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Genetic Control of Chloroform Toxicity in Mice

Abstract. *Mouse strain differences suggest intermediate or multifactorial genetic control of chloroform-induced renal toxicity and death. The chloroform dose lethal to 50 percent of animals was four times higher in C57BL/6J males than in DBA/2J males. Twice as much chloroform accumulated in the kidneys of the sensitive as the resistant strain. First generation offspring were midway between parental strains for both parameters.*

Chloroform, which is used widely for medicinal and industrial purposes, is damaging to the liver and kidney of man (*1*) and experimental animals (*2*). Long-term chloroform administration produces liver tumors in mice (*3*), and nonanesthetizing doses are toxic to the fetal rat (*4*). Our experiments indicate that genetic factors control chloroform toxicity in mice, that the degree of toxicological responsiveness correlates with the extent of renal accumulation of chloroform, and that the mode of inheritance differs from that previously described.

Accidental exposure of different mouse strains to chloroform vapor has demonstrated that response varies from one strain to another (*5–7*). We chose to investigate the mode of genetic control of this differential susceptibility in two inbred strains: C57BL/6J, a resistant strain, and DBA/2J, an exceedingly sensitive strain (*6, 7*).

Susceptibility to the lethal effects of chloroform was studied at 9 weeks ± 4 days of age in these two strains and in their F_1 hybrid (B6D2F_1/J). All mice were obtained from Jackson Laboratory in Bar Harbor, Maine. Each group of eight mice received the same dose of chloroform (certified ACS, Spectranalyzed; Fisher Scientific) on the basis of body weight. The compound was dissolved in peanut oil; each mouse received a total volume of solution of 0.1 ml per 10 g of body weight. The drug was given by gavage, and the frequency of death was recorded over a 10-day period.

The C57BL/6J strain was about four times more resistant to the lethal effects of chloroform than the DBA/2J strain: dose lethal to 50 percent of animals (LD_{50}) values for chloroform were 0.33 ml/kg and 0.08 ml/kg, respectively. The value for the B6D2F_1/J animals (0.20 ml/kg) was midway between those of the two parental strains (Fig. 1) (*8*). These results suggest either single gene intermediate inheritance or multifactorial genetic control over chloroform toxicity. The LD_{50} values differ significantly from each other. The three curves in Fig. 1 do not vary significantly from parallelism.

Resistance to chloroform has been reported to behave as a dominant trait: all males of the C3H/He strain died from an injection of chloroform at 0.18 ml/kg, whereas those of the C57BL/6JN strain and the F_1 hybrid between these strains survived (*9*). Similar findings were reported for the resistant BN strain and F_1 animals from the mating of BN and C3H/He strains (*9*). We performed a chloroform lethal dose response study on 9-week-old C3H/HeJ mice in which the LD_{50} value, 0.27 ml/kg (95 percent confidence limits are 0.22 to 0.33 ml/kg), was not significantly different from that of the C57BL/6J strain. Comparison of the log dose–probability plots for the two strains indicated that, in contrast to the above report (*9*), no dose of chloroform which was lethal to all C3H/HeJ males spared all C57BL/6J males.

Microscopic examination of tissues from mice dying of chloroform revealed dose-dependent changes in pathology (*3, 10*). Mice receiving chloroform doses of 0.17 ml/kg or less died with necrosis of the proximal convoluted renal tubules. However, mice of all three genotypes that received more than 0.17 mg/kg exhibited both renal tubular and hepatic centrolobular necrosis. Thus, at high chloroform doses all mice developed hepatic toxicity, whereas strain differences in LD_{50} values involved primarily the dose at which renal damage caused death. The lowest concentration of chloroform given to C57BL/6J mice was 0.27 ml/kg; only one of eight mice died of combined renal and hepatic lesions. By contrast, DBA/2J animals appear to be susceptible to death from renal lesions at doses of chloroform greater than about 0.05 ml/kg (Fig. 1). Males and females of the same strain exhibit similar thresholds to hepatic damage (*3*), but differ in regard to renal toxicity (*7, 11*); females die of chloroform-induced hepatic damage without developing renal lesions.

Sex hormones have been implicated in this sex difference in renal toxicity: immature male mice and castrated adult male mice are resistant to chloroform-induced damage, but are sensitized by testosterone; estrogen-treated males are resistant; and testosterone-treated females become sensi-

Table 1. Accumulation of [^{14}C]chloroform in mouse tissues. Each of four 9-week-old male mice (19 to 21 g) of the three genotypes received intraperitoneally [^{14}C]chloroform (0.07 ml/kg; 10 μc) in 0.2 ml of peanut oil at 7 p.m., before the lights in the room went off; food was withheld thereafter. Animals were killed 12 hours later. Genotype comparisons are presented as the ratio of specific activity in one group of animals to that in C57BL animals. Specific activities for all C57BL fractions are given as the mean ± standard error of the mean.

Tissue	Specific activity relative to that of C57BL			C57BL specific activity	
	DBA	F_1	C57BL	Wet weight (10^4 dpm/g)	Protein (10^2 dpm/mg)
		Tissue homogenates			
Liver	0.82	0.96	1.00	28.8 ± 2.5	
Kidney	2.41*	1.64	1.00	19.0 ± 1.7	
		Subcellular fractions			
Liver					
Nuclei	0.67	0.76	1.00		0.9 ± 0.1
Mitochondria	1.14	1.14	1.00		12.5 ± 2.0
Microsomes	0.64	0.73	1.00		28.0 ± 4.0
Cell sap	0.98	1.09	1.00		13.5 ± 1.3
Kidney					
Nuclei	2.20*	1.67	1.00		0.7 ± 0.1
Mitochondria	3.67*	1.97	1.00		13.9 ± 1.6
Microsomes	1.74*	1.44	1.00		13.7 ± 0.5
Cell sap	1.65*	1.23	1.00		7.8 ± 0.9

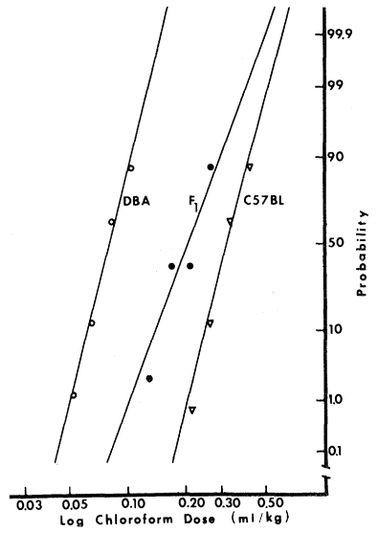
*Difference between DBA and C57BL means significant at $P < .05$, Student's t-test.

tive to renal toxicity (*7, 11*). Mouse strain differences in androgen production and functioning may account for the strain differences in our LD_{50} studies. In support of this concept, indirect measures of androgen function (*12*) suggest that males of the C57BL/10J strain and, to a lesser degree, the C57BL/6J strain are androgen deficient compared to those of the DBA/2J strain.

Chloroform and other halogenated hydrocarbons produce pathological effects by localizing in target tissues and binding covalently to cellular macromolecules (*13, 14*). A metabolite, rather than the parent compound, apparently binds to cellular constituents. Since our studies revealed a genetic difference in renal chloroform sensitivity between the DBA/2J and C57BL/6J strains, we predicted that more chloroform would bind to macromolecules in the kidneys of sensitive DBA/2J animals than in those of the resistant C57BL/6J strain.

Four male mice of these two strains and of their F_1 hybrid received a single intraperitoneal injection of chloroform (0.07 ml/kg) in peanut oil containing 10 μc of [^{14}C]chloroform (original specific activity, 2.0 mc/mmole; New England Nuclear). Animals were killed 12 hours later, a time that results in maximal tissue binding (*13*). Livers and kidneys were immersed in ice-cold 0.25*M* sucrose in TKM buffer (0.05*M* tris-HCl, *p*H 7.5, 0.025*M* KCl, and 5 m*M* $MgCl_2$). All remaining manipulations were carried out at 4°C. Tissues were homogenized in the above buffer (1 : 10, by volume), and a nuclear fraction was isolated (*15*). Supernatants were centrifuged at 9,000*g* for 15 minutes, and the resulting pellet was washed twice in sucrose-TKM to yield a mitochondrial fraction. The 9,000*g* supernatant was spun at 100,000*g* for 90 minutes to form a microsomal pellet; the supernatant was the cell sap. Total radioactivity in each subcellular fraction was determined, since the amount of labeled chloroform bound to cellular constituents varies with total accumulation of label (*16*).

Crude kidney homogenates (Table 1) from DBA/2J animals contained more than twice as much labeled chloroform as did those from C57BL/6J animals, being 45.7×10^4 and 19.0×10^4 disintegrations per minute (dpm) per gram of wet tissue, respectively ($P < .05$). Homogenates from F_1 males were intermediate in chloroform accumulation. Significant strain differences were also noted in each of the kidney subcellular fractions; those from F_1 animals were midway between the parental values (Table 1). The greatest strain difference was the mitochondrial fraction where 3.7 times more chloroform was present in DBA/2J than in C57BL/6J mice. It is of interest that renal proximal convoluted tubular cells are rich in mitochondria (*17*).



Strain differences in chloroform median lethal dose (ml/kg)

Genotype	Point estimate	95% confidence limits
C57BL/6J	0.33	0.26–0.40
B6D2F_1/J	0.20	0.16–0.24
DBA/2J	0.08	0.07–0.10

Fig. 1. Lethality of orally administered chloroform over a 10-day period. Groups of eight animals received single doses of chloroform in peanut oil (0.1 ml per 10 g of body weight) at 4 p.m.; they were housed four per cage; and food and water were always present. Four to five doses of chloroform were administered within the following ranges: 0.05 to 0.14 ml/kg for DBA, 0.10 to 0.27 ml/kg for the F_1 hybrid, and 0.27 to 0.53 ml/kg for C57BL animals. (Top) Log dose of chloroform versus probability of death. (Bottom) LD_{50} point estimates and their 95 percent confidence limits for animals of the three different genotypes. The analysis was performed according to the method of Litchfield and Wilcoxon (*8*).

The distribution of chloroform in the livers of the three groups of mice was opposite to that observed in the kidneys (Table 1). Total radioactivity in crude liver homogenates was highest in C57BL/6J animals, less in F_1, and least in DBA/2J animals. However, neither liver homogenates nor hepatic subcellular fractions showed significant strain differences. Kidneys of male mice are known to accumulate more labeled chloroform than do those of females; the converse is true for livers (*13, 16*).

Polycyclic hydrocarbons are ubiquitous environmental pollutants. Induction by these compounds of the enzyme system responsible for their metabolism is genetically controlled (*18*). Inducible mouse strains are more susceptible than noninducible strains to the cytotoxic and carcinogenic effects of polycyclic hydrocarbons (*19*). Recognition that simple halogenated hydrocarbons contaminate our environment and enter our tissues (*20*) makes the precise genetic mechanism protecting certain individuals, while rendering others more susceptible to the toxicological effects of these compounds, of practical significance.

Since this report was submitted, the Environmental Protection Agency has announced that chloroform is a principal organic chemical impurity in drinking water tested in over 150 American cities (*21*). Because of the predominantly genetic control of large interindividual differences in the disposition of commonly used drugs in normal human volunteers (*22*), our work on genetic control of chloroform toxicity in mice may be relevant if certain individuals develop toxicity from chronic exposure to chloroform in their drinking water.

RICHARD N. HILL, THOMAS L. CLEMENS
DAI KEE LIU, ELLIOT S. VESELL
Department of Pharmacology,
Milton S. Hershey Medical Center,
Pennsylvania State University
College of Medicine, Hershey 17033
W. DEAN JOHNSON
Department of Comparative Medicine,
Milton S. Hershey MedicalCenter

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Monthly Gonadotropin Cycles in Premenarcheal Girls

Abstract. *Patterns of nocturnal excretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were investigated in 11 girls. Autoregressive digital filtering of low- and high-frequency variations was used to make patterns more apparent. Coincident FSH and LH surges, separated by an interval of 20 to 40 days, were seen in specimens from three of six postmenarcheal girls and three to five premenarcheal girls. This suggests that cyclic hypothalamic–pituitary–ovarian interactions occur before menarche.*

After menarche, follicular maturation which results in both ovulation and atresia are associated with distinctive patterns of change in gonadotropin secretion including a gonadotropin surge during each cycle (*1*). In premenarcheal girls surges in gonadotropin secretion have been reported (*2, 3*), but cyclic variations in pituitary gonadotropin secretion similar to those seen in sexually mature girls have not been demonstrated. Having found that gonadotropin concentrations in first morning urine voidings from normally cycling women show patterns consistent with those seen in 24-hour urine specimens and daily blood samples (*4*), we investigated gonadotropin secretion patterns utilizing first morning urine voidings in prepubertal, pubescent, and postpubertal girls. These studies revealed monthly gonadotropin cycles in premenarcheal as well as in postmenarcheal girls.

Eleven healthy girls (five premenarcheal and six postmenarcheal), ages 8 years 11 months to 16 years 7 months, volunteered to collect timed nocturnal and first morning urine specimens for 60 consecutive days. No abnormalities were found in their histories, physical examinations, routine laboratory studies, or endocrine evaluations. None were taking medications or oral contraceptives during the study period. On each of the 60 days, the girls just before retiring would void and discard the urine, noting the time precisely. The next morning, the first urine voiding was added to any urine which may have been collected during the night and the time again was noted precisely. The samples were refrigerated and delivered to the laboratory, where the gonadotropins were precipitated with acetone after *p*H adjustment with acetic acid (*4*). Precipitates, containing both follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were dissolved in buffer and measured by specific radioimmunoassay (*5, 6*). To minimize the effects of interassay variation, all samples from a given subject were examined in the same radioimmunoassay, with IRP2HMG (*7*) as reference preparation.

The excretion rates in these peripubertal girls represent pituitary gonadotropin secretion during sleep. This may be the optimum time to examine gonadotropin activity since serum levels in some pubescent girls have been shown to be higher when the girls were asleep than when they were awake (*8*). Since each specimen was obtained at the same time during the day, the effect of diurnal variation on the data was minimized.

The total gonadotropin excretion during each night was divided by the collection interval and expressed as international units (I.U.) per hour to reduce variation resulting from different collection times. Nevertheless, significant variability remained which may be attributed to (i) variance between assays that is amplified by the small quantities of gonadotropin excreted by premenarcheal girls, (ii) nonsystematic biological variation, in addition to (iii) underlying physiological cycles.

To examine the data for monthly physiological cycles and reduce other sources of variability, a two-stage autoregressive filter in discrete time was used to remove general trends of long duration (periods greater than 45 days) and high-frequency variations (periods less than 10 days). This technique involved transforming the data, $X_1, X_2, \ldots, X_{60}$, according to the following scheme (*9*):

$$Y_i = (1-\alpha)Y_{i-1} + \alpha X_i, \quad i = 2 \text{ to } 60,\ Y_1 = X_1 \qquad (1)$$

$$Z_{i-1} = (1-\alpha)Z_i + \alpha Y_{i-1}, \quad i = 60 \text{ to } 2,\ Z_{60} = Y_{60} \qquad (2)$$

$$U_i = Z_i - \frac{\alpha^2}{1+(1-\alpha)^2} X_i,\ i = 1 \text{ to } 60 \qquad (3)$$

$$V_i = X_i - U_i,\ i = 1 \text{ to } 60 \qquad (4)$$

First, α was chosen to be 0.2, and the autoregression equations, Eqs. 1 through 3, applied twice (the X_i in the second application being the U_i from the first). The residuals V_i were then calculated as the difference between U_i, the final trend, and X_i, the initial data (*10*). This procedure results in removing long-term trends with periods of greater than 45 days. Next, the residuals, V_i, were similarly treated once (V_i was substituted for X_i in Eq. 1) with α set at 0.4 so as to remove variations with periods of less than 10 days.

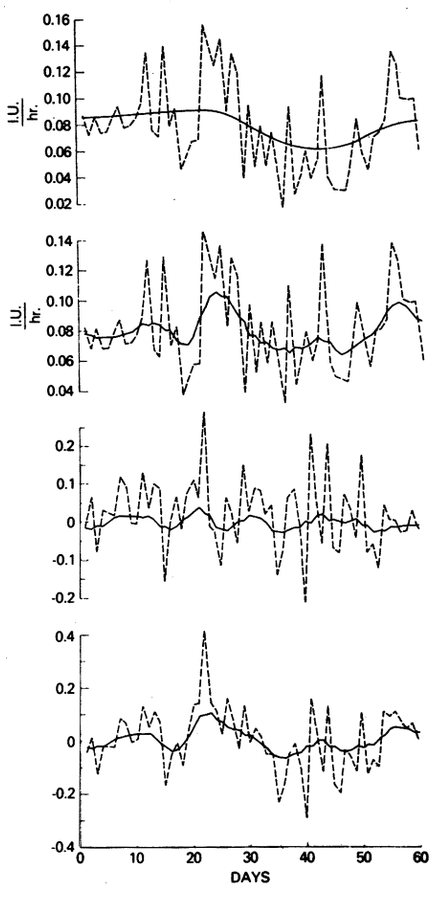

Fig. 1. Autoregression analysis of data and random numbers. The top panel shows FSH data from subject 1 as a broken line and the general trend after double autoregression with $\alpha = 0.2$ as the solid line; periods less than 45 days do not contribute substantially to this trend. The second panel shows the residual between this general trend and the data as a broken line; the solid line represents autoregression of these residuals using $\alpha = 0.4$ and hence emphasizes the remaining components of the data with periods of greater than 10 days. The third panel shows a set of 60 random numbers as the broken line and the ultimate result of analyzing these numbers with the same program used to analyze the data; thus the solid line is comparable to the solid line in panel 2 but shows no evidence of periodicity with wave lengths between 20 and 40. The broken line in the bottom panel shows the same set of random numbers but with the derived signal (solid line) from panel 2 added to it after scaling appropriately; the autoregression analysis results in the solid line in which the signal is seen to reemerge at a decreased amplitude.