for basal cells of the hamster trachea epithelium. Both antigens were visualized by means of fluorescence.

Table 1 *^{32}P-postlabeling analysis of B[a]P-DNA adduct levels in WBC, exposed to various concentrations B[a]P*[a]

Treatment	Concentration (μM)	Total adduct level (add/10^6n)[b]
control	-	ND[c]
B[a]P	10	2.2 ± 0.1
B[a]P	25	4.2 ± 0.2
B[a]P	50	9.3 ± 1.3
B[a]P	100	11.8 ± 2.0

[a]: Human WBC were exposed to B[a]P in the presence of 3-MC-induced rat-liver microsomes for 5 h at 37°C. The control comprised an identical treatment without B[a]P but with microsomes.
[b]: WBC-DNA was pooled from three experiments. The total adduct levels are given in add/10^6n as determined by two independent postlabeling assays ± range of values. Adduct 1 comprised >95% of the total adduct level.
[c]: Not detectable.

First, the sensitivity of the rabbit antiserum, W2/01, was tested in a model system, in which human WBC were exposed to various concentrations of B[a]P (0 to 100 μM) in the presence of 3-MC-induced rat-liver microsomes. After the incubations, WBC were either used for isolation of DNA that was subjected to the ^{32}P-postlabeling procedure to assay adduct levels, or processed for analysis by IFM. As can be seen in Table 1, adduct levels in B[a]P-treated WBC could be readily measured, and ranged from 2.2 ± 0.1 after treatment with 10 μM B[a]P to 11.8 ± 2.0 adducts per 10^6 nucleotides (add/10^6n) after 100 μM B[a]P. The adduct pattern, depicted in Figure 1a, shows one major spot (adduct 1), which comigrated with a reference adduct, *viz.* the adduct between (+)-*anti*-B[a]P-7,8-dihydrodiol-9,10-epoxide and deoxyguanosine (BPDE-N^{2}dG).

With both the FITC- and the TRITC-labeled GaR-conjugate as a second antibody, an increase in nuclear fluorescence could be observed with increasing concentration of B[a]P (see Figure 2a,b). The nuclear fluorescence in WBC that had been treated with 10 μM B[a]P (resulting in 2.2 ± 0.1 add/10^6n according to postlabeling data) was significantly

higher than that in untreated WBC, with both GaR-FITC and GaR-TRITC (Student's t-test; $p=0.0001$ and $p=0.0003$, respectively). In WBC that had been treated with 100 μM B[a]P, the nuclear fluorescence was about four times as high as that in untreated WBC. The fluorescence data show a good correlation with the results of the adduct quantification by postlabeling (Figure 2c).

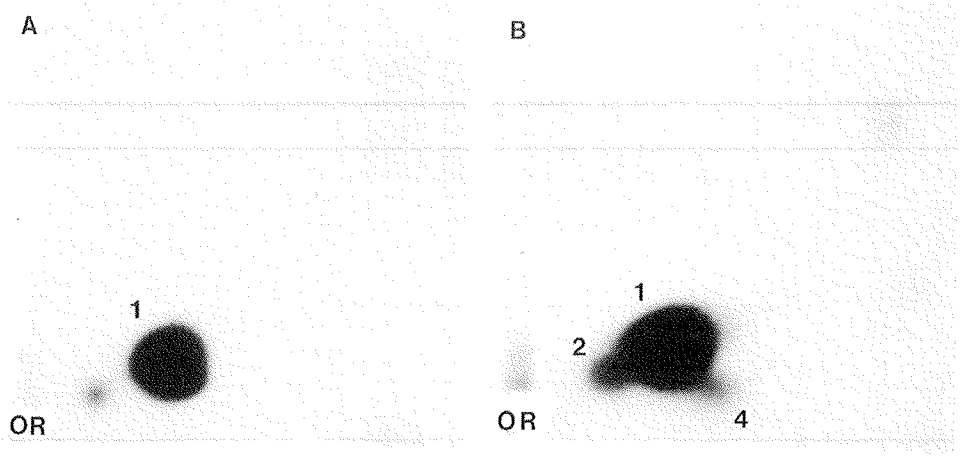

Figure 1 *Adduct patterns, obtained by autoradiography of ^{32}P-postlabeling TLC chromatograms, of DNA isolated from (A) WBC treated with B[a]P (100 μM) in the presence of 3-MC-induced rat-liver microsomes and (B) hamster tracheas exposed to B[a]P (20 μM) for 48h. Films (A) and (B) were exposed at -70°C with intensifying screens for 3 and 6 h, respectively. The D3 (bottom-top)-D4 (left-right) part of the chromatograms is shown. Adduct 1 comigrates with the adduct between (+)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide and deoxyguanosine. Adducts 2 and 4 are minor adducts. OR=origin.*

In the experiment with hamster trachea in organ culture, trachea rings were exposed to B[a]P (40 μM) for 48 h and kept in B[a]P-free culture medium for another 3 days. A typical adduct pattern after ^{32}P-postlabeling of DNA isolated from B[a]P-exposed hamster trachea epithelial cells is depicted in Figure 1b. As was already shown before (22), the major DNA adduct formed during this treatment also comigrates with BPDE-N^{2}dG. Moreover, a few minor adducts, marked 2 and 4 in Figure 1b, could be detected. The IFM analysis of the epithelial cells isolated from the exposed rings, involved immunocytochemical staining of the cytokeratin of the basal cells. This procedure required fixation of the cells in a medium from which acetic acid was omitted, because this interfered with the subsequent staining. Therefore methanol fixation was applied. As can be seen in Figure 3 the use of the RCK102 antibody resulted mainly in perinuclear FITC-staining. This FITC-image was used during interactive image-processing to distinguish basal cells from non-basal cells.

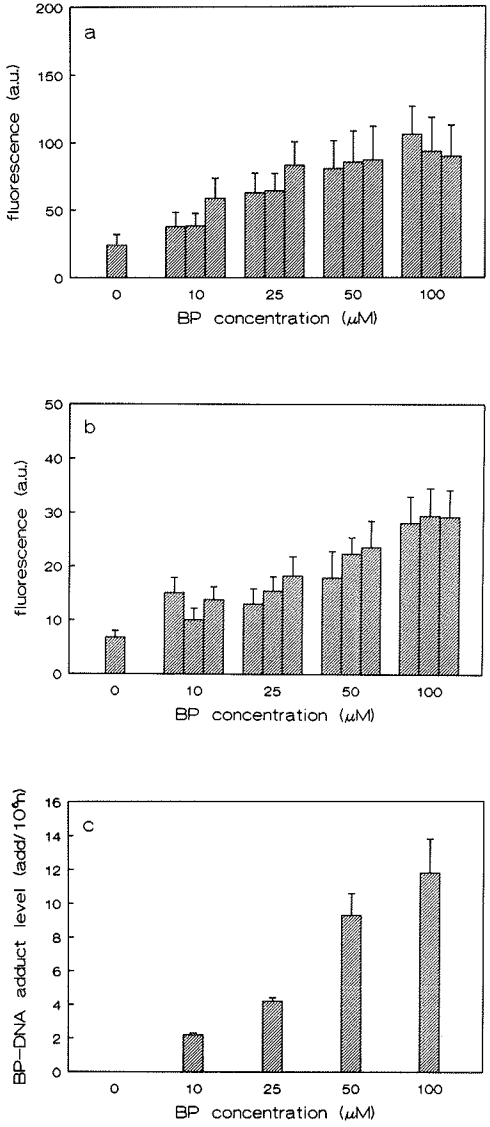

Figure 2 *B[a]P-DNA adduct formation in WBC treated with various concentrations of B[a]P in the presence of 3-MC-induced rat-liver microsomes. Panels a and b show the results after nuclear immunofluorescence detection. Rabbit antiserum W2/01 was used as a first antibody and GaR-FITC (a) or GaR-TRITC (b) as a second antibody. Each B[a]P-treatment was carried out in triplicate. Error bars represent SD (the number of cells varied from 30 to 50). a.u. = arbitrary units. Panel c shows the results after ^{32}P-postlabeling. Triplicate B[a]P-treated WBC-samples were pooled, whereafter DNA was isolated. Bars represent the mean of two postlabeling assays ± range of values. Add/10^{6}n = adducts per 10^6 nucleotides.*

In addition to this cell-type specific staining, a DAPI-staining was used to visualize the nuclear DNA and a TRITC-stain to detect the adducts (Figure 3). The DAPI-image was used to locate the nucleus containing the adduct-specific fluorescence (TRITC-image). After interactive image-processing the computer calculated the mean nuclear fluorescence per cell type (see Table 2). The data were also presented as histograms, in which the individual cells were grouped into classes of nuclear TRITC-fluorescence (Figure 4).

Table 2 *Mean nuclear fluorescence ± SD of basal and non-basal epithelial cells after exposure of tracheal rings to B[a]P (40 μM) for 48 h in organ culture, whereafter B[a]P was removed and tracheas were cultured for 3 more days.*

Group	All cells		Basal cells		Non-basal cells	
	n[a]	mean ± SD[b]	n	mean ± SD	n	mean ± SD
48h B[a]P	84	27.6 ± 9.2	28	28.1 ± 10.1	56	27.4 ± 8.9
24h wash	86	15.8 ± 7.1	28	13.7 ± 5.8[c]	58	17.2 ± 7.4
48h wash	89	14.3 ± 10.1	24	10.8 ± 6.3[d]	65	15.8 ± 10.7
72h wash	106	11.0 ± 5.4	26	9.9 ± 3.4	80	11.4 ± 5.8
control[e]	103	6.4 ± 1.2[f]	27	6.5 ± 1.2[f]	76	6.3 ± 1.2[f]

a. Number of cells of which the nuclear fluorescence was determined. During IFM, emphasis was put on selection of basal cells for recording. Therefore n does not represent the actual relative number of basal and non-basal cells.
b. Mean nuclear fluorescence in arbitrary units ± SD.
c. Significantly different (Student's t-test, p=0.0295) from mean of non-basal cells on the same day.
d. Significantly different (Student's t-test, p=0.0332) from mean of non-basal cells on the same day.
e. Control tracheal rings were incubated for 24 h in exposure medium without B[a]P.
f. Significantly different (Student's t-test, p<0.002) from all other experimental values.

There was no significant difference between basal and non-basal cells with respect to the amount of B[a]P-DNA adducts, after 48 h of exposure to B[a]P (Table 2). However, the subsequent disappearance of B[a]P-DNA adducts from basal cells is significantly faster than that from non-basal cells, as is clear from Table 2 and the histograms obtained on day 3 (24 h after removing B[a]P; Student's t-test, p=0.0295) and on day 4 (48 h after removing B[a]P; Student's t-test, p=0.0332). On day 5, 72 h after removing B[a]P, there no longer was a significant difference in B[a]P-DNA adduct level between the two cell types. However, still a significant difference could be observed with the nuclear fluorescence of the untreated epithelial cells, which indicates that B[a]P-DNA adducts are still present on day 5 in both basal and non-basal cells.

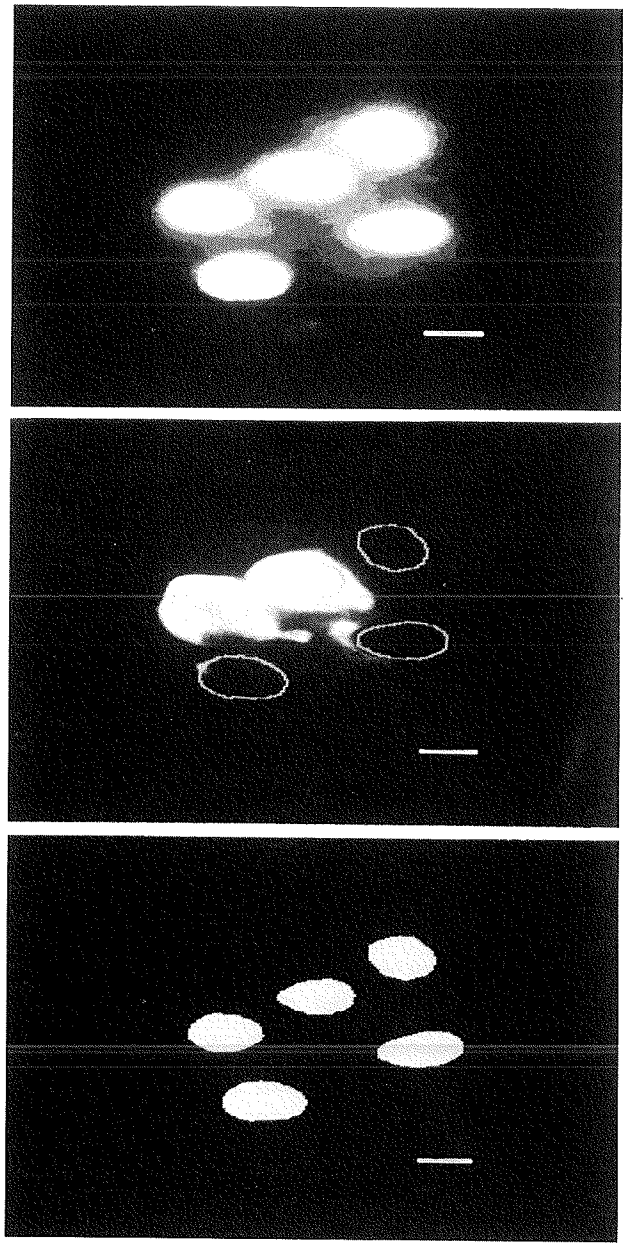

Figure 3 *Detection by IFM of B[a]P-DNA adducts in epithelial cells derived from B[a]P-exposed tracheal rings. The processed image in the upper panel shows the TRITC-image, which is indicative of the amount of B[a]P-DNA adducts. The middle panel shows the FITC-image, which presents RCK102 positive (=basal) cells, and the lower panel depicts the DAPI-image, which shows all the nuclei and is used as an image for the adduct calculations. Bar = 10 μm.*

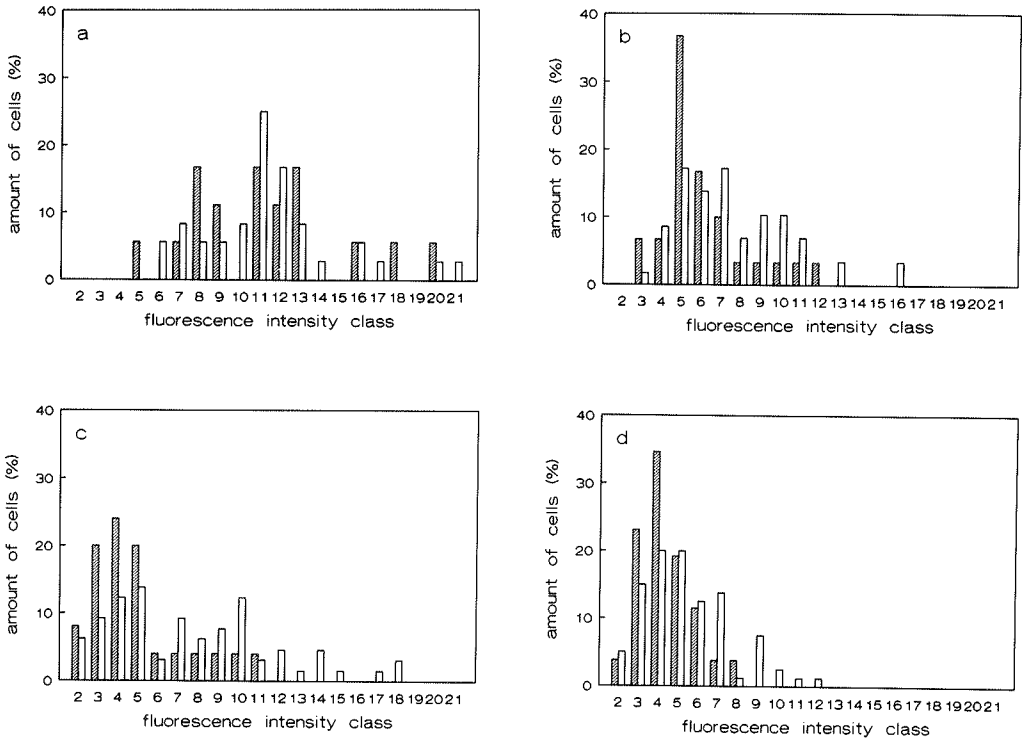

Figure 4 *Detection by IFM of B[a]P-DNA adducts in different cell types of B[a]P-exposed tracheal rings. Hamster trachea rings were exposed to B[a]P (40 μM) for 48 h (panel (a)), whereafter the rings were transferred to B[a]P-free medium and cultured for another 3 days. Panels (b), (c) and (d) represent the histograms obtained after 24, 48 and 72 h culturing in B[a]P-free medium. Histograms are shown of the relative number of cells present within a class of B[a]P-DNA adduct-specific nuclear fluorescence. Each fluorescence class represents 2.6 arbitrary units. Hatched bars refer to basal cells and open bars to non-basal cells.*

Discussion

A procedure was developed that allowed the quantification of B[a]P-DNA adducts in one specific cell type of the hamster tracheal epithelium, *viz.* the basal cell. The procedure involves the use of two antibodies: one adduct-specific, *i.e.*, rabbit antiserum W2/01 and one cytokeratin-specific, *i.e.*, mouse monoclonal antibody RCK102. Following incubation of trachea rings with B[a]P, cytospin preparations were made of epithelial cell isolates and each of the two antigens was stained by use of a fluorescent marker. Interactive image-processing made it possible to determine the level of B[a]P-DNA adducts in basal and non-basal cells of the tracheal epithelium.

In the studies on the detection of B[a]P-DNA adduct levels in WBC, the polyclonal rabbit antiserum W2/01 was shown to be well suited for application in IFM. The IFM data on adduct formation showed a good correlation with the respective postlabeling data. This was indeed expected because the major adduct formed, *i.e.*, BPDE-N^2dG was the adduct present in the B[a]P-modified DNA against which the polyclonal antiserum had been raised. The lower detection limit reached with the rabbit antiserum for B[a]P-DNA adducts appeared to be 1 add/10^6n. This value agrees with the results of our previous studies with the mouse monoclonal antibody II.E4, on human fibroblasts (14) and human WBC (16) that had been treated with the (±)-*anti*-B[a]P-diolepoxide. Van Schooten *et al* (17) reported a lower detection limit of 7 add/10^8n for the immunocytochemical adduct detection with their rabbit antibody, directed against B[a]P-DNA. Their quantification was based on peroxidase-anti-peroxidase/DAB staining, as described by Scherer *et al* (23). In general, the lower detection limit of an immunofluorescence detection method is based on a) the specificity and affinity of the antibody for the adduct, b) the extent of nonspecific binding of the FITC- or TRITC-conjugate and c) the autofluorescence of the cells. The use of preimmune serum, instead of W2/01, as a negative control during *in situ* assays resulted in a nuclear fluorescence that was comparable to that of the control cells that had been incubated with W2/01. Furthermore, there was a good correlation of staining with W2/01 and that with the established monoclonal antibody II.E4. Therefore, it is reasonable to assume that autofluorescence is the main factor that determines the lower detection limit in this type of assay. This autofluorescence does not interfere with the detection of B[a]P-DNA adducts when DAB precipitation is used. In our double-staining procedure, however, the use of colourigenic substrates, such as AEC or DAB, would be impractical: the AEC-derived stain is relatively unstable towards the reaction conditions during DNA denaturation, while the use of DAB results in an intense staining that obscures the nuclear fluorescence (unpublished observations). Consequently, the use of two fluorescent markers was found to be the most suitable.

The B[a]P-DNA adduct levels in hamster trachea epithelial cells were determined in cytospin preparations, rather than in frozen sections of the trachea. The use of the latter was avoided for two reasons. First, the morphology of frozen sections was found to deteriorate during processing the section for IFM, which made it hard to obtain good-quality images. Second, because the preparation of frozen sections implies that cells are cut and nuclei are damaged, an unknown amount of DNA will be lost during the subsequent processing for IFM, which may introduce a substantial uncertainty as to the proportion of adducts still detectable. Cytospin slides, on the other hand, are made with intact cells, which maintain a good morphology. Making these preparations involves

dissociation of the epithelial cells from the tracheal tissue by trypsinization; to avoid DNA repair the trypsin treatment was done at 4°C. In earlier control experiments to determine the relative contribution of non-epithelial cells in the cytospin preparations, cells were stained with monoclonal antibodies RCK102 and RGE53, which specifically recognize basal cells and mucous and ciliated cells, respectively (18). It appeared that approximately 10% of the cells were not stained and therefore not of trachea-epithelial origin. These contaminating cells may be fibroblasts, endothelial cells, granulocytes or lymphocytes.

With regard to the amount of B[a]P-DNA adducts formed, it was surprising that there was no difference between basal and non-basal cells. From the work of Plopper *et al* (24) it is known that the secretory type of cell in the respiratory epithelium (*i.e.*, the mucous cell in the hamster trachea) is the main site of cytochrome P450 activity, known to be required for B[a]P activation. Therefore, it was reasonable to expect the highest B[a]P-DNA adduct level in the non-basal cell fraction. However, at the administered dose of B[a]P (40 μM) this was not the case. This may be explained by intercellular transport of reactive B[a]P-metabolites. Ginsberg *et al* (25) demonstrated that B[a]P-diolepoxide is stable enough to be transported via the serum. Furthermore, the presence of conjugating enzymes should also be considered; relatively high GST-μ levels in non-basal cells may reduce the formation of B[a]P-DNA adducts by conjugating the active B[a]P-diolepoxide. The GST-μ isozyme is known for its high affinity towards epoxides (26).

To avoid the occurrence of mutations, it is of vital importance for a cell to remove DNA adducts before entering into the S-phase of the cell-cycle. The exact contribution of each cell type in the proliferation and differentiation of the tracheal epithelium is not known. The basal cell can give rise to all major cell types in the tracheal epithelium (27). However, non-basal cells, such as the secretory cell (28) and the ciliated cell (29) are also capable of dividing. In our study the disappearance of B[a]P-DNA adducts from the basal cells was significantly faster than that from the non-basal cells. With respect to the role of the basal cell in cell renewal in tracheal epithelium, it may be of interest for the epithelium as a tissue to give preference to DNA repair in basal cells. However, on day 5, 72 h after removing B[a]P, a considerable amount of adducts was still present also in basal cells. Most probably this is related to a reduction in repair capacity by that time, in accordance with results obtained in a previously published induction-repair experiment (30).

The method described in this paper offers the possibility to determine - at the cellular level - the amount of B[a]P-DNA adducts in a specific cell type. This is of great importance for studies on the relation between DNA damage and carcinogenesis, because in the process of carcinogenesis often specific cell types are involved. Then, the amount

of DNA damage in these specific cell types would be far more relevant than the adduct level in the overall tissue, which is determined in the more common procedures using DNA isolated from homogenized tissue. At the moment, studies are in progress in which hamster tracheas in organ culture are exposed to B[a]P continuously. The development of squamous metaplasia and hyperplasia (precursors of neoplasia) is studied in this model system, in relation to B[a]P-DNA adducts and cell proliferation in basal and non-basal cells.

Acknowledgements

Mr MM Schellekens is gratefully acknowledged for his work on the immunization of the rabbit. The valuable comments of Dr F Berends on this manuscript are appreciated. The help of Mr MJM Boermans in preparing the photographs is also acknowledged.

References

1. International Agency for Research on Cancer (1973) Monographs on the evaluation of carcinogenic risk of chemicals to man. Certain polycyclic aromatic hydrocarbons and heterocyclic compounds. Lyon, IARC.
2. Jankowiak R, Cooper RS, Zamzow D, Small GJ (1988) Fluorescence line narrowing-nonphotochemical hole burning spectrometry: femtomole detection and high selectivity for intact DNA-PAH adducts. *Chem Res Toxicol*, **1**, 60-68.
3. Park J-W, Cundy KC, Ames BN (1989) Detection of DNA adducts by high-performance liquid chromatography with electrochemical detection. *Carcinogenesis*, **10**, 827-832.
4. Vähäkangas K, Haugen A, Harris CC (1985) An applied synchronous fluorescence spectrophotometric assay to study benzo(a)pyrene-diolepoxide-DNA adducts. *Carcinogenesis*, **6**, 1109-116.
5. Weston A, Bowman ED (1991) Fluorescence detection of benzo(a)pyrene-DNA adducts in human lung. *Carcinogenesis*, **12**, 1445-1449.
6. Randerath K, Reddy MV, Gupta RC (1981) ^{32}P-Labeling test for DNA damage. *Proc Natl Acad Sci USA*, **78**, 6126-6129.
7. Gupta RC, Reddy MV, Randerath K (1982) ^{32}P-Postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis*, **3**, 1081-1092.
8. Gupta RC (1985) Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen-DNA adducts. *Cancer Res*, **5**, 5656-5662.
9. Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **9**, 1543-1551.
10. Baan RA, Zaalberg OB, Fichtinger-Schepman AMJ, Muysken-Schoen MA, Lansbergen MJ, Lohman PHM (1985) Use of monoclonal and polyclonal antibodies against DNA adducts for the detection of DNA lesions in isolated DNA and in single cells. *Env Health Persp*, **62**, 81-88.
11. Baan RA, Lohman PHM, Fichtinger-Schepman AMJ, Muysken-Schoen MA, Ploem JS (1986) Immunochemical approach to detection and quantitation of DNA adducts resulting from exposure to genotoxic agents. In Sorsa M, Norppa H, eds. Monitoring of occupational genotoxicants. New York: Alan R Liss, 135-146.
12. Santella RM, Gasparo F, Hsieh L-L (1987) Quantitation of carcinogen-DNA adducts with monoclonal antibodies. *Prog Exp Tumor Res*, **31**, 63-75.

13. Shamsuddin AKM, Gan R (1988) Immunocytochemical localization of benzo(a)pyrene-DNA adducts in human tissue. *Hum Pathol*, **19**, 309-315.
14. Baan RA, van den Berg PTM, Watson WP, Smith RJ (1988) *In situ* detection of DNA adducts formed in cultured cells by benzo(a)pyrene diolepoxide (BPDE), with monoclonal antibodies specific for the BP-deoxyguanosine adduct. *Toxicol Env Chem*, **16**, 325-339.
15. Baan RA, van den Berg PTM, Steenwinkel M-JST, van der Wulp CJM (1988) Detection of benzo(a)pyrene-DNA adducts in cultured cells treated with benzo(a)pyrene diol-epoxide by quantitative immunofluorescence microscopy and ^{32}P-postlabelling; immunofluorescence analysis of benzo(a)pyrene-DNA adducts in bronchial cells from smoking individuals. In Bartsch H, Hemminki K, O'Neill IK, eds. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Lyon, IARC, 146-154.
16. Baan RA, van den Berg PTM, van Delft JHM, Roza L, van der Meer EG, van der Wulp CJM (1990) *In Situ* detection of DNA damage in single cells or tissue sections by quantitative immunofluorescence microscopy. In Mendelsohn ML, Albertini RJ, eds. Mutation and the environment, part A: Basic mechanisms, progress in clinical and biological research, vol 340A, New York: Wiley-Liss, 101-112.
17. van Schooten FJ, Hillebrand MJX, Scherer E, den Engelse L, Kriek E (1991) Immunocytochemical visualization of DNA adducts in mouse tissues and human white blood cells following treatment with benzo(a)pyrene or its diol epoxide. A quantitative approach. *Carcinogenesis*, **12**, 427-433.
18. Rutten AAJJL, Bruyntjes JP, Ramaekers FCS (1988) Effect of vitamin A-deprivation and cigarette smoke condensate on keratin expression patterns in cultured hamster tracheal epithelium. An immunohistomorphological study using monoclonal antibodies to keratins. *Virch Archiv B Cell Pathol*, **56**, 111-117.
19. Roggeband R, Wolterbeek APM, Rutten AAJJL, Baan RA (1993) Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* upon activation of benzo[a]pyrene by human, rabbit, and rodent liver microsomes. *Carcinogenesis*, **14**, 1945-1950.
20. Steenwinkel M-JST, Roggeband R, van Delft JHM, Baan RA (1993) Improvements in the ^{32}P-postlabelling procedure to quantify bulky aromatic adducts, in: Postlabelling methods for detection of DNA adducts, Phillips DH, Castegnaro M, Bartsch H, Eds, IARC Scientific Publications No 124, Lyon, pp 65-70.
21. Lesko SA, Li W, Zheng G, Callahan D, Kaplan DS, Midden WR, Strickland PT (1989) Quantitative immunofluorescence assay for cyclobutyldithymidine dimers in individual mammalian cells. *Carcinogenesis*, **10**, 641-646.
22. Wolterbeek APM, Roggeband R, Steenwinkel M-JST, Baan RA, Rutten AAJJL (1993) Formation and repair of benzo(a)pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis. *Carcinogenesis*, **14**, 463-467.
23. Scherer E, van Benthem J, Terheggen PMAB, Vermeulen E, Winterterp HHK, den Engelse L (1988) Immunocytochemical analysis of DNA adducts in single cells: a new tool for experimental carcinogenesis, chemotherapy and molecular epidemiology. In Bartsch H, Hemminki K, O'Neill IK, eds. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Lyon, IARC, 286-291.
24. Plopper CG, Cranz DL, Kemp L, Serabjit-Singh CJ, Philpot RM (1987) Immunohistochemical demonstration of cytochrome P-450 monooxygenase in clara cells throughout the tracheobronchial airways of the rabbit. *Exp Lung Res*, **13**, 59-68.
25. Ginsberg GL, Atherholt TB (1989) Transport of DNA-adducting metabolites in mouse serum following benzo(a)pyrene administration. *Carcinogenesis*, **10**, 673-679.
26. Vos RME, van Bladeren PJ (1990) Glutathione-S-transferases in relation to their role in the biotransformation of xenobiotics. *Chem Biol Int*, **75**, 241-265.
27. Inayama Y, Hook GER, Brody AR, Cameron GS, Jetten AM, Gilmore LB, Gray T, Nettesheim P (1988) The differentiation potential of tracheal basal cells. *Lab Invest*, **58**, 706-717.
28. Nettesheim P, Jetten AM, Inayama Y, Brody AR, George MA, Gilmore LB, Gray T, Hook GER (1990) Pathways of differentiation of airway epithelial cells. *Env Health Persp*, **85**, 317-329.
29. Rutten AAJJL, Beems RB, Wilmer JWGM, Feron VJ (1988) Ciliated cells in vitamin A-deprived cultured hamster tracheal epithelium do divide. *In Vitro Cel Develop Biol*, **24**, 931-935.

30. Roggeband R, Wolterbeek APM, Melis OWM, Wittekoek ME, Rutten AAJJL, Feron VJ, Baan RA (1994) DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture. *Carcinogenesis*, **15**, 661-665.

Chapter

6 *Cell proliferation and DNA adducts in hamster tracheal epithelium exposed to benzo[a]pyrene in organ culture*

R Roggeband, APM Wolterbeek[1], PTM van den Berg, AAJJL Rutten[1], VJ Feron[1] and RA Baan

TNO Nutrition and Food Research Institute, Department of Genetic Toxicology, PO Box 5815, 2280 HV Rijswijk, and [1]Department of Biological Toxicology, PO Box 360, 3700 AJ Zeist, The Netherlands.

Carcinogenesis, submitted

Abstract

The effect of benzo[a]pyrene (B[a]P) on cell proliferation in cultured hamster tracheal epithelium was studied in relation to the formation of B[a]P-DNA adducts. To this end, tracheas were isolated from Syrian golden hamsters, cut into rings and cultured in Ham's F12 medium. Then, the tracheal rings were exposed to 2 or 20 μM B[a]P, either continuously for 7 days or for 2 days followed by a 5-day recovery period without B[a]P. At intervals rings were sampled for determination of both cell proliferation (by means of the labeling index), and the B[a]P-DNA adduct level using *in situ* detection via immunofluorescence microscopy. After 2 days of exposure to 2 or 20 μM B[a]P there was a significant increase in B[a]P-DNA adduct level. A further, linear increase in B[a]P-DNA adduct level, however, was only observed after continuous exposure to 20 μM B[a]P, whereas the adduct level in the 2 μM continuous exposure group remained virtually the same. In unexposed tracheal epithelium an initial peak of cell proliferation was observed. This initial proliferation was significantly lower in the exposed samples. Only continuous exposure to 20 μM B[a]P steadily decreased the labeling index from day 2 to 7. It is concluded that there is a correlation between the B[a]P-DNA adduct level and the reduction of cell proliferation in hamster tracheal epithelium exposed to B[a]P in Ham's F12 medium.

Presently, the role of cell proliferation in carcinogenesis is widely discussed in the literature (1,2). It is clear that clonal expansion by cell proliferation is a prerequisite for tumor development. Apart from the risk of cancer after exposure to DNA-damaging genotoxic agents, non-genotoxic compounds that induce cell proliferation also may increase the risk of tumor development, simply by increasing the likelihood that endogenous DNA damage results in carcinogenic mutations (3,4). Furthermore, in non-dividing cells in adults, such as cardiomyocytes and nerve cells, tumors are rarely observed (5). However, cell proliferation is certainly not the only important factor in carcinogenesis, as follows from the observation that tumors do not occur more frequently in tissues with a relatively high proliferative activity than in those where the proliferative activity is relatively low (6).

The formation of DNA adducts is generally considered to be a necessary initial step in the process by which many chemical carcinogens exert their biological effect (7). DNA adducts, when they are not repaired or erroneously repaired, give rise to mutations in DNA and thus may eventually lead to tumor development. On the other hand, induction of DNA damage in cells is known to retard DNA replication and depress cell proliferation.

In the present study we investigated the effect of incubation with two different concentrations of benzo[a]pyrene (B[a]P) on cell proliferation in hamster trachea in *in vitro* organ culture, in relation to the presence of DNA adducts. The original *in vivo* hamster trachea model (the Saffiotti-model), in which hamsters are intratracheally exposed to B[a]P, proved to be a useful model for respiratory tract carcinogenesis (8). The morphological characteristics of the trachea that precede the formation of tumors in the Saffiotti-model can also be induced in *in vitro* organ culture of the trachea. The genotoxic compound B[a]P, a known respiratory tract carcinogen in rodents (9), has been found to induce cell proliferation *in vivo*, which is considered to be of regenerative origin, as it is often preceded by cell death (10). A similar effect of B[a]P was observed in the *in vitro* system, both in explants of hamster trachea and in organ culture (11,12). However, whether a correlation may exist between the extent of DNA-adduct formation and cell proliferation has not yet been investigated. To the best of our knowledge, this is the first study on the relationship between cell proliferation and DNA adducts in the hamster tracheal epithelium in organ culture.

Tracheas were isolated from ten-week old male Syrian golden hamsters. Tracheal rings of 2 to 3 mm thickness were cultured as described before (13) in serum-free, hormone-supplemented Ham's F12 medium containing 2 mM L-glutamine, 1 μM hydrocortisone, 5 μg/ml bovine pancreatic insulin, 5 μg/ml human transferrin, 25 ng/ml epidermal growth factor, and 50 μg/ml gentamycin. In two independent experiments

tracheal rings were exposed to B[a]P (Sigma Chemicals, St. Louis, MO). In the first, the B[a]P concentration was 2 μM; exposure was either continuously for 7 days or for 2 days followed by a 5-day recovery period. In the second experiment the B[a]P concentration was 20 μM while the exposure regimen was the same as described above. In both experiments tracheal rings exposed to medium containing only DMSO (0.1% v/v) were used as controls. A schematic representation of the experimental design is depicted in Figure 1.

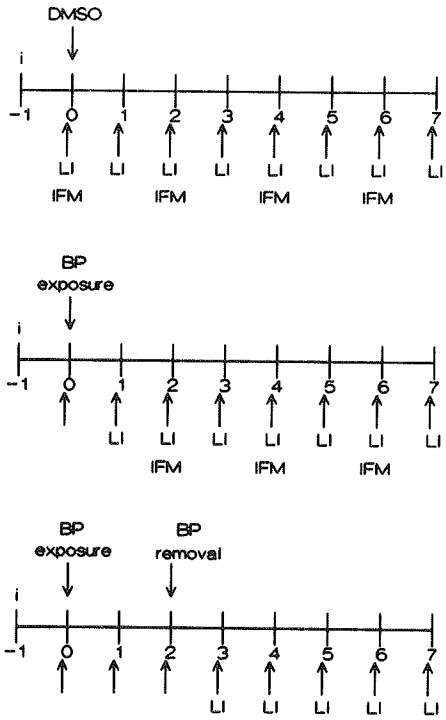

Figure 1 *Experimental design. In two independent experiments, hamster tracheal rings were exposed to B[a]P (2 or 20 μM) in culture medium either for 2 days followed by incubation in B[a]P-free medium, or continuously. Each experiment included an identically treated control, but for the presence of B[a]P. The medium with or without B[a]P was renewed each day. At day −1 the tracheas were isolated, at day 0 the incubation of the tracheal rings started, a number of which were sampled at each of the subsequent 7 days. After sampling, tracheal sections were prepared for cell proliferation by autoradiography (LI), or trachea epithelial cells were isolated and processed for DNA-adduct detection by immunofluorescence microscopy (IFM).*

At the time points indicated in Figure 1, tracheal rings were processed for the quantification of B[a]P-DNA adducts and cell proliferation. The level of B[a]P-DNA

adducts in hamster tracheal cells was determined in cytospin preparations, by means of immunofluorescence microscopy (IFM). Epithelial cells were separated from the cartilage by incubation with trypsin. Next, cytospin preparations were prepared of total cell isolates (basal and non-basal cells). DNA-adduct levels were determined using the B[a]P-adduct-specific rabbit antiserum W2/01 and fluorescein isothiocyanate-labeled goat-anti-rabbit immunoglobulin. Cell proliferation was determined in both basal and non-basal cells on the basis of radiolabeled thymidine incorporation (18 h labeling) as described by Wolterbeek *et al* (14). The labeling index (LI), defined as the percentage of labeled cells amongst the total number of cells, was determined in autoradiographs of semi-thin sections of the trachea. The criteria for the discriminative recognition of basal and non-basal cells were adapted from Rutten *et al* (12).

By use of the ^{32}P-postlabeling procedure it had been shown previously that in DNA obtained from hamster tracheal epithelial cells exposed to B[a]P in organ culture, mainly (>95%) one type of adduct is formed, namely the adduct between (+)-*anti*-B[a]P-diolepoxide and the exocyclic amino group of deoxyguanosine (BPDE-N^{2}dG; ref 14). The rabbit antiserum W2/01 which had been raised against the BPDE-N^{2}dG adduct in DNA, appeared suitable for *in situ* adduct detection in cytospin preparations of hamster trachea epithelial cells. Figure 2 shows the level of the nuclear fluorescence, which is indicative of the amount of B[a]P-DNA adducts, as determined by IFM with W2/01.

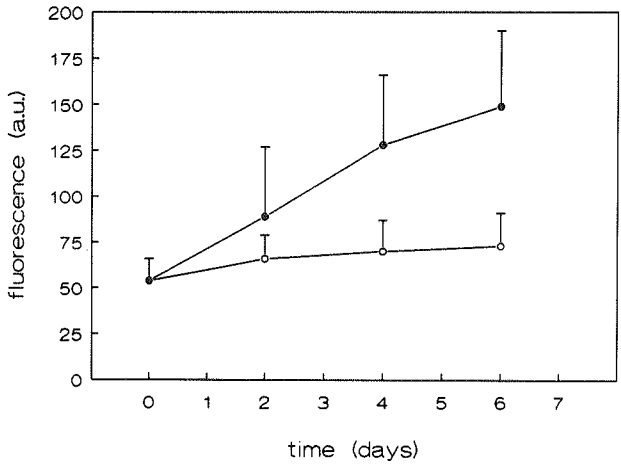

Figure 2 *B[a]P-DNA adduct level in hamster trachea epithelial cells after continuous exposure to B[a]P, as determined by IFM. The closed symbols refer to 20 μM B[a]P, the open symbols to 2 μM B[a]P. Error bars represent SD of the fluorescence distribution among 50 to 80 cells.*

A slight, although statistically significant (Student's t-test; $p<0.05$) increase in adduct level from day 0 (unexposed control) to day 2 could be observed in hamster trachea epithelial cells exposed continuously to 2 μM B[a]P. However, continued exposure to this B[a]P concentration did not result in a further increase in adduct level on days 4 and 6. This could not be due to the fact that B[a]P had been consumed during the incubation, as the culture medium, containing freshly dissolved B[a]P, was replaced each day. In contrast, continuous exposure to 20 μM B[a]P caused a significant increase of the adduct level during the entire culture period (Student's t-test; $p<0.01$). The nuclear fluorescence observed in unexposed trachea epithelial cells (day 0) is due to autofluorescence of the cells and/or aspecific binding of the antibodies to DNA.

Figures 3 a-c and d-f show the labeling indices of the cells exposed to 2 and 20 μM B[a]P, respectively. Data are given separately for basal and non-basal cells. In the first experiment (Figure 3 a-c), the overall LI in the unexposed tracheas increased from about 10% on day 0 to about 20% on day 1, while in the second (Figure 3 d-f) it rose from about 6% on day 0 to about 25% on day 1. In both experiments, the LI in non-basal cells was 3 to 4-fold as high as that in basal cells. The relatively high peak of initial cell proliferation in unexposed tracheas was followed by a gradual decrease in LI to about 5% towards the end of the culture period. Figure 3b and c show that on day 1 the LI remained significantly (Student's t-test; $p<0.02$) lower in the presence of 2 μM B[a]P than the LI on the same day in unexposed tracheas (Figure 3a). This lower level is mainly due to the absence of an increase in proliferation of the non-basal cells. In the tracheas exposed to 20 μM B[a]P (Figure 3e and f) there also was a significantly (Student's t-test; $p<0.02$) lower value of LI, as compared to that in the unexposed control (Figure 3d). The absolute difference in LI of exposed tracheas on day 1 with the corresponding controls on that day was approximately the same for both B[a]P concentrations, which indicates that the same number of cells were prevented from entering the S-phase in both cases. In the 2 μM B[a]P experiment the LI recovered, predominantly in the basal cells, already on day 2, still in the presence of B[a]P (Figure 3b and c). In the 20 μM B[a]P experiment, however, the LI only recovered - mainly in the non-basal cells - after the transfer of the tracheal rings to a B[a]P-free medium (Figure 3e). When tracheal rings were exposed to 20 μM B[a]P continuously, the LI rapidly decreased to a level below 5% and remained low during the entire culture period (Figure 3f). Interestingly, a similar decrease in LI did not occur in tracheas exposed to 2 μM B[a]P continuously (Figure 3c).

The interference of B[a]P with cell proliferation in hamster tracheal epithelium may be related to the presence of B[a]P itself, the formation of B[a]P-metabolites and/or the formation of B[a]P-DNA adducts. In concanavalin-A-stimulated T-cells from human peripheral blood, B[a]P inhibited DNA synthesis and cell proliferation at exposure

concentrations as low as 0.01 μM. This inhibition was dependent on the presence of cytochrome P450 activity (15), which suggests a role for either B[a]P-metabolites and/or B[a]P-DNA adducts in these T-cells. In a study in which mice were given weekly topical applications of B[a]P, the formation of DNA adducts was compared to cell cycle parameters and tumor initiation. It was found that B[a]P exposure resulted in an increase in epidermal thickness (regenerative cell proliferation), while the DNA synthesis was significantly depressed (16). When the LI's during continuous exposure to 20 μM B[a]P measured in our study are compared with the corresponding B[a]P-DNA adduct levels, it is clear that the relatively high adduct levels at later time points are associated with unremitting low values of LI. In contrast, during continuous exposure to 2 μM B[a]P, adduct levels remain relatively low, allowing fluctuations in LI around levels comparable to those of the control cells during the entire period of culture. It is plausible therefore to assume that the observed decrease in LI is the direct effect of the presence of a certain level of B[a]P-DNA adducts. Bulky adducts, such as those of B[a]P, form an obstacle for DNA polymerase (17), and may thus prolong the S-phase of the cell cycle.

Recently, evidence has accumulated that tumor-suppressor genes also are involved in the regulation of the cell cycle (18,19). In that respect, the proposed role of the tumor-suppressor gene p53 as a guardian of the genome is interesting. A p53-mediated block in the G_1-phase of the cell cycle may prevent cells that contain too heavily damaged DNA from entering the S-phase. The function of this block in G_1 may be that it allows the cell more time to repair DNA lesions before it proceeds into the S-phase (20). When this block is not long enough to have a substantial amount of lesions repaired, p53 eventually may induce apoptosis, *i.e.*, programmed cell death. Thus, the potentially carcinogenic effects to be feared when adducts are induced in cellular DNA, appear to be counteracted by a cell-proliferation-repressing action of p53 that is evoked by the presence of these adducts. A possible role of the tumor-suppressor gene p53 in the effects of B[a]P in our *in vitro* model seems well conceivable.

Our results are in contrast, however, with data in the literature. *In vivo* intratracheal exposure of hamsters to B[a]P significantly increased cell proliferation, probably as a result of initial cell death followed by regenerative hyperplasia and growth-promoting activities of the complete carcinogen. In *in vitro* cultures of the hamster trachea, cell proliferation also increased after exposure to B[a]P (11) or to cigarette-smoke condensate (12). A major difference between the latter studies and the present work is the culture medium used. In CMRL medium, which was used in the two studies mentioned above, cell proliferation in unexposed tracheal epithelium is relatively low; exposure to B[a]P increases cell proliferation.

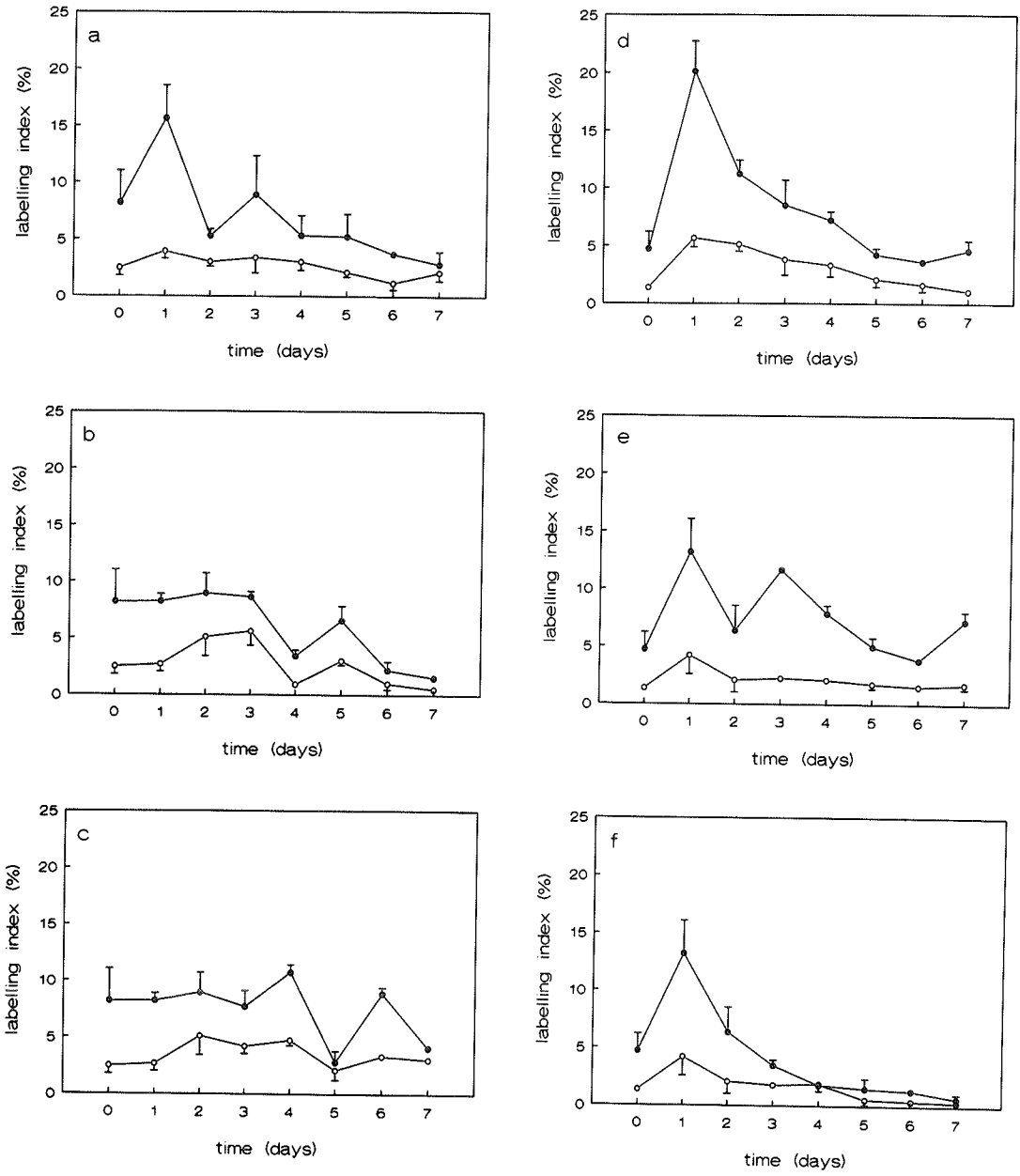

Figure 3 *Labeling index determined in sections of hamster tracheas after exposure to B[a]P. In the first experiment (panels a, b and c) tracheal rings were exposed to (a) control medium, (b) medium with 2 μM B[a]P for 2 days or (c) continuously. In the second experiment (panels d, e and f) tracheal rings were exposed to (d) control medium, (e) medium with 20 μM B[a]P for 2 days or (f) continuously. The closed symbols refer to non-basal cells and the open symbols to basal cells. Error bars represent SD of the LI's determined in 6 to 20 tracheal rings.*

In Ham's F12 medium, used in our study, cell proliferation in unexposed tracheal epithelium is relatively high and exposure to B[a]P decreases cell proliferation. To study this interesting aspect in more detail, experiments are now in progress to determine the relative importance of some constituents of the culture medium as to the effect of B[a]P on cell proliferation in hamster tracheal epithelium, together with the possible involvement of the tumor-suppressor gene p53 in this respect.

References

1. Weinstein IB (1992) Toxicity, cell proliferation, and carcinogenesis. *Mol Carcinogenesis*, **5**, 2-3.
2. Cohen SM, Ellwein LB (1991) Genetic errors, cell proliferation, and carcinogenesis. *Cancer Res*, **51**, 6493-6505.
3. Ames BN, Gold LS (1990) Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science*, **249**, 970-971.
4. Ames BN, Gold LS (1991) Endogenous mutagens and the causes of aging and cancer. *Mutat Res*, **250**, 3-16.
5. Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE (1990) Increased cell division as a cause of human cancer. *Cancer Res*, **50**, 7415-7421.
6. Iversen OH (1992) Is a high rate of cell proliferation carcinogenic in itself? *Human & Exp Toxicol*, **11**, 437-441.
7. Phillips DH (1990) Modern methods of DNA adduct determination. In: Chemical carcinogenesis and mutagenesis, Cooper CS, Grover PL, Eds, Springer Verlag, Berlin, pp 503-546.
8. Saffiotti U, Cetis F, Kolb L (1968) A method for the experimental induction of bronchogenic carcinoma. *Cancer Res*, **28**, 104-124.
9. IARC (1973) Monographs on the evaluation of carcinogenic risk of chemicals to man. Certain polycyclic aromatic hydrocarbons and heterocyclic compounds. Lyon, IARC.
10. McDowell EM, Trump BF (1983) Histogenesis of preneoplastic and neoplastic lesions in tracheobronchial epithelium. *Surv Synth Pathol Res*, **2**, 235-279.
11. Chopra DP, Cooney RA (1985) Histogenesis of benzo(a)pyrene-induced lesions in tracheal explants. *Virch Arch B Cell Pathol*, **48**, 299-315.
12. Rutten AAJJL, Wilmer JWGM, Beems RB (1988) Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture I. A cell proliferation study. *Virch Arch B Cell Pathol*, **55**, 167-175.
13. Roggeband R, Wolterbeek APM, Melis OWM, Wittekoek ME, Rutten AAJJL, Feron VJ, Baan RA (1994) DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture. *Carcinogenesis*, **15**, 661-665.
14. Wolterbeek APM, Roggeband R, Steenwinkel M-JST, Baan RA, Rutten AAJJL (1993) Formation and repair of benzo(a)pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis. *Carcinogenesis*, **14**, 463-467.
15. Mudzinski SP (1993) Effects of benzo(a)pyrene on concanavalin A-stimulated human peripheral blood mononuclear cells in vitro: inhibition of proliferation but no effect on parameters related to the G_1 phase of the cell cycle. *Toxicol Appl Pharmacol*, **119**, 166-174.
16. Albert RE, Miller ML, Cody T, Andringa A, Shukla R, Baxter CS (1991) Benzo[a]pyrene-induced skin damage and tumor promotion in the mouse. *Carcinogenesis*, **12**, 1273-1280.
17. Reardon DB, Bigger CAH, Dipple A (1990) DNA polymerase action on bulky deoxyguanosine and deoxyadenosine adducts. *Carcinogenesis*, **11**, 165-168.
18. Cobrinik D, Dowdy SF, Hinds PW, Mittnacht S, Weinberg RA (1992) The retinoblastoma protein and the regulation of cell cycling. *TIBS*, **17**, 312-315.
19. Yonish-Rouach E, Grunwald D, Wilder S, Kimchi A, May E, Lawrence J-J, May P, Oren M (1993) p53-mediated cell death: relationship to cell cycle control. *Mol and Cell Biol*, **13**, 1415-1423.
20. Lane DP (1992) p53, guardian of the genome. *Nature*, **358**, 15-16.

Chapter

7 DNA adducts and proliferation of basal and non-basal cells in relation to the development of precancerous lesions in the hamster trachea exposed to benzo[a]pyrene in organ culture

R Roggeband, APM Wolterbeek[1], PTM van den Berg, AAJJL Rutten[1], VJ Feron[1], F Berends and RA Baan

TNO Nutrition and Food Research Institute, Department of Genetic Toxicology, PO Box 5815, 2280 HV Rijswijk and [1]Department of Biological Toxicology, PO Box 360, 3700 AJ, Zeist, The Netherlands

Cancer Research, submitted

Abstract

Induction of tumors in the trachea of Syrian golden hamsters has been proven to be a valid model to study characteristics of tumor development in human lung. Morphological features that precede tumor formation in hamster trachea *in vivo*, such as squamous metaplasia and nodular hyperplasia, also can be induced during *in vitro* organ culture. In tracheal epithelium exposed to benzo[a]pyrene (B[a]P) in Ham's F12 medium we studied the relationship between DNA-adduct formation and proliferation of basal and non-basal cells, along with the development of precancerous lesions. Tracheal rings were exposed *in vitro* to 4 or 20 μM B[a]P for 7 days, or to 20 μM B[a]P for 2 days followed by a 5-day recovery period. Only in case of continuous exposure to 20 μM B[a]P there was a sustained increase in B[a]P-DNA adduct level; no significant differences were found in adduct formation between basal and non-basal tracheal epithelial cells. Effects on cell proliferation were predominantly observed in the non-basal cells. Continuous exposure to either 4 or 20 μM B[a]P reduced cell proliferation. In tracheal rings exposed to 20 μM B[a]P for only 2 days, cell proliferation rapidly recovered in B[a]P-free medium. Only in this group the extent of nodular hyperplasia clearly increased during the recovery period. Squamous metaplasia was seen to precede nodular hyperplasia, and its severity was more marked during or after B[a]P exposure as compared to the control. The present findings lead to the hypothesis that the observed precancerous lesions in tracheal epithelium cultured in Ham's F12 medium mainly originate from non-basal cells.

Lung cancer is the leading cause of cancer death in western countries (1-3). With respect to the etiology of lung cancer, epidemiological data revealed a link between the incidence of lung cancer and active smoking. About 85% of all lung cancer deaths in the US are associated with tobacco smoking (4,5). Tobacco smoke contains, amongst other compounds, polycyclic aromatic hydrocarbons (PAHs), some of which have been proven to be carcinogenic in rodents (6). Apart from the presence in tobacco smoke, PAHs are ubiquitous in our environment as pollutants of air, soil, and water (7).

A critical role for the genetic material with respect to chemical carcinogenesis was first put forward by the Millers (8,9). It is now generally accepted that the induction of DNA modifications in specific genes involved in the regulation of cellular growth is a necessary step in the process by which genotoxic carcinogens initiate carcinogenesis (10). In DNA isolated from lung tumors of smokers, the predominant mutation found in the activated K-*ras* oncogene is a G:C to T:A transversion (11). PAHs are known to preferentially react with guanine in DNA. Therefore, it is most likely that these compounds play an important role in the development of lung tumors.

In 1968 an *in vivo* hamster trachea model for respiratory tract carcinogenesis was introduced, which appeared useful to study the main characteristics of the development of human lung cancer (12). By repeated intratracheal instillations of benzo[a]pyrene (B[a]P), a well-studied carcinogen within the group of PAHs and a component of tobacco smoke, neoplastic lesions were induced in the hamster trachea, the morphology of which closely resembled that of neoplasms in human lung (13-16). The morphological characteristics that precede the formation of neoplasms *in vivo*, *i.e.* squamous metaplasia and hyperplasia, could also be observed during *in vitro* organ culture of the hamster trachea after exposure to cigarette-smoke condensate (17).

The aim of the work presented here was to study the relationship between DNA-adduct formation and cell proliferation of basal and non-basal cells in hamster tracheal epithelium on the one hand, and the development of precancerous lesions on the other hand, following exposure of the trachea to B[a]P during organ culture. Tracheal rings were continuously exposed to two different concentrations of B[a]P. The formation of B[a]P-DNA adducts in both basal and non-basal epithelial cells was determined in cytospin preparations of total cell isolates by use of immunofluorescence microscopy (IFM). To determine the effect of a temporary exposure to B[a]P on the extent of cell proliferation and the development of precancerous lesions, tracheal rings were exposed to B[a]P for 2 days, followed by a 5-day recovery period, a protocol that is similar to the one used in our previous studies, in which B[a]P-DNA adduct levels had been determined

by means of ^{32}P-postlabeling. In parallel experiments, semi-thin sections of tracheal rings were used to determine the proliferation of basal and non-basal cells by means of incorporation of radiolabeled thymidine. These sections were also examined for the presence of squamous metaplasia and nodular hyperplasia.

Materials and methods

Culturing of tracheal rings

Ten-week old male Syrian golden hamsters, obtained from Harlan/CPB, Zeist, The Netherlands, were sacrificed by an *i.p.* overdose of Nembutal (Ceva, Paris, France). Tracheas were isolated aseptically, cut into rings of 2 to 3 mm thickness, and placed in serum-free, hormone-supplemented Ham's F12 medium containing 2 mM L-glutamine (Flow Laboratories, Herts, UK), 1 μM hydrocortisone (Sigma Chemicals, St Louis, MO), 5 μg/ml bovine pancreatic insulin (Sigma), 5 μg/ml human transferrin (Sigma), 25 ng/ml epidermal growth factor (Sigma), and 50 μg/ml gentamycin (Flow). Tracheal rings were cultured in 24-well plates (Costar, Cambridge, MA) in a humidified atmosphere containing 40% oxygen, 55% nitrogen and 5% carbon dioxide at 37°C. Further details with regard to the culture conditions have been published before (18,19).

Exposure to B[a]P

B[a]P (Sigma) was dissolved in DMSO (Merck, Darmstadt, Germany); the final concentration of DMSO in culture medium was 0.1% (v/v). As depicted in Figure 1, tracheal rings were exposed to B[a]P according to 3 different protocols. After a 1-day incubation in medium only, rings were either exposed continuously to 4 or 20 μM B[a]P for 7 days, or to 20 μM B[a]P for 2 days followed by a 5-day recovery period. Control tracheal rings were cultured in medium containing DMSO only (0.1% v/v). The medium, with or without B[a]P, was refreshed daily. At various time points, indicated in Figure 1, rings were processed for the determination of the labeling index (LI) as a measure of cell proliferation, for assessment of the precancerous effects squamous metaplasia and nodular hyperplasia (S/H), and for the quantification of B[a]P-DNA adducts by IFM.

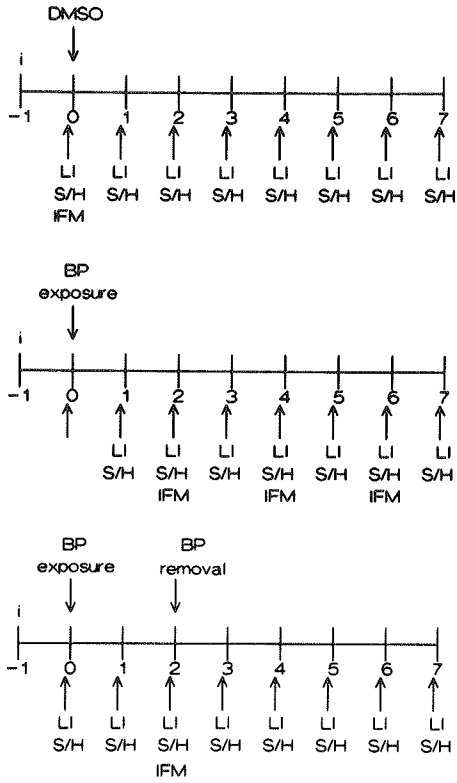

Figure 1 *Experimental design. Tracheal rings were exposed to B[a]P (4 or 20 μM) continuously (middle panel) or to 20 μM B[a]P for 2 days followed by incubation in B[a]P-free medium (lower panel). Control tracheal rings were cultured in medium containing DMSO (0.1% v/v) (upper panel). Medium with or without B[a]P was refreshed daily. At day −1 tracheas were isolated, at day 0 the incubation of the tracheal rings started, a number of which were sampled on each of the subsequent 7 days. After sampling, tracheal sections were prepared for analysis of cell proliferation by autoradiography (LI) and for the assessment of histological changes (S/H), or tracheal epithelial cells were isolated and processed for DNA-adduct determination by immunofluorescence microscopy (IFM).*

Quantification of B[a]P-DNA adducts

The level of B[a]P-DNA adducts in hamster tracheal epithelial (HTE) cells was determined in cytospin preparations by means of IFM. At each time point indicated in Figure 1, HTE cells were separated from the cartilage by incubation with trypsin. Next, cytospin preparations were prepared of total cell isolates. A recently developed triple-staining procedure (20), which involves concurrent staining of B[a]P-DNA adducts, a basal-cell specific cytokeratin and DNA, was used to determine the B[a]P-DNA adduct

level in basal cells and non-basal cells within the same preparation. The rabbit antiserum W2/01 raised against the (+)-*anti*-B[a]P-7,8-diol-9,10-epoxide-dG adduct in DNA, was used in combination with goat-*anti*-rabbit TRITC conjugate to detect this adduct in methanol-fixed HTE cells. To distinguish between basal and non-basal cells, HTE cells were also stained with RCK102 (Organon Teknika, Eindhoven, The Netherlands), a murine monoclonal antibody that recognizes a basal-cell specific cytokeratin. Binding of the monoclonal antibody was visualized with goat-*anti*-mouse FITC conjugate. DNA was localized by counterstaining with DAPI. All images were recorded with the use of a fluorescence microscope (Leitz, Wetzlar, Germany) and a liquid-nitrogen-cooled CCD camera (Astromed, Cambridge, England). Recorded images were processed with in-house developed image-processing software, based on SCIL-Image (CBP, Delft, The Netherlands). The details of this procedure will be published elsewhere (20).

Determination of cell proliferation and histological changes

Cell proliferation was measured by counting the number of labeled basal cells (in contact with the basal lamina and with no part of the cell wall reaching the tracheal lumen) and non-basal cells (all other cells), in autoradiographs of semi-thin (2 μm), toluidine blue-stained sections of the tracheal rings embedded in plastic after incorporation of radiolabeled thymidine (spec.act. 1.81 TBq/mmol). Labeling occurred during the 18-h period before each sampling time point (18,19). The labeling index (LI) was defined as the percentage of labeled cells amongst the total number of cells.

The extent of squamous metaplasia and nodular hyperplasia were assessed by histological examination of the same sections that were used for the determination of cell proliferation. Squamous metaplasia was morphologically defined as loss of normal columnar cells with progressive cell enlargement, flattening and then heaping up as in stratified squamous epithelium. Nodular hyperplasia was defined as clearly distinguishable nodules of more than 30 epithelial cells. The number of tracheal rings in which both cell proliferation and morphology were studied varied between 6 and 12 per time point.

Results

The development of precancerous lesions in hamster tracheal epithelium during organ culture as a consequence of exposure to B[a]P was studied in relation to the induction of B[a]P-DNA adducts and the effects on proliferation of basal and non-basal cells. To this end tracheal rings were exposed to 4 or 20 μM B[a]P continuously for 7 days. The level of DNA adducts was determined in intact cells in cytospin preparations

by use of an adduct-specific antiserum applied in IFM. Cell proliferation and the development of precancerous lesions were studied in sections of tracheal rings exposed simultaneously with those used for adduct analysis, and also in a series of samples that were allowed to recover for 5 days in B[a]P-free medium following a 2-day exposure to 20 μM B[a]P (Figure 1).

Table 1 *Mean nuclear fluorescence ± SD of basal and non-basal epithelial cells after continuous exposure of tracheal rings to B[a]P in organ culture.*

	Basal cells		Non-basal cells		All cells	
Group	n[a]	mean ± SD[b]	n	mean ± SD	n	mean ± SD
B[a]P 4 μM						
day 2	30	13.3 ± 4.3	78	14.5 ± 5.5	108	14.1 ± 5.3[f]
day 4	30	15.7 ± 6.0	49	17.4 ± 7.0	79	16.6 ± 6.7[cf]
day 6	38	13.7 ± 3.4	96	14.7 ± 4.3	134	14.4 ± 4.1[f]
B[a]P 20 μM						
day 2	31	15.1 ± 5.2	67	17.3 ± 7.8	98	16.5 ± 7.1[f]
day 4	30	21.1 ± 6.7	119	21.6 ± 7.4	149	21.5 ± 7.2[df]
day 6	35	25.4 ± 6.6	128	27.9 ± 7.7	163	27.1 ± 7.6[df]
control[e]	37	7.2 ± 1.9	85	7.3 ± 1.4	122	7.2 ± 1.6

a. Number of cells of which the nuclear fluorescence was determined. During IFM, emphasis was put on selection of basal cells for recording. Therefore n does not represent the actual relative number of basal and non-basal cells.
b. Mean nuclear fluorescence in arbitrary units ± SD.
c. Significantly different (Student's t-test, $p<0.005$) from mean of all cells on day 2.
d. Significantly different (Student's t-test, $p<0.001$) from mean of all cells on day 2.
e. Control tracheal rings were incubated for 24 h in exposure medium without B[a]P.
f. Significantly different (Student's t-test; $p<0.0001$) from mean of control cells.

Table 1 shows the level of nuclear FITC fluorescence, indicative of the level of B[a]P-DNA adducts, at the various time points in basal and non-basal cells, as well as in all HTE cells together. With respect to the formation of B[a]P-DNA adducts, no significant differences were found between basal and non-basal cells for both B[a]P concentrations and at all time points (Table 1). After 2 days of exposure to either 4 or 20 μM B[a]P there was a significant increase in B[a]P-DNA adduct level; the level of nuclear fluorescence in the exposed HTE cells was about twice that in unexposed cells. During continuous exposure to 4 μM B[a]P, the adduct level showed a small but significant further increase (Student's t-test; $p<0.005$) on day 4, but on day 6 it had returned to the value of day 2. In contrast, in the HTE cells continuously exposed to 20

μM B[a]P there was a sustained, linear increase in the adduct level from day 2 to days 4 and 6. After 6 days the mean nuclear fluorescence was 4 times as high in B[a]P-exposed as that in unexposed control nuclei. In previously published experiments, in which hamster tracheas were exposed to B[a]P for 2 days followed by a recovery period, DNA adducts had been determined by use of ^{32}P-postlabeling. Upon transfer to B[a]P-free medium the adduct level dropped to approximately 60% of the maximum value (19).

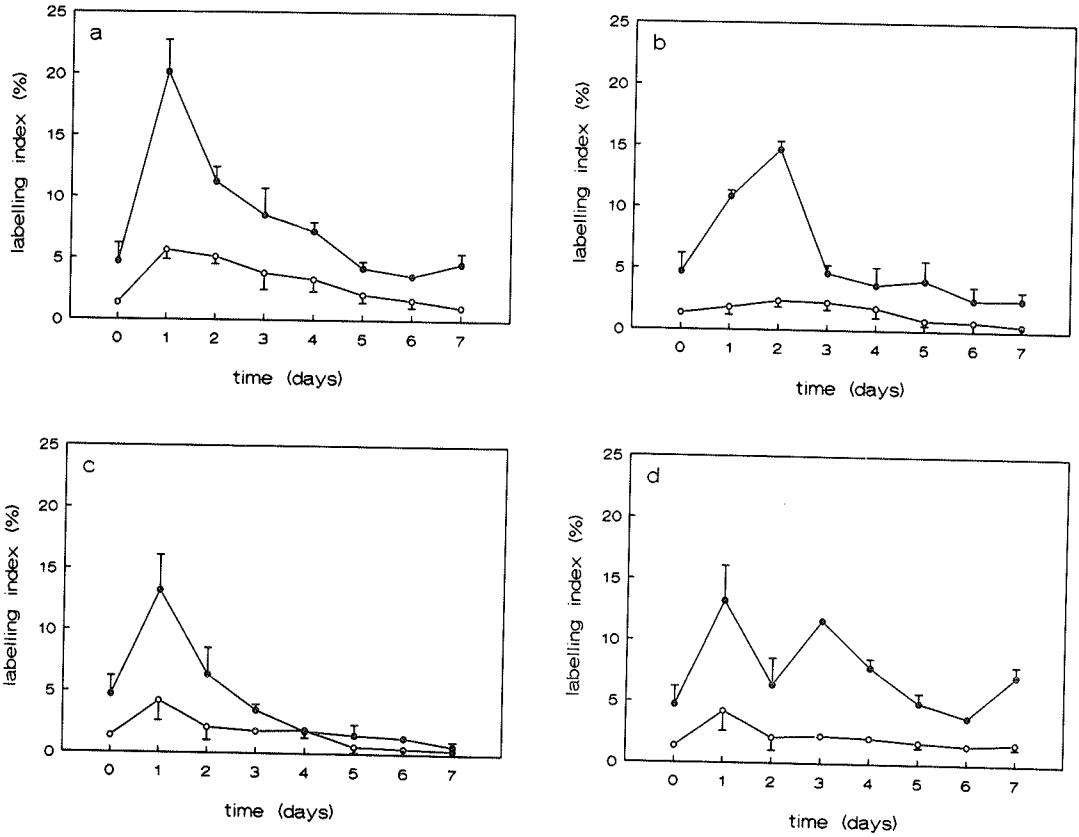

Figure 2 *Labeling index (LI) determined in sections of hamster trachea. Tracheal rings were exposed to (a) control medium, (b) medium with 4 μM B[a]P, continuously, (c) medium with 20 μM B[a]P, continuously, (d) medium with 20 μM B[a]P for 2 days, followed by a 5-day recovery period. Closed symbols refer to non-basal cells and the open symbols to basal cells. Error bars represent SD of the LI's determined in 6 to 12 tracheal rings.*

The labeling indices (LI), a measure of cell proliferation, are presented in Figures 2a-d. In unexposed tracheal epithelium (Figure 2a) the overall LI (basal and non-basal cells) increased from about 6% on day 0 to about 25% on day 1, followed by a gradual decrease to about 6% towards the end of the culture period. The LI in non-basal cells was 3- to 4-fold as high as that in basal cells. On day 1, the LI remained significantly (Student's t-test; $p=0.0001$) lower in the presence of 4 or 20 μM B[a]P, than the LI on the same day in unexposed tracheas (Figures 2a-c). Continuous exposure to 20 μM B[a]P resulted, after the initial peak of cell proliferation on day 1, in a rapid decrease in LI thereafter; it remained low and dropped below 2% on days 5 to 7 (Figure 2c). In case of continuous exposure to 4 μM B[a]P, the LI recovered after the first day, showing a somewhat delayed peak of about 16% on day 2. Subsequently, the LI decreased to about 7% on day 3, and remained relatively low, about 5%, *i.e.* below control level, during days 4 to 7 (Figure 2b); the value of LI was still significantly higher (Student's t-test; $p<0.04$), however, than that on the corresponding days in tracheal epithelium exposed to 20 μM B[a]P. Two days of exposure to 20 μM B[a]P, followed by a 5-day recovery period in a B[a]P-free medium, allowed the LI to rapidly recover to a level observed in unexposed tracheas (Figure 2d).

Morphological changes in the tracheal epithelium were assessed in semi-thin sections. After incubation for 24 h in Ham's F12 medium (day 0), a moderate degree of cell death was observed, extending to about 10 to 15% of the tracheal surface (data not shown). In unexposed tracheal epithelium a moderate degree of squamous metaplasia was observed on days 4 to 7 (Table 2). In the B[a]P-exposed tracheal epithelium, a similar degree of squamous metaplasia was already observed on day 1, but it was less pronounced on day 2 than on day 1. Towards the end of the culture period, on days 5 and 6, severe squamous metaplasia was observed in the continuously exposed tracheas, as well as in the tracheas that were exposed to B[a]P for only 2 days. On day 7 metaplasia was less in all groups - including the control group - as compared to that on days 4 to 6. However, it should be noted that on day 7 it was more difficult to assess the extent of squamous metaplasia, due to the increased extent of dysplasia in the tracheal epithelium. Nodular hyperplasia was scored across the entire tracheal surface as clearly visible nodules of more than 30 cells. An example of nodular hyperplasia is shown in Figure 3.

Table 2 *Squamous metaplasia in hamster tracheal epithelium in organ culture. Tracheas were exposed to B[a]P continuously (4 or 20 μM), or for 2 days (20 μM) followed by culturing in B[a]P-free medium for another 5 days. Control tracheas were cultured in medium containing 0.1% DMSO (v/v).*

			% of tracheal organ cultures with Squamous metaplasia[a]			
Treatment	Day	n[b]	None	Mild	Marked	Severe
control	0	8	100			
	1	10	100			
	2	8	100			
	3	8	75	25		
	4	8		50	50	
	5	8	38	12	50	
	6	10		50	50	
	7	8	25	75		
B[a]P 4 μM cont.	1	10	40	10	50	
	2	12	83	17		
	3	8	50	50		
	4	12		17	83	
	5	8				100
	6	8	17	83		
	7	8	25	75		
B[a]P 20 μM cont.	1	12	42	8	50	
	2	10	50	50		
	3	8			100	
	4	8	38	12	50	
	5	8		12	50	38
	6	8			88	12
	7	10		10	90	
B[a]P 20 μM 2 days	1	12	42	8	50	
	2	10	50	50		
	3	6	67	33		
	4	8	12	38		50
	5	8			88	12
	6	8		12	38	50
	7	8		12	88	

[a] Squamous metaplasia is graded as severe if more than 40% of the epithelial surface is affected; as marked if between 10 and 40%; as mild if less than 10%.

[b] n is the number of tracheal rings examined.

Table 3 *Nodular hyperplasia in hamster tracheal epithelium in organ culture. Tracheas were exposed to B[a]P continuously (4 or 20 μM), or for 2 days (20 μM) followed by culturing in B[a]P-free medium for another 5 days. Control tracheas were cultured in medium containing 0.1% DMSO (v/v).*

			% of tracheal organ cultures with Nodular hyperplasia[a]			
Treatment	Day	n[b]	None[c]	1-2	3-4	> 5
control	0	8	100			
	1	10	100			
	2	8	100			
	3	8	100			
	4	8	50	38	12	
	5	8	50	50		
	6	10	50	50		
	7	8	50	50		
B[a]P 4 μM	1	10	50	50		
cont.	2	12		100		
	3	8		50	50	
	4	12			100	
	5	8			100	
	6	8		25	75	
	7	8		38	62	
B[a]P 20 μM	1	12	100			
cont.	2	10	50	50		
	3	8		88	12	
	4	8		88	12	
	5	8		50	50	
	6	8	50	38	12	
	7	10	50	40	10	
B[a]P 20 μM	1	12	100			
2 days	2	10	50	50		
	3	6		50	50	
	4	8			88	12
	5	8			25	75
	6	8			25	75
	7	8			12	88

[a] Nodular hyperplasia was scored as number of focal hyperplastic lesions per tracheal ring.
[b] n is the number of tracheal rings examined.
[c] Number of nodules per tracheal ring.

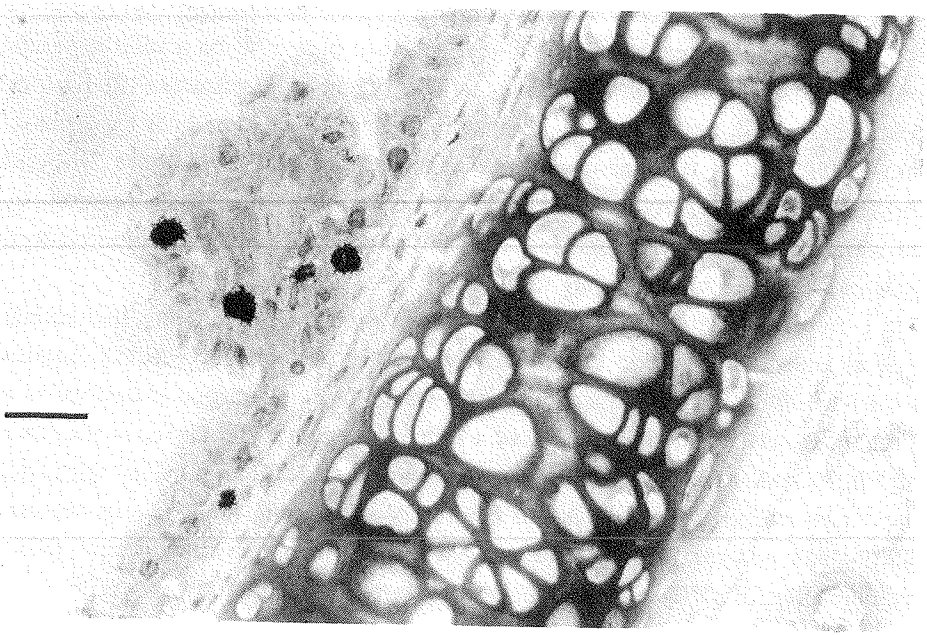

Figure 3 *Nodular hyperplasia in hamster tracheal epithelium exposed to 4 μM B[a]P for 3 days. Bar = 20 μm.*

Nodular hyperplasia was observed to a limited extent in unexposed tracheal epithelium after 4 days, probably as a result of the relatively high initial cell proliferation on day 1 (Table 3). In all three B[a]P-exposure groups nodular hyperplasia occurred earlier and for the first 5 days it increased during culture. In tracheal epithelium exposed continuously to 4 μM B[a]P, all tracheal rings contained 3-4 nodules on days 4 and 5. However, on days 6 and 7 the number of nodules per ring actually decreased, probably as a result of exfoliation of cells in the continuous presence of B[a]P. In case of continuous exposure to 20 μM B[a]P, only 50% of the tracheal rings contained 3-4 nodules on day 5. Also in this group the number of nodules per ring decreased on days 6 and 7. Only in tracheal epithelium exposed to 20 μM B[a]P followed by a 5-day recovery period, there was a steady increase in the number of nodules per ring; on day 7, at the end of the culture period, 88% of the rings contained more than 5 nodules.

The *in vivo* hamster trachea model has been proven to be a valid experimental system to study the morphological characteristics of human lung cancer (12). Lesions that precede tumor formation *in vivo*, *i.e.*, squamous metaplasia and nodular hyperplasia, can also be induced *in vitro* using cultured hamster tracheas. To compare DNA-adduct formation and cell proliferation of basal and non-basal cells in relation to the development of precarcinogenic lesions, hamster tracheal rings were exposed to B[a]P in Ham's F12 medium according to different experimental designs.

In previous studies it had been shown, by use of ^{32}P-postlabeling, that mainly one type of adduct is formed in DNA of HTE cells exposed to B[a]P (18,19). Against this particular adduct in DNA (the adduct of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine) a rabbit antiserum had been generated, which appeared suitable for the *in situ* detection of B[a]P-DNA adducts in cytospin preparations of HTE cells. The triple-staining procedure recently developed in our laboratory allowed the simultaneous detection of B[a]P-DNA adducts in basal and non-basal cells within the same preparation. For quantification of DNA adducts, the use of cytospin preparations of HTE cells was preferred over that of tracheal sections, as in the latter specimens the nuclei are likely to be damaged, which leads to uncertainty with respect to the amount of DNA that is lost during processing of the sections for IFM. The proportion of non-epithelial cells in the cytospin preparations, as determined by immunocytochemical staining, was below 10% at all time points.

No statistically significant differences were found in the extent of B[a]P-DNA adduct formation between basal and non-basal cells at both B[a]P concentrations (4 and 20 μM). This has also been found previously with HTE cells exposed to 40 μM B[a]P for 2 days (20). As the secretory cell (a non-basal cell) is known for its rather extensive endoplasmic reticulum, which contains the activating enzyme P450 (21), whereas the basal cell contains relatively little cytochrome P450, one would expect the highest adduct levels to be present in the non-basal cell fraction. That many adducts were found in basal cells may be due to intercellular transport of reactive B[a]P metabolites, capable of binding to DNA. In this respect, it is interesting to note that (+)-*anti*-B[a]P-diolepoxide, the ultimate carcinogen of B[a]P, can be transported through the animal body by serum, which indicates that this epoxide may be rather stable under certain conditions (22,23). On the other hand, adduct formation in non-basal cells may have been limited by the presence of a relatively high level of detoxicating enzymes, such as glutathione S-transferases, which can inactivate B[a]P metabolites before they bind to DNA.

Only during continuous exposure to 20 μM B[a]P there was a sustained increase

in adduct level in hamster tracheal epithelium. Previous experiments have shown that the adduct level in HTE cells, determined by ^{32}P-postlabeling, decreased to about 60% of the maximum level after transfer of tracheal rings to B[a]P-free medium and remained virtually the same for another 3 days (19). This makes it likely that in the HTE cells recovering from the 2-day exposure to 20 μM B[a]P a significant number of adducts remained, even after a few days of culturing in B[a]P-free medium, although the level probably rapidly dropped below that of the cells continuously exposed to 4 μM B[a]P.

The inhibitory effect of B[a]P on the level of cell proliferation in Ham's F12 medium reported here is in contrast with data in the literature. Both *in vivo* and *in vitro* exposure to B[a]P stimulated cell proliferation in hamster tracheal epithelium; *in vivo* this effect of B[a]P probably was a result of cell death. In *in vitro* explant cultures of hamster tracheas continuously exposed to 20 μM B[a]P in CMRL medium, initially the mitotic index increased in both basal and non-basal cells; after 7 days of culture the mitotic index was still elevated in basal cells, whereas in non-basal cells it was at control levels again (24). In CMRL medium, exposure to cigarette-smoke condensate enhanced proliferation of basal cells of tracheal epithelium, although in this case also the proliferation of non-basal cells was elevated (17). Cigarette-smoke condensate may also have cytotoxic properties, besides the presence of many genotoxic compounds. In the present study, cell death occurred in unexposed tracheal epithelium upon transfer of the tracheal rings to the culture medium (day -1 to 0), which may explain the high initial level of cell proliferation in unexposed tracheal epithelium. Because additional cell death was not observed during the first days of B[a]P exposure, a stimulatory effect of B[a]P on proliferation due to cell death may not be expected at all. The extent of the inhibition of cell proliferation in the experiments presented here appears to be inversely correlated with the level of DNA adducts in the cells. Probably, these two phenomena are causally connected, since B[a]P-DNA adducts are a block for DNA polymerase (25) and may therefore hamper cell proliferation. Furthermore, the presence of a substantial number of adducts may cause a block in the G1 phase of the cell cycle, possibly mediated by the action of the tumor-suppressor gene p53 (26).

Squamous metaplasia was observed to a moderate extent in unexposed tracheal epithelium, which may be related to the absence of a stimulatory effect of vitamin A on cell differentiation during culture (17). Nodular hyperplasia was also observed to a limited extent in unexposed tracheas, probably as a result of the high regenerative proliferation on day 1. During continuous exposure to both concentrations of B[a]P there was a marked increase in the extent of nodular hyperplasia as compared to the control. However, in tracheal rings exposed to B[a]P for 2 days followed by a 5-day period which allowed cell proliferation to recover, the effects on nodular hyperplasia were most

pronounced.

The idea that both the presence of DNA adducts and a certain degree of cell proliferation is critical for precancerous effects to develop has been reported before: repeated administration of N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) induces pancreatic tumors in female Syrian golden hamsters (27). In the liver of these hamsters, the highest adduct levels were found. At the same time, DNA synthesis was suppressed in hepatocytes but increased in non-parenchymal liver cells. In the pancreas, the adduct levels were relatively low, but high levels of DNA synthesis were maintained over a relatively long period, and coincided with maximal adduct levels in the pancreas, allowing development of precancerous effects to develop.

The basal cell is generally regarded as the cell type from which all major cell types in the tracheal epithelium originate (28,29), although non-basal cells - including both the secretory and the ciliated cell - also are capable of dividing (30,31). In the experiments reported here with tracheal epithelium cultured in Ham's F12 medium, there were actually more non-basal cells in S-phase than there were basal cells. However, it should be noted that according to our definitions of basal and non-basal cells, only about 10 to 20% of all tracheal cells can be regarded as basal cells. Therefore, the number of basal cells in S-phase, relative to the total number of basal cells, is actually quite large and comparable to the relative number of non-basal cells in S-phase. Nevertheless, the absolute number of non-basal cells in S-phase exceeds by far that of basal cells. As to the formation of B[a]P-DNA adducts, there were no significant differences in basal and non-basal cells. Previous experiments have shown that upon transfer of tracheal rings to B[a]P-free medium, repair in basal cells is faster than that in non-basal cells. With respect to both the extent of proliferation of non-basal cells and the kinetics of induction and repair of DNA-adducts in basal and non-basal cells, as observed in a previous study (19), it is reasonable to assume that the non-basal cell is the major cell type responsible for the effects observed in tracheal epithelium after exposure to B[a]P in organ culture with Ham's F12 medium.

Acknowledgements

The authors wish to thank Mr CJM van der Wulp for the development of the image-processing software and his assistance during the processing of the recorded images. Mr MJM Boermans is acknowledged for his help in making the photographs.

References

1. Mitsudomi T, Oyama T, Kusano T, Osaki T, Nakanishi R, Shirakusa T (1993) Mutations of the *p53* gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J Natl Cancer Inst*, **85**, 2018-2023.
2. Wattenberg LW (1993) Prevention-therapy-basic science and the resolution of the cancer problem: presidential address. *Cancer Res*, **53**, 5890-5896.
3. Peto R, Lopez AD, Boreham J, Thun M, Heath JrC (1992) Mortality from tobacco in developed countries: indirect estimation from national vital statistics. *Lancet*, **339**, 1268-1278.
4. Loeb LA, Ernster VL, Warner KE (1984) Smoking and lung cancer: an overview. *Cancer Res*, **44**, 5940-5958.
5. Fielding JE (1985) Smoking: health effects and control. *N Eng J Med*, **313**, 491-498.
6. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans (1986). Tobacco smoking. Volume 38, IARC, Lyon.
7. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans (1983). Polynuclear aromatic compounds, Part 1, Chemical, environmental and experimental data. Volume 32, IARC, Lyon.
8. Miller EC (1978) Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. *Cancer Res*, **38**, 1479-1496.
9. Miller JA (1970) Carcinogenesis by chemicals: an overview. GHA Clowes memorial lecture. *Cancer Res*, **30**, 559-576.
10. Phillips DH (1990) Modern methods of DNA adduct determination. In: Cooper CS, Grover PL (eds.), Chemical carcinogenesis and mutagenesis, pp 503-546. Berlin: Springer Verlag.
11. Rodenhuis S, Slebos RJC, Boot AJM, Evers SG, Mooi WJ, Wagenaar SS, van Bodegom PC, Bos JL (1988) Incidence and possible clinical significance of K-*ras* oncogene activation in adenocarcinoma of the human lung. *Cancer Res*, **48**, 5738-5741.
12. Saffiotti U, Cefis F, Kolb LH (1968) A method for the experimental induction of bronchogenic carcinoma. *Cancer Res*, **28**, 104-124.
13. Trump BF, McDowell EM, Glavin F, Barret LA, Becci PJ, Schürch W, Kaiser HE, Harris CC (1978) The respiratory epithelium. III. Histogenesis of epidermoid metaplasia and carcinoma *in situ* in the human. *J Natl Cancer Inst*, **61**, 563-575.
14. McDowell EM, McLaughlin JS, Merenyi DK, Kieffer RF, Harris CC, Trump BF (1978) The respiratory epithelium. V. Histogenesis of lung carcinomas in the human. *J Natl Cancer Inst*, **61**, 587-606.
15. Becci PJ, McDowell EM, Trump BF (1978) The respiratory epithelium. IV. Histogenesis of epidermoid metaplasia and carcinoma *in situ* in the hamster. *J Natl Cancer Inst*, **61**, 577-586.
16. Becci PJ, McDowell EM, Trump BF (1978) The respiratory epithelium. VI. Histogenesis of lung tumors induced by benzo[a]pyrene-ferric oxide in the hamster. *J Natl Cancer Inst*, **61**, 607-618.
17. Rutten AAJJL, Beems RB, Wilmer JWGM (1988) Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture II. A histomorphological study. *Virch Archiv B Cell Pathol*, **55**, 177-186.
18. Wolterbeek APM, Roggeband R, Steenwinkel M-JST, Baan RA, Rutten AAJJL (1993) Formation and repair of benzo(a)pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis. *Carcinogenesis*, **14**, 463-467.
19. Roggeband R, Wolterbeek APM, Melis OWM, Wittekoek ME, Rutten AAJJL, Feron VJ, Baan RA (1994) DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture. *Carcinogenesis*, **15**, 661-665.
20. Roggeband R, van den Berg PTM, van der Wulp CJM, Baan RA (1994) Detection of DNA-adducts in basal and non-basal cells of the hamster trachea exposed to benzo[a]pyrene in organ culture. *J Histochem Cytochem*, in press.
21. Plopper CG, Cranz DL, Kemp L, Serabjit-Singh CJ, Philpot RM (1987) Immunohistochemical demonstration of cytochrome P-450 monooxygenase in Clara cells throughout the tracheobronchial airways of the rabbit. *Exp Lung Res*, **13**, 59-68.

22. Ginsberg GL, Atherholt TB (1989) Transport of DNA-adducting metabolites in mouse serum following benzo(a)pyrene administration. *Carcinogenesis*, **10**, 673-679.
23. Ginsberg GL, Atherholt TB (1990) DNA adduct formation in mouse tissues in relation to serum levels of benzo(a)pyrene-diol-epoxide after injection of benzo(a)pyrene or the diol-epoxide. *Cancer Res*, **50**, 1189-1194.
24. Chopra DP, Cooney RA (1985) Histogenesis of benzo(a)pyrene-induced lesions in tracheal explants. *Virch Arch B Cell Pathol*, **48**, 299-315.
25. Reardon DB, Bigger CAH, Dipple A (1990) DNA polymerase action on bulky deoxyguanosine and deoxyadenosine adducts. *Carcinogenesis*, **11**, 165-168.
26. Lane DP (1992) p53, guardian of the genome. *Nature*, **358**, 15-16.
27. Kokkinakis DM, Subbarao V (1993) The significance of DNA damage, its repair and cell proliferation during carcinogen treatment in the initiation of pancreatic cancer in the hamster model. *Cancer Res*, **53**, 2790-2795.
28. Nettesheim P, Jetten AM, Inayama Y, Brody AR, George MA, Gilmore LB, Gray T, Hook GER (1990) Pathways of differentiation of airway epithelial cells. *Env Health Persp*, **85**, 317-329.
29. Inayama Y, Hook GER, Brody AR, Cameron GS, Jetten AM, Gilmore LB, Gray T, Nettesheim P (1988). The differentiation potential of tracheal basal cells. *Lab Invest*, **58**, 706-717.
30. Jetten AM (1991) Growth and differentiation factors in tracheobronchial epithelium. *Am J Physiol*, **260**, L361-L373.
31. Rutten AAJJL, Beems RB, Wilmer JWGM, Feron VJ (1988) Ciliated cells in vitamin A-deprived cultured hamster tracheal epithelium do divide. *In Vitro Cell & Develop Biol*, **24**, 931-935.

Chapter

8 General Discussion

The main objective of the experiments described in this thesis was to assess the relationship between DNA-adduct kinetics, cell proliferation and the development of precancerous lesions in the trachea of the Syrian golden hamster, exposed to benzo[a]pyrene (B[a]P) in organ culture. This *in vitro* system is considered to be a suitable model to investigate human lung cancer development. To study the formation and removal of B[a]P-DNA adducts, two techniques were used, namely the ^{32}P-postlabeling and the immunocytochemical assay. Despite the fact that the ^{32}P-postlabeling assay *per se* does not provide detailed structural information, cochromatography with authentic adduct standards may indicate the identity of unknown adduct spots. Chapter 2 describes the results of some major improvements with respect to adduct quantification in ^{32}P-postlabeling. These improvements were included in the postlabeling experiments described in all other Chapters in this thesis. An interesting application of the postlabeling technique is illustrated by experiments described in Chapter 3: microsomal activation of polyaromatic test compounds in the presence of either isolated DNA or white blood cells provides insight in the stability and the possible reactivity towards DNA of intermediates of these chemicals.

In Chapter 4 the results are presented of experiments in which hamster trachea rings were continuously or temporarily exposed to B[a]P in organ culture. ^{32}P-postlabeling analysis revealed that the predominant adduct formed in hamster tracheal epithelial cells was that of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine, irrespective of exposure time. Upon continuous exposure to B[a]P, adducts steadily accumulated. In a repair experiment it was found that transfer of tracheal rings to a B[a]P-free medium resulted in a decrease to about 60% of the maximum adduct level. This decrease correlated with an increase in unscheduled DNA synthesis indicating repair processes, while cell proliferation, as determined by the incorporation of radiolabeled thymidine, appeared not to be the cause of the reduction in B[a]P-DNA adduct level. Rats are known to be less susceptible to tracheal tumors after instillation of B[a]P than are hamsters. When rat tracheas were exposed to B[a]P in organ culture a different adduct pattern was obtained on TLC after ^{32}P-postlabeling, while B[a]P-DNA adducts did not accumulate upon continuous exposure to B[a]P.

To be able to determine B[a]P-DNA adduct levels in specific cell types, a triple-staining procedure was developed, which involved the simultaneous staining of DNA adducts and basal-cell specific cytokeratins, both immunochemically, and of DNA. A

rabbit antiserum, W2/01, was generated against B[a]P-DNA adducts in DNA, and was found to be suitable for *in situ* adduct detection on cytospin preparations of epithelial cells. Applications of this procedure are described in Chapters 5-7. It was rather surprising to see that the initial induction of B[a]P-DNA adducts in basal and non-basal cells was comparable. However, the repair of B[a]P-DNA adducts in basal cells was significantly faster as compared to that in non-basal cells. Finally, B[a]P-DNA adduct kinetics and cell proliferation of basal and non-basal cells were related to the development of precancerous lesions (Chapter 7). B[a]P-DNA adducts were found to decrease proliferation of epithelial cells. Precancerous lesions, such as squamous metaplasia and nodular hyperplasia, were most pronounced after temporary exposure to B[a]P followed by a recovery period, during which cell proliferation returned to control values.

First, some major aspects of the two methods to determine DNA-adduct levels will be discussed below. Furthermore, ongoing developments in DNA-adduct dosimetry at the gene level are furthermore discussed. Second, the hamster trachea model is evaluated on the basis of the results described in the Chapters 4 to 7.

8.2.1 Critical evaluation

The ^{32}P-postlabeling procedure has proven to be an attractive method to detect B[a]P-DNA adducts. The method is extremely sensitive; at the same time it should be realized that this sensitivity makes the technique susceptible to confounding factors. The ^{32}P-postlabeling comprises many different steps, each with specific efficiencies and effectivities. Furthermore, in the practical application of the ^{32}P-postlabeling technique, one should be aware of the risks of working with relatively high levels of radioactivity. Several beta-shielding devices have been described helping to avoid excessive exposure to radiation (Reddy and Blackburn 1990). In this respect, the use of ^{33}P- instead of ^{32}P-labeled phosphate would ensure a substantial reduction of the radiation exposure. Although ^{33}P-labeled phosphate is commercially available, so far its use has been limited.

One of the main conclusions of a recently held workshop on postlabeling methods at IARC in Lyon stressed the need to standardize the individual steps within the postlabeling protocol (IARC 1993). A questionnaire which was completed by all groups attending the workshop, revealed that several groups that routinely applied the ^{32}P-postlabeling procedure employed suboptimal conditions during DNA digestion and/or adduct enrichment and labeling (see Chapter 1 for general outline of the procedure). The most critical steps in the ^{32}P-postlabeling procedure are evaluated below.

- DNA digestion

With respect to enzymatic DNA degradation, it should be noted that optimal hydrolysis conditions are to be determined for each type of DNA modification, in order to ensure complete digestion to mononucleotides. The most commonly used digestion of DNA to 3'-monophosphates involves the use of two enzymes, namely micrococcal endonuclease (MN) and spleen phosphodiesterase (SPD). MN should be used at the minimum possible concentration, to ensure optimal adduct recovery. SPD is relatively unstable; therefore DNA digestion may be incomplete if an insufficient amount of this enzyme is present. In general, the number of freeze-thaw cycles of both MN and SPD should be limited to two or three. In case of B[a]P-modified DNA, optimal hydrolysis conditions were published (Reddy *et al* 1981, Reddy and Randerath 1986).

- Adduct enrichment

Enrichment of modified nucleotides is in most cases performed with nuclease P1 treatment or butanol extraction (see Chapter 1, Figure 5). The enrichment step is very critical in its consequences for the sensitivity of the assay, as remaining unmodified nucleotides can also be labeled with γ-[^{32}P]ATP. The effectivity of nuclease P1 treatment, which leads to dephosphorylation of normal nucleotides, can easily be verified on TLC after the application of an aliquot of the labeled digest: there should not be a significant amount of radioactivity migrating with unmodified nucleoside bisphosphates. When using nuclease P1 as an enrichment step, it is assumed that adducts are completely resistant to nuclease P1 digestion, which will not be correct for many of the smaller adducts. Critical to the butanol enrichment is the number of back-extractions with water, needed to recover remaining unmodified nucleotides in the aqueous phase. When relatively large amounts of DNA are used in this procedure, more than two back-extractions with water may be needed. In this enrichment step, it is assumed that adducts are quantitatively extracted into the butanol phase, which will be valid only for strongly hydrophobic adducts such as B[a]P's.

- Adduct labeling

The ^{32}P-labeling of adducted nucleotides is performed at a relatively high pH (~9), to suppress 3'-phosphatase activity of the polynucleotide kinase enzyme. Some adducts may be labile in alkaline solution. However, the availability of a kinase that is free of 3'-phosphatase activity may permit reactions at neutral pH. Many adducts are rather poor substrates to the kinase, which results in low labeling efficiencies. The labeling efficiency is furthermore negatively affected by relatively high levels of unmodified nucleotides, which can still be present despite enrichment (Vaca *et al* 1992). These results indicate that it is important to reduce the remaining amount of unmodified nucleotides as much as possible and to check that some excess γ-[^{32}P]ATP remains in the labeling mixtures after the kinase reaction. The labeled DNA digest should be applied on the TLC plate carefully and slowly, so as to obtain a small spot which ensures optimal resolution. When the DNA digests containing polyaromatic adducts have been applied onto TLC plates, it should be realized that on these plates they are particularly sensitive to daylight (see Chapter 2); in some cases, the UV-A component of daylight may reduce the amount of adduct on the chromatogram to 30-40%. Therefore, ^{32}P-postlabeling experiments with light-sensitive adducts should be carried out under conditions of subdued light.

- Adduct quantification

The first attempts to quantify DNA adducts were performed with the relative adduct labeling (RAL) procedure, in which a small part of the total DNA digest, containing both adducted and normal nucleotides, is labeled. The counts detected in the total nucleotides, are then compared with the counts in the adducted nucleotides, determined after resolution on TLC. Later on, this RAL was specified to indicate the amount of adducts expressed as fmol/μg DNA (or adducts per 10^8 nucleotides) by determination of the specific activity of γ-[^{32}P]ATP, the total radioactivity of the adducts and the amount of DNA that was used in the assay. The determination of the specific activity is performed by the labeling - under identical conditions - of a known amount of deoxyadenosine-3'-monophosphate (dAp); it is assumed that adducts and dAp are labeled with the same efficiency, which is often not the case. The RAL method gives erroneous results when adducts do not label with the same efficiency as dAp.

The amount of input DNA is commonly measured by spectrophotometry. The accuracy of this determination was found to vary up to 50% due to differences in molecular weight of DNA or the presence of contaminants in the DNA preparation. An alternative method to determine the input DNA and to quantify DNA adducts was described recently (Shields *et al* 1993a,b). This procedure involves the determination of the efficiency of digestion by means of HPLC analysis, the dGp peak being taken as a measure of DNA input. Furthermore, the addition of deoxyguanosine-3'monophosphate (dGp) as an internal standard, mixed together with known amounts of chemically synthesized reference adducts, provides a more reliable way of quantification, as the labeling efficiency can easily be determined by comparison of the molar ratio between adduct and dGp before and after labeling. However, a shortcoming of this method is the decrease in sensitivity, as a result of dGp present during labeling.

We have introduced anion-exchange FPLC analysis after DNA digestion (see Chapter 2); this analysis provides a check on the efficiency of digestion, while possible contamination with RNA can also be detected in the chromatogram. On the basis of the dGp peak in the FPLC chromatogram, even very small amounts of DNA can be exactly quantified (lower detection limit of dGp ~ 0.01 nmol). When adducts present in DNA modified with [^{3}H]B[a]P-diolepoxide are run through the entire ^{32}P-postlabeling procedure, the overall recovery of [^{3}H] label varies between 40 and 80% (Gupta *et al* 1982, Reddy and Randerath 1987, Shields *et al* 1993b). The kinase labeling is probably the most crucial step in the postlabeling procedure, as in this type of experiments there were no significant differences between the recovery of adducts after nuclease P1 or the butanol enrichment (Segerbäck and Vodicka 1993).

- Use of modified DNA standards

The method of adduct quantification we developed in the ^{32}P-postlabeling (see Chapters 2 and 3) involves the use of B[a]P-DNA standards with known amounts of modification. The modification levels of the B[a]P-DNA standards were determined by an independent technique, *i.e.* synchronous fluorescence spectrophotometry (see Chapter 1). Routinely, two of these standards, with modification levels in the range expected in the samples to be analyzed, are included in each postlabeling experiment. Because the standard adducts are - in case of B[a]P exposure - of very similar or identical chemical structure as the adducts present in the sample to be tested, differences in labeling efficiency will be minimal with the use of our standards. A limitation of our method would be the necessity to synthesize modified DNA samples for each compound studied. Furthermore, one has to be aware of possible deterioration and adduct loss during storage of these standards. Nevertheless, it was the general conclusion of the workshop on postlabeling methods held at IARC in Lyon, that well-defined DNA standards should be used more widely to facilitate comparison of results from different laboratories. In Table 1 the different adduct-quantification procedures used in ^{32}P-postlabeling are listed with their respective advantages and limitations.

Table 1 *Advantages and limitations in adduct quantification with ^{32}P-postlabeling analysis*

Method	Advantages	Limitations	References
relative adduct labeling (RAL)	no synthesis of adducts required	only relative values are obtained	Gupta *et al* 1982 Randerath *et al* 1985 Randerath *et al* 1986
	no structural information of adduct required	no check on accuracy of procedure	
RAL and labeling of dAp	adducts are quantified per 10^x nucleotides	labeling of dAp may be different from that of adducts	Reddy and Randerath 1986 Randerath *et al* 1989
	no structural information of adduct required	spectrophotometric analysis of DNA is unreliable	
	no synthesis of adducts required	no check on accuracy of procedure	
molar ratio between adduct and dGp (+ HPLC analysis)	check on digestion and exact quantification of input DNA (HPLC profile)	synthesis of adducts required	Shields *et al* 1993a,b
		decreases sensitivity	
	check on labeling	standard is present only in the labeling step	
our procedure B[a]P-DNA adducts in DNA (+ FPLC analysis)	standard is present during entire procedure	requires preparation of modified DNA samples	Steenwinkel *et al* 1993 Roggeband *et al* 1993, 1994
	check on digestion and exact quantification of input DNA (FPLC profile)	standard DNA samples may deteriorate in time	
	check on labeling		

Our method of adduct quantification was applied in a study in which adduct patterns were compared that had been obtained after ^{32}P-postlabeling of DNA treated with B[a]P in the presence of liver microsomes from different species, including man (see Chapter 3). Furthermore, human white blood cells (WBC) were incubated with B[a]P in the presence of 3-methylcholanthrene (3-MC)-induced rat liver microsomes, whereafter DNA was isolated and subjected to ^{32}P-postlabeling. Comparison of adduct patterns obtained after incubation of B[a]P with microsomes and DNA in solution with those obtained after incubation of B[a]P with microsomes and intact cells may provide insight into the biological reactivity of metabolites and their capability to reach DNA in the nucleus. The assays described in Chapter 3, with the use of both isolated DNA and WBC, may provide a rapid screening method to assess the stability and DNA-binding capacities of reactive metabolites formed after microsomal activation of a test compound. To avoid the rather laborious chromatographic steps, a one-directional separation in the ^{32}P-postlabeling would be sufficient. Within the framework of interspecies comparisons, and for the purpose of extrapolation to the human situation, the inclusion in the experiments of human microsomes as a source of activation would be desirable. However, due to the wide interindividual variation this source of microsomes is difficult to standardize, so the use of 3MC-induced rat-liver microsomes may be an acceptable alternative.

The recent development of the fluorescence postlabeling for methyl adducts may obviate the use of radiolabeled compounds for this type of DNA modification (Jain and Sharma 1993). In this assay, modified nucleotides are separated from unmodified nucleotides by means of HPLC, whereafter they are labeled with dansyl chloride. A second separation of the labeled nucleotides, followed by excitation with light of the appropriate wavelength, allows quantification of the dansyl chloride-derived fluorescence. In case of the B[a]P-diolepoxide-N^2dG adduct, the need for derivatization may even be absent, as in this particular adduct the pyrene moiety, which is also fluorescent when irradiated at the appropriate wavelength, is still intact.

One relatively new approach is the separation of postlabeled nucleotides by means of HPLC (Pfau and Phillips 1991, Lecoq *et al* 1992, Möller *et al* 1993) instead of the more commonly used TLC separation. The major advantage of the HPLC separation is the increased resolution, which makes it possible to distinguish adducts that would not have been resolved on TLC. By cochromatography with authentic standards, adducts can be characterized. Also, in the case of complex adduct patterns, as often encountered with exposure to mixtures, diffuse and overlapping adduct spots on TLC can be cut out, eluted off the TLC support and reanalyzed on HPLC. The only potential drawback of the use

of HPLC in combination with on-line counting of radiolabel is the decrease in sensitivity, which is about 10-fold as compared to TLC separation (Pfau and Phillips 1991). The collection and subsequent off-line counting of HPLC fractions of a certain minimal volume would impair the resolution and is therefore not desirable.

A limitation of the ^{32}P-postlabeling analysis is that it does not provide detailed information with respect to the structure of the adducts. Obviously, it would be interesting to identify individual adduct spots. One possible way to do this is by scraping off individual spots and analyze these with a technique of appropriate sensitivity. The absolute amount of material that is present in an adduct spot on a ^{32}P-postlabeling chromatogram (subfmol to fmol quantities) is still below the detection limits of the techniques currently available to provide structural data. However, the use of sensitive identification methods, together with separation techniques, is in full development. In this respect, the NMR technique still requires relatively large amounts of adducts: for characterization with this method about 10 nmol of adduct is needed (Harris *et al* 1988). The advent of fast atom bombardment (FAB) mass spectrometry (MS) has eliminated the need to derivatize compounds in order to achieve adequate volatility, which allows relatively large molecules like DNA adducts to be analyzed. Also the use of tandemly arranged mass spectrometers contributes to the possibility to detect DNA adducts (Grant and Cooks 1990). However, analysis by mass spectrometry still requires subpicomol quantities of adduct (Gross and Cerny 1993, Kaur *et al* 1993). Another most promising technique that may be used in combination with the ^{32}P-postlabeling is the fluorescence line-narrowing spectroscopy (FLNS, see Chapter 1). With this technique intact adducts can be investigated, either as single compounds or attached to DNA. Recently, FLNS has been applied in studies in which the relative importance of two pathways to activate B[a]P, *viz.*, the one-electron oxidation route and the monooxygenase dependent pathway, was determined (Rogan *et al* 1993). In the major adduct, formed via the latter route, the pyrene moiety is still intact, whereas in adducts derived from the former pathway this is not the case. On this basis, the fluorescence spectra could be clearly distinguished. Recent experiments at the Free University of Amsterdam showed that the B[a]P-diolepoxide-N^{2}dG adduct, when spotted on TLC, could be detected by means of FLNS (Velthorst, unpublished observations). The lower detection limit was around 100 fmol of adduct. Although the sensitivity should be improved further, this is a most promising observation with respect to structural identification after postlabeling.

8.3.1 Critical evaluation

In situ detection assays such as those described in this thesis, may allow the analysis of DNA adducts in specific cell types. This is of interest with regard to the role of certain cell types in the process of carcinogenesis. In tissue sections, cells can be characterized relatively easily by their morphology and relative position in the tissue. However, when cryostat sections are processed for adduct detection, morphology often deteriorates. Furthermore, because it cannot be avoided that cells are damaged by the sectioning itself, some DNA may be lost during processing. It is therefore preferable to use whole cells when *in situ* quantification is pursued. In order to achieve cell-specific adduct detection, the immuno-staining with an adduct-specific antibody can be combined with the staining by antibodies directed against specific cell components, such as cytokeratins (Vink *et al* 1993).

The triple-staining procedure we developed involves the simultaneous visualization of B[a]P-DNA adducts, basal-cell specific cytokeratins and DNA (Chapter 5). Both adducts and cytokeratins were stained by use of fluorochrome-labeled second antibodies while DNA was counterstained with DAPI for localization purposes. The use of colorigenic substrates, such as DAB, appeared not suitable because it obscured fluorescence staining. Critical to the triple-staining procedure is the choice of fixative; the presence of acetic acid in the fixative appeared to destroy the antigenic determinant of cytokeratins, as staining was no longer observed. Therefore, fixation with methanol only was preferred. Proteinase K treatment during processing of the cells for immunofluorescence microscopy may, to some extent, also interfere with the antibody-binding to cytokeratins. However, the staining of cytokeratins was found to be still sufficient to identify the basal cells with the use of image-processing software. Throughout culturing of hamster tracheas, the antibody against the basal cell-specific cytokeratin stained basal cells only (Rutten *et al* 1988a), which indicates that the antibody is highly specific.

The rabbit antiserum W2/01, raised against (±)-*anti*-B[a]P-diolepoxide-modified DNA, appeared suitable to detect B[a]P-DNA adducts *in situ* in cytospin preparations of hamster tracheal epithelial cells, whereas the monoclonal antibody II.E4, which had been raised against the adduct of (±)-*anti*-B[a]P-diolepoxide to deoxyguanosine, and had been selected in ELISA procedures, did not. Polyclonal antibodies are generally

considered to have a rather broad range of specificities, although it has recently been reported that the adduct of (+)-*anti*-B[a]P-diolepoxide to dG was recognized by a polyclonal antiserum with a 40-fold preference over the adduct of (−)-*anti*-B[a]P-diolepoxide to dG (Venkatachalam and Wani 1994). When W2/01 was used in *in situ* assays on human WBC exposed to various concentrations of B[a]P in the presence of 3-MC-induced rat liver microsomes, a good correlation was seen between the fluorescence signal obtained with IFM and the level of the (+)-*anti*-B[a]P-diolepoxide deoxyguanosine adduct, determined by ^{32}P-postlabeling. The lower detection limit in immunofluorescence microscopy for this type of adduct was found to be around 1 adduct per 10^6 nucleotides. This may be adequate in animal studies, in which single compounds are administered at a relatively high dose; however, in biomonitoring studies to estimate human exposure this limit may still be too high.

8.3.2 Prospects

A disadvantage of the use of antibodies to detect DNA adducts *in situ* is the relatively high value of the detection limit which makes this type of immunoassays less suitable for biomonitoring purposes. It should, however, be noted that progress in fluorescence microscopy happens in a relatively high rate. A promising development with respect to the detection of fluorochromes with overlapping emission spectra is confocal fluorescence lifetime imaging: differences in fluorescence lifetime of fluorochromes can be detected and used for imaging purposes. This makes it possible to differentiate between autofluorescence and the fluorochrome in one single cell. Furthermore, confocal microscopy eliminates out of focus contribution of the background.

In order to improve the sensitivity by other means, without loss of cell-specific information, it would be desirable to select the cell type of interest, isolate the DNA of this specific cell type, and apply a technique for DNA-adduct detection that is more sensitive than immunocytochemical assays, *e.g.*, the ^{32}P-postlabeling. In the case of WBC, lymphocytes can be easily separated from granulocytes by use of specific density-gradient centrifugation. This separation is particularly useful in biomonitoring studies: in case of human exposure to PAH, a considerable difference in adduct levels was found between these two cell types (Jahnke *et al* 1990, Savela and Hemminki 1991, Grzybowska *et al* 1993). For cell types from other tissues, separation may be more difficult. In that case, cell separation may be achieved with the use of antibodies; specific cell types within cell isolates from whole tissues, can be stained with antibodies directed against cell-specific membrane components. These cells can then be guided through a fluorescence automated cell sorter, which selects the cell type of interest, using positive staining as a

criterion. An even more simple way of selecting cells may be the use of magnetic beads, coated with antibodies directed against cell-specific membrane components. After cell separation, DNA can be isolated and subjected to ^{32}P-postlabeling.

Immunoaffinity chromatography (IAC) with adduct-specific antibodies may serve as an enrichment step in the ^{32}P-postlabeling analysis (Shields *et al* 1993a, King *et al* 1993) or the SFS technique (Manchester *et al* 1990, Weston and Bowman 1991). Typically, DNA is digested to mono-, di- or oligonucleotides and applied onto an IAC column, to which adduct-specific mono- or polyclonal antibodies are bound. The adducts of interest are retained on the column. Of importance in such applications is the specificity of the antibodies used. The capability of both the mouse monoclonal antibody II.E4 and the rabbit antiserum W2/01 to recognize structurally-related adducts derived from PAH compounds other than B[a]P was determined in an experiment in which human WBC were exposed to various individual PAH compounds in the presence of 3-MC-induced rat liver microsomes. Part of these WBC were processed for IFM and from another part DNA was isolated and subjected to ^{32}P-postlabeling, which served to calibrate the immunoassays. With respect to PAH-DNA adduct levels it was found that the antiserum W2/01 cross-reacted with DNA adducts derived from fluoranthene, chrysene, benz[k]fluoranthene, benz[b]fluoranthene and benz[c]phenanthrene, while II.E4 only recognized adducts derived from benzo[a]pyrene. In view of this specificity, the monoclonal antibody II.E4 may be suitable to selectively enrich B[a]P-DNA adducts by IAC. II.E4 was indeed successfully applied in IAC: compared to its affinity for *anti*-B[a]P-diolepoxide-modified DNA in IAC, set at 100%, the affinity of II.E4 for *syn*-B[a]P-diolepoxide- and chrysene-modified DNA was 68% and 6%, respectively (Booth *et al* 1994).

In general, it should be realized that the apparent cross-reactivity of an antibody depends on the technique used. It may well be that a monoclonal antibody directed against one particular adduct, does not show cross-reactivity towards other PAH-DNA adducts in immunocytochemical assays, but does reveal cross-reacting properties in IAC. Therefore, before DNA digests are subjected to IAC analysis, the cross-reactivity of the antibody under these conditions should be known. It is evident that the selectivity of IAC may be combined with the sensitivity of the ^{32}P-postlabeling or synchronous fluorescence spectrophotometry, which would make these techniques even more suitable for use in biomonitoring studies. As an alternative to IAC, specific adducts may be also selected by use of magnetic beads, precoated with antibodies directed against the F_c part of mouse or rabbit immunoglobulin. Incubation of these beads with adduct-specific antibody allows direct selection of the DNA adducts of interest. This approach is most promising, since it obviates the need to prepare IAC columns.

There are indications that DNA damage is not induced or repaired randomly in the genome. Furthermore, the exact location of damage in DNA appears to be a critical determinant of the effect of that specific damage. Therefore, rather than determining the overall genomic damage, it may be of more interest to determine DNA damage in specific genes. The introduction of the polymerase chain reaction (PCR) technique allows the selective study of any gene, provided that the appropriate primers are available. An elegant method to determine DNA-adduct levels in specific genes involves digestion of DNA with restriction enzymes, followed by enrichment of adduct-containing fragments by use of antibodies directed against the DNA adduct (Hochleitner *et al* 1991). PCR is then performed on a selected, relatively small region of the gene of interest in all isolated fragments. Due to the low adduct density, it can be virtually excluded that an obstructing adduct will be present in the fragment to be amplified. Quantification of the PCR product is achieved by hybridization with a ^{32}P-labeled oligonucleotide. Adduct formation and repair can thus be determined in any gene.

Bulky DNA lesions, such as those induced by B[a]P, may be an obstacle for DNA polymerase during PCR. A so-called PCR-stop assay was recently developed with cisplatin-modified DNA (Jennerwein and Eastman 1991). Cisplatin lesions in DNA also inhibit the progress of the DNA polymerase. The degree of inhibition of the PCR provides a direct quantification of the level of DNA damage in a selected gene. The PCR-stop assay may also be applied on cell lysates, derived from as few as 1,000 cells (Oshita and Eastman 1993). This method is excellently suited for small samples and does not require DNA purification and quantification. It is not yet possible to study the presence of DNA adducts at the level of the single cell by use of PCR techniques. However, the recent introduction of the so-called *in situ* reverse transcriptase PCR (RT-PCR) (Heniford *et al* 1993) makes it possible to detect variations in cellular mRNA expression of specific genes at the single-cell level, which may be a consequence of DNA-adduct induction. With regard to the study of carcinogenesis, it would be interesting to investigate changes in oncogene mRNA expression at the single-cell level by use of this *in situ* RT-PCR technique.

8.5.1 *The various forms of human lung cancer*

Malignant human lung cancers can be classified in four major categories, according to morphological criteria: (i) squamous-cell carcinomas, which express many markers associated with squamous cells in general, (ii) adenocarcinomas, which form a rather heterogenous group, some of which express mucous, (iii) small-cell carcinomas, originating from neuroendocrine cells, and (iv) large-cell carcinomas, which represent poorly differentiated forms of other tumor types. It is difficult to exactly determine the incidence of each type of lung cancer. However, squamous-cell carcinoma is the most frequent type of tumor (30 to 35% of all cases), followed by adenocarcinoma, large-cell carcinoma and small-cell carcinoma. Small- and squamous-cell carcinoma are the two types of lung cancer that are strongly associated with tobacco smoking: the relative risk of lung cancer among cigarette smokers compared to non-smokers is 31 for these types of tumors, whereas for adenocarcinomas the relative risk is only about 4. It should be noted, however, that the relative proportion of adenocarcinomas is increasing, *e.g.*, in the US. In certain populations, the risk of adenocarcinoma is exceptionally high, even when exposure to tobacco smoke has been low and short in duration. These populations include women in some areas in China. It is possible that in these populations the etiological factors involved are not related to cigarette smoking but to other life-style and environmental factors, *e.g.*, exposure during cooking of food (IARC 1986).

Increased genetic susceptibility to squamous- and small-cell lung cancer has been described for people who lack the GST Mu isozyme, combined with a specific genotype of CYP1A1 (Hayashi *et al* 1992, Shields *et al* 1993a). Mutations in the p53 tumor-suppressor gene were present in more than 60% of all squamous-cell carcinomas, while only 28% of all adenocarcinomas contained the p53 mutation (Mitsudomi *et al* 1993). With respect to the histological type, the distribution of different forms of lung cancer among non-smokers is similar to that in smokers. However, at the molecular level differences were found. In non-smokers G:C to A:T transitions were more common than G:C to T:A transversions, most often found in DNA of the p53 gene from lung cells of smokers (Takeshima *et al* 1993).

As was already discussed in Chapter 1, the hamster trachea appeared a valid model for the study of the main characteristics of human lung cancer. *In vivo*, intratracheal instillations of B[a]P mainly resulted in the formation of squamous-cell carcinomas and papillomas, while small-cell carcinomas were rarely observed. The main adduct, formed in hamster trachea epithelial cells, was the adduct between (+)-*anti*-B[a]P-diolepoxide and deoxyguanosine. We exposed primary human bronchial epithelial cells, obtained via a bronchial biopsy and cultured in a specific medium (de Jong *et al* 1993) to 20 μM B[a]P for 24 h at 37°C (results not shown). Epithelial cells were harvested, DNA was isolated and subjected to the ^{32}P-postlabeling procedure. The adduct pattern on TLC revealed one major spot, derived from the adduct between (+)-*anti*-B[a]P-diolepoxide and deoxyguanosine. This adduct is the same as found in hamster tracheas exposed to B[a]P in organ culture (see Chapter 4). That the choice for hamster trachea is not indiscriminate among rodent tissue follows from the interesting comparison of B[a]P-DNA adduct kinetics between hamster and rat tracheas. There is a marked difference in tracheal tumor response between the hamster and the rat when both species are intratracheally treated with B[a]P (Schreiber *et al* 1975). Rat tracheas were relatively insensitive to B[a]P-related effects, while in the hamster trachea tumors could be observed. *In vitro* DNA binding studies revealed that the B[a]P-DNA adduct level in hamster trachea epithelial cells, exposed to B[a]P in culture, was much higher than the adduct level in exposed rat trachea epithelial cells (Daniel *et al* 1983, Mass and Kaufman 1983).

The *in vitro* results mentioned above were confirmed in our own experiments, as presented in Chapter 4. A rather large difference was observed in DNA-adduct kinetics between hamster and rat tracheas, exposed to B[a]P in organ culture. It is generally accepted that the secretory cell type in the respiratory tract is responsible for metabolizing xenobiotic compounds (Plopper 1983, Plopper *et al* 1987). With respect to the relative amount of specific secretory cell types, there are some major differences between the hamster and the rat. The major secretory cell type in the hamster trachea is the small mucous granule cell, in contrast to the rat trachea, in which the major secretory cell type is the serous cell. The type of secretory cell and its respective secretion product may thus be of importance *in vivo* with respect to the effect of B[a]P; the chemical composition of the excretion product may, *e.g.*, prevent B[a]P from penetrating the epithelial cells, although also the rat epithelium appeared capable of bioactivation of B[a]P. In the trachea of the Bonnet monkey, a species close to man, the serous cell type was also absent; however, both the Clara-like cell type and the mucous cell type

could be observed, which is comparable to the situation in the hamster trachea (Plopper *et al* 1983).

In the *in vitro* organ culture, B[a]P-DNA adducts were detected in rat tracheal epithelial cells. However, no accumulation of B[a]P-DNA adducts was observed in these cells, whereas in hamster trachea epithelial cells a linear increase in B[a]P-DNA adduct level was found upon continuous exposure to B[a]P. With respect to the pattern of ^{32}P-postlabeled adducts on TLC, it was found that the adduct of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine was virtually the only adduct found in hamster tracheal epithelial cells, whereas in the rat, besides this adduct, also a substantial amount of another adduct derived from (±)-*syn*-B[a]P-diolepoxide, was found. This may indicate that in the rat trachea other CYP isozymes and biotransformation routes play an important role in B[a]P metabolism. The constant, relatively low adduct level in rat trachea epithelial cells was accompanied by a relatively high cell proliferation. Therefore, the development of precancerous lesions in the rat trachea might be expected to occur. Evidence for this is given by the fact that *in vivo* exposure of rats to cigarette smoke, indeed induced precancerous lesions in tracheal epithelium. This indicates that compounds of cigarette smoke were in fact capable of penetrating epithelial cells *in vivo*. Apparently, these lesions do not develop into tumors in this species.

8.6.1 Toxicity and cell proliferation

There is general consensus that the development of neoplasia is a multistep process that involves multiple mutational events (Bishop 1987). Recently there has been a lively debate in the literature on the role of cell proliferation in the process of carcinogenesis. It is evident that cell proliferation is a prerequisite for mutations to occur. The crucial role of cell proliferation in carcinogenesis is further emphasized by the fact that non-dividing cells in adults, such as nerve cells and cardiomyocytes, rarely develop tumors (Preston-Martin *et al* 1990). Taking into account the surprisingly high endogenous rate of DNA-damage induction, which is estimated to be 10^4 hits per cell per day in humans, it was postulated that stimulation of cell division, *i.e.*, mitogenesis may be mutagenic (Ames and Gold 1990, 1991). The standard rodent bioassays, in which chemicals are often tested at the maximum tolerated dose, may lead to toxic effects in specific tissues that give rise to extensive cell proliferation as a regenerative response. This relatively high level of cell proliferation may accelerate the rate of DNA mutation and, as a result, the process of tumor formation, which would lead to an increased number of false positives in rodent bioassays. However, in a recent evaluation of 31 chemicals with respect to their carcinogenic potential by the National Toxicology Program, evidence was provided that toxic injury, defined as any deleterious change that can be discerned by histopathology in the tissues of the exposed animals, was not always associated with induction of chemical carcinogenesis (Tennant *et al* 1991). Furthermore, if we consider that 10^{11} cell divisions take place daily in the human body, and that spontaneous mutations occur once in a million cell divisions, about 10^5 cells would mutate daily. Even if we assume that only a fraction of these cells would give rise to cancer cells, it would still mean that tumors predominantly occur in tissues with a relatively high proliferation rate. This is not reflected in cancer incidence data (Iversen 1992).

We therefore must consider the three inequalities in cancer risk assessment: carcinogenesis can not be regarded as identical to mutagenesis and/or cell proliferation, neither is toxicity equivalent to cell proliferation. This does not mean that cell toxicity, cell proliferation and carcinogenesis are unrelated, but only emphasizes the fact that the carcinogenic process is complex, involving intricate interactions between various biological variables (Cohen and Ellwein 1991, Weinstein 1991, 1992).

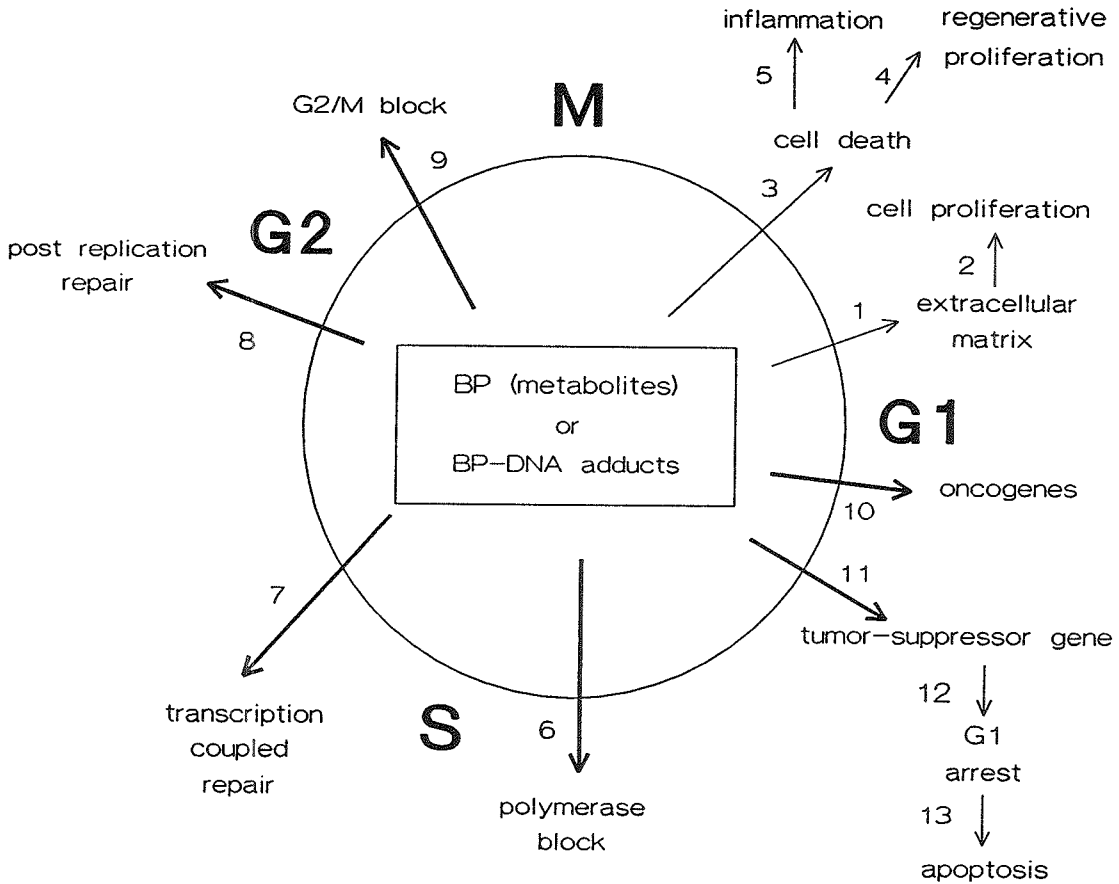

Figure 1 *Possible interference of adducts with cell proliferation. The position of the bold arrows refer to their respective position in the cell cycle. The biological significance of all arrows is explained in the text.*

In Chapter 6 results are described of a study on the effect of B[a]P-DNA adducts on cell proliferation in hamster tracheal epithelium. The negative correlation between B[a]P exposure and cell proliferation we observed is in contrast to data in the literature: cell proliferation increased in epithelial cells in hamster tracheas, exposed to B[a]P in explant culture (Chopra and Cooney 1985). Furthermore, exposure to cigarette-smoke condensate stimulated cell proliferation in hamster tracheas in organ culture (Rutten *et al* 1988b). The possible ways by which B[a]P, B[a]P metabolites or B[a]P-DNA adducts may interfere with cell proliferation are discussed below (*cf* Fig 1).

As was already mentioned in Chapter 1, cells of the normal tracheal epithelium have a rather low turnover rate. There is a strict balance between cell proliferation and differentiation, presumably controlled by growth factors EGF-α, TGF-α and TGF-β. Recently, the role of the extracellular matrix, *i.e.* the basal membrane in case of tracheal epithelium, has been the subject of investigation (reviewed by Haralson 1993). The basal membrane is not only a stable structure that provides support to epithelial cells, but it may also regulate the balance between proliferation and differentiation. Therefore, damage to the basal membrane as a result of B[a]P treatment, may also affect this balance (arrows 1 and 2).

In vivo and *in vitro* treatment with carcinogens or mechanical trauma may enhance cell proliferation in the tracheal epithelium (Boren 1970, Keenan *et al* 1982, Chopra and Cooney 1985, Boucher *et al* 1988, Rutten *et al* 1988b), which is accompanied by well-characterized morphological changes. The increase in cell proliferation is of a regenerative nature, as it is usually preceded by cell death (arrows 3 and 4). A more general consequence of cell death is the initiation of inflammation (arrow 5). The cells that are involved in the process of acute inflammation, *i.e.* the granulocytes, also specifically contribute to B[a]P metabolism by myeloperoxidase-mediated reactions (see also Chapter 1), which may eventually lead to increased B[a]P-DNA adduct formation.

Bulky adducts, such as those induced by B[a]P, are an obstacle to DNA polymerase (arrow 6). This block may thus prolong the S-phase of the cell cycle, allowing transcription-coupled repair (arrow 7) to take place. Recent studies have shown that helicase enzymes are involved in transcription and repair, which both require strand separation (Buratowski 1993). DNA lesions still present after the termination of the S-phase, can be removed by a process called post-replication repair (Kaufmann 1989, Kaufmann and Kaufman 1993; see arrow 8). Even after this repair process has been completed, a so-called checkpoint in the G_2 phase of the cell cycle monitors DNA for the presence of possibly remaining damage (Pardee 1993). Cells that still contain too heavily

damaged DNA, remain arrested in a G_2/M block, and are prevented to enter mitosis (arrow 9).

The proliferation of normal cells is thought to be regulated by growth-promoting proto-oncogenes counterbalanced by the activity of growth-constraining tumor-suppressor genes (Cobrinik *et al* 1992). Mutations that potentiate the activities of proto-oncogenes and convert them to oncogenes evoke uncontrolled cell growth. Certain oncogene products (arrow 10) are known to be specific substitutes for growth factors. Normally, the sustained presence of specific external growth factors is needed to allow a cell to proceed through the G_1 phase. Expression of oncogenes may compensate for this, thereby stimulating cells to proliferate autonomously (Aaronson 1991). Conversely, genetic lesions that inactivate tumor-suppressor genes liberate the cell from the constraints imposed, which leads to the unrestricted growth of the cancer cell (Weinberg 1991).

Among the tumor-suppressor genes (arrow 11), p53 is probably one of the most extensively studied. Mutations of the p53 tumor-suppressor gene are the most frequently detected genetic alterations in human cancers (Hollstein *et al* 1991, Levine *et al* 1991, Allred *et al* 1993, Frebourg and Friend 1993). The function of the p53 gene has been implicated in the regulation of the cell cycle: when DNA damage occurs, the genome-guarding function of p53 is induced, which becomes apparent as accumulation of the p53 protein. This gives rise to an arrest of the cell cycle in the G_1 phase (arrow 12; Kastan *et al* 1991, Lane 1992, Yonish-Rouach *et al* 1993). Accumulation of p53 protein in response to the presence of DNA damage is not the result of enhanced gene expression, but rather of increased p53 protein stability (Fritsche *et al* 1993). Recently it was found that the p53 protein turns on the gene for a 21 kilodalton protein that blocks cyclin-dependent kinases and thus cell division (Marx 1993). When DNA damage is properly repaired during the arrest in G_1, the cell may proceed through the S phase of the cell cycle. When DNA damage is not properly repaired, the p53 gene activity may eventually lead to apoptosis, a process in which cells die in a programmed manner (Lane 1992; arrow 13). Programmed cell death is essentially different from the process of necrosis, as in apoptosis no inflammatory responses are evoked as a consequence of cell death (Fawthorp *et al* 1991).

Evidence that p53 expression is altered *in vivo* after exposure to a carcinogen was recently presented (Bjelogrlic *et al* 1994). Mice were topically exposed to B[a]P; at different time intervals skin DNA was analyzed for the presence of DNA adducts by SFS, while the presence of p53 protein was determined with the use of a polyclonal antibody. It was found that in the highest dose group, *i.e.* 500 μg B[a]P per mouse, 9 out of 17 skin samples were positive for p53 staining at 12 to 48 h after treatment. The highest B[a]P-DNA adduct levels were also detected in this time interval. This finding provides further

evidence for the connection between DNA damage and p53 expression. It may thus well be that in our *in vitro* model B[a]P elicited an increase in the intracellular level of p53 protein, thereby delaying the cell cycle.

8.6.3 DNA adducts, cell proliferation and precancerous lesions

The formation of B[a]P-DNA adducts was determined in two cell populations of the hamster tracheal epithelium, namely the basal cells and the non-basal cells, the latter population comprising small mucous granule cells, mucous goblet cells and ciliated cells. The criterion to distinguish basal cells *in situ* in cytospin preparations was positive staining with RCK102. The mouse monoclonal antibody RCK102 recognizes basal-cell specific cytokeratins, which appeared stable enough during the entire immunofluorescence protocol, to produce reliable staining. It is possible that exposure to B[a]P influences cytokeratin expression, thereby resulting in non-basal cells that stain positively with RCK102, or basal cells that do not stain with RCK102. Because the fraction of cells that were RCK102 positive in the control tracheas was comparable to that in all exposed tracheas, misclassification is not likely to have occurred.

The secretory cell, which is a non-basal cell, is known for its rather extensive endoplasmic reticulum, which contains cytochrome P450, in contrast to both the basal and the ciliated cell. It is intriguing to note that, despite this observation, we did not find any significant difference in B[a]P-DNA adduct level between basal and non-basal cells under the conditions as described in Chapters 5 and 7. Furthermore, no evidence was found for the presence of a subpopulation of cells with low adduct levels - which might have been the ciliated cells - within the class of non-basal cells. On the one hand this may be explained by intercellular transport of reactive B[a]P metabolites. On the other hand, the presence and activity of detoxifying enzymes, such as glutathione S-transferases, may be different from one cell type to the other. By use of the fluorescence automated cell sorter, it may be feasible to separate basal cells from non-basal cells on the basis of the difference in RCK102 staining. In the respective cell populations, the presence and activity of the GST Pi isozyme can then be determined. It should be realized that in hamster tracheas the level of GST isozymes of the Pi class is about 10-fold higher than the level of GST Mu isozymes (Bogaards *et al* 1992). In view of the known affinity of Pi-class isozymes for B[a]P-diolepoxides, this particular isozyme probably plays a more important role in detoxification of B[a]P metabolites in the hamster trachea than does the Mu class isozyme.

Under the experimental conditions described in Chapter 5, *in situ* detection in cytospin preparations of hamster tracheal epithelial cells exposed to 40 μM B[a]P

revealed that B[a]P-DNA adducts disappeared more rapidly in basal cells (from 49% of the maximum adduct level at 24 h after exposure to 35% at 72 h after exposure) than in non-basal cells (from 63% of the maximum adduct level at 24 h after exposure to 42% at 72 h after exposure). The question may be raised whether this removal is due to active DNA repair or is the result of cell proliferation. Because the extent of proliferation is not large enough to explain the loss of DNA adducts, it is likely that active DNA repair occurred. Under the conditions described in Chapter 4, about 60% of the maximum adduct level could still be detected at 24 to 96 h after exposure to 20 μM B[a]P, as determined by ^{32}P-postlabeling. Proliferation of non-basal cells was higher than that of basal cells, while removal of adducts from non-basal cells was significantly slower. The biological explanation for this faster repair of B[a]P-DNA adducts in basal cells may be that this cell type is generally considered to be the major stem cell in tracheal epithelium (see Chapter 1) and is most probably the only cell type that can give rise to all other major cell types in this tissue (Nettesheim *et al* 1990). Thus it is of interest for the epithelium to give preference to DNA repair in basal cells.

Cell proliferation was determined for both basal and non-basal cells by use of the labeling index, *i.e.*, the relative number of labeled cells amongst the total number of cells. The labeling index of non-basal cells was significantly higher than that of basal cells, also in control tracheal epithelium. Misclassification of cells may have occurred, *e.g.*, as a result of upward migration of basal cells towards the tracheal lumen during the 18h-period of [^{3}H]thymidine incorporation. It is, however, not plausible to assume that an extensive migration of basal cells has taken place. A possible explanation for the relatively high labeling index in non-basal cells, even in untreated tracheas, may be the response of the epithelium to the transition from an air-environment to an aqueous environment.

By use of our definition of basal cells, *i.e.*, cells in contact with the basal lamina and with a cytoplasm that does not reach the tracheal lumen, only about 15 to 20% of all epithelial cells within one tracheal ring were considered to be of the basal cell type. When this low proportion is taken into account, the number of basal cells in S phase relative to the total number of basal cells was actually the same or even larger than the number of non-basal cells in S phase, relative to the total number of non-basal cells. This stresses the importance of basal cell proliferation in the tracheal epithelium. However, to evaluate the consequences of cell proliferation on the process of tumor development, one should not only consider the relative number of cells in S phase, but rather the total number of cells in S phase. In that case, the relatively high number of non-basal cells that are in S phase may indeed be of great importance as to the consequences of a high cell proliferation rate.

The relationship between cell proliferation and precancerous lesions that was observed in Chapter 7 was not surprising: cell proliferation is a prerequisite for precancerous lesions to develop. What did appear surprising was the effect of continuous exposure to B[a]P on cell proliferation. Possible reasons for the inhibitory effect of B[a]P exposure on cell proliferation were already discussed in 8.6.2. A clear increase in the severity of precancerous lesions was only observed in our experiments in case of a 2-day exposure to B[a]P, followed by a 5-day recovery period, during which the cell proliferation recovered to control levels. In view of DNA-adduct kinetics and the extent of proliferation, it can be assumed that the non-basal cell type is responsible for the observed precancerous lesions.

In Western societies, lung cancer is the major cause of death of cancer in both men and women. There is substantial epidemiological evidence that tobacco smoking is related to the incidence of lung cancer. Polycyclic aromatic hydrocarbons (PAHs), of which some have been proven to be carcinogenic in rodents, form as such a constituent of tobacco smoke. The type of mutations frequently observed in DNA of lung tumor tissue indicates that PAHs may play an important role in the initiation of tobacco-smoke-related lung cancer. PAHs are also found in the environment as a consequence of traffic (exhaust fumes), industrial activity, residential wood combustion, food preparation, etc.

The respiratory tract of the Syrian golden hamster has proven to be a suitable model to study the main characteristics of carcinogenesis in the human lung. Morphologically, the respiratory tract of the hamster closely resembles that of humans. Furthermore, intratracheal instillations of benzo[a]pyrene (B[a]P), a model compound within the group of PAHs, induce lesions in the hamster respiratory tract that have close similarities to those observed during the development of lung cancer in humans. The lesions that precede cancer formation *in vivo* can also be induced *in vitro*: in hamster tracheas that were exposed to cigarette-smoke condensate, squamous metaplasia and hyperplasia could be observed. We used this *in vitro* model to study DNA-adduct formation and repair, proliferation of both basal and non-basal cells and the development of precancerous lesions, in hamster tracheas exposed to B[a]P in organ culture. The evaluation of all these parameters was expected to provide more insight into the role of different cell types in the process of tumor formation.

We employed two techniques to determine the level of B[a]P-DNA adducts in hamster trachea epithelial cells, namely the ^{32}P-postlabeling and an immunocytochemical assay. With respect to the ^{32}P-postlabeling procedure, several methodological improvements were introduced. First, FPLC analysis of DNA digests was applied to check the efficiency of DNA digestion; this analysis has the added advantage that the absence of RNA-derived nucleotides can be verified, and the amount of input-DNA can be exactly determined. Second, the use of B[a]P-DNA standards with known modification levels was introduced, which allows a more accurate way of adduct quantification, compared to the traditional relative adduct labeling (RAL) method. These B[a]P-DNA standards were prepared by reaction of different amounts of (±)-*anti*-B[a]P-diolepoxide with isolated DNA, and analyzed by means of synchronous fluorescence spectrophotometry to determine the modification level. Routinely, two B[a]P-DNA

standards are included in each postlabeling experiment; the modification level of these two standards is chosen within the range expected for the samples to be analyzed. Third, it was found that polyaromatic adducts, when present in DNA digests on TLC, are sensitive to light, especially when the TLC plates are air-dried in between the chromatographic steps. Therefore, postlabeling experiments were carried out under subdued light conditions.

The ^{32}P-postlabeling procedure, with the improvements as described above, was applied in a study in which liver microsomes - *i.e.* subcellular fractions that contain bioactivating enzymes - from different species, including man, were tested for their capacity to metabolize B[a]P to DNA-reactive intermediates, with either isolated DNA or human white blood cells (WBC) as a target. Postlabeling of the modified DNA revealed some major interspecies differences in the metabolic conversion of B[a]P into DNA-binding compounds. The adduct pattern, obtained after incubation of WBC with microsomes and B[a]P, more closely resembled the pattern obtained after *in vivo* adduct formation. Experiments like these may offer an attractive system for initial screening of chemical compounds, in order to determine whether specific metabolites are formed that possess DNA-binding activity. ^{32}P-postlabeling analysis of modified DNA may in this case be limited to one-directional chromatography on TLC or separation by HPLC, to obtain an indication of the total modification level in a fast and relatively simple way.

The major advantage of the ^{32}P-postlabeling procedure is its sensitivity (1 adduct in 10^9 nucleotides) and the possibility to separate different adduct types. However, it does not allow the study of DNA-adduct formation in individual cells. This is of importance in view of the role of certain cell types in carcinogenesis. *In situ* methods, using antibodies directed against DNA adducts, are well suited to this purpose. We generated antibodies in rabbits by immunization with B[a]P-modified DNA. The antiserum obtained, W2/01, appeared suitable for adduct detection *in situ* in cytospin preparations of whole-cell isolates of hamster trachea epithelial cells. The lower detection limit was 1 B[a]P-DNA adduct in 10^6 nucleotides. We developed a procedure that allows the discriminative detection of B[a]P-DNA adducts in both basal and non-basal cells, within one preparation. This procedure involves the use of the adduct-specific antiserum W2/01 and a mouse monoclonal antibody RCK102, which recognizes basal-cell specific cytokeratins, in addition to a DNA staining to localize cell nuclei. The presence of B[a]P-DNA adducts and cytokeratins were visualized by use of two different fluorochromes. Image-processing software that was developed and optimized for this application allowed the quantification of adduct-specific staining in distinct cell populations, *viz.* basal and non-basal cells.

Both adduct-detection techniques described above were used in the *in vitro* hamster trachea model. In hamster tracheas continuously exposed in organ culture for

15 days to 20 μM B[a]P, DNA adducts were found to steadily accumulate in time. In contrast, at two lower doses of B[a]P, *i.e.* 2 and 4 μM, no sustained increase in B[a]P-DNA adduct level was observed. Irrespective of exposure time and dose, the major (>95%) adduct found after ^{32}P-postlabeling of DNA from hamster tracheal epithelial cells, was that of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine, which is considered to be very likely the DNA lesion that initiates carcinogenesis. In DNA of hamster tracheal epithelial cells exposed for two days to 20 μM B[a]P in organ culture followed by a recovery period, the B[a]P-DNA adduct level decreased to about 60% of the maximum level, within 24h after termination of exposure, as determined by ^{32}P-postlabeling. The reduction in the B[a]P-DNA adduct level was most probably due to active DNA repair - measured by unscheduled DNA synthesis - rather than to 'dilution' caused by ongoing cell proliferation. The rat, in contrast to the hamster, does not develop tracheal tumors after intratracheal instillations of B[a]P. In rat tracheas continuously exposed to 20 μM B[a]P for 15 days in organ culture, no accumulation of B[a]P-DNA adducts was observed. With respect to the adduct pattern on TLC, determined by ^{32}P-postlabeling, it was found that an adduct derived from interaction of *syn*-B[a]P-diolepoxide with deoxyadenosine formed a substantial part of the total adduct level (about 30%), while the adduct of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine comprised about 60%. In human bronchial epithelial cells exposed to 20 μM B[a]P in culture for 24h, only the adduct of (+)-*anti*-B[a]P-diolepoxide to deoxyguanosine was detected with ^{32}P-postlabeling. Taken together, these findings indicate that the metabolic pathways leading to conversion of B[a]P into DNA-binding metabolites in hamster epithelial cells and human bronchial cells are quite similar, while activation of B[a]P in rat tracheal epithelial cells also occurs via other routes. The observation that B[a]P-DNA adducts in rat tracheal epithelial cells do not accumulate, whereas a linear increase was observed in hamster tracheal epithelial cells, is in line with the difference in tracheal tumor susceptibility between the two species. However, it should be noted that in rat tracheas still a substantial level of the adduct, derived from the ultimate carcinogen of B[a]P, was formed. With respect to the different cell types in tracheal epithelium, it is interesting that the major secretory cell type - *i.e.* the cell type mainly responsible for metabolism of xenobiotic compounds - found in the rat trachea is the serous cell, while in both the hamster and the monkey, a species more close to man, this particular cell type is absent. The type of secretion product found in the rat trachea may thus determine the extent of B[a]P-DNA adduct formation in the epithelial cells.

In cytospin preparations of B[a]P-exposed hamster tracheal epithelial cells, no significant difference was found in B[a]P-DNA adduct formation between basal and non-basal cells after continuous exposure to 4 or 20 μM B[a]P, or a 2-day exposure to 40 μM

B[a]P. Removal of B[a]P-DNA adducts, as determined at the single-cell level, was faster in basal cells than in non-basal cells. This preference for DNA repair in basal cells is in line with the relative importance of the basal cell population, being essentially the only cell type that can give rise to all other major cell types in tracheal epithelium. Cell proliferation was furthermore found to be significantly higher in non-basal cells than in basal cells, irrespective of treatment. The presence of B[a]P-DNA adducts was negatively correlated with proliferation of epithelial cells. The extent of precancerous lesions, such as squamous metaplasia and nodular hyperplasia was more marked in tracheas in which cell proliferation had been allowed to recover after B[a]P exposure, as compared to tracheas in which cell proliferation had been suppressed by continuous exposure to B[a]P. In view of B[a]P-DNA adduct kinetics and cell proliferation, the data strongly suggest that the non-basal cell is responsible for the observed precancerous effects in hamster tracheas exposed to B[a]P in organ culture.

In westerse landen is longkanker de belangrijkste oorzaak van kankersterfte bij zowel mannen als vrouwen. Epidemiologisch onderzoek heeft aangetoond dat roken geassocieerd is met de incidentie van longkanker. Polycyclische aromatische koolwaterstoffen (PAKs), waarvan sommige carcinogeen blijken te zijn in proefdieren, vormen een bestanddeel van tabaksrook. Uit het type mutatie dat vaak wordt aangetroffen in DNA uit longtumoren kan worden afgeleid dat PAKs een rol kunnen spelen in de initiatie van aan het roken gerelateerde longkanker. Behalve in tabaksrook worden PAKs ook gevonden in uitlaatgassen van verbrandingsmotoren, en komen ze vrij bij industriële activiteit, bij het stoken van de open haard, bij de voedselbereiding, enz.

De *tractus respiratoricus* van de Syrische goudhamster blijkt een goed model te zijn om de karakteristieken van de ontwikkeling van humane longkanker te bestuderen: in de eerste plaats zijn er grote morfologische overeenkomsten tussen de bovenste luchtwegen van de hamster en die van de mens. Verder is gebleken dat intratracheale toediening van benzo[a]pyreen (B[a]P), een modelstof uit de groep van PAKs, lesies induceert in de hamstertrachea die lijken op de lesies die gevonden worden bij de ontwikkeling van humane longtumoren. Morfologische veranderingen die voorafgaan aan tumorvorming *in vivo*, de zogenoemde precarcinogene lesies, kunnen ook *in vitro* worden geïnduceerd in orgaankweek van de hamstertrachea: zowel plaveiselcel-metaplasie als hyperplasie ontstaan in hamstertrachea's die zijn blootgesteld aan sigaretterook-condensaat. In dit proefschrift zijn experimenten beschreven met dit *in vitro* hamstertrachea-model; daarbij is specifiek gekeken naar DNA-adductvorming en -herstel, proliferatie van basaal- en niet-basaalcellen en de ontwikkeling van precarcinogene lesies in hamstertrachea's blootgesteld aan B[a]P in orgaankweek. Het bestuderen van de relatie tussen deze parameters kan tot meer inzicht leiden in de rol van verschillende celtypen in het proces van tumorvorming.

Om de hoeveelheid B[a]P-DNA-adducten in epitheelcellen van de hamstertrachea epitheelcellen te bepalen, werd gebruik gemaakt van twee technieken, te weten de ^{32}P-postlabeling en een immunocytochemische analyse. Voor wat betreft de eerste techniek werden verschillende methodologische verbeteringen geïntroduceerd: in de eerste plaats werd een FPLC-analyse toegepast om de efficiëntie van de DNA-afbraak te controleren. Deze analyse maakt het tevens mogelijk na te gaan in hoeverre het DNA vrij is van RNA, terwijl aan de hand van het elutiepatroon de hoeveelheid DNA gebruikt in de ^{32}P-postlabeling nauwkeurig kan worden bepaald. In de tweede plaats werden B[a]P-DNA-

standaarden met bekende adduct-niveau's gesynthetiseerd en gebruikt bij het quantificeren van B[a]P-DNA-adducten in onbekende monsters. Deze manier van quantificeren leverde een substantiële verbetering op ten opzichte van de traditionele manier, die gebaseerd is op relatieve adduct-labeling (RAL). Deze B[a]P-DNA-standaarden werden verkregen door DNA *in vitro* te laten reageren met verschillende concentraties van het (±)-*anti*-B[a]P-diolepoxide en vervolgens het adductniveau te bepalen met behulp van synchrone fluorescentie-spectrofotometrie, onafhankelijk van de ^{32}P-postlabeling. Twee van deze B[a]P-DNA-standaarden worden in ieder experiment meegenomen; het adductniveau van die standaarden wordt in dezelfde grootte-orde gekozen als verwacht kan worden in de te analyseren monsters. In de derde plaats is gebleken dat polycyclische aromatische adducten op dunnelaag-chromatogrammen zeer gevoelig zijn voor dag-licht, vooral wanneer deze aan de lucht worden gedroogd tussen de verschillende chromatografische stappen. Postlabeling-experimenten dienen daarom te worden uitgevoerd in een van daglicht afgeschermde omgeving.

De ^{32}P-postlabelingmethode werd toegepast in experimenten waarbij levermicrosomen - oftewel subcellulaire fracties die bioactiverende enzymen bevatten - van verschillende species, waaronder de mens, werden getest op hun vermogen om B[a]P te metaboliseren tot intermediairen die met DNA of met witte bloed cellen (WBC) kunnen reageren. Na ^{32}P-postlabeling van het gemodificeerde DNA werden aanzienlijke interspecies-verschillen gevonden. Het adductpatroon dat werd waargenomen na incubatie van WBC met microsomen en B[a]P kwam echter het meest overeen met patronen die werden gevonden na *in vivo* adductvorming. Dit type experimenten vormt een attractief modelsysteem voor een eerste screening van potentieel genotoxische stoffen. In dit geval zou de analyse van gemodificeerd DNA op het dunnelaag-chromatogram beperkt kunnen blijven tot één stap, om op een snelle en relatief eenvoudige wijze een indruk te krijgen van het adductniveau.

Het grootste voordeel van de ^{32}P-postlabeling is de gevoeligheid (1 adduct per 10^9 nucleotiden) en de mogelijkheid om verschillende typen adducten te scheiden. Het is echter niet mogelijk de hoeveelheid adducten per cel te quantificeren, hetgeen van belang kan zijn gezien de mogelijke rol van bepaalde celtypen in het proces van tumorvorming. *In situ* methoden, waarbij gebruik wordt gemaakt van antilichamen tegen DNA-adducten, bieden dit voordeel wel. Het door ons ontwikkelde antiserum W2/01 bleek in staat B[a]P-DNA-adducten *in situ* aan te tonen in cytospin-preparaten van de epitheelcellen van de hamstertrachea (detectie limiet circa 1 adduct per 10^6 nucleotiden). Met de drievoudige kleuringsmethode die werd ontwikkeld bleek het verder mogelijk B[a]P-DNA-adducten in basaal- en niet-basaalcellen te bepalen. De methode bestaat uit het aankleuren DNA-adducten met W2/01, het zichtbaar maken van basaalcel-specifieke

cytokeratinen met RCK102, een commercieel verkrijgbaar monoclonaal antilichaam (muis), en het tegenkleuren van DNA voor lokalisatie-doeleinden. Software voor de beeldbewerking werd geoptimaliseerd om adduct-specifieke kleuring in zowel basaalcellen als niet-basaalcellen te quantificeren.

Beide hierboven beschreven methoden werden toegepast in het *in vitro* hamstertrachea-model. In trachea's die continu werden blootgesteld aan 20 μM B[a]P gedurende 15 dagen, bleken DNA-adducten te accumuleren in de tijd. Daarentegen werd bij twee lagere doses, te weten 2 en 4 μM, geen continue verhoging van het adduct-niveau gevonden. Met behulp van co-chromatografie na ^{32}P-postlabeling kon het voornaamste adduct (>95%) in DNA uit epitheelcellen van de hamstertrachea met zeer grote waarschijnlijkheid worden geïdentificeerd als het adduct tussen (+)-*anti*-B[a]P-diolepoxide en deoxyguanosine. Dit adduct wordt algemeen beschouwd als de lesie die de carcinogenese initieert. In het DNA van epitheelcellen van trachea's die 2 dagen waren blootgesteld aan 20 μM B[a]P in orgaankweek, gevolgd door een herstelperiode, verminderde het adduct-niveau na 24 uur tot 60% van het maximum. Deze vermindering is waarschijnlijk het gevolg van actief DNA-herstel, dat gemeten werd met UDS, en niet van celdeling. In tegenstelling tot de hamster, ontwikkelt de rat geen tracheale tumoren na intratracheale toediening van B[a]P. In rattetracheas die continu werden blootgesteld aan 20 μM B[a]P gedurende 15 dagen, werd geen accumulatie van B[a]P-DNA-adducten gevonden. Het adductpatroon na postlabeling liet zien dat een substantieel deel van de totale hoeveelheid adducten (~30%) afkomstig was van de interactie tussen *syn*-B[a]P-diolepoxide en deoxyadenosine, terwijl het adduct tussen (+)-*anti*-B[a]P-diolepoxide en deoxyguanosine ongeveer 60% van het totaal uitmaakte. In humane bronchiaal-epitheelcellen blootgesteld aan 20 μM B[a]P gedurende 24 uur, werd na ^{32}P-postlabeling alleen het adduct tussen (+)-*anti*-B[a]P-diolepoxide en deoxyguanosine gevonden. Deze resultaten bevestigen dat de metabole routes die B[a]P omzetten in DNA-reactieve metabolieten in epitheelcellen van de hamstertrachea vergelijkbaar zijn met die in humaan bronchiaalepitheel, terwijl activering van B[a]P in rattetrachea kennelijk ook via andere routes verloopt. Het feit dat B[a]P-DNA-adducten bij de rat niet accumuleren, in tegenstelling tot de situatie in hamster trachea-epitheel, is in overeenstemming met het verschil in gevoeligheid voor tracheatumoren tussen beide species. Echter, een aanzienlijk deel van de totale hoeveelheid adducten in rattetrachea-epitheel is wel degelijk afkomstig van het 'ultimate carcinogen' van B[a]P. Ook bij de rat dringen reactieve B[a]P-metabolieten door tot in de kern van de trachea-epitheelcellen. De voornaamste secreterende cel - het celtype waarin de belangrijkste metabole omzettingen plaatsvinden - die wordt aangetroffen in de rattetrachea is de sereuze cel. Dit specifieke celtype wordt niet gevonden in de hamstertrachea. Het type secretie-produkt dat wordt aangetroffen

in de rattetrachea zou als zodanig de vorming van B[a]P-DNA-adducten kunnen beïnvloeden.

In cytospin-preparaten van epitheelcellen uit met B[a]P behandelde hamstertrachea werd geen significant verschil gevonden in B[a]P-DNA-adductvorming tussen basaal- en niet-basaalcellen na continue blootstelling aan 4 of 20 μM B[a]P, of na een tweedaagse blootstelling aan 40 μM B[a]P. Verwijdering van B[a]P-DNA-adducten, bepaald op enkel-cel-niveau, verliep sneller in basaalcellen dan in niet-basaalcellen. Deze voorkeur voor herstel van DNA-schade in basaalcellen is in overeenstemming met de rol van de basaalcel in het epitheel: de basaalcel is de enige cel van waaruit alle overige celtypen in het epitheel kunnen ontstaan. Proliferatie bleek voornamelijk te geschieden in de niet-basaalcel fractie, zowel in behandelde als onbehandelde trachea's. De hoeveelheid B[a]P-DNA-adducten was negatief gecorreleerd met de proliferatie van de epitheelcellen. De ernst van de precarcinogene lesies, plaveiselcel-metaplasie en nodulaire hyperplasie, was het meest uitgesproken in trachea's waarin de celproliferatie hersteld was, in vergelijking met trachea's waarin celproliferatie onderdrukt bleef door de continue blootstelling aan B[a]P. Gezien de kinetiek van B[a]P-DNA-adducten en celproliferatie lijkt de conclusie gerechtvaardigd dat de niet-basaalcel fractie verantwoordelijk is voor de precarcinogene effecten die worden gevonden in hamstertrachea's blootgesteld aan B[a]P in orgaankweek.

Aaronson SA (1991) Growth factors and cancer. *Science*, **254**, 1146-1153.

Allred DC, Clark GM, Elledge R, Fuqua SAW, Brown RW, Chamness GC, Osborne CK, McGuire WL (1993) Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst*, **85**, 200-206.

Ames BN, Gold LS (1990) Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science*, **249**, 970-971.

Ames BN, Gold LS (1991) Endogenous mutagens and the causes of aging and cancer. *Mutat Res*, **250**, 3-16.

Bishop JM (1987) The molecular genetics of cancer. *Science*, **235**, 305-311.

Bjelogrlic NM, Mäkinen M, Stenbäck F, Vähäkangas K (1994) Benzo[a]pyrene-7,8-diol-9,10-epoxide-DNA adducts and increased p53 protein in mouse skin. *Carcinogenesis*, **15**, 771-774.

Bogaards JJP, van Ommen B, van Bladeren PJ (1992) Purification and characterization of eight glutathione S-transferase isoenzymes of hamster. *Bioch J*, **286**, 383-388.

Boren HG (1970) Pulmonary cell kinetics after exposure to cigarette smoke. Inhalation carcinogenesis, Proceedings of a biology division, Gatlinburg, TN, October 8-11, 1969, 229-241.

Boucher RC, van Scott MR, Willumsen N, Jackson Stutts M (1988) Mechanisms and cell biology of airway epithelial injury. *Am Rev Respir Dis*, **138**, S41-S44.

Buratowski S (1993) DNA repair and transcription: the helicase connection. *Science*, **260**, 37-38.

Chopra DP, Cooney RA (1985) Histogenesis of benzo(a)pyrene-induced lesions in tracheal explants. *Virch Arch B Cell Pathol*, **48**, 299-315.

Cobrinik D, Dowdy SF, Hinds PW, Mittnacht S, Weinberg RA (1992) The retinoblastoma protein and the regulation of cell cycling. *TIBS*, **17**, 312-315.

Cohen SM, Ellwein LB (1991) Genetic errors, cell proliferation, and carcinogenesis. *Cancer Res*, **51**, 6493-6505.

Daniel FB, Schut HAJ, Sandwisch DW, Schenck KM, Hoffmann CO (1983) Interspecies comparisons of benzo(a)pyrene metabolism and DNA-adduct formation in cultured human and animal bladder and tracheobronchial tissues. *Cancer Res*, **43**, 4723-4729.

Fawthorp DJ, Boobis AR, Davies DS (1991) Mechanisms of cell death. *Arch Toxicol*, **65**, 437-444.

Frebourg T, Friend SH (1993) The importance of p53 gene alterations in human cancer: is there more than circumstantial evidence? *J Natl Cancer Inst*, **85**, 1554-1557.

Fritsche M, Haessler C, Brandner G (1993) Induction of nuclear accumulation of the tumor-suppresor protein p53 by DNA-damaging agents. *Oncogene*, **8**, 307-318.

Grant ER, Cooks RG (1990) Mass spectrometry and its use in tandem with laser spectroscopy. *Science*, **250**, 61-68.

Gross ML, Cerny RL (1993) Structure determination of DNA adducts of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene by using tandem four-sector mass spectrometry. In Garrigues P, Lamotte M, Eds. Polycyclic aromatic compounds. Synthesis, properties, analytical measurements, occurrence and biological effects, Gordon and Breach Science Publishers, Langhorne, 749-756.

Grzybowska E, Hemminki K, Szeliga J, Chorazy M (1993) Seasonal variation of aromatic DNA adducts in human lymphocytes and granulocytes. *Carcinogenesis*, **14**, 2523-2526.

Gupta RC, Reddy MV, Randerath K (1982) ^{32}P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis*, **3**, 1081-1092.

Haralson MA (1993) Extracellular matrix and growth factors: an integrated interplay controlling tissue repair and progression to disease. *Lab Invest*, **69**, 369-372.

Harris TM, Stone MP, Harris CM (1988) Applications of NMR spectroscopy to studies of reactive intermediates with nucleic acids. *Chem Res Toxicol*, **1**, 79-96.

Hayashi S-I, Watanabe J, Kawajiri K (1992) High susceptibility to lung cancer analyzed in terms of combined genotypes of P450IA1 and Mu-class glutathione S-transferase genes. *Jpn J Cancer Res*, **83**, 866-870.

Heniford BW, Shum-Siu A, Leonberger M, Hendler FJ (1993) Variation in cellular EGF receptor mRNA expression demonstrated by *in situ* reverse transcriptase polymerase chain reaction. *Nucl Acid Res*, **21**, 3159-3166.

Hochleitner K, Thomale J, Nikitin AY, Rajewsky MF (1991) Monoclonal antibody-based, selective isolation of DNA fragments containing an alkylated base to be quantified in defined gene sequences. *Nucl Acid Res*, **19**, 4467-4472.

Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991) p53 mutations in human cancers. *Science*, **253**, 49-53.

IARC (1986) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Tobacco smoking. Volume 38, IARC, Lyon.

IARC (1993) Postlabelling methods for detection of DNA adducts. Phillips DH, Castegnaro M, Bartsch H, Eds, IARC Scientific Publications No.124, Lyon.

Iversen OH (1992) Is a high rate of cell proliferation carcinogenic in itself? *Human Exp Toxicol*, **11**, 437-411.

Jahnke GD, Thompson CL, Walker MP, Gallagher JE, Lucier GW, DiAugustine RP (1990) Multiple DNA adducts in lymphocytes of smokers and nonsmokers determined by ^{32}P-postlabeling analysis. *Carcinogenesis*, **11**, 205-211.

Jain R, Sharma M (1993) Fluorescence postlabeling assay of DNA damage induced by N-methyl-N-nitrosourea, *Cancer Res*, **53**, 2771-2774.

Jennerwein MM, Eastman A (1991) A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. *Nucl Acid Res*, **19**, 6209-6214.

de Jong PM, van Sterkenburg MAJA, Kempenaar JA, Dijkman J, Ponec M (1993) Serial culturing of human bronchial epithelial cells derived from biopsies. *In Vitro Cell Dev Biol*, **29A**, 379-387.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, **51**, 6304-6311.

Kaufmann WKK (1989) Pathways of human cell post-replication repair. *Carcinogenesis*, **10**, 1-11.

Kaufmann WKK, Kaufman DG (1993) Cell cycle control, DNA repair and initiation of carcinogenesis. *FASEB J*, **7**, 1188-1191.

Kaur S, Pongracz K, Bodell WJ, Burlingame AL (1993) Bis(hydroxyphenylethyl) deoxyguanosine adducts identified by [^{32}P]-postlabeling and four-sector tandem mass spectrometry: unanticipated adducts formed upon treatment of DNA with styrene 7,8-oxide. *Chem Res Toxicol*, **6**, 125-132.

Keenan KP, Combs JW, McDowell EM (1982) Regeneration of hamster tracheal epithelium after mechanical injury. *Virch Arch B Cell Pathol*, **41**, 193-214.

King MM, Cuzick J, Jenkins D, Routledge MN, Garner RC (1993) Immunoaffinity concentration of human lung DNA adducts using an *anti*-benzo(a)pyrene-diol-epoxide-DNA antibody. Analysis by ^{32}P-postlabelling or ELISA. *Mutat Res*, **292**, 113-122.

Lane DP (1992) p53, guardian of the genome. *Nature*, **358**, 15-16.

Lecoq S, Pfau W, Grover PL, Phillips DH (1992) HPLC separation of ^{32}P-postlabelled DNA adducts formed from dibenz[a,h]anthracene in skin. *Chem Biol Int*, **85**, 173-185.

Levine AJ, Momand J, Finlay CA (1991) The p53 tumour suppressor gene. *Nature*, **351**, 453-456.

Manchester DK, Wilson VL, Hsu IC, Choi JS, Parker NB, Mann DL, Weston A, Harris CC (1990) Synchronous fluorescence spectroscopic, immunoaffinity chromatographic and ^{32}P-postlabeling analysis of human placental DNA known to contain benzo[a]pyrene diol epoxide adducts. *Carcinogenesis*, **11**, 553-559.

Marx J (1993) How *p53* suppresses cell growth. *Science*, **262**, 1644-1645.

Mass MJ, Kaufman DG (1983) A comparison between the activation of benzo(a)pyrene in organ cultures and microsomes from the tracheal epithelium of rats and hamsters. *Carcinogenesis*, **4**, 297-303.

Mitsudomi T, Oyama T, Kusano T, Osaki T, Nakanishi R, Shirakusa T (1993) Mutations of the p53 gene as a predictor of poor prognosis in patients with non-small-cell lung cancer. *J Natl Cancer Inst*, **85**, 2018-2023.

Möller L, Zeisig M, Vodicka P (1993) Optimization of an HPLC method for analyses of ^{32}P-postlabeled DNA adducts. *Carcinogenesis*, **14**, 1343-1348.

Nettesheim P, Jetten AM, Inayama Y, Brody AR, George MA, Gilmore LB, Gray T, Hook GER (1990) Pathways of differentiation of airway epithelial cells. *Env Health Persp*, **85**, 317-329.

Oshita F, Eastman A (1993) Gene-specific damage produced by cisplatin, ormaplatin and UV light in human cells as assayed by the polymerase chain reaction. *Oncol Res*, **5**, 111-118.

Pardee AB (1993) Toxins and checkpoints. *CIIT Activities*, **13**, 1-5.

Pfau W, Phillips DH (1991) Improved reversed-phase high-performance liquid chromatographic separation of ^{32}P-labelled nucleoside 3'-5'-bisphosphate adducts of polycyclic aromatic hydrocarbons. *J Chrom*, **570**, 65-76.

Plopper CG (1983) Comparative morphologic features of bronchiolar epithelial cells. *Am Rev Respir Dis*, **128**, S37-S41.

Plopper CG, Mariassy AT, Wilson DW, Alley JL, Nishio SJ, Nettesheim P (1983) Comparison of nonciliated tracheal epithelial cells in six mammalian species: ultrastructure and population densities. *Exp Lung Res*, **5**, 281-294.

Plopper CG, Cranz DL, Kemp L, Serabjit-Singh CJ, Philpot RM (1987) Immunohistochemical demonstration of cytochrome P-450 monooxygenase in clara cells throughout the tracheobronchial airways of the rabbit. *Exp Lung Res*, **13**, 59-68.

Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE (1990) Increased cell division as a cause of human cancer. *Cancer Res*, **50**, 7415-7421.

Randerath E, Agrawal HP, Weaver JA, Bordelon CB, Randerath K (1985) ^{32}P-postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis and dermis of mice treated topically with 7,12-dimethylbenz[a]anthracene. *Carcinogenesis*, **6**, 1117-1126.

Randerath E, Avitts TA, Reddy MV, Miller RH, Everson RB, Randerath K (1986) Comparative ^{32}P-analysis of cigarette smoke-induced DNA damage in human tissues and mouse skin. *Cancer Res*, **46**, 5869-5877.

Randerath E, Miller RH, Mittal D, Avitts TA, Dunsford HA, Randerath K (1989) Covalent DNA damage in tissues of cigarette smokers as determined by ^{32}P-postlabeling assay. *J Natl Cancer Inst*, **81**, 341-347.

Reddy MV, Gupta RC, Randerath K (1981) ^{32}P-base analysis of DNA. *Anal Biochem*, **117**, 271-279.

Reddy MV, Randerath K (1986) Nuclease P1-mediated enhancement of sensitivity of ^{32}P-postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543-1551.

Reddy MV, Randerath K (1987) ^{32}P-postlabeling assay for its carcinogen-DNA adducts: nuclease P1-mediated enhancement of its sensitivity and applications. *Env Health Persp*, **76**, 41-47.

Reddy MV, Blackburn GR (1990) ^{32}P-Postlabeling assay for carcinogen-DNA adducts: description of beta shielding apparatus and semi-automatic spotting and washing devices that facilitate the handling of multiple samples. *Carcinogenesis*, **11**, 683-687.

Rogan EG, Devanesan PD, RamaKrishna NVS, Higginbotham S, Padmavathi NS, Chapman K, Cavalieri EL, Jeong H, Jankowiak R, Small GJ (1993) Identification and quantitation of benzo[a]pyrene-DNA adducts formed in mouse skin. *Chem Res Toxicol*, **6**, 356-363.

Roggeband R, Wolterbeek APM, Rutten AAJJL, Baan RA (1993) Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* upon activation of benzo[a]pyrene by human, rabbit and rodent liver microsomes. *Carcinogenesis*, **14**, 1945-1950.

Roggeband R, Wolterbeek APM, Melis OWM, Wittekoek ME, Rutten AAJJL, Feron VJ, Baan RA (1994) DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture. *Carcinogenesis*, **15**, 661-665.

Rutten AAJJL, Bruyntjes JP, Ramaekers FCS (1988a) Intermediate filament expression in normal and vitamin A-deprived cultured hamster tracheal epithelium as detected by monoclonal antibodies. A study with emphasis on histological changes. *Virh Arch B Cell Pathol*, **56**, 103-110.

Rutten AAJJL, Wilmer JWGM, Beems RB (1988b) Effects of all-*trans* retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture I. A cell proliferation study. *Virch Archiv B Cell Pathol*, **55**, 167-175.

Savela K, Hemminki K (1991) DNA adducts in lymphocytes and granulocytes of smokers and nonsmokers detected by the ^{32}P-postlabelling assay. *Carcinogenesis*, **12**, 503-508.

Schreiber H, Martin DH, Pazmino N (1975) Species differences in the effect of benzo(a)pyrene-ferric oxide on the respiratory tract of rats and hamsters. *Cancer Res*, **35**, 1654-1661.

Segerbäck D, Vodicka P (1993) Recoveries of DNA adducts of polycyclic aromatic hydrocarbons in the ^{32}P-postlabelling assay. *Carcinogenesis*, **14**, 2463-2469.

Shields PG, Bowman ED, Harrington AM, Doan VT, Weston A (1993a) Polycyclic aromatic hydrocarbon-DNA adducts in human lung and cancer susceptibility genes. *Cancer Res*, **53**, 3486-3492.

Shields PG, Harris CC, Petruzelli S, Bowman ED, Weston A (1993b) Standardization of the ^{32}P-postlabeling assay for polycyclic aromatic hydrocarbon-DNA adducts. *Mutagenesis*, **8**, 121-126.

Steenwinkel M-JST, Roggeband R, van Delft JHM, Baan RA (1993) Improvements in the ^{32}P-postlabelling procedure to quantify bulky aromatic DNA adducts. In: Postlabelling methods for detection of DNA adducts, Phillips DH, Castegnaro M, Bartsch H, Eds, IARC Scientific Publications No 124, Lyon, pp 65-70.

Takeshima Y, Seyama T, Bennett WP, Akiyama M, Tokuoka S, Inai K, Mabuchi K, Land CE, Harris CC (1993) p53 mutations in lung cancers from non-smoking atomic-bomb survivors. *Lancet*, **342**, 1520-1521.

Tennant RW, Elwell MR, Spalding JW, Griesemer RA (1991) Evidence that toxic injury is not always associated with induction of chemical carcinogenesis. *Mol Carcinogenesis*, **4**, 420-440.

Vaca CE, Löfgren M, Hemminki K (1992) Some quantitative considerations about DNA adduct enrichment procedures for ^{32}P-postlabelling. *Carcinogenesis*, **13**, 2463-2466.

Venkatachalam S, Wani AA (1994) Differential recognition of stereochemically defined base adducts by antibodies against *anti*-benzo[a]pyrene diol-epoxide-modified DNA. *Carcinogenesis*, **15**, 565-572.

Vink AA, Berg RJW, de Gruijl FR, Lohman PHM, Roza L, Baan RA (1993) Detection of thymine dimers in suprabasal and basal cells of chronically UV-B exposed hairless mice. *J Invest Dermatol*, **100**, 1-6.

Weinberg RA (1991) Tumor suppressor genes. *Science*, **254**, 1138-1146.

Weinstein IB (1991) Mitogenesis is only one factor in carcinogenesis. *Science*, **251**, 387-388.

Weinstein IB (1992) Toxicity, cell proliferation, and carcinogenesis. *Mol Carcinogenesis*, **5**, 2-3.

Weston A, Bowman ED (1991) Fluorescence detection of benzo[a]pyrene -DNA adduct in human lung. *Carcinogenesis*, **12**, 1445-1449.

Yonish-Rouach E, Grunwald D, Wilder S, Kimchi A, May E, Lawrence JJ, May P, Oren M (1993) p53-mediated cell death: relationship to cell cycle control. *Mol Cell Biol*, **13**, 1415-1423.

In waarschijnlijk de meest gelezen paragraaf wil ik iedereen bedanken die een bijdrage heeft geleverd aan de totstandkoming van het in dit boekje beschreven werk.

In de eerste plaats is dat mijn co-promotor, Dr RA Baan. Beste Rob, ik realiseer mij goed dat zonder jouw inspanning mijn loopbaan er misschien heel anders uit had gezien. Onze eerste kennismaking vond plaats in Nijmegen, bij een PAK-dag, waar ik onder de indruk was van de dia's van de 'fluorescerende' cellen. Dit was de aanzet tot een stage plaats in de sectie Genetische Toxicologie van het toenmalige Medisch Biologisch Laboratorium. Een instituut, dat naar later bleek, één grote 'familie' was. Mijn verblijf kon, mede door jouw inzet, worden verlengd, eerst als ROAG-er in het kader van de militaire dienstplicht en later als promovendus. Jouw nauwgezette manier van wetenschap bedrijven heeft een hoop indruk op mij gemaakt. Ook de niet-wetenschappelijke discussies tijdens onze 'ritjes' naar Zeist zal ik niet gauw vergeten.

Mijn beide promotoren, de professoren VJ Feron en WRF Notten. Beste Vic, jouw 'nuchtere' kijk op de wetenschap sprak mij zeer aan. Tijdens onze 'luchtpijp'-beraden, of zoals jij het noemde het 'Baan-project', wist jij steeds de kernvraag te formuleren, waardoor ik beter in staat was om vervolgexperimenten op te zetten. Beste Wilfried, ik denk dat door de reorganisaties binnen TNO jouw pogingen om met mij in contact te treden zeer beperkt zijn gebleven. Via deze weg wil ik je toch bedanken voor de medewerking die je verleend hebt om mijn promotie onderzoek binnen het MBL te starten.

Dr F Berends, beste Frits, de snelheid waarmee jij manuscripten doorwerkt grenst aan het onwaarschijnlijke. Soms ging er door de hele discussie één grote streep en herschreef jij de discussie, op kladpapier bij de printer vandaan, met de opmerking, 'lijkt je dit iets', of, 'voorstel voor discussie'. Na de diverse nakijksessies van mijn eerste artikel herkende ik op een gegeven moment van het origineel niets meer terug.

André Wolterbeek, beste André, het knopje van de '4' op mijn telefoontoestel is meer aan slijtage onderhevig geweest dan de andere knopjes. Er is een waar communicatie netwerk ontstaan tussen ons, bestaande uit fax, brief, bedrijfsauto en piepschuim dozen. Vaak heb jij in de weekeinden trachea kweekjes voor mij doorgezet, onder het motto van, ik ben er toch. De samenwerking is in al die jaren altijd perfect geweest. Er was nooit onenigheid over wie wat zou gaan opschrijven, ondanks het feit dat de aard van onze werkzaamheden steeds dichter bij elkaar kwam.

De studentes Olga Melis en Janneke Wittekoek. Bedankt voor jullie enthousiaste

manier van werken, waarvan de resultaten zijn verwerkt in hoofdstuk 3.

Fons Rutten, beste Fons, van jouw inzicht om bepaalde dingen kort en bondig op papier te zetten heb ik veel geleerd.

Max Latuhihin, van de Dienst Getijdewateren van Rijkswaterstaat, voor zijn geduld bij alle SFS metingen aan BP-tetraolen.

Dr Dipple of the Basic Research Program, National Cancer Institute in Frederick, thank you for promptly sending us some *syn*-benzo[a]pyrene-diolepoxide, that was of great help to us in identifying some of the adduct spots on TLC in the experiments described in Chapters 2 and 3.

De firma Harlan CPB, de leverancier van de Syrische goudhamsters, voor hun financiële bijdrage aan de drukkosten van mijn proefschrift.

Last but certainly not least, de gentox-ers Agnes, Anita, Anja, Ank, Annemarie, Edwin, Esmeralda, Govert, Helma, Jaap, Jacqueline, Joost, Jos, Kees, Len, Marianne, Marie-José, Marij, Marjan, Mia, Paula, Roos en Sjef, van wie ik er enkele in het bijzonder wil bedanken. Marie-José, beste José, in die 6 jaar dat ik in het MBL heb gewerkt grensde mijn bureau altijd aan dat van jou. Jij leerde mij de in's en out's van de postlabeling. Vooral door jouw toedoen heeft de wereld van de postlabelaars in Bordeaux en in Lyon met ons kennis gemaakt. Een meid met principes, alhoewel, de laatste tijd..... In discussies had jij altijd een uitgesproken mening, waar je misschien later wel eens op terug moest komen, maar ja. Je kon overal over mee praten, of je praatte gewoon overal over mee, of je had het zelf al meegemaakt. Misschien zal ik je gezelschap nog gaan missen ook! Rognon a l'ancienne zal ik in ieder geval niet gauw meer bestellen. Paula van den Berg, beste Paulus, samen hebben we aardig wat WBC's behandeld. Onze samenwerking ging altijd perfect, behalve dan die ene keer midden op het Velperplein in Arnhem toen jouw Kadett City, met mij achter het stuur, afsloeg. Bij het voorbereiden van experimenten stonden altijd alle benodigdheden klaar, bijna zonder iets tegen elkaar te zeggen. Ondanks de tegenslag die we hebben gehad bij onze speurtocht naar de juiste antilichamen tegen B[a]P-DNA adducten, hebben we uiteindelijk een goed antilichaam te pakken gehad, waarmee we ook nog leuke resultaten hebben kunnen boeken. Jacqueline, Jaap, Helma en Roos, de Efteling, Parijs en het Gare du Nord, Duinrell, bedrijven volleybal, slechts een greep uit activiteiten die buiten werktijd plaatsvonden. Al die jaren hebben jullie geprobeerd mijn recept voor cake bakken te ontfutselen; het blijft het geheim van de chef.

De mensen die het genot hebben gehad met mij in de portocabine te mogen zitten. Anita, jij wist met verjaardagen altijd wel iets te verzinnen waar je eigenlijk niets aan had. Mijn gezeur tijdens de fietsrit op weg naar huis heeft zelfs jou, een fervent fietsliefhebster, tijdelijk in de auto gedreven. Vooral in mijn laatste jaar op het lab heb

ik nog geprobeerd om een soort voorbeeld functie voor je te vervullen, we maakten afspraken over deadlines voor artikels. Afspraken, daar bleef het bij. Edwin, beste Ed, jouw ideeën over bepaalde zaken lagen soms zo dicht bij die van mij dat je een broer van mij had kunnen zijn. Bij ons was in principe alles bespreekbaar. Het begrip **'chaos'** in de wetenschap, waar zelfs boeken over geschreven zijn, lijkt onlosmakelijk met jou verbonden te zijn. Men beweert dat de grote breinen uit de geschiedenis allemaal iets chaotisch hadden, maar ja, men zegt zo veel. Jouw streven naar maximale entropie op het lab staat echter in schril contrast met jouw gedrag in de fitness-zaal, waar je een goddelijke **perfectie** van je lichaam nastreefde. Volhouden Ed!

De tafeltennis 'club', beste Ton, Bas, Rinus, Ferry, Jan en later Martin, Priscilla, en Roos. Het spelletje was voor mij een bijna onmisbare en welkome bezigheid voor tussen de middag. Hierbij wil ik mijn excuses maken voor al die onvermijdelijke nederlagen die ik jullie heb bezorgd. Ik realiseer me eigenlijk nu pas dat ik hiermee veel psychologische schade aangericht zou kunnen hebben. Des te groter is echter mijn waardering voor het feit dat jullie iedere middag toch weer achter de tafel verschenen.

De afdeling Fotografie, Ronald en Michel, voor al hun opnamen van de autoradiogrammen. Meestal waren de foto's sneller klaar dan gepland. Sjaan - 's morgens altijd de geur van verse koffie - en Herbert - hasta luego amigo - uit de 'keuken'. Rinus, chef 'rollend' materiaal en Ferry, plv chef 'rollend' materiaal, beste Rinus en Ferry, met jullie vertrek uit het MBL verdween tegelijkertijd de samenhang uit het lab. Lo, aan het radiologische gebeuren op het lab had jij je handen vol. Voor de diensten in het algemeen geldt dat het jammer is dat de meeste mensen eerder geneigd zijn te kijken naar wat bepaalde zaken kosten, in plaats van te kijken naar wat ze waard zijn.

Mijn ouders, die mij in de gelegenheid stelden om een studie te volgen. Hun belangstelling voor mijn resultaten was altijd groot en vormde tegelijkertijd ook een stimulans.

Patricia, vooral door jouw toedoen heb ik het laatste jaar van mijn promotie in 'relatieve rust' in de avonden thuis veel lees- en schrijfwerk kunnen doen. Onbewust zorgde jij voor de nodige deadlines met opmerkingen als 'heb je nu je artikel al af?'. Ik hoop dat je het eindresultaat de moeite waard vindt.

Willems MI, Roggeband R, Baan RA, Wilmer JWGM, de Raat WK, Lohman PHM (1991) Monitoring the exposure of rats to benzo[a]pyrene by the determination of mutagenic activity in excreta, chromosome aberrations and sister chromatid exchanges in peripheral blood cells, and DNA adducts in peripheral blood lymphocytes and liver, *Mutagenesis*, **6**, 151-158

Wolterbeek APM, Roggeband R, Feron VJ, Rutten AAJJL (1992) Vitamin A and respiratory tract cancer: *in vitro* studies and studies in hamsters, *Voeding*, **53**, 175-179

Wolterbeek APM, Roggeband R, Steenwinkel M-JST, Baan RA, Rutten AAJJL (1993) Formation and repair of benzo[a]pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis, *Carcinogenesis*, **14**, 463-467

Roggeband R, Wolterbeek APM, Rutten AAJJL, Baan RA (1993) Comparative ^{32}P-postlabeling analysis of benzo[a]pyrene-DNA adducts formed *in vitro* upon activation of benzo[a]pyrene by human, rabbit and rodent liver microsomes, *Carcinogenesis*, **14**, 1945-1950

Steenwinkel M-JST, Roggeband R, van Delft JHM, Baan RA (1993) Improvements in the ^{32}P-postlabeling procedure to quantify bulky aromatic DNA adducts, in: Postlabelling methods for detection of DNA adducts, Eds DH Phillips, M Castegnaro and H Bartsch, IARC Scientific Publications No 124, Lyon, pp 65-70

Heussen GAH, Bouman HGM, Roggeband R, Baan RA, Alink GM (1994) ^{32}P-postlabelling analysis of DNA adducts in white blood cells of humans exposed to residential wood-combustion particulate matter, *Environmental and Molecular Mutagenesis*, **23**, 121-127

Roggeband R, Wolterbeek APM, Melis OWM, Wittekoek ME, Rutten AAJJL, Feron VJ, Baan RA (1994) DNA-adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture, *Carcinogenesis*, **15**, 661-665

Roggeband R, Wolterbeek APM, van den Berg PTM, Baan RA (1994) DNA adducts in hamster and rat tracheas exposed to benzo[a]pyrene *in vitro*, Proceeding of the Fourth European Meeting on Environmental Hygiene, June 9-11, 1993, Wageningen, The Netherlands, *Toxicology Letters*, **72**, 105-111

Roggeband R, van den Berg PTM, van der Wulp CJM, Baan RA (1994) Detection of DNA-adducts in basal and non-basal cells of the hamster trachea exposed to benzo[a]pyrene in organ culture, *Journal of Histochemistry and Cytochemistry*, in press

Baan RA, Steenwinkel M-JST, van den Berg PTM, Roggeband R, van Delft JHM (1994) Molecular dosimetry of DNA damage induced by polycyclic aromatic hydrocarbons; relevance for exposure monitoring and risk assessment, *Human & Experimental Toxicology*, submitted

Roggeband R, Wolterbeek APM, van den Berg PTM, Rutten AAJJL, Feron VJ, Baan RA (1994) Cell proliferation and DNA adducts in hamster tracheal epithelium exposed to benzo[a]pyrene in organ culture, *Carcinogenesis*, submitted

Roggeband R, Wolterbeek APM, van den Berg PTM, Rutten AAJJL, Feron VJ, Berends F, Baan RA (1994) DNA adducts and proliferation of basal and non-basal cells in relation to the development of precancerous lesions in the hamster trachea exposed to benzo[a]pyrene in organ culture, *Cancer Research*, submitted

Wolterbeek APM, Roggeband R, van Moorsel CJA, Baan RA, Koeman JH, Feron VJ, Rutten AAJJL (1994) Vitamin A and β-carotene decrease the level of benzo[a]pyrene-DNA adducts in hamster tracheal epithelium by increasing DNA-repair activities, *Cancer Research*, submitted

Robert Roggeband was born in Rotterdam on December 11, 1962. After graduation from secondary school at the Scheldemond Scholengemeenschap in Vlissingen in 1981, he began his studies in Human Nutrition at the Agricultural University in Wageningen. He obtained his MSc degree in Human Nutrition (Dr CE West) and Toxicology (Dr PJ van Bladeren) in 1987. From 1988-1994 he was employed at the Department of Genetic Toxicology (Dr RA Baan), Medical Biological Laboratory TNO in Rijswijk (TNO Nutrition and Food Research Institute as of January 1, 1994), initially to fulfil his military service, followed by 4 years as a PhD student. His research encompassed the detection of DNA damage induced by polycyclic aromatic hydrocarbons, by use of biochemical and immunochemical techniques.

1. Het verschil tussen basaal- en niet-basaalcellen in het vermogen om benzo[a]pyreen om te zetten in reactieve metabolieten is niet alléén bepalend voor de hoeveelheid benzo[a]pyreen-DNA adducten die wordt aangetroffen in die twee celtypen, na blootstelling van de luchtpijp van de Syrische goudhamster in orgaankweek aan 4 of 40 μM benzo[a]pyreen gedurende twee dagen.

- *dit proefschrift*

2. De DNA-herstelcapaciteit van basaalcellen in de luchtpijp van de Syrische goudhamster, gemeten na twee dagen blootstelling aan 40 μM benzo[a]pyreen gevolgd door een herstelperiode van 3 dagen, is groter dan die van niet-basaalcellen, hetgeen in overeenstemming is met de centrale rol van de basaalcellen in de luchtpijp.

- *dit proefschrift*

3. Het gebruik van onafhankelijk geijkte standaarden van gemodificeerd DNA in de ^{32}P-postlabeling is onontbeerlijk voor nauwkeurige bepaling van adductniveau's, en maakt gegevens van verschillende laboratoria onderling beter vergelijkbaar.

- *dit proefschrift*

4. De manier waarop sommige onderzoekers, die de ^{32}P-postlabelingmethode gebruiken, adductniveau's berekenen aan de hand van autoradiogrammen van dunnelaag-chromatogrammen is bedenkelijk.

- *Hemminki et al, Carcinogenesis, 15, 1994*

5. De verminderde consumptie van verse groenten en fruit door de Nederlandse bevolking lijkt een zorgwekkende ontwikkeling, gezien de aanwijzingen in de literatuur dat de consumptie van vitamines het risico van kanker op latere leeftijd verlaagt.

- *Mayne et al, JNCI, 86, 1994*
- *Ames et al, PNAS, 90, 1993*

6. Het elfenbankje, de paarse schijnridder en vooral de grijze gaatjeszwam zouden een belangrijke rol kunnen spelen bij het 'schoonmaken' van met PAK's, PCB's en dioxines vervuilde fabrieksterreinen, waardoor miljoenen guldens, nodig voor het afgraven van de grond, bespaard zouden kunnen worden.

- *Volkskrant, 27 november 1993*

7. Het cellentekort in Nederland zou veel groter zijn wanneer bepaalde vormen van witteboordencriminaliteit, zoals de manier waarop sommige 'managers' met hun personeel omgaan, aangepakt zouden worden.

8. Archeologen uit het jaar 3000, die bij bodemonderzoek op de grondlaag uit 1994 stuiten, mogen uit de combinatie van de kleuren paars en vooral oranje geen conclusies trekken met betrekking tot de mode uit die tijd, noch ten aanzien van een mogelijk te nadrukkelijke bemoeienis van het Staatshoofd met de kabinetsformatie.

9. De campagne voor het nieuwe wasmiddel 'Omo Power' geeft een geheel frisse kijk op het woord 'zeper'.

10. De meeste dingen in het leven zouden anders afgelopen zijn als men even gepauzeerd had.

- *Wim Sonneveld*

Robert Roggeband
Utrecht, 13 september 1994