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Black (Air-cured) and Blond (Flue-cured) Tobacco Cancer Risk IV: Molecular Dosimetry Studies Implicate Aromatic Amines as Bladder Carcinogens

H. Bartsch, C. Malaveille, M. Friesen, F.F. Kadlubar and P. Vineis

Tobacco smoking causes a major fraction of male urinary bladder cancers and the relative risk of bladder cancer is reported to be two to three times higher for smoking of black (air-cured) than for smoking of blond (flue-cured) tobacco. In molecular dosimetry studies to examine the hypothesis that aromatic amines in tobacco smoke are primarily responsible for bladder cancer, the higher bladder cancer risk in smokers of black tobacco was correlated with two to five times higher exposure to carcinogenic aromatic amines present in black tobacco smoke, notably 4-aminobiphenyl (ABP). For the same amount of smoking, black tobacco smokers had levels of ABP-haemoglobin (Hb) adducts 1.5 times higher and excreted a 1.8-fold higher level of urinary mutagens. These mutagens were characterised as aromatic amines, and included the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a known mutagen and multiorgan/species carcinogen. In smoking volunteers, the ABP-Hb adduct level depended significantly on the acetylator and P-450IA2 phenotypes, being 1.3- to 1.5-fold lower in fast acetylators, slow/intermediate P-450IA2 individuals. The *N*-(deoxyguanosin-8-yl)-ABP adduct was a major smoking-related DNA adduct in bladder biopsies from surgical patients. It was also tentatively identified in exfoliated urothelial cells of smoking volunteers, who showed a significant and linear correlation between adduct levels of ABP with Hb and with deoxyguanosine in urothelial DNA; both were related to number of cigarettes smoked per day. Levels of several smoking-related DNA adducts in urothelial cells were 2–20 times elevated in smokers. Similar convex dose–response relationships have been found between the number of cigarettes smoked and the relative risk for bladder cancer and between the levels of ABP-Hb adducts and markers of recent smoking. A possible explanation is that fast and slow acetylators have different susceptibility to aromatic amine carcinogens. Case–control studies have consistently revealed an excess of variable magnitude of slow acetylators in subgroups exposed occupationally to carcinogenic aromatic amines. Altogether, results from these studies reinforce the association between cigarette smoking, carcinogen-DNA adducts in urothelial cells, and implicate primary aromatic and possibly heterocyclic amines as bladder carcinogens.
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I. INTRODUCTION

TOBACCO SMOKING is considered to be an important cause of urinary bladder cancer in humans, 50% of male and 25% of female cases in Western societies being attributable to smoking [1]. Tobacco smoke contains at least 3800 constituents [1], among which known carcinogens include benzene, volatile and tobacco-specific *N*-nitrosamines (TSNA), aromatic amines, polynuclear aromatic compounds and polonium-210. In contrast to cancers of the upper respiratory tract, for which tobacco-specific *N*-nitrosamines, polynuclear aromatic compounds and radioisotopes have been implicated as causative agents, bladder cancer seems more likely to be caused by aromatic amines [2], some of which are known bladder carcinogens in both humans and experimental animals [3].

Although the majority of cigarettes smoked in the world are composed of blond (bright or flue-cured) tobacco, black (burley or air-cured) tobacco is still much used in the Mediterranean countries of Europe and in Latin America. Four case–control studies in Italy, France, Argentina and Uruguay have reported the relative risk of urinary bladder cancer to be two to three times higher for smokers of black tobacco than for smokers of blond tobacco [4–7].

Because of the occurrence of several aromatic amines in tobacco smoke, including some known human bladder carcinogens (Table 1), and the excretion of mutagens in smokers' urine [1, 8], a series of molecular dosimetry and biochemical epidemiology studies were conducted to complement existing epidemiological data and to elucidate the mechanisms involved. These studies have attempted to answer the following questions:

(a) Does black tobacco smoke contain higher concentrations of the aromatic amine carcinogens that are common in both tobacco types, or does it contain other carcinogens which are not present in blond tobacco smoke?

(b) Are the differences between the tobacco types in risk for bladder cancer reflected in levels of biomarkers in black and blond tobacco smokers, for example, urinary mutagenicity or macromolecular-carcinogen adducts?

(c) What are the structures of the aromatic amines (the presumed mutagens) that smokers excrete in the urine?

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Table 1. Aromatic amines in the smoke of black or blond tobacco

Carcinogen	Concentration (ng/cigarette) Black tobacco	Blond tobacco	Ratio Black/blond	Reference
Aromatic amines				
o-Toluidine	162.0	32.2	5.0	Patrianakos and Hoffmann, 1979 [62]
4-Aminobiphenyl	4.6	2.4	1.91	Patrianakos and Hoffmann, 1979 [62]
2-Naphthylamine	1.7	1.0	1.7	Patrianakos and Hoffmann, 1979 [62]
Heterocyclic amines				
Amino-α-carboline	110–134	25–260	4.4–0.5	Yoshida and Matsumoto, 1980 [9]
	—	80	—	Matsumoto *et al.*, 1981 [63]
	—	28–48	—	Manabe *et al.*, 1990 [64]
Methylamino-α-carboline	20–22	16–37	1.3–0.6	Yoshida and Matsumoto, 1980 [9]
	—	7	—	Matusmoto *et al.*, 1981 [63]
	—	2.2–2.9	—	Manabe *et al.*, 1990 [64]
PhIP	—	16–23	—	Manabe *et al.*, 1991 [65]
Trp-P-1	—	0.29–0.48	—	Manabe *et al.*, 1990 [64]
Trp-P-2	—	0.82–1.1	—	Manabe *et al.*, 1990 [64]
Glu-P-1	—	0.37–0.89	—	Kanai *et al.*, 1990 [66]
Glu-P-2	—	0.25–0.88	—	Kanai *et al.*, 1990 [66]
IQ	—	0.26	—	Yamashita *et al.*, 1986 [67]

(d) For a given amount of smoking, does genetic polymorphism of drug metabolising enzymes involved in the activation and deactivation of aromatic amines influence their bioavailability and macromolecular binding in the urothelium or in surrogate cells [for example, the haemoglobin (Hb) of red blood cells]?

II. OCCURRENCE OF CARCINOGENS IN TOBACCO SMOKE

Primary aromatic amines occur in smoke at ng/cigarette levels in both black and blond cigarettes (Table 1). In general, mononuclear arylamines such as *o*-toluidine are present at higher levels than binuclear arylamines such as 4-aminobiphenyl (ABP) or 2-naphthylamine. Levels are two to five times higher in black than in blond tobacco smoke.

Nitrogen-containing heterocyclic amines have been associated with cooked foods, but they have also been reported to occur in cigarette smoke condensate (Table 1). Highest levels have been reported for amino-α-carboline and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Few data are available on levels in black and blond tobacco smoke; however, one study reported levels of amino-α-carboline and methylamino-α-carboline to be higher in black tobacco smoke [9].

Volatile nitrosamines such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosopyrrolidine (NPYR) are found at low ng/cigarette levels in tobacco smoke (Table 2). Tobacco-specific nitrosamines such as *N'*-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butonone (NNK) and *N'*-nitrosoanatabine are found at levels 10 to 100 times higher, with higher levels in black than blond tobacco smoke.

Some of the other carcinogens reported in tobacco smoke are present at very high levels (Table 3), with formaldehyde and acetaldehyde levels of up to 36 μg/cigarette and 0.9 mg/cigarette, respectively, and no apparent difference with tobacco type. Benzene, 2-nitropropane and quinoline are also present at the μg/cigarette level. Levels of 2-nitropropane appear somewhat higher in black tobacco smoke.

III. METABOLISM, MACROMOLECULAR BINDING AND CARCINOGENICITY OF AROMATIC AMINES

ABP and 2-naphthylamine are aromatic amines that are known to induce urinary bladder cancer in occupationally exposed humans [3, 10] and in a dog model [11]. The patterns of metabolism and macromolecular binding in exposed subjects and animal models show that aromatic amines require metabolic activation by complex, often competing, pathways before they exert DNA-binding and carcinogenicity [12]. Formation of an electrophilic intermediate in the liver through *N*-hydroxylation

Table 2. Nitrosamines in the smoke of black or blond tobacco

Carcinogen		Concentration (ng/cigarette) Black tobacco	Blond tobacco	Ratio Black/blond	Reference
Volatile nitrosamines					
N-Nitrosodimethylamine	NF	29	6.8–13.8	4.3–2.1	Ruhl *et al.*, 1980 [68]
	NF	38.8–76.4	9.4–48.4	4.1–1.6	Tricker *et al.*, 1991 [69]
	F	4.3	1.8–5.7	2.4–0.8	Ruhl *et al.*, 1980 [68]
N-Nitrosodiethylamine	NF	2.7	<0.1–1.8	27–1.8	Ruhl *et al.*, 1980 [68]
	NF	2.1–6.3	<0.1–7.1	21–0.9	Tricker *et al.*, 1991 [69]
	F	0.5	0.4–1.0	1.3–0.5	Ruhl *et al.*, 1980 [68]
N-Nitrosopyrrolidine	NF	25	11.0–30.3	2.3–0.8	Ruhl *et al.*, 1980 [68]
	NF	22.7–36.1	6.9–41.2	3.3–0.9	Tricker *et al.*, 1991 [69]
	F	10.5	3.1–8.7	3.4–1.2	Ruhl *et al.*, 1980 [68]
N-Nitroso-diethanolamine	NF*	290	30–51	9.7–5.7	Brunnemann and Hoffman, 1981 [70]
Tobacco-specific nitrosamines					
N'-Nitrosonornicotine	NF*	3700	620	6.0	Hoffmann *et al.*, 1979 [71]
	NF	512–625	82–255	6.0–2.5	Fischer *et al.*, 1989 [72]
	NF	203	29	7	Fischer *et al.*, 1990 [73]
	NF	550–800	79–885	7.0–0.9	Tricker *et al.*, 1991 [69]
	F	117–389	213	0.5–1.8	Djorjevic *et al.*, 1991 [74]
4-(*N*-Nitrosomethylamine-1-(3-	NF*	320	420	0.8	Hoffmann *et al.*, 1979 [71]
pyridyl)-1-butanone	NF	108–432	70–156	1.5–2.8	Fischer *et al.*, 1989 [72]
	NF	136	40	3.4	Fischer *et al.*, 1990 [73]
	NF	84–470	62–185	1.4–2.5	Tricker *et al.*, 1991 [69]
	F	13–55	32	0.4–1.7	Djorjevic *et al.*, 1991 [74]
N-Nitrosoanatabine**	NF*	4600	410	11.2	Hoffmann *et al.*, 1979 [71]
	NF	266–353	81–225	3.3–1.6	Fischer *et al.*, 1989 [72]
	NF	108	45	2.4	Fischer *et al.*, 1990 [73]
	NF	225–520	75–380	3.0–1.4	Tricker *et al.*, 1991 [69]
	F	74–196	92	0.8–2.1	Djorjevic *et al.*, 1991 [74]

NF, Non-filter; F, filter. *Experimental cigarette; **NAT contains *N'*-nitrosoanabasine.

is catalysed in humans predominantly by cytochrome P450IA2 (Fig. 1) [13]. This enzyme activity shows an apparently trimodal distribution in different populations and has allowed the tentative designation of slow, intermediate and rapid P-450IA2 (*N*-hydroxylation) phenotypes [14]. However, there is still no proof that the *N*-oxidation phenotype is due to genetic polymorphism [15].

The *N*-hydroxy metabolite of arylamines such as ABP then enters the circulation, reacts covalently with Hb in erythrocytes and is filtered into the bladder lumen where it reacts with urothelial DNA, an event critical for the initiation of bladder carcinogenesis. A competing pathway leading to detoxification of the amine proceeds by *N*-acetylation catalysed by acetyltransferases in the liver [16]. *N*-Acetyltransferase (NAT) activity in humans is coded by two distinct genes, designated NAT1 and NAT2. The former activity is monomorphically distributed, while the latter exhibits genetic polymorphism in humans, allowing the detection of phenotypically slow and rapid *N*-

Table 3. Other carcinogens in the smoke of black or blond tobacco

Carcinogen		Concentration (ng/cigarette) Black tobacco	Blond tobacco	Ratio Black/blond	Reference
Volatile aldehydes					
Formaldehyde	NF*	16100–25100	26800–36300	0.6–0.7	NCI, 1976 [75]
Acetaldehyde	NF*	726000–966000	797000–906000	0.9–1.1	NCI, 1976 [75]
Benzene		12000	27000	0.4	Johnstone *et al.*, 1962 [76]
2-Nitropropane	NF	1430–2180	220–1190	6.5–1.8	Hoffmann and Rathkamp, 1968 [77]
Quinoline	F	1200	620	1.9	Brunnemann and Hoffmann, 1974 [78]
Polycyclic aromatic hydrocarbons					
Benz[a]anthracene	NF*	10.7–16.7	21.0–25.9	0.5–0.6	NCI, 1976 [75]
Benzo[a]pyrene	NF*	24	38–53	0.6–0.5	Wynder and Hoffmann, 1963 [79]
	NF*	7.5–9.6	12–26	0.6–0.4	NCI, 1976 [75]
	NF*	19.7	35.4	0.6	Dontenwill *et al.*, 1973 [80]

NF, Non-filter; F, filter. *Experimental cigarettes.

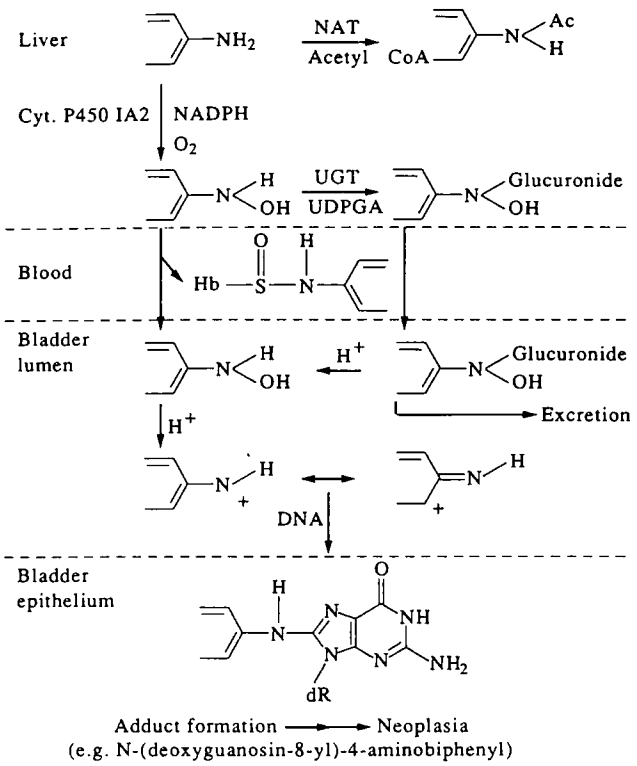

Fig. 1. Proposed biochemical pathways for urinary bladder cancer induction by aromatic amines.

acetylating individuals. Only NAT2 is expressed at high levels in the livers of rapid acetylators. This NAT-encoded, non-inducible polymorphic enzyme is under genetic control: slow acetylators are homozygous for the slow acetylator gene, while rapid acetylators are either homozygous or heterozygous for the rapid gene. The molecular basis for this polymorphism has been partially elucidated [17]. A number of studies have indicated that slow acetylators, particularly among people occupationally exposed to aromatic amines, are at increased risk for bladder cancer [18, 19].

The metabolically formed *N*-hydroxy derivative of ABP undergoes an acid-catalysed reaction with urothelial DNA and produces a C-8 substituted deoxyguanosine adduct (Fig. 1) as well as other minor adducts [12]. Administration of ABP to dogs, in which it produces bladder tumours, resulted in the formation of this major *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl adduct [20]. Subsequently, it was also found as one of the smoking-related DNA adduct in bladder biopsies of surgical patients and in exfoliated urothelial cells (see Section VI), suggesting that aromatic amines are involved in DNA damage and bladder carcinogenesis.

In addition to mono- and binuclear arylamines, several heterocyclic amines have been detected in condensate of mainstream cigarette smoke (Table 1). This class of mutagenic carcinogens is commonly formed in heated food by pyrolysis of proteins or by heating amino acids, glucose and creatinine [21]. Several of these amines also appear to be formed in the combustion of tobacco that contains amino acids [1] and possibly creatinine, known to be a constituent of plant material [22]. PhIP is excreted in the urine [23], and has been characterised as one major DNA-damaging agent that heavy smokers of black tobacco excrete, possibly accounting in part for the mutagenic activity in smokers' urine [24, 25]. PhIP undergoes *N*-oxidation by cytochrome P450IA2 to yield *N*-hydroxy-PhIP, a principal metabolite leading to mutation and DNA damage [26–28]. In experimental animals, this metabolite is conjugated to form semi-stable transportable glucuronides, which are excreted in the bile and urine [29, 30] or may be *O*-acetylated by (colon) acetyltransferase (NAT2) to yield *N*-acetoxy-PhIP, the presumed ultimate carcinogenic metabolite (Kadlubar *et al.*, in preparation). Enzymatic cleavage of glucuronides in extrahepatic tissues or by intestinal bacteria may lead after *O*-acetylation to the formation of PhIP-DNA adducts in extrahepatic tissues, such as in colon, pancreas, kidney, heart and urinary bladder [30, 31]; both pathways could explain the wide range of target tissues of PhIP carcinogenicity compared with other heterocyclic amines [32, 33].

Reaction of *N*-hydroxy-PhIP with DNA bases yields a characteristic adduct pattern [24] with *N*-(deoxyguanosin-8-yl)-PhIP as the major product (Lin *et al.*, in preparation). Other heterocyclic amines (Trp-P-2, Glu-P-1 and IQ) also react with DNA *in vitro* to form C8-deoxyguanosine-substituted products analogous to those found with other aromatic amine carcinogens [34, 35] and similar adducts are found in the livers of rats treated with Trp-P-2 and Glu-P-1 [36]. DNA from bladder biopsies of bladder cancer patients and from exfoliated cells of smokers containing a smoking-related adduct, whose chromatographic properties are compatible with a PhIP-DNA adduct (see Section V).

Because cytochrome P-450IA2- and acetyltransferase-mediated activation and detoxification reactions of ABP exhibit genetic polymorphism, studies have been conducted in smokers to examine whether the large individual variations in adduct formation of aromatic amine carcinogens are due to a particular metabolic phenotypes.

IV. HAEMOGLOBIN ADDUCTS OF AROMATIC AMINES, ASSOCIATIONS WITH SMOKING STATUS, TYPE OF TOBACCO AND METABOLIC PHENOTYPE

The reaction of carcinogens, through electrophilic intermediates, with amino acids of haemoglobin presents a method to quantify a time-rated average of formation of reactive carcinogen metabolites *in vivo*, which in the case of ABP and related amines is the *N*-hydroxylated metabolite (Fig. 1). The level of haemoglobin adducts, therefore, represents a marker of the biologically effective dose [37], but whether or not a correlation exists for macromolecular binding in surrogate and target tissue(s) has to be demonstrated for each carcinogen. The technique depends upon measurement of the covalently bound adduct of the 93-beta cysteine residue formed by interaction of *N*-hydroxyarylamines with haemoglobin. This adduct can be broken down *in vitro* to regenerate the parent amine, quantification of which is accomplished by gas chromatography/mass spectrometric analysis [38].

In a group of cigarette smokers, the level of ABP-Hb adducts was higher than in non-smoking subjects [39]; a convex dose–response curve with the quantity of cigarettes smoked or with urinary nicotine and cotinine concentration has been observed [40] (see Fig. 3a). Hb adducts of ABP and other aromatic amines present in tobacco were measured in smokers of black and blond tobacco [38]. When adjusted for the same amount of smoking, black tobacco smokers had 1.5-fold higher ABP-Hb adduct levels (expressed as pg bound ABP/g Hb) than those of smokers of blond tobacco. Hb adducts of 2-naphthylamine and 4-toluidine were associated with smoking status, but not with tobacco type. Other mono- and binuclear amines showed no significant correlation with smoking status

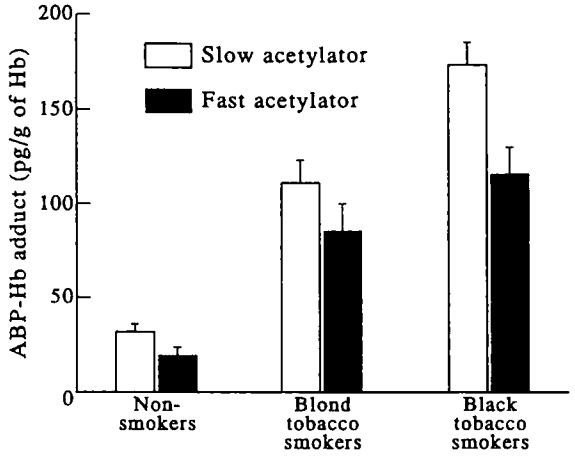

Fig. 2. Mean values (± S.E.) of 4-aminobiphenyl-haemoglobin adducts in non-smokers and smokers, grouped by acetylation phenotype and type of tobacco (data from [40] and replotted).

and tobacco type, emphasising the importance for ABP in bladder cancer causation by tobacco smoke.

Previous studies of aromatic amine-induced bladder cancer in dogs and occupationally exposed humans indicated that the human phenotype at lowest risk for bladder cancer development is likely to be the slow acetylator and rapid acetylator and slow/intermediate *N*-oxidizer [41]. The metabolic scheme in Fig. 1 implies that levels of ABP-Hb adducts in smokers will show a similar phenotype dependence and this has been confirmed in 97 volunteers who were smokers of black and blond tobacco or non-smokers [42]. *N*-Acetylation and *N*-oxidation phenotypes were assigned on the basis of urinary caffeine metabolite ratios after a standard coffee dose. The *N*-oxidation phenotypes show an apparent tri-modal distribution in non-smokers permitting the designation of slow, intermediate and rapid oxidation phenotypes [14].

When smoking habits (quantity, type of tobacco) and metabolic phenotype were analysed in a multi-variate model, an independent and statistically significant contribution of the phenotype to ABP-Hb adduct formation was found [40] (Fig. 3). In smoking subjects grouped as fast and slow *N*-acetylators, the latter had 1.3–1.5-fold higher levels of Hb adducts. When grouped according to both acetylator and *N*-oxidiser phenotypes, the highest level of ABP-Hb adducts was found in the slow acetylators irrespective of their oxidiser status. However, the lowest level was seen in the fast acetylators who were slow/intermediate oxidisers (data not shown). This study also clearly showed an association between ABP-Hb adduct levels, the number of cigarettes and the type of tobacco smoked.

These results provide the first demonstration that the genetically determined slow acetylator phenotype, which is linked to a higher risk for arylamine-induced occupational bladder cancer [18], is also (for the same amount of smoking) associated with a higher level of ABP-Hb adducts; the data support the idea that the biochemical pathways (Fig. 1) are relevant for human arylamine-induced bladder carcinogenesis. This scheme also implies that the metabolic phenotype should affect the macromolecular binding of other aromatic amines present in tobacco smoke (Table 1), for which some indirect support has been provided [43].

A convex dose–response relationship, as seen in Fig. 3b, between the quantity of cigarettes smoked and the relative risk for bladder cancer has been repeatedly observed in case–control studies [1, 44, 45]. The similar convex dose–response curves have been found for correlations of the levels of ABP-Hb adducts with markers of recent smoking, namely with the number of cigarettes and the urinary levels of cotinine plus nicotine (Fig. 3a), which further reinforces the hypothesis that aromatic amines are risk factors. This similarity in the shapes of the curves could arise by coincidence or due to saturation of enzymic pathways in aromatic amine metabolism. Another interpretation is the presence of two sub-groups with different susceptibilities to aromatic amine carcinogenesis [46]: when the ABP-Hb adducts in smokers were examined in relation to acetylation phenotype (Fig. 4), slow acetylators reached a high level of adducts at low cigarette consumption, whereas the levels in fast acetylators increased less rapidly. Superimposition of the two curves leads to a non-linear relationship similar to that observed in Fig. 3a. Whether the relative risks of smoking-associated bladder cancer in fast and slow acetylators follow similar curves needs to be examined. In contrast, metabolic phenotype has not been found to influence the level of mutagens excreted [42].

Levels of ABP-Hb adducts in surrogate tissues can be used to predict DNA adduct levels in urothelial (target) tissue, as shown by an analysis of both endpoints in the same smoking subjects experiencing exposure to ABP. Carcinogen-DNA modifications in exfoliated urothelial cells of smokers were quantified using ^{32}P-postlabelling technique [47]. An adduct, tentatively characterised as *N*-(deoxyguanosin-8-yl)-ABP (Fig. 1), showed the strongest relationship with cigarette consumption and was best and linearly correlated with ABP-Hb adducts [48]. In dogs, the relationship between ABP adducts in bladder DNA and Hb was linear at low, but not at high doses of ABP [48]. It may be

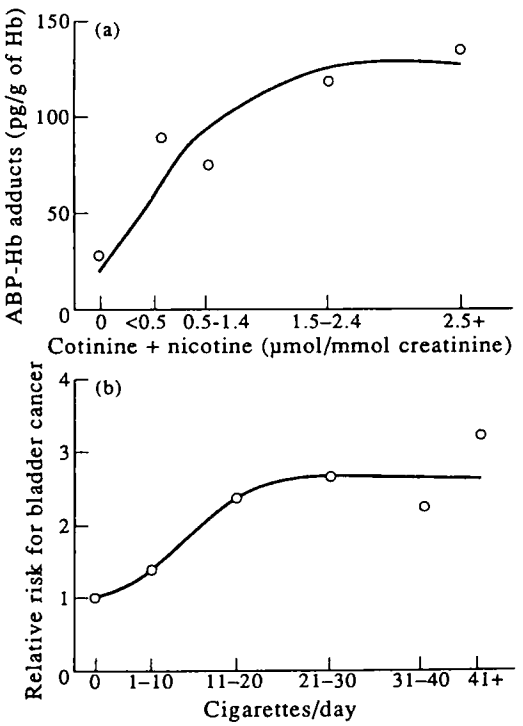

Fig. 3. (a) Mean values (± S.E.) of 4-aminobiphenyl-haemoglobin adducts in healthy volunteers as a function of recent cigarette consumption, measured as level of cotinine plus nicotine in the urine (from [42] and replotted). (b) Dose–response relationship from a case–control study [61] between the number of cigarettes smoked per day and the relative risk for bladder cancer.

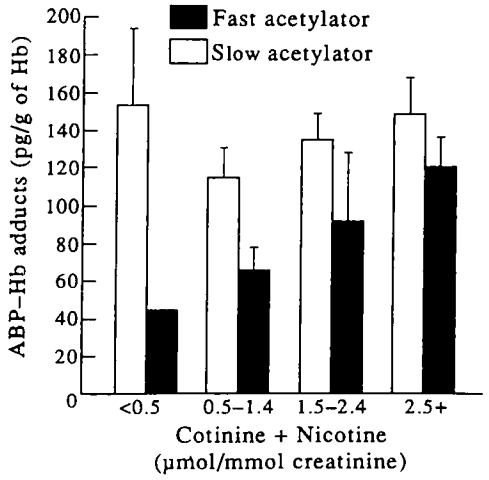

Fig. 4. Mean values (±S.E.) of 4-aminobiphenyl-haemoglobin adducts in smokers grouped according to levels of nicotine plus cotinine in the urine and by acetylator phenotype (from [46] and replotted). No error bar indicates one subject only.

supposed that the metabolic phenotype will also influence DNA adduct formation in urothelial (exfoliated) cells when subjects are exposed to similar levels of (tobacco-derived) aromatic amines, but this still remains to be proven. However, formation of DNA adducts with 2-aminofluorene in the bladder was higher in inbred slow acetylator female mice than in rapid acetylators, as measured by ^{32}P-postlabelling after a dose of this arylamine bladder carcinogen [49].

V. URINARY MUTAGENICITY IN RELATION TO SMOKING STATUS, TYPE OF TOBACCO AND CHARACTERISATION OF PhIP IN URINE OF BLACK TOBACCO SMOKER AS A MAJOR DNA-DAMAGING AGENT

As cigarette smokers excrete mutagenic compounds in their urine [1, 8], experiments were carried out to examine whether or not the difference in risk for bladder cancer between black and blond tobacco smokers is reflected by different levels of urinary mutagens [25]. Mutagenicity of urine extracts was measured in *S. typhimurium* TA98 in the presence of a rat liver S9 activation system. Mutagenicity, nicotine, cotinine and creatinine were measured in 21 non-smokers, 26 smokers of blond tobacco and nine smokers of black tobacco, all consuming a similar diet. Mutagenicity was expressed either per 24-h urine or per mmol creatinine. The sum of urinary nicotine and cotinine levels was used as a measurement of cigarette smoking. Mutagenic activity of the urine extract was found to increase with the nicotine and cotinine concentration and with the number of cigarettes smoked per day. Urinary mutagenicity was also related to tobacco type (Fig. 5): the urine of smokers of black tobacco contained 1.8 times as much mutagenic material as the urine of blond tobacco smokers, after adjustment for the amount of smoking (P = 0.02). The higher mutagenicity in urine of black tobacco smokers implies a two-fold higher exposure of mutagens/carcinogens in the bladder of these smokers, which closely reflects the 2–3-fold variation in bladder cancer risk between those tobacco types. The mutagenic substances in urine were characterised as aromatic amines, since they induced frameshift mutations in bacteria [50], they were easily extractable from urine by blue cotton [51], and a deamination treatment with nitrous acid completely abolished their S9-mediated mutagenicity [25, 50]. In addition, *S. typhimurium* YG1024, a derivative of *S. typhimurium* strain TA98 with high O-acetyltransferase activity, was more sensitive in detecting the mutagenicity in smokers' urine than the parent strain, further implicating aromatic amines as the mutagens [52].

The tobacco-derived mutagen(s) excreted in the urine of black tobacco smokers were characterised by comparing the DNA adducts formed when urinary mutagens with calf thymus DNA (in the presence of a liver metabolic activation system) with those formed by six reference *N*-hydroxy-arylamines, by the ^{32}P-postlabelling procedure. Autoradiography of the adducts on thin-layer plates revealed that *N*-hydroxy-2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]-quinoxaline (N-hydroxy-MeIQx), *N*-hydroxy-2-amino-3-methyl-imidazo[4,5-*f*]-quinoline (*N*-hydroxy-IQ), *N*-hydroxy-2-naphthylamine, *N*-hydroxy-4-aminobiphenyl and *N*-hydroxy-2-amino-6-methyldipyrido[1,2-a: 3',2'-*d*]imidazole (*N*-hydroxy-GLU-P-1) did not contribute to the adduct pattern produced by the urinary mutagens. However, three or four PhIP-related DNA adducts were present among the five or six adducts observed in the autoradiograms of urinary mutagen-adducted nucleotides. These postlabelling data, which were supported by mutagenicity testing combined with HPLC fractionation of urine extracts implicated PhIP as one of the mutagens in the urine of smokers of black tobacco [24, 50].

The known biological activity of PhIP as a DNA-binding agent *in vivo* in many extra-hepatic tissues, including the bladder [30, 31], and its multi-organ carcinogenicity [32, 33] suggest that this amine has a significant role in tobacco smoke-related carcinogenesis, but further studies are needed.

VI. CARCINOGEN-DNA ADDUCTS IN UROTHELIUM OF SMOKERS

The occurrence and nature of smoking-related DNA adducts in the bladder was investigated by ^{32}P-postlabelling in human

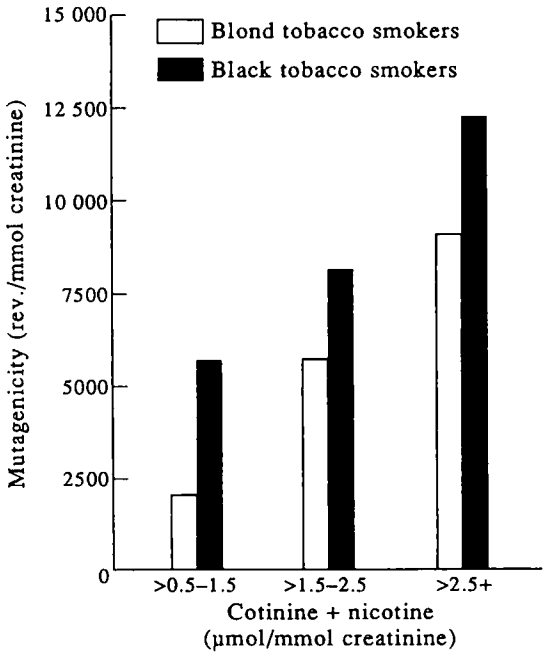

Fig. 5. Urinary mutagenicity (geometric means) as a function of quantity and type of tobacco smoked in volunteers consuming similar diets (from [25] and replotted). Mutagenicity of urine extracts was assayed in the *Salmonella* assay, TA98 strain in the presence of a metabolic activation system.

bladder biopsies and exfoliated urothelial cells from smokers. In bladder biopsies from 42 surgical patients, the levels of total bulky/aromatic adducts and of several individual DNA adducts were significantly elevated in the urothelial DNA of current smokers, compared to ex- and non-smokers [47]. In ex-smokers, after 5 years of abstinence from smoking, no such adducts were detectable, presumably due to DNA repair and cell turnover (human urothelial cells have an estimated half-life of 50–200 days). One major adduct was identified, on the basis of chromatographic behaviour as *N*-(deoxyguanosin-8-yl)-ABP, which together with another unidentified DNA adduct, were statistically significantly correlated with current cigarette smoking. Dogs, which develop bladder tumours following ABP exposure, *N*-oxidise ABP in the liver to its *N*-hydroxy derivative and this is then transported in the blood to the urinary bladder, where it forms the deoxyguanosine ABP-DNA adduct in direct proportion to the parent amine dose [20]. This appears to directly model the situation in smokers exposed to ABP. Exfoliated urothelial cells from 73 volunteers, including smokers of black and blond tobacco and non-smokers who had been investigated for ABP-Hb adducts and urinary mutagenicity were analysed by ^{32}P-postlabelling [53]. Twelve different carcinogen-DNA adducts were detected, of which at least four bulky aromatic (carcinogen) adducts were related to cigarette smoking, having levels 2–20 times higher in smokers than in non-smokers. Two smoking-related adducts were qualitatively very similar to those found in bladder biopsies from bladder cancer patients who smoked. One of these corresponded in its chromatographic behaviour to *N*-(deoxyguanosyl-8-yl)-ABP, which was best and linearly correlated with levels of ABP-Hb adducts ($r = 0.6$, $P < 0.01$), with the number of cigarettes smoked per day ($r = 0.5$, $P < 0.03$) and with urinary mutagenicity ($r = 0.5$, $P < 0.03$) [48, 53]. Levels of the second adduct also showed significant correlation with the amount and type of tobacco smoked [53]; the mean levels being 16 times higher for black tobacco than in non-smokers. In this study, this adduct was found in about 20% of the non-smokers and in those who smoked less than 15 cigarettes/day, but in 57% of the heavy smokers, suggesting that this adduct is a marker of heavy exposure to cigarette smoke. Because its chromatographic properties were similar to those of the adduct produced by *N*-hydroxy-PhIP upon reaction with DNA [53], this second smoking-related adduct in exfoliated cells could be a PhIP-DNA adduct. PhIP has been detected in tobacco smoke condensate (Table 1) and is a major DNA-damaging agent in the urine of heavy smokers of black tobacco [24].

These data reinforce the association between cigarette smoking and DNA damage and suggest a molecular basis for the initiation of human urinary bladder cancer by cigarette smoke in which aromatic amines and possibly heterocyclic amines are involved. However, given the limitations of adduct detection by the ^{32}P-postlabelling method, it is not possible to exclude a contribution to bladder carcinogenesis by tobacco-derived carcinogens that yield DNA adducts other than ones containing bulky aromatic residues.

VII. CONCLUSIONS AND PERSPECTIVES

Molecular dosimetry studies to measure levels of urinary mutagens, Hb-ABP adduction and DNA adducts in urothelial cells have provided support for the hypothesis that the observed 2–3-fold difference in bladder cancer risk between black and blond tobacco smokers is due to the higher concentration of carcinogenic aromatic and possibly heterocyclic amines in the former. In view of the sensitivity of the techniques available and the small number of smoking-related DNA adducts seen in urothelium of smokers, it now appears feasible to identify the adducts formed unequivocally, and to examine qualitatively and quantitatively the differences in the smoking-related DNA adducts formed in the urothelium of black and blond tobacco smokers.

In smokers, it is clear that the *N*-acetylation and possibly the P-450IA2 (*N*-oxidation phenotype) influence the macromolecular binding of 4-aminobiphenyl to Hb. The effect of these phenotypes on levels of aromatic amine-DNA adducts in exfoliated urothelial cells of smokers has not yet been examined. However, the linear and highly significant correlation between adduct levels of ABP with Hb and those with DNA (tentatively identified as *N*-(deoxyguanosin-8-yl)-ABP) in urothelial cells and data from a mouse model, suggest that there should be such a phenotype dependence. It would now be appropriate to conduct a study to measure the levels of aromatic amine-DNA adducts in exfoliated bladder cells in smoking subjects who have been phenotyped for *N*-acetylation and *N*-oxidation polymorphisms.

Aromatic amines form DNA adducts and in almost all instances the major adduct obtained is an arylamine derivative substituted at C8 of deoxyguanosine. Levels of C8-deoxyguanosine-arylamine adducts have been correlated with the induction of mutations in bacteria and mammalian cells. They have also been shown to cause point mutations, of which the majority are G→T transversions. Precisely this type of mutation has been detected at codon 61 of the *ras* protooncogene during the induction of mouse liver tumours by *N*-hydroxy-2-acetylaminofluorene. In addition, a point mutation at codon 61 of the *ras* protooncogene has been shown to occur in a proportion of human bladder cancers [54]. Thus, C8-deoxyguanosine-arylamine adducts may well be involved in the aetiology of aromatic amine-induced bladder cancers in humans.

There are no published data showing an excess of slow acetylators among patients with smoking-associated bladder cancer, but a number of case–control studies have consistently revealed an excess, of variable magnitude, of slow acetylators in sub-groups exposed occupationally to carcinogenic aromatic amines [19]. Figure 1 implies that the levels of aromatic amine-DNA adducts in urothelial cells of smokers should be higher in slow acetylators. Whether this leads for the same level of cigarette consumption to a higher risk of bladder cancer remains to be proven. From Fig. 4 one may infer that such a phenotype dependence might be most pronounced at low levels of cigarette consumption.

The established associations between exposure to aromatic (heterocyclic) amines in tobacco smoke, the levels of their macromolecular adducts and bladder cancer risk in smokers suggest that the stronger carcinogenic effect of black than blond tobacco shown for larynx, lung and oesophagus [7, 55, 56] may also be related to carcinogens that are present at higher concentration in black tobacco smoke. Prime candidates are volatile nitrosamine and tobacco-specific nitrosamines (NNN, NNK) (Table 2). Among the compounds identified in tobacco smoke, only the aromatic amines are associated with bladder cancer in experimental animals and humans. None of the *N*-nitrosamines which are consistently found in tobacco smoke have been shown to be bladder carcinogens in laboratory animals. Tobacco-specific nitrosamines show organ-specific effects in experimental animals, in, for example, the trachea, lung, oesophagus and nasal cavity [57]. Sensitive dosimetry methods for some of these nitrosamines have been developed [58, 59] that could

now be applied in molecular epidemiology studies for various organs in a manner analogous to that used for the urinary bladder. In addition, the analysis of mutational spectra in tumour suppressor genes and oncogenes [60] in these tumour sites could possibly demonstrate a causal link between specific carcinogen exposure, DNA modifications and tumour growth in smoking-related cancers. However, even if carcinogens can be identified as the main causative agents of certain tobacco-related cancers (such as the few aromatic amines that appear to be responsible for bladder cancer), efforts towards cancer prevention must continue to focus on complete avoidance or cessation of tobacco use.

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