Pharmacogenetics (1993) 3, 71–76

Original article

Polymorphic N-acetylation of 2-aminofluorene by cell-free colon extracts from inbred mice

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Received 15 June 1992 and accepted 3 August 1992

The increased risk of rapid acetylator humans for the development of colorectal cancer has created interest in experimental animal models to study the relationship of N-acetyltransferase phenotype to colon cancer. Colon cytosols from inbred mouse lines were assayed for the ability to N-acetylate 2-aminofluorene to determine if the mouse model of the N-acetyltransferase polymorphism could be used to study this relationship. The results indicate that the colon acetylcoenzyme A:2-aminofluorene-N-acetyltransferase activity parallels that of the liver. Colon activity from slow acetylator (A and B6.A) mouse lines is significantly lower than that of rapid acetylator (B6, B6.D, and A.B6) lines. *p*-Aminobenzoic acid N-acetyltransferase activity also differed between colon cytosols from rapid and slow acetylator strains. Isoniazid acetylation in colon and in liver did not differ between phenotypes. Northern blot analysis demonstrated the presence of mRNA for both NAT-1 and NAT-2 in mouse colon as well as in mouse liver. These results indicate that the N-acetyltransferase polymorphism is expressed in mouse colon when 2-aminofluorene or *p*-aminobenzoic acid is used as substrate and therefore the mouse may be a model for study of the effect of acetylator phenotype on development of colorectal cancer in humans.

Introduction

N-Acetylation, a major pathway of arylamine metabolism, has been found to be polymorphic in humans (reviewed in Weber, 1987), rabbits (Weber *et al.*, 1976), mice (reviewed in Levy *et al.*, 1992), hamsters (Hein *et al.*, 1982), and rats (Juberg *et al.*, 1991). On the basis of N-acetyltransferase (NAT) activity in the liver with certain substrates (polymorphically acetylated substrates), individuals can be classified as rapid or slow acetylators. This genetically determined characteristic has important consequences in the individual's encounters with arylamine and hydrazine drugs and arylamine carcinogens (reviewed in Weber, 1987).

The NAT phenotype of an individual as determined by assay of liver cytosol is usually reflected in cytosols of various extrahepatic tissues. In mice, the NAT phenotype expressed in liver has been found in blood, bladder (Mattano & Weber, 1987), kidney, and small intestine (Glowinski & Weber, 1982b). In hamsters, the liver phenotype agrees with the phenotype determined in colon, kidney, and bladder (Hein *et al.*, 1991b). In rats, the extrahepatic tissues with polymorphic NAT activity include kidney, colon, bladder, and prostate (Hein *et al.*, 1991a). An unusual expression of the polymorphism is found in the rabbit where liver and blood phenotypes appear to be reciprocally related (Weber *et al.*, 1976; Szabadi *et al.*, 1978).

Interest in the presence of polymorphic N-acetylation in various tissues is due mainly to the correlation between the slow acetylator phenotype and various drug toxicities and to bladder cancer in humans, particularly those individuals with known exposure to carcinogenic arylamines (Cartwright *et al.*, 1982). Further interest has been generated by reports of an increased risk of colon and rectal cancer in rapid acetylator humans (Lang *et al.*, 1986; Ilett *et al.*, 1987). Potential correlations of other tumours and conditions with the genetically determined acetylator status have also justified examination of various target tissues in humans and experimental animals for the NAT polymorphism.

We have previously identified inbred mouse lines that are rapid or slow acetylators (Glowinski & Weber,

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0960-314X

1982a) and produce acetylator congenic lines in which only a small piece of chromosome surrounding the NAT gene locus differs between rapid and slow acetylators (Mattano *et al.*, 1988). In the rapid acetylator congenic line, A.B6-*Nat*^{r}, the rapid acetylator allele from C57BL/6J mice has been incorporated into the genome of A/J mice replacing the normally present slow acetylator allele. Conversely, in B6.A-*Nat*^{s} mice, the slow acetylator allele of A/J has been incorporated into the normally rapid C57BL/6J mice. The acetylator congenic mice permit examination of the effects of the acetylator polymorphism with minimal interference from the modifier genes of the genetic background. The availability of parental and acetylator congenic lines has permitted us to develop a mouse model for the study of acetylation and bladder tumours. In order to expand our model to the study of colon tumours it was necessary to determine if the NAT polymorphism is expressed in mouse colon.

Materials and methods

Animals

The following inbred and congenic lines were used: C57BL/6J (B6), A/J (A), B6.A-*Nat*^{s} (B6.A), A.B6-*Nat*^{r} (A.B6), and B6.D-*Ah*^{d} (B6.D). Mice were produced in the Department of Human Genetics facilities at the University of Michigan. Mice were allowed food (Purina Mouse Chow 5020) and water *ad libitum*, and were used at between 6 and 8 weeks of age.

Assays for NAT activity

Mice were killed by cervical dislocation and the liver and colon quickly removed. The liver was blotted and then homogenized in five volumes of lysing buffer (20 mM Tris pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 50 μM phenylmethanesulfonyl fluoride, 10 μM leupeptin) using a Polytron homogenizer. The terminal 3 cm of the colon was cleaned in normal saline and homogenized in three volumes of lysing buffer.

Aliquots of the homogenates were centrifuged for 5 min at 10 000 × *g* in Eppendorf tubes and kept on ice. N-Acetyltransferase activity was determined on diluted enzyme aliquots using 2-aminofluorene (AF) (0.1 mM final concentration) and acetyl-coenzyme A (AcCoA) (0.5 mM final concentration), or *p*-aminobenzoic acid (PABA) (0.1 mM) and AcCoA (0.5 mM), or isoniazid (INH) (11.1 mM) and AcCoA (2.8 mM) and the recycling assay described by Andres *et al.* (1985) except that acetyl carnitine and carnitine acetyltransferase were used in place of acetyl phosphate and phosphotransacetylase (Mattano & Weber, 1987). Control tubes received water in place of AcCoA. The final volume of the assay was 90 μl and the final protein concentration was 0.4–1.5 μg ml^{-1}. After 10 min incubation at 37° C, 100 μl of acetonitrile for AF, 100 μl 20% trichloroacetic acid (TCA) for PABA, or 30 μl 20% TCA for INH was added and the tubes centrifuged at 10 000 × *g* for 2 min. Both enzyme assays and controls were performed in triplicate.

HPLC assay of 2-aminofluorene (AF) and 2-acetylaminofluorene (AAF)

An assay adapted from Kawakubu *et al.* (1988) was used. A Varian MCH-NCAP-5 ODS reversed phase column 4 mm × 15 cm was used with a Varian 5060 liquid chromatograph and a UV 100 detector. The solvent system was 57% 20 mM K-PO_4, pH 4.5 and 43% CH_3CN at a flow rate of 1.2 ml min^{-1}. Detection was at 280 nm. Under these conditions the acetylated product, AAF, elutes at about 6 min and unreacted substrate, AF, elutes at 8 min. Quantitation was by reference to an external standard curve. 30 μl of the supernatant fraction of the reaction mixture was injected using a 100 μl loop.

HPLC assay of p-aminobenzoic acid (PABA) and p-acetamidobenzoic acid (N-AcPABA)

The solvent system of 90% 50 mM acetic acid/10% acetonitrile was used at a flow rate of 1.2 ml min^{-1} in the system described for AF. Detection was at 266 nm. PABA eluted at about 3 min and N-AcPABA at about 7 min.

Determination of acetylated isoniazid

One ml of 0.8 M potassium borate pH 9 was added to each tube and the absorbance at 303 nm was measured. Under these conditions increase in absorbance of 6.32 indicates formation of 1 μmol of acetylated isoniazid (Weber, 1971).

Protein assay

Protein was assayed using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

RNA isolation and Northern blotting

Total RNA was isolated from colon, kidney, small intestine, and liver of adult B6 mice using procedures outlined in the RNAgent(TM) total isolation kit, from Promega (Madison, WI). Total RNA (30 μg per lane) was separated according to size by electrophoresis on a 1% agarose gel containing 6% formaldehyde and then transferred to Hybond N (Amersham, Arlington Heights, IL) using standard Northern blotting procedures (Sambrook *et al.*, 1989). Riboprobes specific for either NAT-1 or NAT-2 (Martell *et al.*, 1992) were labelled to a specific activity of 7 × 10^6 cpm μg^{-1} using the Riboprobe II Core system

from Promega and [α-^{32}P]-UTP (800 Ci mmol^{-1}) from Amersham. The Northern blot was prehybridized at 60° C for 4 h in 5% SDS, 400 mM Na-PO_4 pH 7.0, 1 mM EDTA, 1 mg ml^{-1} bovine serum albumin, and 50% formamide. Hybridization was in the same solution at 50° C for 18 h with the riboprobe specific for NAT-1 or NAT-2. The blot was washed sequentially in 0.5 × SET (0.1 M NaCl, 10 mM Tris, 1 mM EDTA pH 8.0) with 0.1% sodium pyrophosphate for 5, 10, and 15 min at room temperature and for 10 min at 55° C. The blot was exposed for 72 h at −70° C using Kodak X-OMAT film overlaid with intensifying screens.

The Northern blot was initially hybridized with the NAT-1 specific riboprobe and then stripped in 0.5 × SET + 0.1% SDS at 80° C for several hours. Removal of the NAT-1 riboprobe was confirmed by the absence of signal on an autoradiogram exposed for 1 week. The blot was then prehybridized, hybridized with the NAT-2 specific riboprobe, washed, and exposed as described for NAT-1.

Statistical treatment

The values obtained from the different lines of mice were compared for statistical significance using the *t*-test with unknown variance.

Results

AF-NAT activity in liver and colon cytosols

Originally, separate data were compiled for males and females, but as no significant differences were found between sexes for either tissue in any of the lines with any of the substrates used, the data for males and females were combined. The results for AF-NAT activity in mouse liver homogenates are shown in Table 1. The lines previously determined to be rapid acetylators (B6, B6.D, and A.B6) differed from the slow acetylators (A and B6.A) ($p < 0.001$).

The colon AF-NAT activities are reported in Table 2. The activity of colon homogenates from A mice differs from the rapid acetylator lines of mice ($p < 0.002$) and the B6.A also differs from the rapid acetylators ($p < 0.001$). Thus the NAT polymorphism for AF is expressed in colon of these mouse lines in parallel to the expression of the polymorphism in liver.

Table 1. 2-Aminofluorene NAT activity in liver cytosols from rapid and slow acetylator mice

Mouse line	*Activity ± SEM (nmol min^{-1} per mg protein)*	*No. of mice tested*
Rapid:		
B6	10.07 ± 0.35	10
B6.D	9.17 ± 0.31	10
A.B6	9.35 ± 0.24	10
Slow:		
A	4.83 ± 0.15[a]	10
B6.A	4.43 ± 0.14[a]	10

[a]Differs significantly from rapid lines $p < 0.001$.

Table 2. 2-Aminofluorene NAT activity in colon cytosols from rapid and slow acetylator mice

Mouse line	*Activity ± SEM (nmol min^{-1} per mg protein)*	*No. of mice tested*
Rapid:		
B6	5.36 ± 0.14	10
B6.D	5.19 ± 0.35	7
A.B6	5.27 ± 0.24	10
Slow:		
A	4.39 ± 0.22[a]	8
B6.A	2.89 ± 0.19[b]	10

[a]Differs significantly from rapid lines $p < 0.002$; [b]differs significantly from rapid lines $p < 0.001$.

PABA-NAT activity in liver and colon cytosols

A small number of B6 and A mice were used to determine if PABA, a specific substrate for NAT-2 in mice (Martell *et al.*, 1992), was polymorphically acetylated in liver and colon. The results shown in Table 3 indicate that, in agreement with several previous studies (Mattano & Weber, 1987; Hein *et al.*, 1988), specific activity of PABA-NAT in B6 liver is about twice that of A liver. Mouse colon homogenates were also able to acetylate PABA. The specific activity of cytosols from B6 mouse colon was approximately twice that of cytosols from A mouse colon.

INH-NAT activity in liver and colon cytosols

Expressed mouse NAT-1 and NAT-2 were both shown to acetylate INH although NAT-1 had six- to seven-fold the specific activity of either the slow or rapid allelic forms of NAT-2 (Martell *et al.*, 1992). Measurement of INH-NAT activity in cytosols of B6 and A mice indicates no significant difference between strains in specific activity for the acetylation of INH in either liver or colon (Table 4). However, unlike the results for

Table 3. *p*-Aminobenzoic acid NAT activity in liver and colon cytosols from B6 and A mice

Mouse line	*Activity ± SEM (nmol min^{-1} per mg protein)*		*No. of mice tested*
	Liver	*Colon*	
B6	6.13 ± 0.20	2.13 ± 0.13	3
A	3.19 ± 0.27[a]	0.93 ± 0.02[a]	3

[a]Differs significantly from B6 $p < 0.001$.

AF and PABA, specific activity towards INH was higher in colon than in liver.

Presence of RNA message for NAT in liver and colon

It was previously demonstrated through use of riboprobes specific for either Nat-1 or Nat-2 that mRNAs for NAT-1 and NAT-2 were present in B6 and A mouse livers (Martell *et al.*, 1992). Figure 1 shows the presence of NAT-2 mRNA in colon as well as liver, kidney, and small intestine of B6 mice. In the figure, the band at approximately 1.4 kb corresponds to NAT-2 mRNA. Bands near 5.3 and 1.9 kb are due to non-specific interaction with large amounts of 28S and 18S ribosomal RNA present in the total RNA preparation applied to the gel. Probing the Northern blot with an NAT-1 specific riboprobe showed a weak signal for NAT-1 in colon and liver migrating beyond the 1.6 kb marker to approximately 1.4 kb (data not shown).

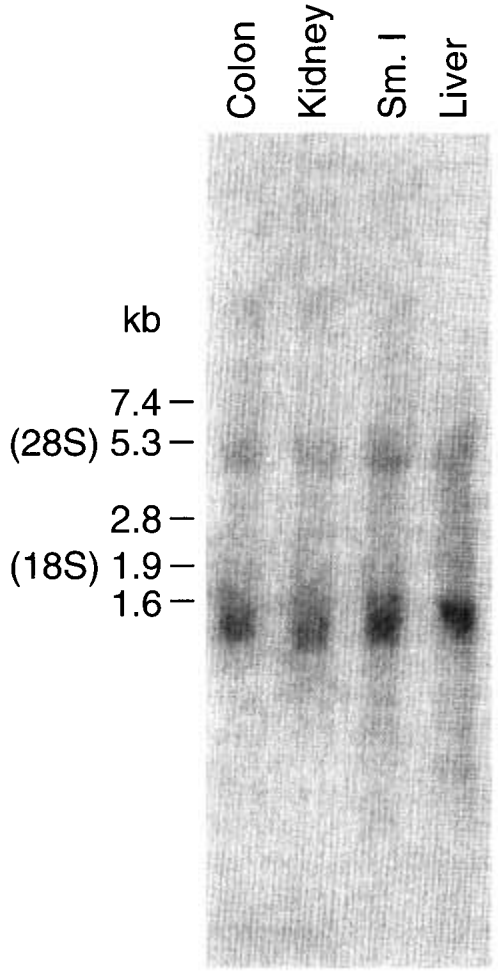

Fig. 1. Northern blot analysis of NAT-2 expression. Sample lanes were loaded with 30 μg of total RNA from colon, kidney, small intestine (Sm. I) and liver of B6 mice. Molecular weight markers to the left of the blot indicate the size in kilobases (kb) and the migration of the RNA. The blot was hybridized with a riboprobe specific for NAT-2 as described in Materials and methods.

Discussion

Inbred mice, and in particular acetylator congenic inbred mice, have been used to study the relationship between acetylator phenotype and arylamine induced DNA damage in liver (Levy & Weber, 1989) and urinary bladder (Levy & Weber, 1992), two of the targets of arylamine carcinogens in mice. The interest in the relationship of acetylator phenotype to DNA damage arises from the association of bladder cancer in humans with the slow acetylator phenotype who are occupationally exposed to arylamines (Cartwright *et al.*, 1982), and from the known importance of NAT in the metabolic activation of arylamines (Garner *et al.*, 1982). Studies with the mouse model of NAT polymorphism have shown higher DNA-AF adduct levels in livers of rapid acetylator mice than in slow acetylators after exposure to AF (Levy & Weber, 1989). Conversely, slow acetylator mice had higher DNA-AF adduct levels in urinary bladder DNA than did rapid acetylators after AF exposure, although age and sex exerted considerable influence on bladder DNA adduct formation (Levy & Weber, 1992). The distribution of AF-DNA adducts between liver and bladder in male and female mice agreed with the distribution of benzidine-induced (Nelson *et al.*, 1982) and 4-aminobiphenyl-induced (Schieferstein *et al.*, 1985) tumours found in chronic tumourigenesis studies: males had a preponderance of bladder tumours while females developed mainly liver tumours.

Reports showing an association of human colon tumours with the rapid acetylator phenotype (Lang *et al.*, 1986; Ilett *et al.*, 1987) have raised the question of using the acetylator congenic mice to study colon cancer. The situation for colon cancer differs somewhat from that for bladder cancer in that for bladder cancer documented exposure to carcinogenic arylamines such as benzidine or β-naphthylamine is an important factor in the slow acetylator-bladder cancer relationship. For colon cancer, no exposure to

Table 4. Isoniazid NAT activity in liver and colon cytosols from B6 and A mice

Mouse line	Activity ± SEM (nmol min^{-1} per mg protein)		No. of mice tested
	Liver	Colon	
B6	0.64 ± 0.21	2.20 ± 0.30	3
A	0.44 ± 0.14	2.94 ± 0.44	3

known carcinogens has been established. It has been speculated that heterocyclic amines produced by cooking of protein-containing foodstuffs could be the carcinogens responsible for colon cancer since it is known that many of the heterocyclic amines in cooked foods are mutagenic and carcinogenic in mammals (Kato & Yamazoe, 1987).

As a preliminary experiment in developing a mouse model for study of the human colon cancer-acetylator phenotype question, we examined colon NAT activity towards the carcinogenic arylamine AF. The colon AF-NAT activity of slow acetylators was found to be statistically significantly lower than that of rapid acetylators, similar to the results found in liver.

Mice have at least 2 NAT activities, NAT-1 and NAT-2. NAT-1 is often referred to as monomorphic because its activity is identical in livers of rapid and slow acetylator mouse lines. NAT-2 is polymorphic as its activity differs between rapid and slow acetylator strains. The nucleotide sequence of the coding region for NAT-1 has been shown to be identical in B6 and A mice whereas the coding region of NAT-2 differs at nucleotide 296 by having an adenine in B6 replaced by a thymine in A mice (Martell *et al.*, 1991). The nucleotide change leads to substitution of isoleucine in A mice for asparagine in B6 at position 99 of the deduced amino acid sequence (Martell *et al.*, 1991). The consequences of the resulting change in hydrophobicity of the surrounding area of NAT-2 include a drastic decrease in stability of the enzyme from A mice at 37° C (Martell *et al.*, 1992). The nucleotide sequence of NAT-2 from A.B6 was identical to B6 and the sequence of NAT-2 from B6.A was identical to A (DeLeon *et al.*, 1992).

Expression studies of rapid, slow, and monomorphic NAT's in COS-1 cells showed that the NAT-2 enzymes were active towards AF and p-aminobenzoic acid (PABA), whereas NAT-1 was active towards AF but not towards PABA. Isoniazid (INH) was a relatively good substrate for NAT-1 but was poorly and equally acetylated by both the NAT-2 enzymes (Martell *et al.*, 1992). Northern blots of colon and liver RNA probed with specific NAT-1 and NAT-2 riboprobes showed the presence of message for both NAT enzymes in liver and in colon. The presence of message for the polymorphic NAT-2 confirms the results based on activity (Table 2), that the mouse colon is indeed polymorphic for AF-NAT activity. The results of the experiment with PABA (Table 3) also agree with this conclusion.

The acetylation of INH by mouse colon cytosols can not be definitely ascribed to NAT-1 or NAT-2, as both NAT-1 and NAT-2 showed monomorphic acetylation of this substrate when studied with expressed enzymes (Martell *et al.*, 1992). The low level of liver INH-NAT reported here (Table 4) and previously by Hein *et al.* (1988) may be explained by low activity of NAT-1 in mouse liver. Acetylation of INH is greater in colon than liver for both B6 and A mice (Table 4), suggesting NAT-1, which has high activity for INH, is a significant part of the acetylating activity in the colon of mice. That NAT-1 contributes significantly to acetylation in mouse colon is also supported by the smaller difference between AF-NAT activity in rapid and slow acetylator colons compared to livers. The ratio of AF-NAT activity between rapid liver and slow liver is about 2 while the ratio between rapid and slow colons is about 1.4, suggesting a significant contribution of the monomorphic NAT-1 activity in mouse colon. PABA is not a substrate for NAT-1 (Martell *et al.*, 1992) and the PABA-NAT activity in both liver and colon shows a two-fold difference between rapid and slow strains (Table 3), as is to be expected in the absence of a significant contribution from a monomorphic NAT activity.

While the Northern blots indicated that both NAT's are produced in colon, no information as to quantities of the two enzymes, their rates of turnover, or the efficiency of translation of their mRNAs in colon is available at present. The use of specific antibodies able to distinguish NAT-1 from NAT-2, when available, may provide an answer to the question of how much of which activity is present in the various tissues.

The mouse colon has now been shown to express the NAT polymorphism similar to liver, blood, urinary bladder, kidney, and small intestine. Use of the mouse model to study a relationship between acetylator phenotype and heterocyclic amine induced colon cancer next requires determining if heterocyclic amines or some metabolite of heterocyclic amines are polymorphically acetylated and if the difference in acetylation correlates with some measure of DNA damage in colonic mucosa.

Acknowledgements

We thank Ms Lily Hu for excellent technical assistance. This work was supported by USPHS grants OH 00081, CA 39018, and GM 44965.

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