[CANCER RESEARCH 43, 3120–3123, July 1983]

Role of the Acetylation Polymorphism in Determining Susceptibility of Cultured Rabbit Hepatocytes to DNA Damage by Aromatic Amines[1]

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ABSTRACT

In humans and rabbits, differences in the rate of *N*-acetylation of aromatic amines are under polymorphic genetic control. Individuals are classified as either rapid or slow acetylators. In the current study, the relationship between acetylator phenotype and susceptibility to the genotoxicities of benzidine, 4-aminobiphenyl, 4,4′-methylenebis-2-chloroaniline, and 2-naphthylamine was investigated. Cultured hepatocytes isolated from rapid and slow acetylator rabbits were exposed to a dose range of the aromatic amines, and genotoxicity was determined by the autoradiographic measurement of DNA repair synthesis. Hepatocytes from rapid acetylator rabbits were more susceptible to the genotoxic effect of benzidine than were cells from slow acetylators. 4-Aminobiphenyl and 4,4′-methylenebis-2-chloroaniline were both weakly genotoxic, but no clear correlation was seen with acetylator phenotype. No genotoxicity was observed with 2-naphthylamine. These results thus demonstrate that differences in acetylation rates can affect the genotoxicity of benzidine. This study provides further evidence for the role of the genetically determined acetylator polymorphism in determining susceptibility to the effects of certain aromatic amine carcinogens. Since the acetylator polymorphism is a human trait, a similar susceptibility may be displayed in humans.

INTRODUCTION

The acetylator polymorphism is a genetically determined difference in the *N*-acetylation of a variety of aromatic amines and hydrazines. In both humans and rabbits, individuals are either rapid or slow acetylators, and slow acetylation is the recessive trait (4, 6, 7, 9, 16). Sensitivity to the toxicity of drugs metabolized by polymorphic *N*-acetyltransferase (EC 2.3.1.5) has been linked to the acetylator phenotype (3, 26). For example, slow acetylators are more likely to develop drug-related systemic lupus erythematosus (3, 28, 38) or peripheral neuropathy during isoniazid therapy (12, 13).

Differences in the capacity for *N*-acetylation have also been related to the species and tissue specificity of carcinogenic aromatic amines. A species such as the dog, lacking *N*-acetyltransferase activity (18, 29), develops only bladder tumors after receiving a nonacetylated aromatic amine. If the acetylated derivative is given, both liver and bladder tumors develop (18). It seems, then, that acetylation is necessary for the formation of liver tumors by aromatic amines. Lower *et al.* (17, 19) have proposed that acetylator phenotype could affect susceptibility to the tumorigenic effects of aromatic amines in humans. Indeed, an excess of slow acetylators has been observed among patients who presented with late-stage bladder cancer and had been exposed to aromatic amines previously (2).

Carcinogenic substrates of polymorphic *N*-acetyltransferase can be further metabolized to products capable of reacting with DNA. DNA damage, a probable critical action of certain of these carcinogens (10, 31), has been observed with benzidine (20, 21, 27, 30, 34), 2-aminofluorene (15, 24, 30), 4-aminobiphenyl (15, 34), 4,4′-methylenebis-2-chloroaniline (23), and hydralazine (24, 37). In order to investigate the relationship between acetylator phenotype and genotoxicity, an *in vitro* system was developed that permits measurement of *N*-acetyltransferase activity and DNA damage in the same cell (24). This system uses rabbit hepatocytes, since the rabbit displays acetylator polymorphism (6, 7, 9), and liver is a major source of polymorphic *N*-acetyltransferase (8, 18). In primary cultures of hepatocytes derived from both phenotypes, *in vivo* differences in acetylation rates are maintained, and DNA repair, an indicator of DNA damage, is readily measured. This system was previously used to study the effect of differences in acetylation rates on the genotoxicity of 2-aminofluorene and hydralazine. Hepatocytes from rapid acetylators were more sensitive to the genotoxic effect of 2-aminofluorene, while hydralazine induced greater damage in hepatocytes from slow acetylators (24).

In the present study, the genotoxicities of benzidine, 4-aminobiphenyl, 4,4′-methylenebis-2-chloroaniline, and 2-naphthylamine were investigated in hepatocytes from rapid and slow acetylator rabbits. A phenotype-dependent difference in susceptibility to benzidine was demonstrated. 4,4′-Methylenebis-2-chloroaniline and 4-aminobiphenyl were weakly genotoxic, but no clear correlation could be made with acetylator phenotype. No genotoxicity was observed with 2-naphthylamine.

MATERIALS AND METHODS

Materials. Williams' Medium E, calf serum, Hanks' balanced salt solution, collagenase, Linbro dishes, and Thermanox coverslips were obtained from Flow Laboratories, McLean, Va. [*methyl*-^{3}H]thymidine, 60 to 80 Ci/mmol, was purchased from New England Nuclear, Boston, Mass. NTB emulsion, D-19 developer, and fixer were obtained from Eastman Kodak Co., Rochester, N. Y. Benzidine, 4-aminobiphenyl, and 2-naphthylamine were obtained from Sigma Chemical Co., St. Louis, Mo.; 2-aminofluorene was obtained from Aldrich Chemical Co., Milwaukee, Wis.; and 4,4′-methylenebis-2-chloroaniline was donated by Dr. Wendell W. Weber, University of Michigan.

Animals. New Zealand White rabbits, selectively bred for acetylator phenotype, were provided by Dr. Wendell W. Weber, University of Michigan. The animals were maintained and phenotyped as described previously (24).

Hepatocyte Isolation and Culture. Hepatocytes were isolated by a 2-step perfusion of the liver of rapid and slow acetylator rabbits. The

[1] This work was partially supported by USPHS Grants CA 33144 and RR 05775 awarded by the National Cancer Institute, Department of Health and Human Services.

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Received June 28, 1982; accepted April 5, 1983.

procedure described for rats (35, 36) was modified to accommodate the larger animal (22, 24). Perfusion with 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution for 6 min at 70 ml/min was followed by perfusion with collagenase, 100 units/ml, in Williams' Medium E for 10 min at 50 ml/min (22, 24). Only preparations with a viability, determined by trypan blue exclusion, of greater than 80% were used. Approximately 5×10^5 viable cells were plated in Linbro dishes with each well containing a 25-sq-mm round Thermanox coverslip and Williams' Medium E supplemented with 10% calf serum. The cells were allowed to attach for 2 hr at 37° in a 5% CO_2 humidified incubator, then washed, and refed with Williams' Medium E.

Hepatocytes cultured from rabbits of both phenotypes maintain the differences observed *in vivo* (24). Enzyme activity in these primary cultures was monitored by the rate of disappearance of sulfamethazine from the culture medium as described previously (24). Sulfamethazine concentrations were determined spectrophotometrically (11). The $t_{1/2}$ for sulfamethazine ranged from 2 to 3 hr in hepatocytes from rapid acetylators and 49 to 83 hr in cells from slow acetylators (24).

DNA Repair Assay. Immediately following cell attachment, the cultures were simultaneously exposed to 10 μCi[^{3}H]thymidine/ml, and the test chemical dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the cultures was 1%. After 18 hr, the coverslips were processed as described previously (33, 34, 36). Air-dried coverslips were mounted on slides and dipped in NTB emulsion and were then developed and stained after 10 days (36). Nuclear and cytoplasmic grain counts were determined using an Artek Model 880 counter (36). Net nuclear counts were determined by subtracting the highest cytoplasmic count from the nuclear count. Negative net nuclear counts were considered as zero in calculations of the mean counts. A net nuclear count of at least 5 grains was necessary for a positive result.

Cytotoxicity of the test chemical was determined both by nuclear morphology and the absence of S-phase cells. Misshapen, pyknotic, or isolated nuclei were not counted. The use of these morphological criteria of toxicity has been supported by studies with rat hepatocytes which quantify cytotoxicity by the release of intracellular lactate dehydrogenase (25).

RESULTS

The genotoxicity of 4 aromatic amine carcinogens was tested in cultured hepatocytes from 3 rapid and 3 slow acetylator rabbits. The *N*-acetyltransferase activities of these hepatocytes was reported previously (24).

A phenotype-dependent difference in susceptibility to DNA damage was observed with benzidine. Hepatocytes from rapid acetylator rabbits showed substantial DNA repair synthesis at 10^{-6} M benzidine and maximum repair at 10^{-5} M (Table 1). At higher concentrations, a decrease in DNA repair was seen, presumably due to the cytotoxicity of benzidine. Hepatocytes from slow acetylator rabbits showed no significant repair up to 10^{-4} M and only a low level at 10^{-3} M. At the concentration of benzidine that elicited maximum repair in rapid acetylator hepatocytes, *i.e.*, 10^{-5} M, 100% of the hepatocytes displayed repair synthesis (5 or more net grains/nucleus). However, at the concentration inducing maximum repair in slow acetylator hepatocytes, *i.e.*, 10^{-3} M, only approximately 50% of the cells were positive (data not shown).

4,4'-Methylenebis-2-chloroaniline elicited a weakly positive response in hepatocytes from 3 of the 6 rabbits studied (Table 2). Net nuclear grain counts of at least 5 were only observed in 2 rapid and one slow acetylator. There was variation among the individual rabbits, but there was no correlation between acetylator phenotype and the genotoxicity of 4,4'-methylenebis-2-chloroaniline.

With 4-aminobiphenyl, results were similar to 4,4'-methylenebis-2-chloroaniline (Table 3). This chemical induced only a weakly positive response in rabbit hepatocytes. Although this positive response was observed in hepatocytes from all 6 animals, 50% showed only the minimum of 5 net grains/nucleus. There was no relationship between acetylator phenotype and susceptibility to the genotoxicity of 4-aminobiphenyl.

Table 1

Genotoxicity of benzidine in rabbit hepatocytes

		Net grains/nucleus						
Animal	Phenotype	0	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
230	Rapid	0.4 ± 0.6[a]	15.2 ± 8.5	44.4 ± 2.2	>150	>150	T[b]	NT
237	Rapid	0 ± 0	NT	16.3 ± 0.7	>150	>150	3.9 ± 1.7	T
321	Rapid	0.5 ± 0.5	NT	45.6 ± 9.7	>150	78.9 ± 14.0	21.9 ± 2.6	T
243	Slow	0 ± 0	NT	1.2 ± 0.5	2.5 ± 1.5	2.0 ± 1.7	7.5 ± 4.1	T
260	Slow	0 ± 0	NT	0.9 ± 0.5	4.6 ± 2.1	2.5 ± 1.6	11.6 ± 2.6	T
262	Slow	0.4 ± 0.4	2.2 ± 1.5	0.6 ± 0.3	1.2 ± 0.5	1.1 ± 0.6	2.0 ± 1.5	NT

[a] Mean ± S.D. of triplicate coverslips.
[b] T, toxic; NT, not tested.

Table 2

Genotoxicity of 4,4'-methylenebis-2-chloroaniline in rabbit hepatocytes

		Net grains/nucleus					
Animal	Phenotype	0	10^{-5} M	10^{-4} M	5×10^{-4} M	10^{-3} M	10^{-2} M
230	Rapid	0.4 ± 0.6[a]	7.4 ± 0.7	12.9[b]	T[c]	T	T
237	Rapid	0 ± 0	1.5 ± 1.6	1.7 ± 0.8	1.2 ± 1.2	3.1 ± 0.6	T
321	Rapid	0.5 ± 0.5	1.1[b]	10.9 ± 2.8	T	T	T
243	Slow	0 ± 0	1.0 ± 0.6	0.6 ± 0.4	NT	0.6 ± 0.5	T
260	Slow	0 ± 0	NT	2.5 ± 1.0	13.7 ± 1.7	12.7[b]	T
262	Slow	0.4 ± 0.4	0.3 ± 0.6	0.5 ± 0.2	1.9 ± 1.2	0.8 ± 0.9	T

[a] Mean ± S.D. of triplicate coverslips.
[b] Average of duplicate coverslips.
[c] T, toxic; NT, not tested.

Table 3

Genotoxicity of 4-aminobiphenyl in rabbit hepatocytes

Animal	Phenotype	Net grains/nucleus					
		0	10^{-5} M	10^{-4} M	5×10^{-4} M	10^{-3} M	10^{-2} M
230	Rapid	0.4 ± 0.6[a]	0.9 ± 0.3	1.8 ± 0.2	5.9 ± 2.2	T[b]	T
237	Rapid	0 ± 0	1.0 ± 0.2	1.8 ± 0.2	5.0[c]	5.9 ± 2.3	T
321	Rapid	0.5 ± 0.5	1.2 ± 0.6	NT	16.2 ± 8.4	T	T
243	Slow	0 ± 0	1.6 ± 1.3	3.2 ± 1.0	NT	10.4 ± 4.7	T
260	Slow	0 ± 0	0.5[c]	2.5 ± 3.6	23.6[c]	T	T
262	Slow	0.4 ± 0.4	0.2 ± 0.3	0.2 ± 0.4	5.1 ± 2.6	T	T

[a] Mean ± S.D. of triplicate coverslips.
[b] T, toxic; NT, not tested.
[c] Average of duplicate coverslips.

Table 4

Genotoxicity of 2-naphthylamine in rabbit hepatocytes

Animal	Phenotype	Net grains/nucleus				
		0	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
224	Rapid	0 ± 0[a]	NT[b]	NT	0.3[c]	T
237	Rapid	0 ± 0	1.3 ± 0.9	0.4 ± 0.3	0.5 ± 0.4	T
321	Rapid	0.5 ± 0.5	0.3[c]	0.7 ± 0.7	4.0 ± 1.2	T
243	Slow	0 ± 0	1.4 ± 1.1	2.4 ± 0.3	1.5 ± 0.4	T
260	Slow	0 ± 0	1.1 ± 1.0	0.6 ± 0.3	T	T
262	Slow	0.4 ± 0.4	0 ± 0	0.3 ± 2.8	2.5 ± 1.2	T

[a] Mean ± S.D. of triplicate coverslips.
[b] NT, not tested; T, toxic.
[c] Average of duplicate coverslips.

The final chemical tested was 2-naphthylamine. Although 2-naphthylamine was cytotoxic at 10^{-2} M, it was not genotoxic to hepatocytes from either phenotype (Table 4).

DISCUSSION

The role of *N*-acetylation in the formation of the ultimate carcinogenic metabolites of aromatic amines has been investigated in hepatocytes from rapid and slow acetylator rabbits. Hepatocytes in primary culture allow both *N*-acetyltransferase activity and DNA damage, a probable mechanism of action of some carcinogens (10, 31), to be studied in a single system.

A relationship between acetylator phenotype and genotoxicity of certain xenobiotics has been demonstrated previously. Hepatocytes from rapid acetylator rabbits were more susceptible to the genotoxic effect of 2-aminofluorene, an aromatic amine, than hepatocytes from slow acetylators (24). The results obtained in the current study show a similar relationship for another aromatic amine, benzidine. Since chemicals of this class require metabolism to reactive products that can damage DNA (32), these results also provide evidence for the metabolic capabilities of primary cultures of rabbit hepatocytes.

Several pathways for the metabolism of benzidine have been proposed from *in vitro* studies with liver preparations. One pathway would involve conversion of benzidine to *N*-acetylbenzidine, then *N*,*N*′-diacetylbenzidine, followed by *N*-hydroxy-*N*,*N*′-diacetylbenzidine and nucleic acid binding (27). In a second pathway, *N*-acetylbenzidine is hydroxylated to *N*-hydroxy-*N*-acetylbenzidine, which is then acetylated to *N*-hydroxy-*N*,*N*′-diacetylbenzidine, which is subsequently converted to *N*′-hydroxy-*N*-acetylbenzidine (5). Both *N*′-hydroxy-*N*-acetylbenzidine and *N*-hydroxy-*N*,*N*′-diacetylbenzidine are suggested as proximate carcinogens of benzidine, and *N*-(deoxyguanosin-8-yl)-*N*′-acetylbenzidine has been identified as a DNA adduct (5, 20). In a third pathway, cooxidative metabolism of benzidine and binding to nucleic acid has also been demonstrated in renal medullary microsomes (39). Acetylation, then, seems to be an important step in the proposed metabolic pathways of benzidine in liver. In a study with isolated rat hepatocytes, inhibition of *N*-acetylation resulted in a reduction in the genotoxicity of benzidine (1). The results of the present study, in which hepatocytes with a rapid rate of acetylation had a greater amount of DNA damage at a lower dose than hepatocytes from slow acetylators, are thus supportive of these previous observations.

Of the other aromatic amines studied, 4,4′-methylenebis-2-chloroaniline and 4-aminobiphenyl were weak inducers of DNA repair. Although there was variation among individual rabbits, no correlation was seen between acetylator phenotype and genotoxicity. One possible source of variability in response to chemicals is the genetic heterogeneity of the animals. The rabbits used in this study were selectively bred for acetylator phenotype, but they were not an inbred strain. Interestingly, these 2 carcinogens were more active in rat hepatocytes (23, 34) and, in the instance of 4,4′-methylenebis-2-chloroaniline, in mouse and hamster hepatocytes (23). DNA repair was not induced by 2-naphthylamine, a chemical with little or no carcinogenicity in the rabbit (14).

In our studies on the role of acetylator polymorphism, phenotype-dependent differences in susceptibility to the genotoxic effect of 3 chemicals, 2-aminofluorene (24), hydralazine (24), and benzidine, have now been identified. The susceptible phenotype varied with the structure of the genotoxic chemical. Hepatocytes from rapid acetylators were more sensitive to the aromatic amines, 2-aminofluorene (24) and benzidine, than were hepatocytes from slow acetylators. In contrast, hydralazine induced DNA repair in hepatocytes from slow acetylators, while little or no repair was seen in the rapid phenotype (24). These results provide evidence for the importance of genetically controlled

differences in *N*-acetylation capacity in determining susceptibility to aromatic amine carcinogens.

ACKNOWLEDGMENTS

We thank Dr. Wendell W. Weber for providing the rabbits; S. Crescenzi, B. Merrill, and M. J. Miller for their contributions to this study; and S. Dorn and J. Benvin for editorial assistance.

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Cancer Res 1983;43:3120-3123.