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Relationship between the genetically determined acetylator phenotype and DNA damage induced by hydralazine and 2-aminofluorene in cultured rabbit hepatocytes

(systemic lupus erythematosus/drug-related lupus erythematosus/unscheduled DNA synthesis/liver/genotoxicity)

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ABSTRACT **The relationship between acetylation rates of rabbit hepatocytes and their susceptibility to genotoxicity by DNA-damaging chemicals that undergo *N*-acetylation was studied in primary cultures of hepatocytes from New Zealand White rabbits that have a genetically determined difference in acetylation rates. Hepatocytes from rapid and slow acetylator rabbits maintained in culture the difference in acetylation rates that existed *in vivo*. DNA repair, an index of DNA damage, was produced by hydralazine in hepatocytes from slow acetylator rabbits but not in those from rapid acetylators. In contrast to these results, hepatocytes from rapid acetylators were more sensitive than those from slow acetylators to toxicity from the carcinogen 2-aminofluorene and displayed greater amounts of DNA repair. The amount of DNA repair measured with either chemical was dose dependent. These phenotype-dependent differences in the genotoxicity of two DNA-damaging chemicals provide evidence for the role of the acetylation polymorphism as a factor in determining susceptibility to toxicity, and perhaps carcinogenicity, of these chemicals.**

N-Acetylation rates of xenobiotics are under polymorphic genetic control in both humans and rabbits, resulting in individuals being either rapid or slow acetylators (1–5). This trait has been linked to toxicity and damage to DNA by chemicals of the aromatic amine or hydrazine type (6). For example, slow acetylator individuals are more likely than rapid acetylators to develop drug-related systemic lupus erythematosus (6–8). Individuals that develop this reaction have antinuclear antibodies as well as antibodies to DNA and nucleoproteins (6–13). *In vitro* studies have also demonstrated interaction of systemic lupus erythematosus-inducing drugs with DNA (11, 14, 15).

In the metabolism of xenobiotics, *N*-acetylation is a step that can be followed by reactions such as *N*-hydroxylation and esterification, resulting in the generation of reactive metabolites that undergo covalent binding with cellular macromolecules, including DNA (16, 17). Chemicals that can be acetylated and that also form covalent adducts with DNA include procainamide (18, 19), isoniazid (20), and hydralazine (15), as well as the aromatic amine carcinogens, benzidine (21, 22), 2-aminofluorene, and 4-aminobiphenyl (23). Adduct formation by chemicals can be mediated by the enzymatic removal of the *N*-acetyl moiety (24, 25), and evidence in the rabbit suggests that this reaction and the initial acetylation step are properties of the same enzyme (26).

Because a difference in the acetylation rate can alter the proportion of specific metabolites that are formed (27), it is possible that genotoxicity—i.e., damage to DNA (28)—by substrates of *N*-acetyltransferase could be affected by the amount of acetylation. In order to investigate this possibility, we developed a model system that permitted measurement of both *N*-acetyltransferase activity and DNA damage in the same cells, using hepatocytes, which represent a major tissue of acetylation (29–32), derived from rapid and slow acetylator rabbits.

In the present study, rabbit hepatocytes in primary culture were shown to be capable of *N*-acetylation and to express the acetylator polymorphism. The genotoxicity of hydralazine or 2-aminofluorene, as determined by the autoradiographic measurement of DNA repair in hepatocytes from both acetylator phenotypes, was found to differ. Hydralazine induced greater damage in hepatocytes from slow acetylators, whereas 2-aminofluorene was more genotoxic to rapid acetylator hepatocytes.

MATERIALS AND METHODS

Materials. Williams' medium E and calf serum were obtained from Flow Laboratories, McLean, VA. Sulfamethazine and hydralazine were purchased from Sigma, acetylsulfamethazine from ICN, and 2-aminofluorene from Aldrich. [*methyl*-^{3}H]Thymidine (60–80 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear. NTB emulsion, D-19 developer, and fixer were supplied by Eastman Kodak.

Animals. New Zealand White rabbits, selectively bred for acetylator phenotype, were maintained at the University of Michigan. The animals were caged individually and fed sulfonamide-free chow and water ad lib. Males 6–8 months old and weighing 2.7–3.6 kg were used.

Acetylator Phenotyping. Acetylator phenotype was determined by measuring the half-life ($t_{1/2}$) of sulfadiazine in blood (33). Rabbits with a $t_{1/2}$ less than 50 min were classified as rapid acetylators, and those with values greater than 70 min were classified as slow acetylators. Classification was confirmed by pedigree information and *N*-acetyltransferase liver activity *in vitro*.

Hepatocyte Isolation and Culture. Rabbits were anesthetized with 50 mg of sodium pentobarbital per kg of body weight and a lobe of the liver was removed to be used for determination of *N*-acetyltransferase activity. The remainder of the liver was perfused *in situ* with collagenase. The procedure described for isolation of rat hepatocytes (34–36) was modified by altering the flow rate and speed of the perfusate to accommodate the larger animal. Cell viability was determined with trypan blue exclusion, and only preparations with a viability greater than 80% were used. For *N*-acetyltransferase assays, 10×10^6 viable cells were plated into a 100-mm Petri plate containing Williams' medium E plus 10% calf serum plus gentamicin. After 2 hr at 37°C in a humidified incubator with 5% CO_2, unattached cells

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were removed, and the cultures were re-fed with Williams' medium E. Sulfamethazine was added to the cultures, which were then incubated in a 37°C CO_2 incubator. Aliquots of medium were removed at intervals and stored at −20°C. For DNA repair assays, 5×10^5 viable cells were seeded in a Linbro dish with each well containing a 25-mm^2 round Thermanox plastic coverslip. After 2 hr in a 37°C humidified CO_2 incubator, the cells were washed and re-fed with Williams' medium E.

Preparation of Liver Cytosols. Rabbit liver samples were homogenized and a 105,000 × *g* fraction was prepared as described (32).

***N*-Acetyltransferase Activity.** Enzyme activity in hepatocytes in primary cultures was monitored by measuring spectrophotometrically the sulfamethazine concentration of either medium or lysed cell samples by a modification of the Bratton–Marshall method (31). The presence of acetylsulfamethazine in the medium was confirmed by thin-layer chromatography. Aliquots of medium were spotted on silica gel plates and chromatographed in ethylene dichloride and methanol (9:1, vol/vol) (32). Both free (unmetabolized) and total sulfamethazine contents were determined.

N-Acetyltransferase activity in the 105,000 × *g* fraction was determined by a radioactive assay (32).

DNA Repair Assay. After the attachment period, hepatocytes were simultaneously exposed to [^{3}H]thymidine and either the compound to be tested or ultraviolet light. Incubations were carried out in the dark. Eighteen hours later, the coverslips were processed for emulsion autoradiography as described (34). Nuclear and cytoplasmic counts were determined by using an Artek model 880 counter. Net nuclear counts were calculated by subtracting the highest cytoplasmic count from the nuclear count for each cell. A net nuclear count of greater than 5 grains is considered indicative of DNA repair (36).

RESULTS

The *N*-acetyltransferase activity of slow and rapid acetylator rabbits was determined both in cytosolic fractions (105,000 × *g*) prepared from portions of liver taken prior to perfusion and hepatocytes in primary cultures prepared from dissociated cells after perfusion. Enzyme activity was readily measured in cytosols from rapid acetylator rabbits but was not detectable in cytosols from slow acetylator rabbits, even when protein or incubation time was increased (Table 1). *N*-Acetyltransferase activity in hepatocytes in primary culture was determined by monitoring the disappearance of sulfamethazine from the incubation medium. The detection of acetylsulfamethazine by thin-layer chromatography confirmed that this disappearance was due to acetylation. In the absence of hepatocytes, there was no change in sulfamethazine concentration. The rate of acetylation ($t_{1/2}$) of sulfamethazine in the cultured hepatocytes clearly reflected the phenotype of the animal determined either *in vivo* or in liver cytosols (Table 1). The hepatocytes acetylated sulfamethazine without the addition of exogenous acetyl-coenzyme A and were capable of sustaining a linear metabolic rate for at least 24 hr as evidenced by hepatocytes from slow acetylator rabbits (Fig. 1).

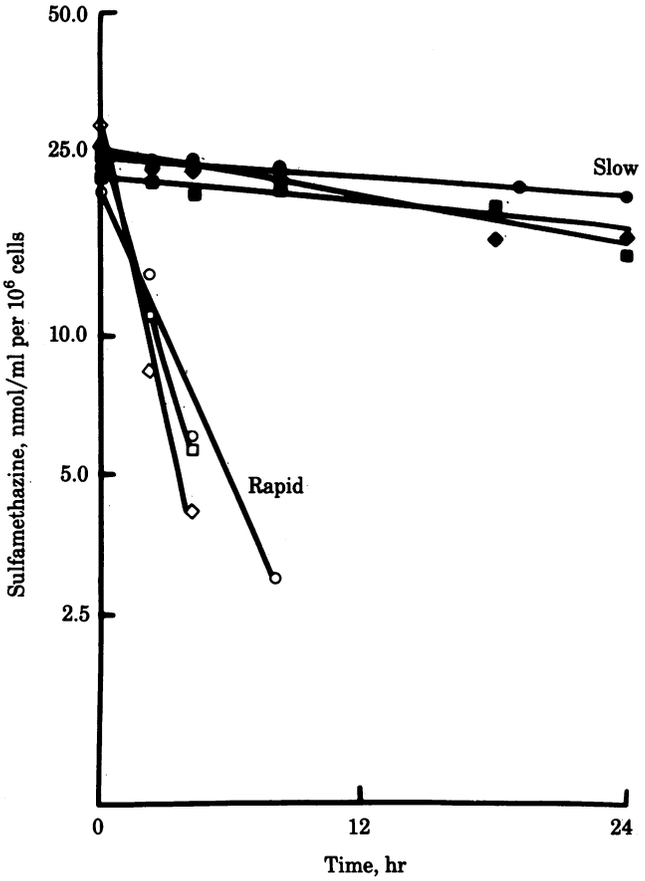

FIG. 1. Rate of disappearance of sulfamethazine from the medium of rabbit hepatocyte primary cultures. The sulfamethazine concentration in aliquots of medium was determined by a modification of the Bratton–Marshall method (22). Each symbol represents a different rabbit.

As an index of DNA damage, the amount of DNA repair resulting from exposure of hepatocytes from rapid and slow acety-

Table 1. Acetylator phenotype determinations in primary cultures of rabbit hepatocytes

		In vivo	*In vitro*	
Animal	Acetylator phenotype	Sulfadiazine $t_{1/2}$, min	Liver cytosol, nmol acetylsulfamethazine/min per mg protein	Hepatocyte sulfamethazine $t_{1/2}$, hr
224	Rapid	40	2.98	3
230	Rapid	41	3.56	2
237	Rapid	36	4.07	2
243	Slow	111	ND	49
260	Slow	87	ND	32
262	Slow	79	ND	83

ND, not detectable; sensitivity of the assay is 0.17 nmol of acetylsulfamethazine formed.

Table 2. DNA repair in rapid and slow acetylator rabbit hepatocytes exposed to hydralazine

		Net grains per nucleus			
Animal	Acetylator phenotype	0	1 mM	5 mM	10 mM
224	Rapid	0	4.8 ± 2.4	—	Toxic
230	Rapid	0	1.5 ± 0.8	Toxic	Toxic
237	Rapid	0	1.7 ± 0.8	2.2 ± 0.2	Toxic
243	Slow	0	17.7 ± 4.0	—	Toxic
260	Slow	0	11.4 ± 3.4	31.2 ± 3.7	Toxic
262	Slow	0	5.6 ± 2.4	15.5 ± 1.8	Toxic

Hydralazine concentrations are given in the headings. The results are mean ± SD of triplicate slides. A — indicates the experiment was not done.

Table 3. DNA repair in rapid and slow acetylator rabbit hepatocytes exposed to 2-aminofluorene

Animal	Acetylator phenotype	Net grains per nucleus		
		0	0.1 mM	1 mM
224	Rapid	0	33.8 ± 6.7	Toxic
230	Rapid	0	57.7 ± 12.3	Toxic
237	Rapid	0	48.4 ± 4.7	Toxic
243	Slow	0	19.2 ± 5.4	33.6 ± 5.4
260	Slow	0	26.4 ± 12.5	58.4 ± 13.9
262	Slow	0	7.7 ± 0.5	31.1 ± 13.1

2-Aminofluorene concentrations are given in the headings. The results are mean ± SD of triplicate slides.

lator rabbits to hydralazine or 2-aminofluorene was determined. Hydralazine at 10 mM was toxic to hepatocytes from rabbits with both phenotypes (Table 2). At lower concentrations, a dose-dependent repair response was evident in slow acetylator hepatocytes, whereas no DNA repair was evident in rapid acetylator hepatocytes. Phenotype-dependent differences in DNA repair were also observed with 2-aminofluorene (Table 3). However, in contrast to the results with hydralazine, rapid acetylator hepatocytes were more sensitive than those from slow acetylators. A high level of repair was elicited by 0.1 mM 2-aminofluorene in rapid acetylator hepatocytes, whereas toxicity was seen at 1 mM. In slow acetylator hepatocytes, comparable levels of repair were not achieved until 1 mM.

The capacity of hepatocytes from both phenotypes to carry out DNA repair was determined by using exposure to ultraviolet light. Hepatocytes from a rapid acetylator, rabbit no. 237, had 45.6 ± 1.1 grains per nucleus after exposure to 50 J/m^2, whereas hepatocytes from a slow acetylator, no. 243, had 46.9 ± 10.6 grains per nucleus. Thus, because the repair capacity of hepatocytes from both phenotypes was essentially the same, the differences observed in chemically induced repair can reasonably be attributed to phenotypically related differences in metabolism.

DISCUSSION

Hepatocytes in primary culture were found to provide an *in vitro* system in which the relationship between the acetylation polymorphism and genotoxicity of xenobiotics that are substrates for *N*-acetyltransferase could be examined. Cultured hepatocytes from New Zealand White rabbits maintained the differences observed *in vivo* and in liver cytosolic preparations. Moreover, the linear rate of acetylation throughout 24 hr in slow acetylator hepatocytes indicated that primary cultures of these cells preserve *N*-acetyltransferase activity in a functional state and maintain the cofactor, acetyl-coenzyme A, necessary for activity. This permitted the extended incubation time necessary to detect low levels of enzyme activity that could not be measured in cytosolic preparations.

Studies with rat hepatocyte suspensions have demonstrated a linear rate of *N*-acetylation of sulfamethazine for up to 4 hr (37, 38). In one study (38), individual rabbit hepatocyte suspensions were shown to have up to a 10-fold difference in acetylating activity for sulfamethazine, a variation that may have been due to differences in the acetylator phenotype, although animal phenotypes had not been determined. Our studies demonstrate that rabbit hepatocytes in primary culture express and maintain the sulfamethazine acetylator polymorphism.

Slow acetylators have been shown to be more susceptible to a variety of toxic reactions to drugs that are *N*-acetylated (6). Hydralazine, an antihypertensive drug, induces the development of anti-hydralazine antibodies, anti-DNA antibodies, systemic lupus erythematosus in humans (9, 10), and tumors in mice (39). *In vivo* studies showed that hydralazine-induced antinuclear antibodies and systemic lupus erythematosus were more common in slow acetylators (2, 7, 40–42). Although the mechanism of antibody production is not well understood, hydralazine has been shown to bind to isolated DNA, altering its tertiary structure (19). Specifically, hydralazine was found to bind to the pyrimidine bases, thymine and cytosine (43). It has been suggested that enhancement of the immunogenic properties of DNA results from the interaction of DNA and systemic lupus erythematosus-inducing drugs, such as hydralazine (39).

We have demonstrated that the interaction of hydralazine or its metabolites with DNA is dependent on acetylator phenotype, because DNA damage, as measured by DNA repair, was detectable in slow acetylator hepatocytes but not in cells from rapid acetylators. Hydralazine was also genotoxic in rat hepatocytes, which have levels of acetylation comparable to those of slow acetylator rabbits (15). This difference in the amount of DNA damage induced by the drug may be implicated in the mechanism for increased susceptibility of slow acetylators to hydralazine-related systemic lupus erythematosus.

Many chemical carcinogens, including aromatic amines, require activation to form reactive metabolites (16, 17). Differences in activities of the enzymes necessary for biotransformation can account for tissue and species specificity. For example, the dog, lacking *N*-acetyltransferase activity (44, 45), develops only bladder tumors after receiving an unacetylated aromatic amine. However, administration of the acetylated derivative results in liver and bladder tumors (45). Although an association between acetylator phenotype and susceptibility to aromatic amine carcinogenesis has been proposed from an epidemiologic study (46, 47), and biochemical evidence obtained in the rabbit suggests a plausible basis for such an association (32), conclusive evidence for any of these possibilities is lacking. We have now demonstrated that the magnitude of 2-aminofluorene-induced DNA damage differs in the two acetylator phenotypes. In contrast to the results with hydralazine, liver cells with the rapid acetylator phenotype were found to be more sensitive to both the toxic and genotoxic effects of 2-aminofluorene. The differences in the amount of unscheduled DNA synthesis elicited were not due simply to differences in the capacity of the hepatocytes for DNA repair, because both phenotypes responded equally to ultraviolet irradiation, which produces direct damage to DNA. Moreover, as shown by the different responses to hydralazine and 2-aminofluorene, the susceptible phenotype varied with the structure of the genotoxic chemical. Because causing DNA damage appears to be a mechanism of action of some carcinogens (28, 48), the demonstration of phenotype-dependent differences in the amount of DNA damage induced by aromatic amines or hydrazine derivatives offers evidence supporting the role of genetic variability in *N*-acetylating capacity—i.e., the acetylation polymorphism—as a factor in determining susceptibility to toxicity, including carcinogenesis, of these chemicals.

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