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Monomorphic and Polymorphic Human Arylamine *N*-Acetyltransferases: A Comparison of Liver Isozymes and Expressed Products of Two Cloned Genes

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SUMMARY

A genetic polymorphism of human liver arylamine *N*-acetyltransferase (NAT; EC 2.3.1.5) enzyme activity divides populations into distinguishable "slow acetylator" and "rapid acetylator" phenotypes. Two human genes, *NAT1* and *NAT2*, encoding NAT proteins [*DNA Cell Biol.* 9:193–203 (1990)] were transiently expressed in cultured monkey kidney COS-1 cells, and the resulting recombinant NAT1 and NAT2 proteins were compared with *N*-acetyltransferase activities in human liver cytosol with respect to their stability, chromatographic behavior on anion exchange columns, electrophoretic mobility, and arylamine acceptor substrate specificity. NAT1 was far less stable *in vitro* than NAT2. Under conditions designed to optimize enzyme stability, anion exchange chromatography experiments revealed that enzymes corresponding to both recombinant NAT1 and NAT2 were expressed in human liver. Recombinant and human liver NAT1 enzymes showed the same characteristic selectivity (low apparent K_m, high V_{max}) for the "monomorphic" substrates *p*-aminosalicylic acid and *p*-aminobenzoic acid. Such substrates fail to discriminate between the acetylator phenotypes *in vivo*. The same criteria established that recombinant NAT2 was indistinguishable from one of two previously observed *N*-acetyltransferases (NAT2A and NAT2B) whose liver contents correlate with acetylator phenotype in human populations. Recombinant NAT2 and the liver NAT2 isoforms NAT2A and NAT2B selectively *N*-acetylated the "polymorphic" substrates sulfamethazine and procainamide, whose disposition *in vivo* is affected by the acetylation polymorphism. Interestingly, the carcinogen 2-aminofluorene was very efficiently metabolized by both NAT1 and NAT2. Independent regulation of *NAT1* and *NAT2* genes was suggested by a lack of correlation of NAT1 and NAT2 enzyme activities in cytosols from 39 human livers. The results provide strong evidence that the *NAT2* locus is the site of the human acetylation polymorphism. In addition, the use of recombinant NAT1 and NAT2 will allow us to predict whether any given arylamine will be polymorphically acetylated in humans.

The CoASAc-dependent *N*-acetylation of many primary arylamine and hydrazine xenobiotics by liver cytosolic NAT (CoASAc:arylamine *N*-acetyltransferase, EC 2.3.1.5) plays a key role in their inactivation and/or elimination from the body. The reaction involved is a classical two-step substituted-enzyme (ping-pong) kinetic mechanism (1), as shown below (where Ac is acetyl and Ar is aryl).

$$\text{NAT} + \text{CoASAc} \rightarrow \text{AcNAT} + \text{CoASH} \quad (1)$$

$$\text{Ac-NAT} + \text{Ar-NH}_2 \rightarrow \text{Ar-NHAc} + \text{NAT} \quad (2)$$

$$\text{Arylamine} \rightarrow \text{arylamide}$$

Of particular significance to clinicians and toxicologists has been the recognition for over 30 years that a genetic polymorphism leading to wide interindividual variations in *N*-acetylation capacity in human populations often results in the occurrence of drug- and chemical-induced toxicity (2–4). This so-called acetylation polymorphism, which affects the disposition of such diverse therapeutic agents as isoniazid, many sulfonamides, procainamide, hydralazine, dapsone, and caffeine, as well as several potential carcinogenic amines, divides the Caucasian population into almost equal proportions of phenotypically "rapid" and "slow" acetylators (4).

Paradoxically, certain arylamines, such as PAS, PABA, and sulfanilamide, are acetylated at a high rate yet are unable to distinguish between rapid and slow acetylators either *in vivo* (5, 6) or in liver (7) or blood (8) *in vitro*. Such compounds came to be known as "monomorphic" substrates, in distinction to "polymorphic" substrates such as SMZ and PA, which could indeed be used as probe drugs in simplified clinical tests to determine acetylator phenotype.

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ABBREVIATIONS: CoASAc, acetylcoenzyme A; NAT, arylamine *N*-acetyltransferase; PAS, *p*-aminosalicylic acid; PABA, *p*-aminobenzoic acid; SMZ, sulfamethazine; PA, procainamide; AF, 2-aminofluorene; KDL, kidney donor liver; HPLC, high performance liquid chromatography.

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Jenne (6), in the first detailed biochemical studies of drug acetylation in humans, proposed that two different *N*-acetyltransferase enzymes were responsible for the metabolism of these distinct classes of arylamines. Moreover, it appeared that the observed genetic variation in acetylation of polymorphic substrates was related to differences in the quantity of one of these enzymes present in human liver cytosol rather than to altered kinetic characteristics of a structural variant. However, subsequent work using the rabbit as an animal model for the human acetylation polymorphism shed doubt on this concept, because kinetic, biochemical, and immunological experiments in rabbits (9–11) suggested 1) that a single species of enzyme protein mediates the *N*-acetylation of both monomorphic and polymorphic substrates and 2) that liver cytosols from slow and rapid acetylator rabbits possess equal quantities of enzyme molecules with different substrate kinetic characteristics.

We have recently conducted experiments, both in humans and in the rabbit model, that were designed to elucidate the biochemical and molecular mechanisms responsible for producing the observed interindividual variations in arylamine *N*-acetylation. These studies have so far yielded the following results. Firstly, contrary to the abovementioned predictions for the rabbit model, it is now clear that defective arylamine *N*-acetylation in slow acetylator rabbits is caused by the specific absence of a gene encoding a 33-kDa *N*-acetyltransferase protein, leading to a complete absence of both the enzyme and its transcript (12). Secondly, *in vivo/in vitro* correlation studies using an antibody of high specificity raised against a purified human *N*-acetyltransferase (13) indicate that the slow acetylator phenotype in humans is associated with a marked decrease in the quantity of two closely related acetylating enzymes, NAT2A and NAT2B[2] (14). Most recently, we have employed molecular cloning methods to isolate two human gene sequences, designated *NAT1* and *NAT2*, which encode functional *N*-acetyltransferase enzymes; a third isolated region contains a related pseudogene, *NATP* (15).

In the present study we have characterized the NAT1 and NAT2 proteins produced from the cloned human genes via heterologous expression in cultured mammalian cells, and we have compared their substrate specificity, chromatographic behavior, electrophoretic mobility, and immunoreactivity with those of *N*-acetyltransferase enzymes detectable in human liver cytosol. We demonstrate 1) that recombinant NAT1 and NAT2 show significant differences in *in vitro* stability and electrophoretic mobility, that both can be detected in human liver cytosol, and that only NAT2 corresponds to one of the two previously detected isozymes (13, 14); 2) that NAT1 and NAT2 show marked selectivity for monomorphic and polymorphic arylamine substrates, respectively; 3) that the carcinogen AF is an extremely good substrate for both enzymes; and 4), as previously suggested (15), that *NAT2* is the polymorphic gene responsible for the human acetylation polymorphism.

Materials and Methods

Chemicals and reagents. The arylamine acceptor substrates SMZ, PA, PAS, PABA, and AF, the acetylated metabolite acetyl-AF, and all enzyme assay components were obtained from Sigma Chemical Co. Other *N*-acetylated drug metabolites were synthesized as described in Ref. 16, and their purity was verified by HPLC. DEAE-Sephacel was from Pharmacia, and all other chemicals and reagents were analytical grade, from standard suppliers.

Sources of enzymes. Human liver samples were obtained as previously described (14, 17). KDL 26 provided the source of liver cytosol for anion exchange chromatography experiments. Human recombinant NAT1 and NAT2 were obtained by transient expression of cloned *NAT1* and *NAT2* genomic DNA fragments (15) in COS-1 monkey kidney cells, using the expression vector p91023(B) (18), according to methods outlined recently (12). Cytosols from transfected cell cultures were prepared as described (12) and used without further purification. Cytosols for activity comparisons between human livers were prepared using a micromethod adapted from Ref. 14, in which cytosols were isolated from tissue homogenates with a rapid (12-min) ultracentrifugation in a Beckman Airfuge and were then used immediately for enzyme assays.

***N*-Acetyltransferase enzyme assay.** Measurements of enzyme activity were performed essentially as described (13, 14), with minor modifications as follows. Incubations (90 μl) contained 40 μl of enzyme source (cytosolic preparations or partially purified anion exchange column fractions, suitably diluted in the previously described homogenization buffer containing 1 mg/ml bovine serum albumin); 20 μl of acetyl-DL-carnitine/carnitine acetyltransferase cofactor-regenerating system (19) dissolved in 225 mM triethanolamine-HCl, 4.5 mM DL-dithiothreitol, 4.5 mM EDTA, pH 7.5; 20 μl of CoASAc (450 μM in water); and varying amounts of substrate added in 10 μl of 25% dimethyl sulfoxide to start reactions. Formation of *N*-acetylated products was quantified by HPLC using the conditions previously described (13), except that the UV detection wavelength was 270 nm for the PA, PAS, PABA, and AF acetylation assays and the acetonitrile content of the mobile phase was increased to 40% (w/w) for determination of acetyl-AF. Under these conditions, retention times of each substrate and its acetylated metabolite were, respectively, SMZ, 3.0 and 4.2 min; PA, 1.3 and 2.7 min; PAS, 2.5 and 3.8 min; PABA, 1.7 and 3.0 min; and AF, 1.2 and 3.0 min. Following initial pilot experiments, enzyme velocity measurements were made using 15 acceptor substrate concentrations ranging from approximately $0.1K_m$ to $10K_m$, except where limited by substrate solubility or by the detection limits of the HPLC assay. Kinetic parameters (apparent K_m, V_{max}) were determined by the method of Hofstee (20), and protein concentrations were measured using a dye-binding method (21).

Anion exchange chromatography. The following modifications to the published protocol (14) were made to improve the recovery and resolution of eluted enzymes. Cytosols were applied to DEAE-Sephacel columns directly following overnight dialysis, omitting the ammonium sulfate and pH-shift prepurification steps. Column length was increased from 25 to 30 cm, and the volume of collected fractions was changed from 2 to 1.5 ml. Aliquots of column fractions were analyzed for enzyme activity using SMZ (500 μM), PA (1000 μM), PAS (100 μM), PABA (100 μM), and AF (100 μM) as acceptor substrates. One unit of enzyme activity was defined as that producing 1 μmol of acetylated product/min at 37°, using the substrate concentrations given above. Appropriate peak fractions from anion exchange column eluates were pooled, concentrated, and used for kinetic studies and detection of immunoreactive proteins.

Immunodetection of proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, immunoreaction with a polyclonal rabbit antiserum raised against a purified human NAT protein (13), and densitometric quantitation of autoradiogram intensities in arbitrary units of area density were performed using standard protocols, as recently described (14).

Results

Enzyme stability. Fig. 1 illustrates the marked difference in intrinsic stability between the two recombinant gene prod-

[2] We had previously isolated two closely related isozymes of NAT, using SMZ as acceptor substrate (13), and designated them as "NAT-1" and "NAT-2" (13–15). On the basis of the results in the present study, we have renamed these two activities NAT2A and NAT2B, to indicate that both of these activities arise from the *NAT2* gene (15) and to distinguish them from the single *NAT1* gene product, NAT1.

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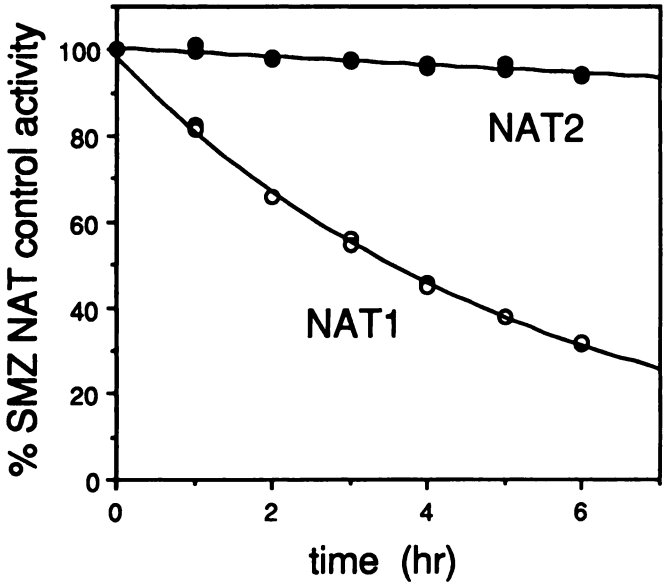

Fig. 1. *In vitro* stability of recombinant human NAT1 and NAT2. Cytosols from transfected COS-1 cells were diluted to 0.08 mg/ml with TEDK buffer (10 mM triethanolamine·HCl, 1 mM EDTA, 1 mM DL-dithiothreitol, 50 mM KCl, pH 7.0), containing 1 mg/ml bovine serum albumin, and were incubated at 37°. At the times indicated, duplicate aliquots were removed and assayed for enzyme activity, using SMZ (500 μM) as acceptor amine. Control product formation rates (time = 0) were 13.9 and 26.5 nmol/min/mg of COS-1 cell cytosol protein for NAT1 and NAT2, respectively.

ucts. NAT2 was about 17-fold more heat stable at 37° ($t_{1/2}$ = 61 hr) than NAT1 ($t_{1/2}$ = 3.5 hr) under the enzyme dilution conditions we employed. In addition, we observed a far higher correlation between SMZ-NAT and AF-NAT activities in cytosols from different human livers when the cytosols were previously frozen and thawed (r = 0.95, n = 24) than when they were assayed immediately after preparation (r = −0.12, n = 17). This suggested the possibility that a second, more labile, enzyme that acetylates AF is present in human liver, in addition to the one that catalyzes the acetylation of both AF and SMZ.

Anion exchange chromatography of human liver cytosol and recombinant gene products. Using the described modifications to the original conditions for preparing cytosols for anion exchange chromatography, we detected a large peak of *N*-acetyltransferase activity in human liver cytosol using low concentrations (100 μM) of PABA or PAS as the acceptor amine (Fig. 2A), in addition to the two (Fig. 2B) that had previously been observed with SMZ or with much higher concentrations (10 mM) of PABA and PAS (13, 14). This PABA/PAS activity peak coincided with the elution profile of recombinant NAT1 applied to an identical anion exchange column (Fig. 2A). Recombinant NAT2 eluted at a position similar to that of liver NAT2A (Fig. 2B); the isoforms NAT2A and NAT2B had been previously observed and designated "NAT-1" and "NAT-2."[2] Of particular interest was the finding that the first activity peak was selectively detected using the 'monomorphic' substrates PAS and PABA as acceptor amines, whereas the two peaks eluting at higher salt concentrations were seen with the "polymorphic" substrates SMZ and PA. On the other hand, all three peaks of activity were detected with AF as substrate (Fig. 2C). The enzyme activity peak detected by PAS or PABA in liver cytosol was far less stable than those measured using SMZ or PA; overnight dialysis of cytosol at 4°

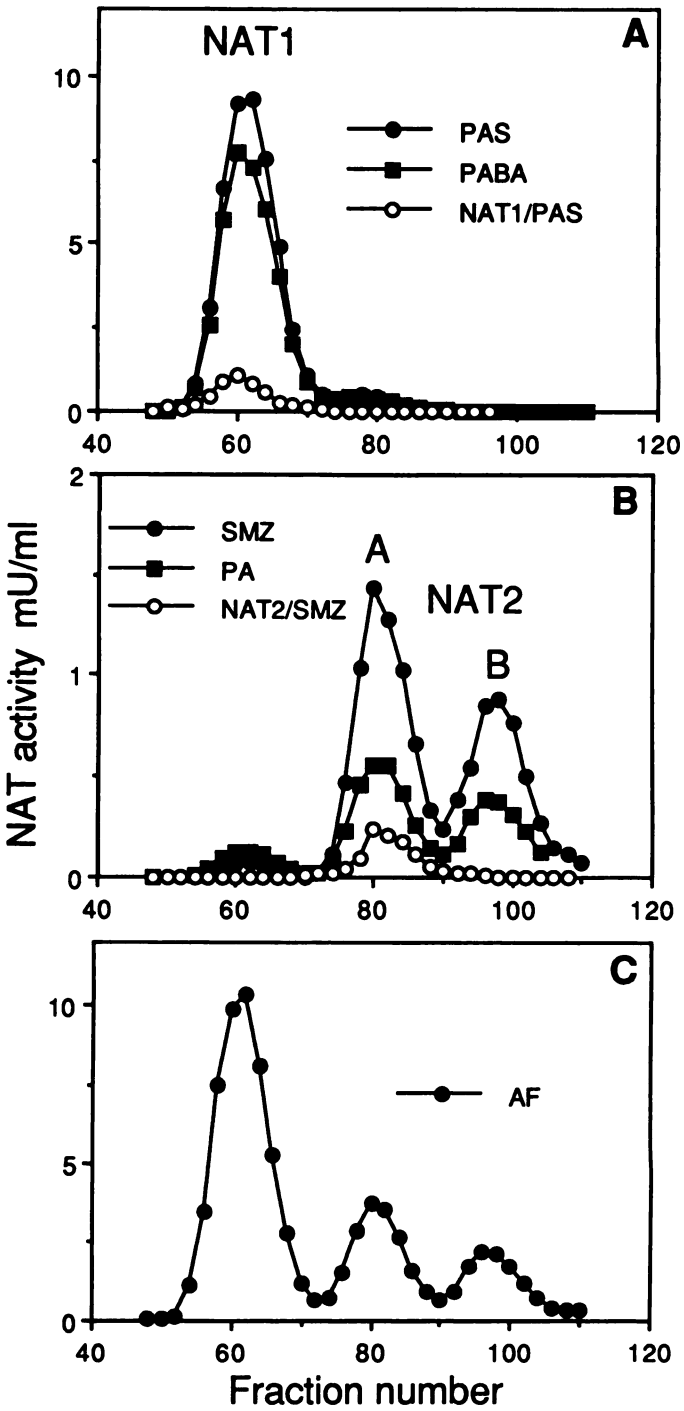

Fig. 2. DEAE-Sephacel anion exchange chromatography of cytosols from human liver KDL 26 (●, ■) and from COS-1 cells expressing recombinant human *NAT1* and *NAT2* gene products (○). Columns (1.6 × 30 cm) were equilibrated with TEDK buffer (see legend to Fig. 1), dialyzed cytosols (194, 0.8, and 1.8 mg of cytosol protein from KDL 26, COS/NAT1, and COS/NAT2, respectively) were applied, and enzyme activities were eluted with a linear 200-ml gradient from 50 to 200 mM KCl. Column eluates (1.5 ml) were analyzed for enzyme activity using the monomorphic acceptor amines PAS or PABA (A), the polymorphic substrates SMZ or PA (B), and the carcinogen AF (C).

before anion exchange chromatography led to a 42% loss of PAS-NAT activity and only a 5% loss of SMZ-NAT activity.

Fig. 3 shows a comparison between recombinant and human liver *N*-acetyltransferase protein immunoreactivity, using a

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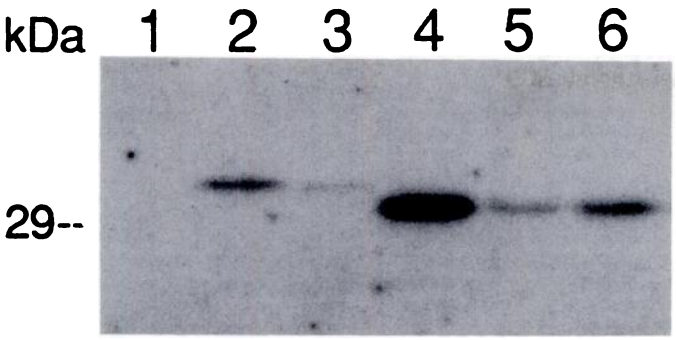

Fig. 3. Immunochemical comparison of recombinant and human liver *N*-acetyltransferase proteins. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gels (12%), electrophoretically transferred to nitrocellulose, and reacted with a polyclonal rabbit antiserum raised against human NAT2A (13). *Lane 1*, COS-1 cell cytosol (10 μg of protein) from mock-transfected cells; *lane 2*, COS-1 cell cytosol (10 μg of protein, 4.2 milliunits of PAS-NAT) from cells transfected with human *NAT1; lane 3*, aliquot from fraction 60 of KDL 26 anion exchange column elution (50 μg of protein, 0.7 milliunits of PAS-NAT); *lane 4*, COS-1 cell cytosol (10 μg of protein, 0.3 milliunits of SMZ-NAT) from cells transfected with human *NAT2; lane 5*, aliquot from fraction 80 of KDL 26 anion exchange column elution (10 μg of protein, 0.05 milliunits of SMZ-NAT); *lane 6*, aliquot from fraction 96 of KDL 26 anion exchange column elution (10 μg of protein, 0.07 milliunits of SMZ-NAT).

polyclonal rabbit antiserum raised against NAT2A (13). Immunoreactive protein with electrophoretic mobility equal to that of recombinant NAT1 (apparent molecular mass, 33 kDa) could be detected in the first eluting activity peak from human liver cytosol (Fig. 3, *lanes 2* and *3*), whereas recombinant NAT2 and human liver NAT2A and NAT2B showed the same apparently lower molecular mass of about 31 kDa (Fig. 3, *lanes 4–6*). We do not know the reason for the difference in electrophoretic mobility between NAT1 and NAT2, given that their deduced molecular masses are almost identical [33.8 and 33.5 kDa, respectively (15)]. NAT1-associated immunoreactivity in human liver was very weak (Fig. 3, *lane 3*) even after the partial purification, although the catalytic activity was considerable. This suggests that NAT1 possesses a very high specific activity with PAS and PABA.

Kinetic properties of human *N*-acetyltransferase enzymes. Tables 1 and 2 summarize the results of studies to functionally characterize the recombinant and human liver *N*-acetyltransferase enzymes, respectively. Three important results were obtained from these experiments.

Firstly, comparison of the substrate affinities of the recombinant enzymes with those of liver cytosol confirmed the results from the anion exchange chromatography and immunochemical experiments described above, that the *NAT1* gene product is in fact expressed in human liver. Enzyme with substrate specificity identical to that of recombinant NAT1 could be detected in human liver when requirements for preservation and detection of its activity were met.

Secondly, the kinetic results verified that recombinant NAT2 likely corresponds to only one of the two peaks (NAT2A) of enzyme activity detected with SMZ after anion exchange chromatography of human liver cytosol (Fig. 2B). This leaves open the question of the origin of the additional peak of *N*-acetyltransferase activity (NAT2B) observed in human liver. Because our cloning results (15) strongly suggested that no other closely related gene sequences exist in the human genome, the similarity in kinetic properties (Table 2), electrophoretic mobility (Fig. 3), and between-liver expression (14) of these two activities lead us to propose that NAT2A and NAT2B are both products of the *NAT2* locus; furthermore, NAT2B appears to arise from the primary *NAT2* gene product (i.e., NAT2A) either by post-translational processing or as an *in vitro* artifact of the purification procedure. For this reason we have renamed NAT-1 and NAT-2 (13) as NAT2A and NAT2B, respectively, to denote that they both arise from *NAT2*.[2]

Thirdly, both the recombinant and liver NAT1 possessed a much higher affinity (lower apparent K_m) than NAT2 for the monomorphic substrates PAS and PABA, whereas NAT2 had a higher affinity than NAT1 for SMZ and PA. NAT2 had a remarkably high affinity for the carcinogen AF ($K_m < 2$ μM), and the affinity of NAT1 for AF was comparable to that for PAS and PABA.

Tables 1 and 2 also show estimates of intrinsic clearance [$Cl_i = V_{max}/K_m$ (22)] for the expressed and liver enzymes, respectively. In Table 1, maximal velocities were expressed per arbitrary unit of immunoreactive enzyme protein (Fig. 3, *lanes 2* and *4*). Assuming that the NAT1 and NAT2 protein molecules present in the transfected cell cytosols possess similar immunoreactivity with the polyclonal serum used for their detection, the values in Table 1 represent a comparison of the molecular efficiencies of the two enzymes for the substrates listed. NAT1 possessed much higher Cl_i values for PAS and PABA than for SMZ and PA, whereas NAT2 cleared the latter two compounds more efficiently. AF was cleared at a very high rate by both of the *N*-acetyltransferase enzymes. An identical pattern of relative Cl_i values (NAT2: SMZ > PA > AF ≫ PAS, PABA :NAT1)

TABLE 1

Enzyme kinetic constants for recombinant human NAT1 and NAT2 expressed in COS-1 cells

Kinetic parameters for recombinant NAT1 and NAT2 were determined in cytosols from COS-1 cells transfected with *NAT1* and *NAT2* gene vectors. Maximal velocities were expressed per arbitrary unit of immunoreactive protein, determined by densitometric quantitation of autoradiograms after immunoreaction on Western blots (Fig. 3)

Substrate	NAT1			NAT2			Cl_i ratio, NAT2/NAT1
	K_m	V_{max}	Cl_i (V_{max}/K_m)	K_m	V_{max}	Cl_i (V_{max}/K_m)	
	μM	nmol/min/unit	ml/unit/min	μM	nmol/min/unit	ml/unit/min	
SMZ	1,160	28	0.024	120	37	0.31	12.9
PA	29,700	510	0.017	2,750	27	0.01	0.6
PAS	11	1,280	116	2,900	14	0.005	<0.0001
PABA	15	1,250	83	—[a]	—[a]	—[a]	<0.001
AF	18	1,395	78	1.1	36	33	0.4

[a] PABA showed apparent substrate activation with NAT2 (logarithmic increase in product formation rate at increasing PABA concentrations to 10 mM, followed by enzyme degradation at higher concentrations), preventing any determination of kinetic constants. The rate of acetyl-PABA formation by NAT2 at 10 mM PABA was 83% of that using an equivalent concentration of PAS as substate.

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TABLE 2

Enzyme kinetic constants for NATs from human liver KDL 26

V_{max} values are in nmol/min/mg of protein in partially purified activity peaks from anion exchange chromatography of KDL 26 liver cytosol (Fig 2), and $C1_i$ values (V_{max}/K_m) are in ml/mg/min. The arithmetic mean of clearances by NAT2A and NAT2B was used to calculate NAT2/NAT1 clearance ratios.

Substrate	NAT1			NAT2A			NAT2B			C_l ratio, NAT2/NAT1
	K_m	V_{max}	$C1_i$	K_m	V_{max}	$C1_i$	K_m	V_{max}	$C1_i$	
	μM	nmol/min/mg	ml/mg/min	μM	nmol/min/mg	ml/mg/min	μM	nmol/min/mg	ml/mg/min	
SMZ	1,530	0.42	0.0003	117	5.50	0.047	87	7.49	0.086	220
PA	20,300	5.97	0.0003	2,740	6.19	0.002	1,640	8.82	0.005	12
PAS	9	14.75	1.64	3,000	2.61	0.0009	3,900	4.53	0.001	0.0006
PABA	12	13.85	1.15	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]	<0.001
AF	15	16.40	1.09	1.7	11.4	6.706	1.4	13.8	9.857	7.6

[a] See footnote to Table 1.

was observed using the partially purified enzymes from human liver KDL 26 (Table 2), although absolute values were different as a consequence of the natural levels of enzyme expression in human liver. Thus, in at least one human liver the actual levels of expression of NAT1 and NAT2 proteins strongly favored selective clearance of SMZ and PA by NAT2 (both A and B forms) and of PAS and PABA by NAT1. Again, AF was very efficiently converted to its acetylated metabolite by all three of the enzymes present in KDL 26.

The ratios of intrinsic clearances by NAT1 and NAT2 for each substrate are also given in Tables 1 and 2. In human liver, a high NAT2/NAT1 clearance ratio, characteristic of selective clearance by NAT2, was displayed by SMZ, PA, and AF, whereas PAS and PABA showed a very low ratio, characteristic of highly selective clearance by NAT1.

Expression of NAT1 and NAT2 in different human livers. Our cloning studies (15) demonstrated that the *NAT1* and *NAT2* gene loci are both on chromosome 8 but that their protein-coding regions are separated by at least 30 kilobases. The question that then naturally arose was whether NAT1 and NAT2 expression is independently regulated in human liver. From the kinetic data given above, we chose concentrations of PAS and PABA (100 μM) or of SMZ (500 μM) that detect almost exclusively NAT1 or NAT2 activity, respectively, in unpurified human liver cytosol. Fig. 4 shows the results of an enzyme activity screen of cytosolic fractions from 39 human livers with the abovementioned substrates, using cytosol preparation conditions to optimize detection of the labile NAT1 enzyme activity. As expected, activities using the two monomorphic substrates PAS and PABA, specific for NAT1, correlated very highly (Fig. 4, *top*). The NAT1 activities varied over a wide range among the different livers, with product formation rates in several livers that were 4–5-fold higher than activities in rapid acetylator liver for the polymorphic substrate SMZ (14). On the other hand, comparison of PAS-NAT and SMZ-NAT activities in human liver cytosols (Fig. 4, *bottom*) showed no evidence of any correlation, suggesting that NAT1 and the genetically polymorphic NAT2 are independently expressed in human liver.

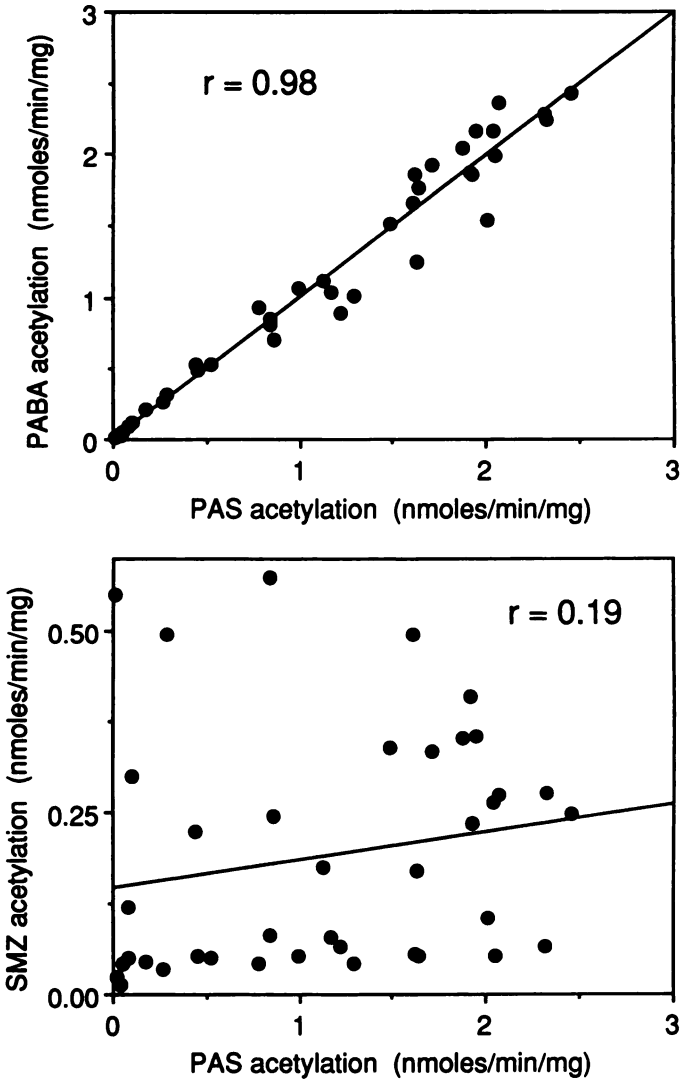

Fig. 4. Expression of NAT1 and NAT2 in cytosols from 39 human livers. *Top*, correlation between enzyme activities with the two monomorphic (NAT1) substrates PAS and PABA, each measured at an acceptor amine concentration of 100 μM. *Bottom*, correlation between enzyme activities with PAS (NAT1) and SMZ (NAT2) as acceptor amines.

Discussion

Our results provide convincing evidence that both of the two human genes *NAT1* and *NAT2* are expressed in human liver, that their enzyme products show marked selectivity for monomorphic and polymorphic arylamine substrates, respectively, and, consequently, that the *NAT2* locus is the site of the acetylation polymorphism in humans.

Our original studies concerning the purification of *N*-acetyltransferase from human liver (13) revealed the presence of two acetylating enzymes, NAT2A and NAT2B,[2] with identical electrophoretic mobility and immunoreactivity but distinguishable with respect to their substrate kinetics and retention on anion

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exchange columns. The recent discovery of two human genes, *NAT1* and *NAT2*, with the potential to encode functional *N*-acetyltransferase enzymes in a eukaryotic transient expression system (15), therefore provided a logical basis for the present experiments, which were aimed at determining the relationship between the products of these genes and observed liver *N*-acetyltransferase activities. We have already established that recombinant NAT2 possesses an electrophoretic mobility identical to that of both NAT2A and NAT2B from human liver and that the quantity of the latter two proteins varies with acetylator phenotype (13–15). However, no other related enzyme activity or immunoreactive protein corresponding to recombinant NAT1 had been observed. This suggested either that the *NAT1* gene product is not expressed in human liver or that the conditions used for cytosol preparation and enzyme purification prevented us from observing it. The present results make it clear that the latter is true; NAT1 can be specifically detected in human liver cytosol by using low concentrations of PABA or PAS as acceptor substrate, when losses due to its intrinsic instability (Fig. 1) are minimized during sample preparation. It is also important to note that the specific activity of NAT1 for the *N*-acetylation of PABA and PAS is so high that the product formation observed in human liver cytosols is catalyzed by amounts of enzyme protein that are only barely detectable on Western blots using our polyclonal antiserum, even after partial purification by anion exchange chromatography (Fig. 3, *lane 3*).

Whether the wide variation we observed in NAT1 activity (Fig. 4) is real or whether it results from differences in preservation of the labile enzyme protein during storage of different human livers requires verification. Hein *et al.* (23) also observed a much more rapid inactivation of PABA-NAT than of SMZ-NAT in human liver cytosols, as well as a large degree of variation between human livers in the rate of *in situ* enzyme inactivation. This would indeed suggest that a significant fraction of our observed variation in NAT1 activity could be a result of *in situ* enzyme instability. On the other hand, if even a portion of this variation occurs *in vivo*, it could have important implications for predisposition to toxicity from arylamine drugs with structures enabling them to be *N*-acetylated by either or both of the NAT enzymes present in human liver. The carcinogen AF presents one such instance where differences in the relative rates of metabolism by NAT1 and NAT2 might ultimately lead to marked variation in the occurrence of chemically induced cancers. A systematic survey of the substrate specificities of NAT1 and NAT2 promises to uncover other instances in which interindividual differences in levels of NAT isozyme expression may be of therapeutic or toxicological importance.

Our results allow us to conclude that arylamine substrates whose acetylation patterns in human populations *in vivo* do not correlate with the acetylation polymorphism are those that are selectively metabolized by NAT1 and those that do correlate are substrates for the NAT2 isoforms. Conversely, and of greater potential practical significance for new drug development, it should be a straightforward matter to compare rates of *N*-acetylation of a given compound by recombinant human NAT1 and NAT2 *in vitro*, to predict whether its disposition *in vivo* will be influenced by the acetylation polymorphism. Specifically, selective clearance by NAT2 predicts polymorphic acetylation, because the liver content of functionally active NAT2A and NAT2B has been shown to vary with acetylator phenotype (14). From the observed selectivity of the polymorphic NAT2 isoforms for SMZ acetylation (Table 2), it becomes clear in retrospect why this arylamine so effectively discriminates rapid and slow acetylator phenotypes.

Model for arylamine *N*-acetylation in humans. Our past (14, 15) and present studies allow us to propose a biochemical model to explain observed patterns of arylamine *N*-acetylation in humans (Fig. 5). The model may be summarized as follows.

Two gene loci assigned to human chromosome 8, named *NAT1* and *NAT2*, encode the functional acetylating enzymes NAT1 and NAT2. NAT1 is inherently much less stable than NAT2 *in vitro*, leading to potential problems in its accurate detection unless care is taken with tissue preparation methods. Both enzymes are expressed in human liver, with NAT2 being observed as two closely related but distinguishable isoforms, NAT2A and NAT2B. NAT1 and NAT2A/B have marked specificity (both high affinity and high substrate turnover) for monomorphic (e.g., PAS and PABA) and polymorphic (e.g., SMZ and PA) arylamine substrates, respectively. Based on this and on the results of *in vivo/in vitro* correlation studies (14), the *NAT2* locus can be established as the site of the human acetylation polymorphism; in genetically slow acetylators, the liver content of both NAT2A and NAT2B is markedly reduced. The liver content and/or activity of NAT1 may also be highly variable between individuals (Fig. 4) but is unrelated to the classical acetylation polymorphism.

Our model is consistent with numerous clinical and experimental observations in humans (6, 7, 23, 24), including the predictions made 25 years ago by Jenne (6). It is now clear that a relatively unstable (*in vitro*) acetylating activity in human liver, with high specificity for PAS, PABA, and sulfanilamide (7, 24), is contributed by NAT1, whereas enzymes (NAT2A/B) with greater selectivity for SMZ and PA vary in liver content with acetylator phenotype (6, 14). It is interesting to note that Jenne (6) had also observed the two peaks of NAT2-type activity on anion exchange columns that we have now attributed to the polymorphic *NAT2* locus.

From the reported substrate specificity of arylamine-acetylating activity in human peripheral blood cells (8, 25, 26), it would seem likely that the enzyme present in these cell types is almost exclusively monomorphic NAT1. The detailed tissue distribution of NAT1 and NAT2 remains to be determined but, if similarities exist with the rabbit model (27, 28),[3] then it can be expected that the polymorphic NAT2 will have a liver- and

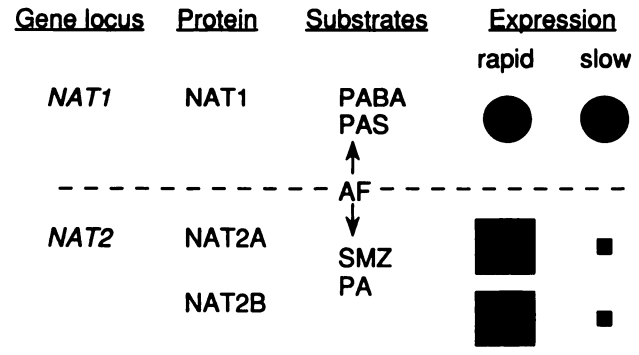

Fig. 5. A model for arylamine *N*-acetylation in humans.

[3] Manuscript in preparation.

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gut-selective pattern of expression, whereas NAT1 will be expressed at significant levels in a wide variety of body tissues. It will, however, be of considerable importance to verify a recent report of polymorphic NAT activity in human bladder (29), in light of considerable epidemiological evidence suggesting an association between the slow acetylator phenotype and bladder cancer (4).

The results presented here also allow for comparisons to be made with predictions based upon considerable data from experiments in animal models provided by the rabbit (12, 30) and the inbred hamster (31). In fact, with the exception of one set of conflicting immunological results (11), all of the data allow for the conclusion that the three species appear to be remarkably similar with respect to the multiplicity of acetylating enzymes and the biochemical manifestations of the genetic defect in arylamine *N*-acetylation. It appears that, in all species, two enzymes exist, one with specificity for substrates whose disposition is affected by the genetic defect and one that metabolizes substrates that are independent of it. The substrate specificities of the two enzymes are similar between human (this study) and rabbit (9, 28), whereas in the hamster the specificities of the monomorphic and polymorphic enzymes are reversed (31, 32). In addition, the phenotype resulting from the genetic defect, at least at the protein level, appears to be similar among the three species; there is a decrease in the amount of the polymorphic NAT enzyme present (12, 14, 31). However, we predict that the mechanisms of the human and rabbit acetylation polymorphisms at the gene level must differ, because in slow acetylator rabbits a gene deletion causes the complete absence of both the NAT protein and its transcript (12), whereas in slow acetylator humans the defective alleles are present (33–35) and a residual NAT2 activity remains (14). The molecular mechanism of defective arylamine *N*-acetylation in the hamster remains to be established, but the recent cloning of a hamster gene encoding a functional NAT (36) should facilitate efforts in this direction.

While this manuscript was in preparation, Ohsako and Deguchi (33) reported the cloning and heterologous expression of cDNAs encoding NATs from human liver. Their result supports our finding that both the *NAT1* and *NAT2* genes are expressed in human liver and provides evidence for the existence of a mutant allele at the *NAT2* locus, which produces a protein with a single amino acid change relative to the putative wild-type. We have recently determined that this mutant allele represents the rarest (about 5% of all mutant alleles) of three major mutant alleles present in a Caucasian population.[4]

In conclusion, we have used kinetic and biochemical methods to establish the existence of NAT enzymes in human liver corresponding to products of both the *NAT1* and *NAT2* gene loci. Moreover, we have presented further evidence that the *NAT2* locus is the site of the acetylation polymorphism. The exact nature of the *NAT2* gene defects causing slow acetylation in human populations is currently being investigated and appears to involve a relatively small number of mutant alleles containing discrete nucleotide substitutions that alter mRNA translation efficiency or enzyme stability (33–35).[4] We are also currently determining the variation in NAT1 expression in human populations and its potential implications for modification of the toxicity of arylamine drugs and other xenobiotics.

[4] M. Blum *et al.* Submitted for publications.

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