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Relationship between "Aromatic Hydrocarbon Responsiveness" and the Survival Times in Mice Treated with Various Drugs and Environmental Compounds

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SUMMARY

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Differences in toxicity caused by various environmental pollutants or drugs were studied in several inbred strains of mice and in siblings of the (C57BL/6N) (DBA/2N)F_1 × DBA/2N backcross, in which the phenotypes aromatic hydrocarbon "responsiveness" or "nonresponsiveness" had been determined. This trait of "responsiveness"—which refers to the capacity for induction of cytochrome P_1450 and numerous monooxygenase activities by certain aromatic hydrocarbons—has been previously shown to segregate almost exclusively as a single gene among offspring of the (C57BL/6N) (DBA/2N)F_1 × DBA/2N backcross. "Responsiveness" is associated with shortened survival times following large (500 mg kg^{-1} day^{-1}) doses of intraperitoneal benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, β-naphthoflavone, or polychlorinated biphenyls; the cause of death in these experiments is not yet certain. "Nonresponsiveness" is associated with shortened survival times following smaller (120 mg kg^{-1} day^{-1}) oral doses of benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, or lindane, yet is associated with a longer survival time following small daily doses of oral polychlorinated biphenyls. All nonresponsive mice ingesting benzo[*a*]pyrene daily die within 4 weeks, whereas the survival of responsive mice ingesting benzo[*a*]pyrene daily is not significantly different from that of control mice; the apparent cause of early death in these experiments is toxic depression of the bone marrow, the pancytopenia leading to death due to hemorrhage or overwhelming infection. When given an intraperitoneal dose of lindane that is lethal to normal mice within 12 hr, 3-methylcholanthrene-treated responsive mice are protected and therefore do not die during this time period. A dose of bromobenzene sufficient to cause considerable necrosis in the liver of a pregnant mouse does not transplacentally cause any detectable necrosis in fetal liver. Genetic differences in aromatic hydrocarbon responsiveness between C57BL/6N and DBA/2N mice are not

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associated with toxicity caused by large intraperitoneal daily doses of bromobenzene, zoxazolamine, diphenylhydantoin, dichlorodiphenyltrichloroethane (*p,p'*-DDT), hexachlorobenzene, butylated hydroxytoluene, chlorpromazine, tetracycline hydrochloride, carbamazepine, or diphenylbarbituric acid, and are not associated with differences in survival time following bromobenzene or *p,p'*-DDT in the diet. These data suggest that the life span of animals exposed to certain environmental compounds can be markedly influenced by a single gene or a very small number of genes, and that the same genetic trait can be either beneficial or detrimental to the animal, depending on whether detoxification or metabolic potentiation occurs.

INTRODUCTION

The importance of aryl oxidations of drugs, polycyclic hydrocarbons, and other environmental contaminants by the cytochrome P450-mediated monooxygenase systems has been recently reviewed (1, 2). The application of these results to the research fields of pharmacology, toxicology, and chemical carcinogenesis is being actively studied in a large number of laboratories. Large differences in the induced levels of about 10 drug-metabolizing enzyme activities in aromatic hydrocarbon-treated mice have been shown (3, 4) to be controlled by a very small number of genes. The difference between aromatic hydrocarbon-treated B6 and D2 mice,[1] with respect to these induced monooxygenase activities and associated increases in cytochrome P_1450, closely approximates a single-gene difference (3–8) in liver. Marked differences between these two strains also exist in such nonhepatic tissues as kidney, bowel, lung, skin (5, 9), and eye.[2] Inbred mouse strains might respond differently to a stimulus for numerous reasons not easily understood; for example, each inbred strain is known to differ from another strain by hundreds of genes which have been identified. However, with the use of siblings from the $B6D2F_1 \times D2$ backcross, one can relate differences in susceptibility to carcinogens or toxic chemicals and relate these specifically to a previously determined phenotype. Such genetic differences among siblings offer a powerful probe—especially in the research areas of toxicology, chemical carcinogenesis, and pharmacology—because many test substances produce nonspecific toxicity, malnutrition, sedation, or other disruptions of normal physiology.

Susceptibility to tumors caused by 7,12-dimethylbenz[*a*]anthracene (10) or benzo[*a*]pyrene (11) was shown not to be associated with the *Ah* locus[3] in B6 and D2 mice; however, 3-methylcholanthrene-initiated tumorigenesis is correlated very closely with aromatic hydrocarbon "responsiveness" in a large number of inbred strains (12–14) and among progeny of the appropriate genetic crosses between B6 and D2 mice (15). When fetal cell cultures derived from the B6 inbred strain are compared with those from the D2 strain (16), 20 μM benzo[*a*]pyrene in the growth medium causes significantly more rapid cytotoxicity and cell death in the aromatic hydrocarbon-"responsive" B6 cells. The interpretation of these data is that increased amounts of the induced monooxygenase system led to a higher steady-state level of a reactive metabolic intermediate, thereby causing increased toxicity. In this report our work is extended to toxicity *in vivo*. Among inbred strains and among siblings of previously determined (17) phenotype for the *Ah* locus, differences in life span are examined during which the mice were exposed to various environmental compounds.

[1] The abbreviations used are: B6, the inbred C57BL/6 strain; D2, the inbred DBA/2 strain; *p,p'*-DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; zoxazolamine, 2-amino-5-chlorobenzoxazole.

[2] H. Shichi, S. A. Atlas, and D. W. Nebert, manuscript submitted for publication.

[3] The term *Ah* locus is used only in a general sense for describing the phenotype aromatic hydrocarbon responsiveness, and we cannot at this time infer a genetic mechanism of action. What at first (5–8) appeared to be a single autosomal dominant trait is now viewed as not exclusively monogenic; a minimum of two or three genes and six alleles is now estimated (3, 4, 9).

MATERIALS AND METHODS

Benzo[*a*]pyrene and 7,12-dimethylbenz-[*a*]anthracene from Sigma and 3-methylcholanthrene from Eastman Organic Chemicals were each purified by recrystallization from benzene. Sodium phenobarbital was purchased from Merck & Company. We bought β-naphthoflavone and *p,p'*-DDT from Aldrich Chemical Company; NADPH, NADH, lindane (γ-hexachlorocyclohexane), and butylated hydroxytoluene, from Sigma; polychlorinated biphenyls, from Analabs, Inc., North Haven, Conn.; bromobenzene, from Fisher Chemical Company; 2-chloro-10-(3-dimethylaminopropyl)phenothiazine HCl (chlorpromazine), from Smith Kline & French Laboratories; tetracycline HCl (Achromycin i/v), from Lederle Pharmaceutical Company; and generally labeled [^{3}H]benzo[*a*]pyrene (25 mCi/μmole), from Amersham-Searle. The remainder of the compounds used in this study were generous gifts: hexachlorobenzene, from Dr. Alan P. Poland, University of Rochester Medical Center; zoxazolamine (2-amino-5-chlorobenzoxazole), from McNeil Laboratories, Inc.; diphenylhydantoin, from Parke, Davis & Company; and diphenylbarbituric acid, from Dr. Arthur Raines, Department of Pharmacology, Georgetown University, Washington, D. C. Tegretol tablets were obtained commercially from a Swiss pharmacy; carbamazepine was extracted from the tablets into methylene chloride and after evaporation of the solvent the molecular weight of the compound corresponded to that of pure carbamazepine as indicated by the manufacturer. The NMR, ultraviolet, and mass spectra corresponded to data published for the authentic compound (18). National Institutes of Health Animal Supply provided us with sexually immature mice of the various inbred strains. In some experiments C57BL/6J and DBA/2J mice from the Jackson Laboratory (Bar Harbor, Me.) were used in place of C57BL/6N and DBA/2N from the National Institutes of Health Animal Supply. The backcross animals were generated within our own mouse colony. The environment in the animal room, feeding of the animals, and preparation of liver microsomes were exactly as previously described (5, 8). Standard preliminary treatment regimens (5, 8) included intraperitoneal doses of 3-methylcholanthrene or β-naphthoflavone (both 80 mg kg^{-1}) in corn oil about 48 hr before assay and sodium phenobarbital in 0.85% sodium chloride every 24 hr for four doses. The first dose of phenobarbital was 30 mg kg^{-1}; the next three doses were 60 mg kg^{-1} (5, 8).

Determination of phenotype among backcross animals. Forty-eight hours after a single intraperitoneal dose of β-naphthoflavone (80 mg kg^{-1}), zoxazolamine (225 mg kg^{-1}) was administered intraperitoneally and the length of paralysis time (17) was recorded. In a typical experiment, mice about 4 weeks of age—three B6, three D2, and about 70 progeny from the B6D2F_1 × D2 backcross—were tested simultaneously. The "nonresponsive" (B6D2)D2 and inbred D2 mice were paralyzed between 60 and 180 min longer than the "responsive" (B6D2)D2 siblings and inbred B6 mice (17). Any questionable individual (e.g., one with a leak at the injection site, or a mouse of some unappreciated genotype) was discarded.

Toxicity by compounds administered intraperitoneally or orally. Three to four weeks after the zoxazolamine paralysis test, (B6D2)D2 mice or animals of various inbred strains were given daily large doses of test compounds dissolved in a minimal amount of corn oil. Toxicity studies involving oral regimens were begun 1 week after the zoxazolamine paralysis test; Wayne Lab-Blox (Allied Mills, Inc., Chicago) were soaked 24 hr or longer in corn oil containing the test compound at a concentration of 10 mg/ml. The corn oil-soaked laboratory chow was accepted eagerly by the mice. Controls in these experiments received the laboratory chow soaked in corn oil alone. By knowing the weight of food ingested daily by a 20-g mouse and by using [^{3}H]benzo[*a*]pyrene in several experiments, we estimated that the oral dosage of benzo[*a*]pyrene ranged between 100 and 125 mg kg^{-1} day^{-1}. The daily dosage of the other test compounds given orally was not determined with this degree of precision but is assumed to be similar to that of benzo[*a*]pyrene. Deaths were recorded at least twice daily.

Enzyme assay. The hydroxylase activity in liver microsomes were determined as previously described (5, 8). One unit of aryl hydrocarbon hydroxylase activity is defined (5, 8) 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. Specific activity denotes units per milligram of microsomal protein.

Histology. During the course of intraperitoneal injections or feeding experiments with the various test compounds, tissues were fixed in formalin and paraffin sections were prepared and stained. Quantitative analysis of liver necrosis was performed on a double-blind basis and scored according to the method of Chalkley (19). During examination of several random fields on two sections from each liver, necrosis was scored as follows: 0 = absent; 1+ = necrosis of less than 6% of the hepatocytes; 2+ = necrosis of 6–25% of the hepatocytes; 3+ = necrosis of 26–50% of the hepatocytes; and 4+ = necrosis of more than 50% of the hepatocytes. For this evaluation the liver sections were stained with hematoxylin and eosin.

Evaluation of benzo[a]pyrene binding to DNA. DNA from B6 and D2 mice which had received [^{3}H]benzo[*a*]pyrene orally or intraperitoneally for 2 days was isolated (20) from liver, bowel, kidney, lung, and adrenal, and the amount of benzo[*a*]pyrene covalently bound per milligram of DNA was determined (21) by the references cited.

Spectrophotometry. The CO difference spectral method (22) was used for determining the concentrations of cytochromes P450 and $P_1$450. The extinction coefficient of 91 mM^{-1} cm^{-1} was used for the difference in absorbance between the Soret maximum and the 490 nm baseline for the cytochrome-CO complex reduced with sodium dithionite (22). The wavelength measurements were standardized with the use of a holmium oxide crystal (Fisher Scientific Company).

RESULTS

Intraperitoneal polycyclic hydrocarbons. When inbred mice were given massive intraperitoneal doses of benzo[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene (Fig. 1), the survival time of the three responsive inbred strains—B6, C3H/HeN, and BALB/cAnN—was significantly ($p < 0.01$) shorter than that of the two nonresponsive inbred strains, D2 and AKR/N. Because this genetic difference was also seen among progeny of the B6D2F_1 × D2 backcross, we conclude that what appears to be a single-gene difference in aromatic hydrocarbon responsiveness is associated with a decreased survival time. We have not yet found a satisfactory explanation for the cause of earlier death in the responsive mice.[4] Similar results were also found with excessive doses (500 mg kg^{-1}) of 3-methylcholanthrene or β-naphthoflavone in B6, D2, and (B6D2)D2 mice (data not illustrated). When intraperitoneal 3-methylcholanthrene at a dose of 300 mg kg^{-1},

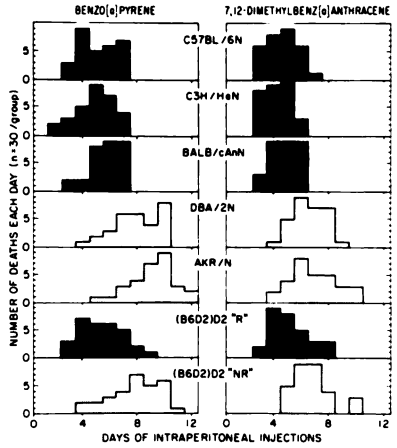

FIG. 1. *Rates of death among five inbred strains and responsive [(B6D2)D2 "R"] or nonresponsive [(B6D2)D2 "NR"] offspring from (B6D2)F_1 × D2 backcross*

Each group consisted of 30 mice 8 weeks of age. At about 9:00 a.m. daily each mouse was given a large (500 mg kg^{-1}) intraperitoneal dose of benzo[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene; the number of deaths was recorded each day at about 9:00 a.m. and again at about 9:00 p.m. The backcross animals were siblings which, as weanlings, had been separated according to phenotype by differences in zoxazolamine paralysis times (17).

[4] Centrolobular necrosis of the liver was not found at the time of death in mice receiving daily massive doses of intraperitoneal polycyclic hydrocarbons.

benzo[a]pyrene at a dose of 100 mg kg^{-1}, or corn oil alone was used with groups of 30 mice each, no significant genetic differences in survival times were seen.

Treatment of weanlings with β-naphthoflavone plus zoxazolamine might cause in some unknown manner the observed genetic differences in response to these excessive doses of polycyclic hydrocarbons 3 or 4 weeks later. To test this possibility, we felt that the phenotype of these backcross animals might be determined even after death. Figure 2 shows that 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity is in fact stable in the livers of B6 mice lying dead for 12 hr at room temperature; the hepatic enzyme in 3-methylcholanthrene-treated D2 mice was much less stable after death. Similar results were found with the kidney hydroxylase activity (data not shown). It therefore is possible to collect from the cage, within 6–12 hr after death, mice which have received large doses of aromatic hydrocarbons, to freeze the livers, and to determine at a later time the phenotype by assaying the hydroxylase activity. By means of this type of experiment with (B6D2)D2 mice receiving excessive doses of 3-methylcholanthrene daily (Fig. 3), we found that those siblings having higher hydroxylase activity were likely to die at earlier times. Hence prior treatment with β-naphthoflavone and zoxazolamine several weeks beforehand does not affect the genetically mediated difference observed when mice receive excessive intraperitoneal doses of polycyclic hydrocarbons. It should be noted that, up to the time of death, all tissues examined from B6 mice receiving β-naphthoflavone or 3-methylcholanthrene at 500 mg kg^{-1} day^{-1} had hydroxylase activities which were about 2 to more than 10 times higher than those in D2 mice treated similarly (9, 23).[5]

Oral polycyclic hydrocarbons. When the dosage and the route of administration of benzo[a]pyrene were changed (Table 1),

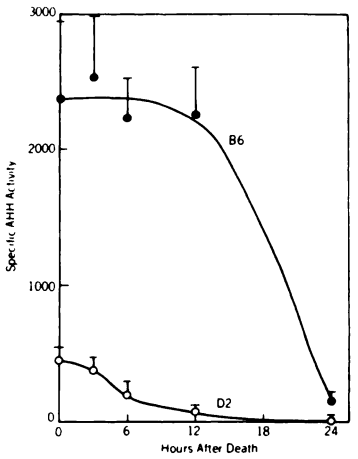

FIG. 2. *Hepatic aryl hydrocarbon hydroxylase (AHH) specific activity as a function of time after death*

The animals were killed by cervical dislocation and allowed to lie in the cage at room temperature for the indicated times, after which the livers were removed, microsomes were prepared, and the enzyme activity was assayed in the usual manner. Both B6 and D2 mice had been treated with 3-methylcholanthrene (500 mg kg^{-1}) 24 hr prior to death. Each point and bracket represents the mean ± standard deviation of four mice.

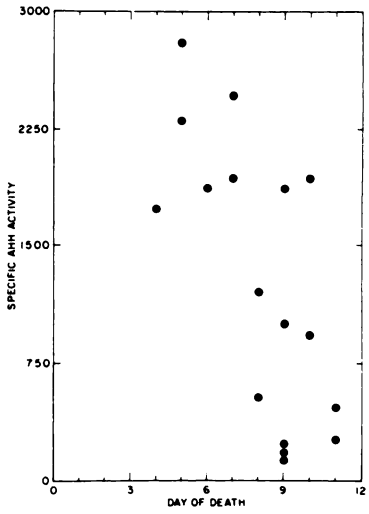

FIG. 3. *Hepatic aryl hydrocarbon hydroxylase (AHH) specific activity in progeny of (B6D2)F_1 × D2 backcross on day of death*

Each point represents a single mouse; until death all 17 mice received daily large (500 mg kg^{-1}) intraperitoneal doses of 3-methylcholanthrene. Twelve hours or less after death, livers were removed and frozen. When all livers had been collected and frozen, the microsomal enzyme activity was assayed in the usual manner. The correlation coefficient r = 0.61 ($p < 0.01$).

[5] Unpublished observations.

TABLE 1

Toxicity of oral benzo[a]pyrene daily

Groups of 30 mice were fed ad libitum laboratory chow which had been soaked previously for at least 24 hr in corn oil containing 10 mg of benzo[a]pyrene per milliliter. The oral dose of benzo[a]pyrene was estimated as 120 mg kg^{-1} day^{-1}. The number of deaths was recorded daily at about 9:00 a.m. The backcross animals had been separated previously according to phenotype by differences in zoxazolamine paralysis times (17).

Strain	*Ah* phenotype	Deaths recorded between				Still alive after 180 days
		0–10 days	11–15 days	16–20 days	21–180 days	
B6	Responsive	0	0	0	2	28
C3H/HeN	Responsive	0	0	0	3	27
BALB/cAnN	Responsive	0	0	0	1	29
D2	Nonresponsive	10	9	11	0	0
AKR/N	Nonresponsive	3	16	11	0	0
(B6D2)D2	Responsive	0	0	1	5	24
(B6D2)D2	Nonresponsive	8	10	9	3	0
B6 controls[a]		0	0	0	1	29
D2 controls[a]		0	1	0	2	27

[a] Fed laboratory chow soaked in corn oil only.

the survival time of the genetically nonresponsive mouse was markedly shortened, whereas no significant earlier death rate was seen in genetically responsive mice, even after 6 months of continuous feeding. Animals receiving food soaked in corn oil alone were included for comparison. This 6-month experiment with B6, D2, and (B6D2)D2 mice was carried out a second time and the results were identical. The animals surviving 6 months on oral benzo[a]pyrene appeared at least as healthy as the control animals. Since the dramatic difference in survival time was found in the nonresponsive (B6D2)D2 mice as well as in the D2 and AKR/N strains, we conclude that this effect is closely associated with the *Ah* locus.

The cause of early death in these nonresponsive mice was investigated. Before death the mice looked ill for several days, and weight loss was considerable (Fig. 4). Hepatic aryl hydrocarbon hydroxylase activity in mice receiving oral benzo[a]pyrene was not statistically significantly different from basal enzyme levels in either B6 or D2 mice. In mice receiving oral benzo[a]pyrene for 5–10 days, lung hydroxylase activity was induced no more than 2-fold in B6 and considerably less in D2. The bowel hydroxylase activity was induced 5–15-fold in both strains, but the absolute specific activity in B6 mice was always at least twice that in D2 mice.

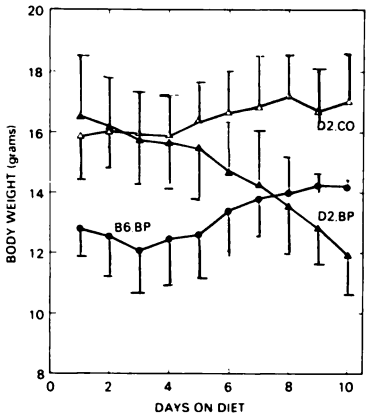

FIG. 4. *Daily weights of B6 and D2 mice on benzo[a]pyrene (BP) diet and D2 mice on laboratory chow soaked in corn oil (CO) alone*

Numbers of mice in each group were three, eight, and 12, respectively. Symbols and brackets denote means ± standard deviations.

The amount of phenolic benzo[a]pyrene per milligram of feces was determined after fasting B6 and D2 mice for 2 days and then administering oral benzo[a]pyrene in the usual manner for several days. In general, the amount of phenolic product per milligram of feces from B6 mice was 2–3

times greater than that from D2 mice; however, an occasional value from a D2 mouse was higher than the lowest values from individual B6 mice. This experiment was therefore not useful in attempting to explain the cause of early death in nonresponsive mice receiving oral benzo[a]pyrene. We also considered this technique as a means for determining the phenotype in (B6D2)D2 animals. Because of individual variation from day to day and overlap between values from B6 and D2 mice, however, this idea was discarded. Between 24 hr and 5 days of oral benzo[a]pyrene, values in D2 and B6 mice ranged between 150 and 900 pmoles of phenolic benzo[a]pyrene per milligram of fecal material.

The amount of benzo[a]pyrene metabolites covalently bound to DNA was determined in the various tissues of B6 and D2 mice (Table 2). The amount bound to B6 DNA was generally higher than that bound to D2 DNA, whether the mice had received oral or intraperitoneal benzo[a]pyrene. In mice ingesting benzo[a]pyrene, however, the amount of polycyclic hydrocarbon presumably bound covalently to DNA from D2 bowel was almost twice as high as that from B6 mice. We also attempted to study adrenal DNA, but the yield of DNA from 10 pairs of mouse adrenals was small, and the amount of radioactivity per milligram of DNA was not significantly above background radioactivity. The significance of the data in Table 2 is unclear, although we have demonstrated that low continuous oral benzo[a]pyrene administration can be almost as effective in causing covalent binding of benzo[a]pyrene metabolites to DNA of various tissues as a daily intraperitoneal benzo[a]pyrene dose which is several times greater. The levels of benzo[a]pyrene binding to bowel DNA are in fact equivalent, or increased, in mice receiving the oral benzo[a]pyrene, compared with those receiving the chemical intraperitoneally.

Microscopic evaluation of liver, lung, bowel, kidney, and adrenal from mice receiving oral benzo[a]pyrene for 5–12 days showed no significant differences between B6 and D2 animals. The bone marrow, however, demonstrated a marked hypocellularity and specifically a decreasing myeloid to erythroid ratio in the D2 and the nonresponsive (B6D2)D2 mice shortly before death. This was not found in responsive mice on the oral benzo[a]pyrene diet or in any mice eating the control (corn-oil soaked) diet. At the time of death the myeloid cells in several mice were estimated to be less than 5% of their normal concentration in the bone marrow. These histological observations were confirmed by examination of the peripheral blood (Table 3). A significant ($p < 0.05$) depression in the white cell count was observed in surviving D2 mice which had eaten benzo[a]pyrene for 12 days. Although no significant ($p > 0.05$) differences in the total red blood cell count, hemoglobin, and hematocrit values between the control and experimental groups were found, a slight fall in these erythroid components was seen in D2 mice shortly before death. The mean corpuscu-

TABLE 2

Benzo[a]pyrene covalently bound to DNA in B6 and D2 mice receiving oral or intraperitoneal benzo[a]pyrene

The mice were fasted for 48 hr and then either placed on the oral [^{3}H]benzo[a]pyrene diet or given daily intraperitoneal injections of [^{3}H]benzo[a]pyrene (500 mg kg^{-1}). After 2 days of either regimen, DNA was prepared and the amount of [^{3}H]benzo[a]pyrene bound per milligram of DNA was determined. The values shown are the means of two experiments each.

Route of administration	Benzo[a]pyrene (presumably metabolites) bound to DNA							
	Liver		Lung		Kidney		Bowel	
	B6	D2	B6	D2	B6	D2	B6	D2
	pmoles/mg		*pmoles/mg*		*pmoles/mg*		*pmoles/mg*	
Oral	13	7.0	<1.0	<1.0	1.1	1.4	16	26
Intraperitoneal	21	14	2.6	<1.0			18	16

TABLE 3

Peripheral white blood cell and red blood cell studies in mice receiving oral benzo[a]pyrene

Groups of B6 and D2 mice were placed on the oral benzo[a]pyrene regimen, and other D2 mice were also fed the control diet. At 8 and 12 days the blood from individual surviving mice was collected in a tube containing calcium oxalate, and the peripheral blood profile was determined in the Clinical Pathology Laboratory, National Institutes of Health. Values are expressed as means ± standard deviations; N = five or six individual mice for each group. B6 mice receiving the control diet (data not shown) were not different from D2 mice receiving the control diet or B6 mice ingesting benzo[a]pyrene.

Strain	Diet	Days on diet	Total white blood cells	Total red blood cells	Hemoglobin concentration	Hematocrit
			$\times 10^{-3}/mm^3$	$\times 10^{-6}/mm^3$	*g/100 ml*	%
D2	Corn oil alone	8	5.5 ± 0.72	8.0 ± 1.4	13.4 ± 1.8	35 ± 5.7
		12	5.3 ± 0.86	8.1 ± 1.1	12.9 ± 1.6	35 ± 5.4
B6	Benzo[a]pyrene	8	4.8 ± 0.98	7.8 ± 1.2	13.4 ± 1.9	35 ± 5.5
		12	5.0 ± 0.90	7.8 ± 1.4	13.0 ± 1.6	34 ± 5.3
D2	Benzo[a]pyrene	8	3.8 ± 1.1	7.2 ± 1.1	12.2 ± 1.4	31 ± 5.1
		12	3.3 ± 0.49[a]	6.6 ± 1.2[b]	11.5 ± 1.5[b]	28 ± 5.2[b]

[a] This value is significantly ($p < 0.05$) different from the first four values in this column.

[b] This value is not significantly ($p > 0.05$) different from the first four values in the respective column.

lar volume ranged between 44 and 46 μm^3, the mean corpuscular hemoglobin ranged between 34% and 49% among the individual mice; no statistically significant difference in these parameters was observed between the control and experimental groups. The results are therefore consistent with chemical toxicity of the bone marrow: first, a depression in myeloid precursors; later, the appearance of a normocytic anemia. Further explanation as to why the bone marrow failure is associated with the nonresponsive mouse ingesting benzo[a]pyrene requires additional study.

The amount of phenolic product per milliliter of heparinized plasma was determined in B6 and D2 mice which had received oral benzo[a]pyrene for 2 days. The average of seven D2 mice was 15, and of seven B6 mice 7.0, pmoles/ml of plasma. The significance of this 2-fold elevation in the D2 strain is unclear but may be related in some manner to the toxic bone marrow depression.

Tables 4 and 5 demonstrate that nonresponsive inbred or backcross mice receiving oral 7,12-dimethylbenz[a]anthracene or 3-methylcholanthrene also die significantly earlier than the responsive inbred and backcross animals. Whereas almost all the responsive mice receiving oral benzo[a]pyrene survived for more than 6 months (Table 1), however, all the responsive mice receiving oral 7,12-dimethylbenz[a]anthracene and 3-methylcholanthrene died within 15 and 30 days, respectively.

Aryl hydrocarbon hydroxylase activity and total CO-binding cytochrome content in mice receiving polychlorinated biphenyls, lindane, and other compounds. Arochlor 1254 behaves like both polycyclic hydrocarbons and phenobarbital with respect to the different induced forms of microsomal CO-binding cytochrome that can be separated electrophoretically (24). We therefore reasoned that the halogenated hydrocarbon would exert a "polycyclic hydrocarbon effect" plus a "phenobarbital effect" in responsive mice, but only the phenobarbital effect in nonresponsive mice. The polycyclic hydrocarbon effect appears as an increase in CO-binding hemoprotein content, a hypsochromic shift in the Soret maximum of the reduced cytochrome-CO complex, and a rise in aryl hydrocarbon hydroxylase activity (5, 23). The phenobarbital effect appears as an increase in the cytochrome concentration and a small rise in hydroxylase activity but without the spectral shift in the Soret peak (5). These results were in fact what was found (Table 6). Lindane was similarly studied. Lindane at 60 mg kg^{-1} caused a small increase in CO-binding cytochrome content but no

TABLE 4

Toxicity of oral 7,12-dimethylbenz[a]anthracene daily

The experiment was carried out exactly as described in the legend to Table 1, except that the laboratory chow was soaked in corn oil containing 10 mg of 7,12-dimethylbenz[a]anthracene per milliliter.

Strain	*Ah* phenotype	Deaths recorded between				
		0–6 days	7–8 days	9–10 days	11–12 days	13–15 days
B6	Responsive	0	6	12	10	2
D2	Nonresponsive	3	23	4	0	0
(B6D2)D2	Responsive	1	7	14	7	1
(B6D2)D2	Nonresponsive	3	18	8	1	0

TABLE 5

Toxicity of oral 3-methylcholanthrene daily

The experiment was carried out exactly as described in the legend to Table 1, except that the laboratory chow was soaked in corn oil containing 10 mg of 3-methylcholanthrene per milliliter.

Strain	*Ah* phenotype	Deaths recorded between					
		0–8 days	9–11 days	12–13 days	14–16 days	17–20 days	21–30 days
B6	Responsive	1	1	9	13	3	3
D2	Nonresponsive	9	18	3	0	0	0
(B6D2)D2	Responsive	0	3	10	14	1	2
(B6D2)D2	Nonresponsive	6	16	7	1	0	0

TABLE 6

Effect of Arochlor 1254 and lindane on hepatic CO-binding cytochrome content and aryl hydrocarbon hydroxylase activity in B6 and D2 mice

Arochlor 1254 (500 mg kg^{-1}) or corn oil alone was given intraperitoneally for 4 consecutive days, and the mice were killed 24 hr after the fourth dose. Lindane was given as a single dose 48 hr prior to hepatic microsomal isolation. Each value represents liver microsomes combined from four individual animals per group.

in vivo Treatment	Strain	CO-binding cytochrome content	Soret maximum	Specific hydroxylase activity
		pmoles/mg microsomal protein	*nm*	*units/mg microsomal protein*
Corn oil	B6	650	450–451	640
Corn oil	D2	610	450–451	660
Arochlor 1254	B6	1640	448–449	3080
Arochlor 1254	D2	980	450–451	1170
Lindane[a]	B6	760	450–451	680
Lindane[a]	D2	590	450–451	600
Lindane[b]	B6	670	450–451	450

[a] Intraperitoneal dose was 60 mg kg^{-1}; none of the four mice per group died at this dose.

[b] Intraperitoneal dose was 100 mg kg^{-1}; one B6 out of four survived this dose, and all D2 animals died.

significant shift to the blue in the Soret band in B6 compared with D2 mice. A nearly lethal (100 mg kg^{-1}) dose of lindane to B6 mice showed no greater effects; in fact, the significant fall in hydroxylase activity suggests toxicity. Other doses of lindane from 12 hr to 5 days produced no greater genetic differences. The data there-

fore suggest that polychlorinated biphenyls, at sufficiently high doses, cause genetic differences associated with the *Ah* locus, whereas lindane, at doses approaching lethality, produces little, if any, genetic difference associated with the *Ah* locus.

Numerous other compounds at doses up to lethal levels were tested in a manner similar to experiments shown in Table 6. No obvious genetic differences between B6 and D2 mice were seen after 2–5 days of treatment with bromobenzene (500–1000 mg kg^{-1}), *p,p'*-DDT (400–800 mg kg^{-1}), butylated hydroxytoluene (1000 mg kg^{-1}), chlorpromazine (1000 mg kg^{-1}),[6] tetracycline hydrochloride (1000 mg kg^{-1}), carbamazepine (8 mg kg^{-1}), hexachlorobenzene (500 mg kg^{-1}), diphenylbarbituric acid (50 mg kg^{-1} for 3 days only), or diphenylhydantoin (150 mg kg^{-1}). Our reason for testing carbamazepine was that an epoxide intermediate of this drug in human urine has been isolated and identified (18).

Intraperitoneal polychlorinated biphenyls. Large daily doses of Arochlor 1254 behaved similarly to benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, and β-naphthoflavone in that the responsive inbred and backcross mice died significantly sooner (Table 7). However, the effect was far more distinct than the few days' difference we had seen (Fig. 1) with intraperitoneal benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, or β-naphthoflavone given daily. The majority of responsive animals died by the 15th day of continuous treatment, whereas one-third to one-half of the nonresponsive mice remained alive at 30 days, at which time the experiment was stopped (Table 7).

Oral polychlorinated biphenyls. Daily feeding of laboratory chow soaked in Arochlor 1254 produced the opposite effect to oral benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, or 3-methylcholanthrene. Table 8 shows that the majority of the responsive inbred and backcross animals died during the first 10 days, yet the majority of the nonresponsive inbred and backcross mice remained alive longer than 20 days.

Oral lindane. Daily administration of lindane-soaked food (Table 9) produced results most similar to those with oral benzo[*a*]pyrene and 3-methylcholanthrene and opposite to the results seen with oral polychlorinated biphenyls. Most nonresponsive mice died within the first 12–15 days of continuous feeding, and most responsive mice were still alive after 15 days, at which time the experiment was terminated.

Other toxicity tests with intraperitoneal or oral compounds. Large daily doses of intraperitoneal lindane (50–200 mg kg^{-1}) caused death during the first 1–5 days of treatment, yet we could not discern any differences between B6 and D2 mice, and therefore (B6D2)D2 animals were not tested for intraperitoneal lindane toxicity. Other test compounds given intraperitoneally in large doses that did not demonstrate differences in survival times between inbred B6 and D2 mice included bromobenzene (500 and 1000 mg kg^{-1}), diphenylhydantoin (100 and 200 mg kg^{-1}), *p,p'*-DDT (250 and 500 mg kg^{-1}), and hexachlorobenzene (500 mg kg^{-1}). When given orally in the same manner as described in Table 1, test compounds which did not show genetic differences in toxicity between B6 and D2 mice included bromobenzene and *p,p'*-DDT. These results are perhaps not surprising, since none of these substances—at levels approaching lethality—appeared to induce cytochrome P_1450 formation or aryl hydrocarbon hydroxylase activity.

Protection against lindane toxicity. Lindane may interact preferentially with cytochrome P_1450, because this insecticide *in vitro* preferentially inhibits the aromatic hydrocarbon-induced hydroxylase activity (26). We therefore questioned whether differences in lindane toxicity could be distinguished between mice having relatively large amounts of cytochrome P_1450 and mice having a relative lack of P_1450. Table 10 shows the results when mice receive a dose of lindane that is normally lethal

[6] As an inducer of aryl hydrocarbon hydroxylase activity in various strains of mice, chlorpromazine was previously found (25) to behave more like phenobarbital than polycyclic hydrocarbons.

TABLE 7

Toxicity of intraperitoneal polychlorinated biphenyls

The experiment was carried out exactly as described in the legend to Fig. 1, except that Arochlor 1254 (500 mg kg^{-1}) in corn oil was given daily.

Strain	*Ah* phenotype	Deaths recorded between				Still alive after 30 days
		0–10 days	11–15 days	16–20 days	21–30 days	
B6	Responsive	4	14	11	1	0
C3H/HeN	Responsive	3	13	10	2	2
D2	Nonresponsive	2	2	3	9	14
AKR/N	Nonresponsive	2	6	4	7	11
(B6D2)D2	Responsive	6	16	8	0	0
(B6D2)D2	Nonresponsive	2	6	7	6	9

TABLE 8

Toxicity of oral polychlorinated biphenyls daily

The experiment was performed exactly as described in the legend to Table 1, except that the laboratory chow was soaked in corn oil containing 10 mg of Arochlor 1254 per milliliter.

Strain	*Ah* phenotype	Deaths recorded between			Still alive after 30 days
		0–10 days	11–20 days	21–30 days	
B6	Responsive	21	9	1	0
C3H/HeN	Responsive	23	7	0	0
BALB/cAnN	Responsive	25	5	0	0
D2	Nonresponsive	1	12	13	4
AKR/N	Nonresponsive	0	3	9	18
(B6D2)D2	Responsive	18	10	2	0
(B6D2)D2	Nonresponsive	0	9	10	11

TABLE 9

Toxicity of oral lindane daily

The experiment was carried out exactly as described in the legend to Table 1, except that the laboratory chow was soaked in corn oil containing 10 mg of lindane per milliliter.

Strain	*Ah* phenotype	Deaths recorded between			Still alive after 15 days
		0–6 days	7–12 days	13–15 days	
B6	Responsive	0	3	3	24
D2	Nonresponsive	3	14	13	0
(B6D2)D2	Responsive	1	1	4	24
(B6D2)D2	Nonresponsive	4	10	13	3

within 12 hr. Previous 3-methylcholanthrene treatment of the responsive B6 and (B6D2)D2 mice protected them against the large dose of this insecticide, whereas almost all control mice or 3-methylcholanthrene-treated nonresponsive D2 or (B6D2)D2 mice died during the first 12 hr.

Hepatic necrosis caused by bromobenzene or zoxazolamine. Hepatic necrosis caused by bromobenzene in rats (27–29) has been shown (30) to be decreased by prior treatment with 3-methylcholanthrene and to be potentiated by previous treatment with phenobarbital, compared with prior treatment with corn oil only. After 3-methylcholanthrene treatment, an *o*-phenolic derivative of bromobenzene is a major metabolite; after phenobarbital treatment, a *p*-phenolic derivative is a major metabolite (30). Table 11 shows that

TABLE 10

Prevention of lindane toxicity by previous treatment with 3-methylcholanthrene

Each mouse received an intraperitoneal dose of 3-methylcholanthrene (80 mg kg^{-1}) or corn oil alone 48 hr prior to the intraperitoneal administration of a large (300 mg kg^{-1}) dose of lindane. The number of deaths was recorded hourly. The backcross animals had been separated previously according to phenotype by differences in zoxazolamine paralysis times (17).

Strain	*Ah* phenotype	Previous treatment	Deaths recorded between				Still alive after 12 hr and after 5 days[a]
			0–3 hr	4–6 hr	7–9 hr	10–12 hr	
B6		Corn oil	2	6	12	9	1
D2		Corn oil	4	10	8	8	0
B6	Responsive	3-Methylcholanthrene	0	1	1	1	27
D2	Nonresponsive	3-Methylcholanthrene	9	8	8	5	0
(B6D2)D2	Responsive	3-Methylcholanthrene	1	2	4	1	22
(B6D2)D2	Nonresponsive	3-Methylcholanthrene	7	7	9	4	3

[a] No animals appeared ill after 5 days, and therefore the experiment was concluded.

such differences in hepatic necrosis found in control and 3-methylcholanthrene-treated rats do not occur in control and 3-methylcholanthrene-treated B6 or D2 mice. There are at least two possible explanations for this difference between rats and mice. Either the *o*-phenolic product is not a major metabolite in 3-methylcholanthrene-treated responsive mice, or, although the *o*-phenolic product is a major metabolite, an increase in its further metabolism (e.g., conjugation of the epoxide with glutathione or conjugation of the phenol with glucuronic acid) results in no decreased hepatic necrosis in 3-methylcholanthrene-treated B6 mice.

Zoxazolamine was introduced into the United States in 1957 as a muscle relaxant and uricosuric agent (31, 32). However, studies implicating this drug as the etiological agent in several instances of fatal hepatic necrosis in man (33) caused removal of zoxazolamine (Flexin) from the American pharmaceutical market in 1963. We have recently shown (17) that zoxazolamine hydroxylation is associated with the *Ah* locus. We therefore tested whether or not zoxazolamine would cause more hepatic necrosis in mice having greater concentrations of cytochrome P_1450. Table 11 shows that no such necrosis could be demonstrated at nearly lethal doses of zoxazolamine.

Protection in fetal liver against transplacental hepatotoxicity caused by bromobenzene. The bottom portion of Table 11 shows experiments with bromobenzene-treated responsive (B6D2)F_1 pregnant mothers and their (F_2) offspring *in utero*. Although bromobenzene produced significant hepatic necrosis in the control and 3-methylcholanthrene-treated pregnant animals, no liver damage could be detected in any of the fetuses. Considerable hematopoietic tissue was present in the fetal livers; however, hepatocytes and hepatic lobules could be clearly recognized and no significant toxicity was seen. The fetal liver therefore appears to be protected against any transplacental toxic effects produced by either bromobenzene alone or with 3-methylcholanthrene.

DISCUSSION

When various chemicals cause toxicity and shortened survival times, there can be numerous explanations for differences between mouse strains, because inbred strains of mice are known to differ at hundreds of genetic loci. The important addition to this study, therefore, was the inclusion of (B6D2)D2 backcross animals, whose phenotype had been determined 1 week or more prior to the toxicity experiments by the zoxazolamine paralysis time (17). Because these backcross mice are, in effect, siblings which can be separated by what appears to be almost exclusively (3–

TABLE 11

Hepatic necrosis caused by bromobenzene or zoxazolamine treatment of B6 or D2 mice

Groups of weanling mice or pregnant (B6D2)F_1 mice received an intraperitoneal dose of 3-methylcholanthrene (80 mg kg^{-1}) or corn oil alone 48 hr prior to the intraperitoneal administration of bromobenzene (500 mg kg^{-1}) or zoxazolamine (200 mg kg^{-1} every 12 hr for four consecutive doses). Previous treatment with phenobarbital is described in MATERIALS AND METHODS; the test compound was then given 24 hr after the last dose of phenobarbital. The livers were removed 48 hr after the bromobenzene or 12 hr after the fourth dose of zoxazolamine. For the pregnant animals, 3-methylcholanthrene or corn oil was given on about day 16 or 17 of gestation, and bromobenzene was given therefore on about day 18 or 19; the livers from the fetuses and pregnant mothers were then examined 48 hr later, on day 20 or 21 of gestation. Paraffin sections were prepared and stained, and histological quantitation of liver necrosis was performed as described in MATERIALS AND METHODS.

Strain and previous treatment	Test compound	No. of animals having extent of necrosis				
		0	1+	2+	3+	4+
B6						
Corn oil	Bromobenzene	3	5	13	8	1
3-Methylcholanthrene	Bromobenzene	4	8	11	3	4
Phenobarbital	Bromobenzene	2	3	8	12	5
D2						
Corn oil	Bromobenzene	4	14	8	3	1
3-Methylcholanthrene	Bromobenzene	8	9	10	3	0
Phenobarbital	Bromobenzene	0	4	8	10	8
B6						
Corn oil	Zoxazolamine	20	0	0	0	0
3-Methylcholanthrene	Zoxazolamine	20	0	0	0	0
Phenobarbital	Zoxazolamine	20	0	0	0	0
D2						
Corn oil	Zoxazolamine	20	0	0	0	0
3-Methylcholanthrene	Zoxazolamine	20	0	0	0	0
Phenobarbital	Zoxazolamine	20	0	0	0	0
Pregnant (B6D2)F_1 mothers						
Corn oil	Bromobenzene	0	0	1	1	0
3-Methylcholanthrene	Bromobenzene	0	1	2	1	0
(B6D2)F_2 fetuses from (B6D2)F_1 mothers						
Corn oil	Bromobenzene	15	0	0	0	0
3-Methylcholanthrene	Bromobenzene	27	0	0	0	0

9) a single-gene difference, we can conclude with confidence that certain responses to toxic environmental agents are associated with this small genetic difference. Hence aromatic hydrocarbon responsiveness was shown to be correlated with earlier death following large daily intraperitoneal doses of benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, β-naphthoflavone, or polychlorinated biphenyls and following small daily oral doses of polychlorinated biphenyls. Aromatic hydrocarbon nonresponsiveness was shown to be correlated with earlier death following small daily oral doses of benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, or lindane; with oral benzo[*a*]pyrene the cause of death appeared to be toxic chemical depression of the bone marrow.

It is of historical interest that carcinogens such as intraperitoneal shale oil to rabbits (34), intraperitoneal benzo[*a*]pyrene, dibenz[*a*,*c*]anthracene, 1:2:5:6-dibenzacridine, and chrysene to rats (35), intraperitoneal dibenz[*a*,*c*]anthracene to rabbits (36, 37), and oral benzo[*a*]pyrene to rats (38) were studied 26–44 years ago.

Weight loss and early death in animals receiving these carcinogens were observed. An increase in liver size combined with a decrease in body weight was reported (37) in rabbits receiving repeated intraperitoneal doses of dibenz[*a*,*c*]anthracene. We similarly found an increased liver weight to body weight ratio shortly before death in nonresponsive mice eating benzo[*a*]pyrene daily (mean ± SD = 0.53 ± 0.0042; $N = 8$), compared with the ratio in nonresponsive mice of the same age eating corn oil-soaked food (mean ± SD = 0.047 ± 0.0063; $N = 12$). This same increase in liver to body weight ratio was also apparent in dying mice receiving intraperitoneal benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, β-naphthoflavone, and especially polychlorinated biphenyls. The toxic and carcinogenic effects of tar were in fact divorced from each other by Polson in 1936 (39).

We cannot understand why responsive mice succumb earlier to polychlorinated biphenyls administered either intraperitoneally or orally, but responsive mice given intraperitoneal polycyclic hydrocarbons die earlier whereas nonresponsive mice on oral polycyclic hydrocarbons die earlier. Hydroxylated derivatives of the polychlorinated biphenyls—presumably formed by the monooxygenases—have been identified (40–43), and effects of polychlorinated biphenyls *in vivo* include gastric mucosal hyperplasia and dysplasia (44), disruption of the respiratory chain (45), and uroporphyrin accumulation (46). Although hexachlorobiphenyl was reported (40) not to be metabolized at all, biphenyls having fewer than 6 chlorine atoms are readily hydroxylated (40–42); recently, however, a metabolite of hexachlorobiphenyl has been described (43).

It is curious that γ-hexachlorocyclohexane (lindane) was found to have genetic differences in its toxic effects, yet hexachlorobenzene did not. A similar difference between these two hexachloro-containing compounds also exists in the preferential inhibition of aryl hydrocarbon hydroxylase activity *in vitro* (26). The greater hydrophobicity and lesser axes of symmetry of lindane, compared with hexachlorobenzene, may both be important factors in its metabolism and/or interaction with cytochrome P_1450. Indeed, it is questionable whether hexachlorobenzene is metabolized at all in the rabbit (47). The finding that a normally lethal dose of intraperitoneal lindane is not lethal in a genetically responsive mouse previously treated with 3-methylcholanthrene (Table 10) also suggests to us that cytochrome P_1450 may metabolize the parent toxic compound to something less detrimental. The importance of bond energy differences (48) and the degree to which a substrate of cytochromes P450 is an uncoupler (49) are two additional factors which may play a role in explaining the toxic effects of halogenated hydrocarbons.

Genetic differences in aryl hydrocarbon hydroxylase induction exist in cultured human lymphocytes (50–52). An association between 3-methylcholanthrene-initiated tumorigenesis (12–15) and the inducible hydroxylase activity exists in mice; a correlation between individual susceptibility to bronchogenic carcinoma and the extent of hydroxylase induction in humans has been suggested (53) but will require corroboration. There is a concern about the degree to which polychlorinated biphenyls (54, 55) and polycyclic hydrocarbons such as benzo[*a*]pyrene (56) are contaminating the biosphere. The concentration of polychlorinated biphenyls in microscope immersion oils, used commonly for light microscopy by pathologists and medical technicians, is extremely high (30–45%) and capable of inducing cytochrome P_1450 in rat liver when minute amounts (1–10 μl) of the substance are applied topically (57). How does the oral dose of about 120 mg of benzo[*a*]pyrene kg^{-1} day^{-1}, which causes bone marrow depression and premature death in nonresponsive mice, compare with present-day doses in our environment? If the estimate of 3.5 ng of benzo[*a*]pyrene per cubic meter of "city air" (56) is used, one can calculate—knowing the tidal volume and respirations per minute—that a 70-kg person in the city will inhale 0.3–0.4 ng kg^{-1} day^{-1}; this value would be considerably greater during a time of temperature

inversion or in habitual smokers. In a smoke-filled hall in Prague, 120 ng of benzo[*a*]pyrene per cubic meter of air were determined (58). A 24-hr exposure to such polluted air would be approximately 10–15 ng kg^{-1} for a 70-kg person. This estimate is still six to seven orders of magnitude below the oral dose shown in this report to cause marked bone marrow depression and premature death. That the survival times and health in mice exposed to various environmental agents are dramatically influenced by a single gene, or small number of genes, should be appreciated in any future studies evaluating the exposure of humans to toxic environmental or industrial chemicals or drugs.

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