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Genetic Differences in the Aromatic Hydrocarbon-Inducible *N*-Hydroxylation of 2-Acetylaminofluorene and Acetaminophen-Produced Hepatotoxicity in Mice

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SUMMARY

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The genetically mediated presence or absence of induction, as well as the magnitude of induction, of aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity is highly correlated ($p < 0.001$) with the *N*-hydroxylation of 2-acetylaminofluorene in the livers of C57BL/6N and DBA/2N inbred mice treated with the microsomal enzyme inducers 3-methylcholanthrene, β-naphthoflavone, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and sodium phenobarbital. The extent of hepatotoxicity caused by acetaminophen (*p*-hydroxyacetanilide) administered intraperitoneally to these two strains of mice is also highly associated with both aromatic hydrocarbon-inducible monooxygenase "activities": aryl hydrocarbon hydroxylase and acetylarylamine *N*-hydroxylase. We suggest that cytochrome P_1450 is involved with the aromatic hydrocarbon-inducible *N*-hydroxylase activity and that these genetic differences among inbred strains of mice offer a valuable experimental model system for studying the mechanism of hepatotoxicity and carcinogenicity among siblings of a defined genotype.

INTRODUCTION

The hepatotoxicity and carcinogenicity of *N*-acetylarylamines such as acetaminophen (*p*-hydroxyacetanilide) and 2-AAF[1] have been shown (2-5) to result from toxic metabolites arising via *N*-oxidation. Recently *N*-oxidation of these compounds has been proven to be mediated by the cytochrome P450-dependent monooxygenase system(s) (6, 7). Inducers of cytochrome P450-associated monooxygenase activities, such as MC and PB, are known (8) to modify both the toxicity and carcinogenicity of *N*-acetylarylamines. In particular, prior treatment of rats, rabbits, and hamsters with MC causes a differential effect with respect to both the metabolism and the carcinogenicity of 2-AAF (8).

Administration of polycyclic hydrocar-

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[1] The abbreviations used are: 2-AAF, 2-acetylaminofluorene; PB, sodium phenobarbital; MC, 3-methylcholanthrene; BNF, β-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; hydroxylase, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; *N*-hydroxylase, acetylarylamine (2-acetylaminofluorene) *N*-hydroxylase; B6, the inbred C57BL/6N mouse strain; D2, the inbred DBA/2N mouse strain.

bons such as MC (9–12) or the noncarcinogenic compound BNF (13) increases the activity of another monooxygenase, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase, in the livers of certain inbred strains of mice but not other strains. In contrast, the extent of hepatic hydroxylase induction by PB is about the same among more than 10 inbred strains of mice (9, 11).

This paper presents evidence that aromatic hydrocarbon responsiveness in the mouse is very closely associated with aromatic hydrocarbon-inducible increases in the *N*-hydroxylation of *N*-acetylarylamines.

MATERIALS AND METHODS

The polycyclic hydrocarbons benzo[*a*]pyrene and MC were obtained from Sigma and J. T. Baker Chemical Company, respectively. BNF was purchased from Aldrich Chemical Company; PB, from Merck and Company, Inc.; 2-AAF and acetaminophen, from Eastman Kodak Company; and NADPH and NADH, from Sigma Chemical Company. [9-^{14}C]-2-AAF (10.5 mCi/mmole), purchased from New England Nuclear, was shown to be more than 99.9% pure by thin-layer chromatography on salicic acid (chloroform–methanol, 97:3, v/v). Authentic *N*-hydroxy-2-acetylaminofluorene was generously given to us by Dr. Elizabeth Weisberger, National Cancer Institute. TCDD was a generous gift of Dr. Alan P. Poland, University of Rochester Medical and Dental School; special precautions were taken in its handling, as emphasized previously (14). All other reagents were of the best available commercial grade. National Institutes of Health Animal Supply provided us with the inbred C57BL/6N (B6) and DBA/2N (D2) mice used in these studies.

Treatment of animals. The mice were kept in standard hardwood bedding in plastic cages and fed Wayne Lab-Blox ad libitum. We attempted to control as completely as possible the animal room environment by maintaining an automatic day-night (12 hr–12 hr) cycle and preventing exposure to pharmacologically active compounds such as cigarette smoke, insecticides, and softwood (e.g., pine or cedar) bedding. At the time of experiment, mice of either sex (9) between 4 and 6 weeks of age were used. For studies involving enzyme induction by the aromatic hydrocarbon MC or BNF, 80 mg of the compound per kilogram of body weight in corn oil were administered intraperitoneally to each mouse 48 hr before sacrifice; controls received corn oil alone. PB-treated mice received the drug intraperitoneally in 0.90% NaCl, 80 mg/kg on each of 3 successive days, and were killed 24 hr after the last dose. TCDD-treated mice were given 100 μg of the chemical in *p*-dioxane per kilogram 48 hr prior to assay; controls received 0.40 ml of *p*-dioxane per kilogram of body weight.

Preparation of microsomes. All animals were killed at the same time of day. Immediately upon exsanguination, the minced livers from individual mice were separately washed as free as possible from blood in ice-cold 20 mM potassium phosphate–KCl buffer, pH 7.4, and homogenized. The homogenate was centrifuged for 20 min at 9000 $\times$ *g*, and the supernatant fluid was carefully decanted and recentrifuged for 60 min at 105,000 $\times$ *g*. The surface of the microsomal pellet was washed twice with the phosphate–KCl buffer, and the pellet was resuspended in the same buffer prior to assay. Protein was determined according to the method of Lowry and co-workers (15).

Enzyme assays. The hydroxylase activity in liver microsomes was estimated as previously described (9, 11). One unit of aryl hydrocarbon hydroxylase activity is defined (9, 11) as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of the 3-hydroxybenzo[*a*]pyrene recrystallized standard.

The rate of *N*-hydroxylation of 2-AAF in liver microsomes was measured as previously described (6). The incubation mixture, in a total volume of 5.0 ml, contained 20 mM potassium phosphate buffer (pH 7.4), 75 mM KCl, 100 mM NaF, 2.0 mM NADPH, and 0.40 mM [9-^{14}C]-2-AAF; the sodium fluoride was included to inhibit deacetylation (16). The microsomal pro-

tein concentration was adjusted to 1.0 mg/ml. The mixture was incubated at 37° for 15 min, during which time the reaction rate remained constant (6). The ^{14}C-labeled hydroxamic acid, *N*-hydroxy-2-acetylaminofluorene, was separated and purified from the radioactive substrate and any other metabolites by thin-layer chromatography (chloroform–methanol, 97:3); authentic N-hydroxy-2-acetylaminofluorene was used as the standard. The radioactivity was determined by liquid scintillation spectrometry. One unit of 2-AAF *N*-hydroxylase activity is defined as that amount of enzyme catalyzing, per minute at 37°, the formation of 1 pmole of *N*-hydroxy-2-acetylaminofluorene. The specific activities of both aryl hydrocarbon hydroxylase and 2-AAF *N*-hydroxylase are expressed in this report as units per milligram of microsomal protein.

Histology. The mice received various doses of acetaminophen intraperitoneally 48 hr after treatment with either MC or corn oil alone. The acetaminophen was administered in 0.90% NaCl as the vehicle; 24 hr after the dose of acetaminophen, paraffin sections of liver were prepared and stained with hematoxylin and eosin (17). Quantitative analysis of the liver necrosis caused by acetaminophen was carried out according to Chalkley (18). During examination of several random fields on two sections from each liver, necrosis was quantitated as follows: 0 = absent; 1+ = necrosis of less than 6% of the hepatocytes; 2+ = 6–25% of the hepatocytes; 3+ = 26–50% of the hepatocytes; 4+ = more than 50% of the hepatocytes.

RESULTS

Relationship between aromatic hydrocarbon-inducible aryl hydrocarbon hydroxylase and 2-AAF N-hydroxylase activities. Figures 1 and 2 show that an increase in the *N*-hydroxylation of 2-AAF following MC treatment *in vivo* appears to segregate as a single autosomal dominant gene in reciprocal matings between B6 and D2 inbred mice and that the *N*-hydroxylase induction is always associated with induction of aryl hydrocarbon hydroxylase activity by MC. The *N*-hydroxylase activity was induced in

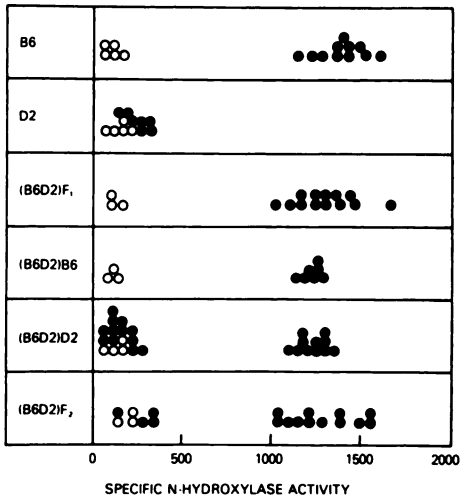

FIG. 1. *Genetic variance of 2-AAF N-hydroxylase in liver induced by MC in appropriate crosses between C57BL/6N and DBA/2N mice*

●, an individual treated with MC 48 hr prior to assay; O, a corn oil-treated control animal.

the (B6D2)F_1 hybrid and in each offspring of the (B6D2)F_1 × B6 backcross to about the same extent as in the inbred B6 parent (Fig. 1). The increased *N*-hydroxylation of 2-AAF occurred in about half the MC-treated progeny from the (B6D2)F_1 × D2 backcross and in about three-fourths of the MC-treated offspring from the (B6D2)F_1 × (B6D2)F_1 intercross. The *N*-hydroxylase specific activity in the livers of the remaining one-half and one-fourth of the MC-treated offspring from the (B6D2)F_1 × D2 backcross and the F_2 generation, respectively, was not statistically significantly ($p > 0.05$) different from that of the MC-treated inbred D2 parent or the basal specific *N*-hydroxylase activity in control animals. In more than 120 MC-treated individuals from these various genetic crosses to date, the presence or absence of induction of these two enzymes has been unequivocally associated in every single mouse.

Table 1 shows the high correlation between the hydroxylase and *N*-hydroxylase activities in the livers of B6 and D2 mice treated with the inducers MC, BNF, TCDD, and PB. Out of the more than 200 mice individually examined for these two enzyme activities, there was a striking correlation not only with the presence or

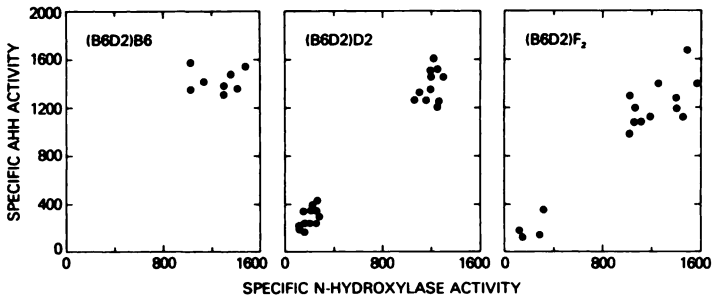

FIG. 2. *Relationship between specific aryl hydrocarbon hydroxylase (AHH) activity and specific 2-AAF N-hydroxylase activity in livers of various mice 48 hr following MC treatment*

Left, offspring from the (C57BL/6N)(DBA/2N)F_1 × C57BL/6N backcross; center, offspring from the (C57BL/6N)(DBA/2N)F_1 × DBA/2N backcross; right, offspring of the (C57BL/6N)(DBA/2N)F_2 generation.

TABLE 1

Relationship between aryl hydrocarbon hydroxylase activity and N-hydroxylation of 2-acetylaminofluorene in mouse liver

Treatments of the mice and the preparation of liver microsomes are described under MATERIALS AND METHODS. The values of both activities are expressed as means ± standard errors. The correlation coefficient, r, for these data is 0.98 ($p < 0.001$, calculated by Student's t-test).

Strain	Previous treatment	N	Aryl hydrocarbon hydroxylase specific activity	2-AAF N-hydroxylase specific activity
			units/mg microsomal protein	
C57BL/6N	None	8	240 ± 30	150 ± 20
	MC	23	1330 ± 120	1430 ± 86
	BNF	6	1530 ± 180	1270 ± 170
	TCDD	4	1630 ± 200	1430 ± 200
	PB	7	450 ± 22	320 ± 17
DBA/2N	None	10	230 ± 34	180 ± 16
	MC	21	270 ± 20	220 ± 30
	BNF	6	280 ± 36	260 ± 30
	TCDD	4	1740 ± 240	1600 ± 140
	PB	6	480 ± 24	280 ± 17

absence of induction, but also with its magnitude. As an inducer of the hydroxylase activity in rat lung, BNF was first shown by Wattenberg and co-workers (19) to be very similar to the carcinogen MC. Subsequently this similarity was demonstrated (13) in mice; BNF induces the enzyme activity in "responsive" individuals, whereas the hydroxylase induction is absent, or much lower, in various tissues of BNF-treated, so-called "nonresponsive" mice. TCDD as an inducer appears to behave the same as MC or BNF, except that both cytochrome P_1450 formation and the hydroxylase induction occur as well in either the responsive mouse or the inbred strains which are nonresponsive to MC or BNF (20). PB as an inducer of the hydroxylase activity, on the other hand, differs in mechanism from MC, BNF, and TCDD in that the liver enzyme is induced equally well in both responsive and nonresponsive mice, and cytochrome P_1450 formation does not occur to any major extent (9, 11, 13, 21).

Acetaminophen-produced hepatic necrosis in B6 and D2 inbred mice. The histological changes caused by acetaminophen overdose, both in humans and in experimental animals, have been extensively described (3, 22, 23), the characteristic lesion being a dose-dependent centrilobular hepatic necrosis. Following treatment with MC (Table 2) the extent of hepatic necrosis and death after various doses of acetaminophen was much more marked in the B6 than in the D2 mouse. In mice receiving no MC previously, no deaths were caused by acetaminophen doses of 200 or 500 mg/kg, and the severity of necrosis was similar in the B6 and D2 mice. Prior treatment with MC potentiated both the incidence and the severity of the acetaminophen-caused hepatic necrosis in the B6 mice, but had little effect on the D2 mice. Administration of 350 mg of acetaminophen per kilogram caused 80% mortality, and all the surviving B6 mice suffered 3+ or 4+ necrosis. Following the 500 mg/kg dose there was 100%

TABLE 2

Effect of prior administration of MC on extent of hepatic necrosis in C57BL/6N and DBA/2N mice 24 hr after treatment with various intraperitoneal doses of acetaminophen

The various regimens of prior treatment are described under MATERIALS AND METHODS. The extent of hepatic necrosis was scored in the survivors by the criteria given in the text.

Strain	Prior treatment	Acetaminophen	N[a]	Mortality	Extent of necrosis				
					0	1+	2+	3+	4+
		mg/kg		%	%	%	%	%	%
C57BL/6N	None	200	20	0	100				
	None	500	26	0			38	52	10
	MC	200	30	10	10		25	45	20
	MC	350	28	80				33	67
	MC	500	35	100					
DBA/2N	None	200	20	0	100				
	None	500	24	0		5	70	25	
	MC	200	31	0	100				
	MC	350	25	0			40	60	
	MC	500	32	0			28	72	

[a] Number of animals in each group at the time acetaminophen was administered.

mortality of the MC-treated B6 mice, compared with no deaths among the MC-treated D2 mice after 24 hr. When death occurred, it usually took place during the first 12 hr after administration of acetaminophen. Liver sections taken 6 hr after the 500 mg/kg dose of acetaminophen to MC-treated B6 mice showed massive necrosis of the entire center and most of the midzonal area of the hepatic lobules.

The effect of prior MC treatment on acetaminophen-produced hepatic necrosis in D2 mice was minimal. There was no mortality after 200 or even 500 mg of acetaminophen per kilogram, and the severity of necrosis was increased only slightly (i.e., more 3+ than 2+ necrosis was observed) at acetaminophen doses of 350 and 500 mg/kg.

DISCUSSION

Since toxicity and carcinogenicity of *N*-acetylarylamines are, in most cases, produced by toxic metabolites, it is of obvious importance to establish the role of the various inducers of monooxygenase activities that might modify the formation of toxic metabolites. Of particular interest to us is the response of these two inbred strains of mice, B6 and D2, which have been successfully used for model studies of aryl hydrocarbon hydroxylase induction.

In this paper we have shown that the induction of 2-AAF *N*-hydroxylase activity by MC appears to segregate as a single autosomal dominant gene in progeny from the appropriate backcrosses and intercross involving B6 and D2 mice (Fig. 1). The *N*-hydroxylation of the *N*-acetylarylamine 2-AAF is also closely linked with the induction of aryl hydrocarbon hydroxylase activity by aromatic hydrocarbons (Fig. 2 and Table 1). In addition, the degree of hepatotoxicity caused by acetaminophen, another *N*-acetylarylamine, correlates well with the MC-induced *N*-hydroxylation of 2-AAF and aryl hydrocarbon hydroxylase induction in B6 and D2 mice (Table 2).

The trait of aromatic hydrocarbon responsiveness, defined previously (13) as the *Ah* locus,[2] has been shown to be associated not only with new cytochrome P_1450

[2] For purposes of this report, appropriate crosses between B6 and D2 inbred strains only were used, because the genetic expression between these two strains (9–13) appears to segregate as a single autosomal dominant trait. Genetic expression between other strains of mice, however, appears to be considerably more complex (24–27); at least two, and probably more than two, nonlinked genetic loci have been suggested (26). Hence the term *Ah* locus should be used only in a general sense for describing the phenotype aromatic hydrocarbon responsiveness, and we cannot at this time infer a mechanism of action.

formation[3] and aryl hydrocarbon hydroxylase induction by such compounds as MC and BNF (9–13, 20, 21), but also with other aromatic hydrocarbon-inducible monooxygenase activities (20, 28), such as *p*-nitroanisole *O*-demethylase, 7-ethoxycoumarin *O*-deethylase, 3-methyl-4-methylaminoazobenzene *N*-demethylase, and zoxazolamine hydroxylase (29). Other monooxygenase activities which we feel are associated with the *Ah* locus, and therefore with cytochrome P_1450, include the formation of *N*-acetyl-*p*-aminophenol from phenacetin (i.e., the *O*-dealkylation of phenacetin) (30) and at least four additional drug-metabolizing enzyme activities.[4] In this report the *N*-hydroxylation of 2-AAF is also clearly associated with the *Ah* locus and therefore with cytochrome P_1450 content. It seems likely that the amount of P_1450 relative to the concentrations of other species of cytochrome(s) P450 in a given tissue could determine the degree of toxicity and/or carcinogenicity of numerous *N*-acetylarylamines.

TCDD causes the induction of 2-AAF *N*-hydroxylase activity of a similar magnitude in both the responsive B6 mice and the so-called nonresponsive D2 mice. This finding is in agreement with studies (20) on aryl hydrocarbon hydroxylase induction by TCDD. Genetically nonresponsive mice apparently have the structural and regulatory genes necessary for expression of these monooxygenase activities associated with new formation of cytochrome P_1450. Possible mechanisms to explain this defect in so-called nonresponsive mice have been discussed (20).

The magnitude of hepatotoxicity, as determined by the histological quantitation of centrolobular necrosis caused by acetaminophen, has been shown (4, 6) to correlate well with the covalent binding of a metabolite(s) to liver macromolecules. Metabolic studies of both acetaminophen and its analogues (6, 31) have strongly suggested that the toxic metabolite(s) arises from *N*-hydroxylation via the monooxygenase activities mediated by membrane-bound CO-binding hemoprotein of the P450 type. The difference in hepatotoxicity of acetaminophen following the administration of MC to B6 and D2 mice, as observed in the present study, further supports that suggestion.

With the genetic difference in induction of the *N*-hydroxylation of 2-AAF by polycyclic aromatic hydrocarbons between inbred strains of mice, plus the technical possibility of separating responsive from nonresponsive phenotypes without sacrificing the animal (29), we feel it is now possible to examine in detail the hepatotoxicity and carcinogenicity of the *N*-hydroxy derivatives of various *N*-acetylarylamines among siblings of a defined genotype.

[3] Cytochrome P_1450 (also called P-448) denotes in this report that species of membrane-bound CO-binding hemoprotein which increases in concentration in response to polycyclic hydrocarbon or BNF treatment.

[4] S. A. Atlas, K. Kumaki, J. W. Daly, and D. W. Nebert, manuscript in preparation.

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