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Genetic Differences in Aryl Hydrocarbon Hydroxylase Induction and Benzo[a]pyrene-Produced Tumorigenesis in the Mouse

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SUMMARY

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Aryl hydrocarbon (benzo[a]pyrene) hydroxylase induction in C57BL/6N and DBA/2N inbred mice and their progeny by aromatic hydrocarbons is most likely controlled by a single autosomal dominant trait in skin, peritoneal lining, and lung, as has been previously illustrated for induction of the enzyme in liver, kidney, and bowel; additional factors may influence the expression in skin, peritoneal lining, and lung, however. The susceptibility to tumorigenesis produced by the topical application of benzo[a]pyrene and promotion by phorbol ester or by the intraperitoneal administration of large doses of benzo[a]pyrene is not correlated with the genetically mediated presence or absence of the hydroxylase induction in littermates of back-crosses and intercrosses among the C57BL/6N, DBA/2N, and NZW/BLN strains. Therefore, if this enzyme is necessary for metabolic activation of benzo[a]pyrene to the proximal carcinogen, the basal levels of the hydroxylase are sufficient. Compared with application of 7,12-dimethylbenz[a]anthracene directly to the skin, the intraperitoneal administration of the carcinogen is at least 25 times more effective in causing tumors at the site of promotion. This phenomenon may reflect either circulation of carcinogens metabolically activated by liver enzyme systems or circulation of existing viruses, perhaps subviral particles, activated by the parent polycyclic hydrocarbon molecule or its metabolism.

INTRODUCTION

The induction of aryl hydrocarbon hydroxylase activity in many tissues of the mouse by aromatic hydrocarbons is expressed as a single autosomal dominant trait, which resides at the *Ah* locus[2] (1-9).

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[2] The locus for aryl hydrocarbon hydroxylase induction by aromatic hydrocarbons is *Ah* (for *a*romatic *h*ydrocarbon responsiveness) (7). The allele Ah^b designates the dominant gene, i.e., expression of the hydroxylase induction to the same extent as that found in the dominant parent—in liver, kidney, or bowel of F_1 progeny from the cross between C57BL/6 and DBA/2, NZW/BLN, or NZB/BLN strains (1, 2, 4); the allele Ah^d represents the recessive gene. The C57BL/6 strain (or B6) is arbitrarily designated the prototype strain for the Ah^b allele, and the DBA/2 strain (or D2) is the prototype strain for the Ah^d allele. We now have evidence that changes

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Thus, in any mouse homozygous or heterozygous for the allele Ah^b, there occurs a substantial increase in the mono-oxygeanse activity from such tissues as the liver, kidney, and bowel within 24 hr after a single dose of polycyclic hydrocarbon such as 3-methylcholanthrene, benz[a]-anthracene, certain flavones, and 2-phenylbenzothiazole derivatives (6). Furthermore, in any inbred or hybrid mouse homozygous for the allele Ah^d, there is little or no hydroxylase induction in these tissues following aromatic hydrocarbon administration (1–6).

Factors which might influence the susceptibility of various inbred mouse strains to polycyclic hydrocarbon-produced tumorigenesis include the basal or inducible activities of aryl hydrocarbon hydroxylase, epoxide hydrase, or glutathione transferase (see refs. 3, 10–12 for review), immunological differences, or the presence or absence of latent viral infections (13–16). If one can determine genetic differences for any of these parameters among progeny in the same litter, various nonspecific events peculiar to certain strains of inbred mice can be canceled. For example, we noted differences in the pH optima of the basal hydroxylase activity between Ah^b/Ah^b and Ah^d/Ah^d mice (1). It is possible that there exist other dissimilarities in polycyclic hydrocarbon metabolism between these strains of mice, e.g., the position[3] or amount of epoxide formation (18) or the amount of covalent interaction with cellular macromolecules (19, 20), and that these factors may affect the susceptibility to cancer. In the F_2 progeny of an $Ah^b/Ah^d \times Ah^b/Ah^d$ intercross or in offspring from the $Ah^b/Ah^d \times Ah^d/Ah^d$ back-cross, it is therefore possible to examine the relative presence or absence of inducible hydroxylase activity among littermates. Using the same reasoning and experimental design, we recently concluded (3) that mouse skin tumorigenesis initiated by DMBA[5] and promoted by repeated applications of phorbol ester is unrelated to genetic differences in the extent of hydroxylase induction by aromatic hydrocarbons in inbred or hybrid C57BL/6N and DBA/2N mice. However, DMBA may be activated through mechanisms not mediated by the hydroxylase system, either at methyl groups (21–23) or at other positions (24–26). For this study the nonmethylated carcinogen benzo[a]pyrene was therefore used. Whether or not these genetic differences in hydroxylase induction are important in the mechanism of tumorigenesis caused by topical or intraperitoneal administration of varying doses of BP is the subject of this report. Also included in this paper is the expression of aryl hydrocarbon hydroxylase induction in those tissues wherein most tumors were found.

MATERIALS AND METHODS

Materials. DMBA was purchased from Eastman Organic Chemicals; MC, from J. T. Baker Company; phorbol ester, from Schuchardt-USA; and NADPH, NADH, and BP, from Sigma Chemical Company. Generally labeled [^{3}H]DMBA (5680 mCi/mmole) was obtained from Amersham/Searle. National Institutes of Health Animal Supply provided us with inbred C57BL/6N, DBA/2N, and NZW/BLN strains, from which back-crosses and intercrosses were accomplished in our laboratory.

Treatment of animals. Strict control of the environment, bedding, diet, and circadian rhythmicity was maintained as described previously (1–3). MC was administered 24 hr before the enzyme assay

in the spin state of cytochrome P-450 iron (8) and the induction of other mono-oxygenase activities by aromatic hydrocarbons (9) are regulated by a single autosomal dominant gene at or near the *Ah* locus.

[3] For example, phenobarbital-induced mono-oxygenase activities preferentially produce 3,4-epoxidation of bromobenzene (17) and ω- and ω-1-hydroxylations of *n*-hexane,[4] whereas the MC-induced enzyme systems cause predominantly 2,3-epoxidation of bromobenzene (17) and ω-2-hydroxylation of *n*-hexane.[4]

[4] V. Ullrich, personal communication.

[5] The abbreviations used are: DMBA, 7,12-dimethylbenz[a]anthracene; phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate; BP, benzo[a]pyrene; MC, 3-methylcholanthrene; B6, the C57BL/6 inbred mouse strain; D2, the DBA/2 inbred mouse strain.

was performed either in 3-week-old weanlings or in 4–10-month-old adults; no significant sex differences were found in either group. An intraperitoneal dose of 80 mg of MC in 0.5 ml of corn oil per kilogram of body weight, or a topical dose of 300 μg of MC in 0.20 ml of acetone to the nape of the neck, was given 24 hr prior to death. Single intraperitoneal doses of less than 40 mg of MC per kilogram of body weight were found to be less than maximally effective; doses of 100 or 200 mg of MC per kilogram of body weight slightly diminished, rather than enhanced, whatever effect was seen at 80 mg/kg. Likewise, topical doses of less than 150 μg or greater than 400 μg were no more effective than the 300-μg dose. The nonhepatic tissues—bowel, lung, skin, and peritoneal lining—were isolated in the manner previously described (1, 27). Liver microsomes were routinely prepared as described previously (1, 5).

Topical administration of polycyclic hydrocarbons. To one ear of each weanling mouse, the carcinogenic initiator in 20 μl of acetone was applied once. With appropriate groups of mice for the next 3–5 months, 1 μg of the promoter phorbol ester in 20 μl of acetone was applied thrice weekly to the ear. Control groups received acetone alone instead of DMBA or BP initially, or acetone alone instead of phorbol ester continuously. Other groups received the initiator BP without any promotion. All papillomas appeared within 2–6 weeks and were generally multiple. Between 4 and 6 weeks after no further tumors appeared, the presence or absence of at least one Ah^b allele was assessed by treating the animal with MC for 24 hr and then determining the extent of hydroxylase induction.

Intraperitoneal administration of polycyclic hydrocarbons. In separate experiments, 3 mg of BP were administered intraperitoneally in 0.5 ml of corn oil to weanling mice from the intercross or back-cross. After some of the mice had developed tumors, the incidence and types of tumors were evaluated with a complete autopsy at the time hydroxylase induction was evaluated.

Topical or intraperitoneal administration of [^{3}H]DMBA. Various doses (1 ng–100 μg) of the radioisotope [^{3}H]DMBA were applied to the ear or were given intraperitoneally, and radioactivity of the ear and various other tissues was evaluated at various times subsequently. Radioactivity remaining in these tissues 2 months after [^{3}H]DMBA treatment, with or without promotion by phorbol ester three times weekly, was also measured. We made no attempt to distinguish between the parent compound and its metabolites.

Enzyme assay. Hydroxylase activity and protein concentration were determined in duplicate, exactly as described previously (1, 5). *One unit* of aryl hydrocarbon hydroxylase activity has been defined (5) as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene. The specific activity of the hepatic oxygenase is expressed throughout this paper as units per milligram of microsomal protein, whereas the specific activities of the enzyme from lung, skin, and other nonhepatic tissue are shown as units per milligram of tissue homogenate protein. The hepatic enzyme was assayed at pH 7.25, and the enzyme activity in nonhepatic tissues was measured at pH 7.55; 0.25 M potassium phosphate–30% glycerol buffer was used in each assay. The limit of sensitivity for the assayed enzyme specific activity was about 0.01 unit/mg of protein, and duplicate determinations normally varied less than 10% (5).

RESULTS

Genetic differences in aryl hydrocarbon hydroxylase induction[6] *in hepatic and nonhepatic tissues after 3-methylcholanthrene treatment.* In many tissues of the C57BL/6N

[6] The process of induction denotes a relative increase in the rate of synthesis *de novo* or in the rate of activation of enzyme activity from preexisting moieties, or in the rate of both, compared with the rate of breakdown. Since this enzyme may be a multicomponent, membrane-bound system, there are technical difficulties in attempting to distinguish between enzyme synthesis *de novo* and activation. Thus the rate of enzyme induction is used here only to express the rate at which induced hydroxylase activity accumulates.

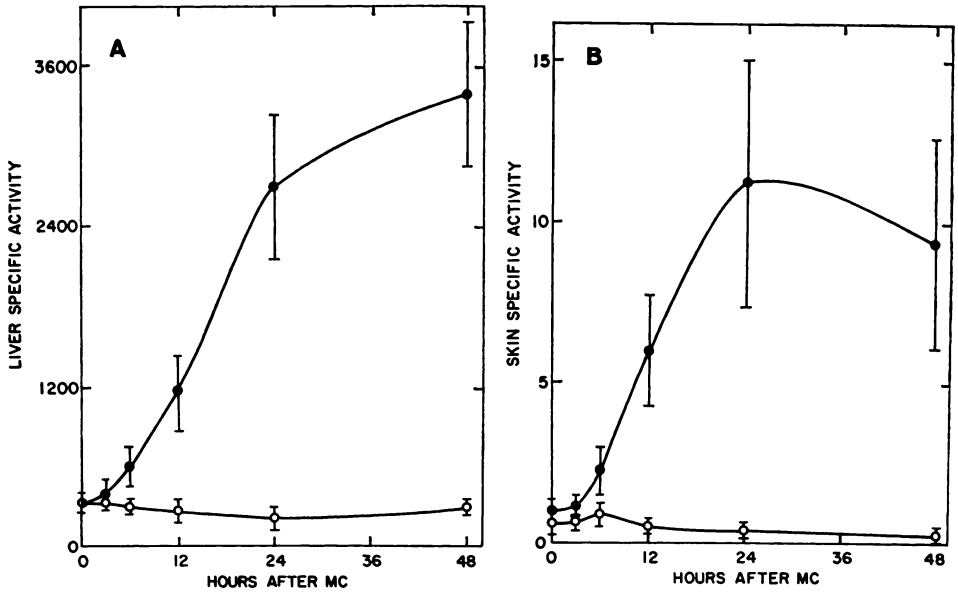

FIG. 1. *Kinetics of aryl hydrocarbon hydroxylase induction by MC in liver (A) and skin (B) from Ah^b/Ah^b, Ah^b/Ah^d, and Ah^d/Ah^d weanling mice*

●, means of Ah^b/Ah^b or Ah^b/Ah^d mice; ○, means of Ah^d/Ah^d mice; brackets depict standard deviations for each mean. At each of the six time points a minimum of six C57BL/6N inbred mice and eight Ah^b/Ah^b or Ah^b/Ah^d offspring from genetic crosses was used in calculating the means shown by ●. A minimum of six DBA/2N or NZW/BLN inbred mice and six Ah^d/Ah^d progeny was used in calculating the means depicted by ○. Each mean ± standard deviation therefore represents 12–23 mice per time point. Each mouse received simultaneously 80 mg of MC per kilogram of body weight intraperitoneally and 300 μg of MC to the nape of the neck, and enzyme activities from liver (1) and skin (3, 27) of each mouse were determined.

inbred mouse, the hydroxylase is inducible by MC, whereas in DBA/2N and NZW/BLN inbred strains the enzyme is relatively nonresponsive to MC (1). The rate at which hydroxylase activity accumulates in liver and skin of MC-treated C57BL/6N, DBA/2N, and NZW/BLN inbred mice and their progeny is shown in Fig. 1. In those inbred mice or offspring having at least one Ah^b allele, the hepatic specific mono-oxygenase activity increased about 3-fold in 12 hr, about 6-fold in 24 hr, and about 8-fold in 48 hr following MC administration. The magnitude of the enzyme induction in skin from these same mice was markedly similar, except for considerably larger standard deviations and some decrease in the value at the 48-hr time point. In the inbred mice or offspring presumably homozygous for the Ah^d allele, both the hepatic and skin hydroxylase specific activities became slightly depressed during the 48-hr period following MC treatment. Similar results were obtained with the use of BP or β-naphthoflavone as the inducer. Similar kinetics, but with a smaller magnitude of hydroxylase induction, was also found with DMBA as the inducer.

Figure 2 shows that, among offspring from the B6D2 F_1 × D2 back-cross, there is a high degree of correlation between the presence of inducible hepatic mono-oxygenase activity and the presence of inducible enzyme in skin,[7] peritoneal lining,

[7] It was recently reported (28) that α-naphthoflavone inhibits DMBA-produced skin tumorigenesis only if the inhibitor is applied less than 12 hr after the application of DMBA to mouse skin. In view of this finding, it must be kept in mind that, if polycyclic hydrocarbon-evoked cancer requires metabolic activation by the aryl hydrocarbon hydroxylase system, changes in the skin-specific enzyme activity during the first 12 hr would be far more important than those occurring

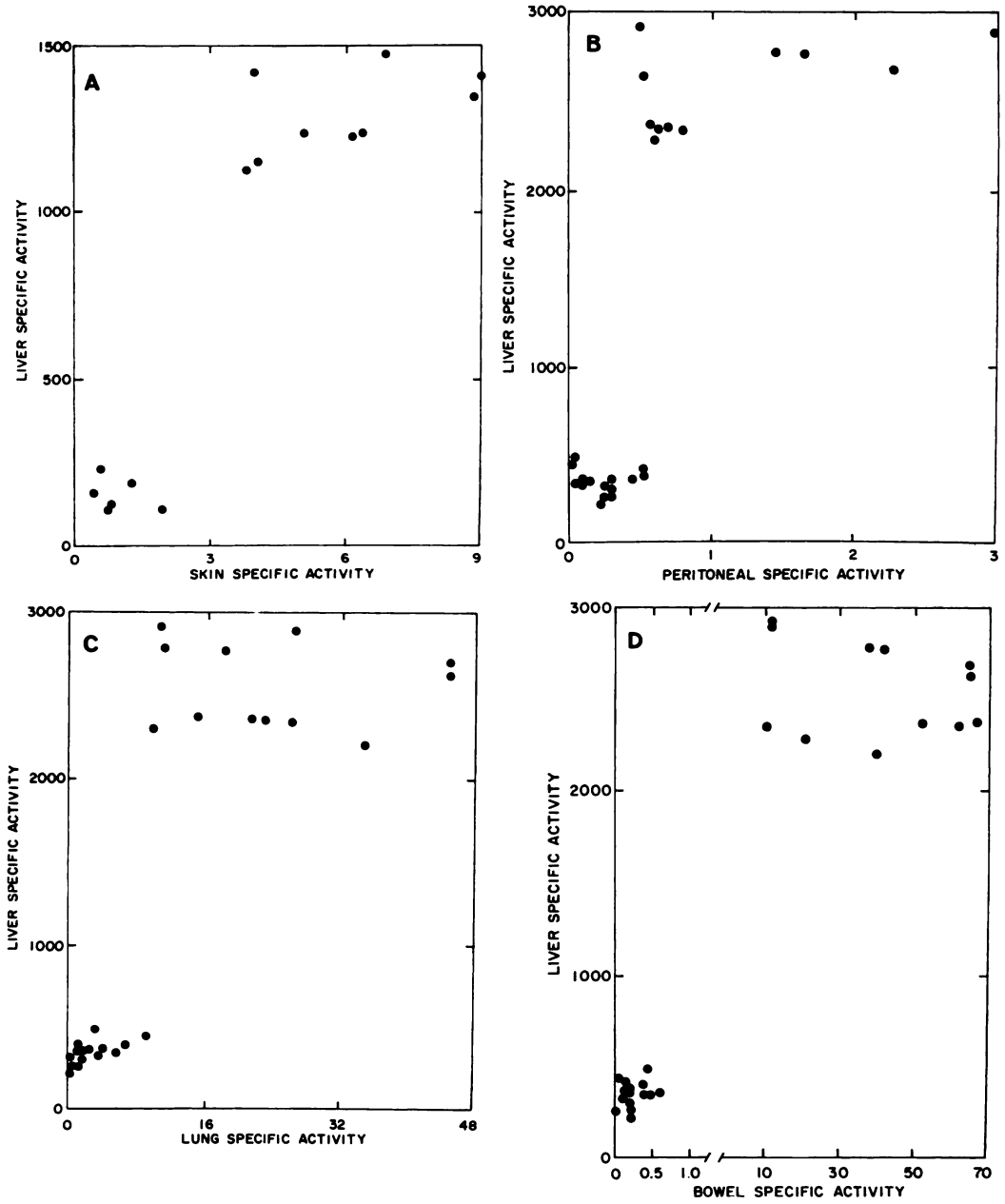

FIG. 2. *Relationship between hepatic specific aryl hydrocarbon hydroxylase activity and enzyme level in skin (A), peritoneal lining (B), lung (C), and bowel (D) from MC-treated Ah^b/Ah^d or Ah^d/Ah^d offspring from B6D2 F_1 × D2 back-cross*

Each point in Fig. 2A depicts a single mouse 12 hr after receiving simultaneously 80 mg of MC intraperitoneally per kilogram of body weight and 300 μg of MC in 0.20 ml of acetone to the nape of the neck. Each point in Fig. 2B, C, and D represents an individual weanling 24 hr after intraperitoneal treatment with MC; the hydroxylase activities from peritoneal lining, lung, and bowel are from the same 27 mice. The correlation coefficients r are 0.89 (A), 0.71 (B), 0.78 (C), and 0.78 (D) ($p < 0.001$ for each of the four relationships). In each tissue the specific hydroxylase activity of control animals was not significantly ($p > 0.05$) different from that found in MC-treated Ah^d/Ah^d mice.

lung, and bowel. As mentioned previousyl (1, 6), the differences in specific hydroxylsae activities between MC-treated Ah^b/Ah^d and Ah^d/Ah^d mice were considerably greater (from 15- to more than 80-fold) in bowel than in skin (2–10-fold).[8] On the other hand, the highest lung hydroxylase activities in some mice possessing the non-inducible hepatic oxygenase overlapped with the lowest lung hydroxylase activities in a few mice having the inducible liver enzyme. This phenomenon was even more evident with the peritoneal enzyme activities (Fig. 2B) and is further illustrated in Fig. 3. It is possible that the magnitude of enzyme induction for each individual animal is difficult to evaluate. For example, with the particular MC-treated mouse having a peritoneal specific hydroxylase activity of 0.50 and a hepatic enzyme activity of 2400, perhaps the specific activity of its peritoneal basal enzyme was 0.05; with the particular MC-treated mouse having a peritoneal enzyme activity of 0.50 and a hepatic enzyme activity of 500, perhaps the specific activity of its peritoneal control enzyme was 0.50. This possibility could best be tested by comparing a control enzyme activity with the enzyme activity from the same tissue after MC treatment of the same mouse. However, such an experiment would require biopsies of lung, peritoneal lining, and skin from a large number of mice.

Thus Figs. 2 and 3 show that the hydroxylase induction in lung and peritoneal lining by MC probably segregates as a single autosomal dominant trait among progeny of the B6 and D2 inbred strains, as is the case for the enzyme expression in liver, bowel, kidney, and skin (1–6). However, other factors apparently influence the extent of hydroxylase induction in lung, peritoneal lining, and perhaps even skin; these factors may include hormonal levels, stress, nutrition, environmental exposure to pharmacologically or toxicologically active substances (29), or differences in the subcellular mechanisms of transcription or translation (5).

As had been found for the enzyme in the peritoneal lining, low, and sometimes overlapping, results were found for the enzyme activity in lymph nodes from MC-treated mice from the B6D2 × D2 back-cross. We tested for the presence or absence of inducible hydroxylase activity in such tissues as peritoneal lining and lymph nodes, because these tissues were exclusively the sites for malignancy following intraperitoneal administration of BP.

Benzo[a]pyrene-produced tumorigenesis among C57BL/6N, DBA/2N, and NZW/BLN inbred mice and their progeny. We examined different doses of the polycyclic hydrocarbon initiator, with and without promotion by phorbol ester (30) (Fig. 4). The reason for the assay, which necessitated killing the animal, was to determine whether or not a particular mouse had at least one Ah^b allele; we are unable to distinguish between the Ah^b/ah^b and Ah^b/Ah^d genotypes. Even in mice with fairly large abdominal wall tumors, it was quite easy to determine the presence or absence of hydroxylase induction in liver[9] and kidney by MC. However, the presence or absence of hydroxylase induction was not evaluated in any mouse which obviously appeared debilitated.

Table 1 summarizes all our tumorigenesis

after 12 hr. It was because of this finding that the changes in liver and skin hydroxylase after 12 hr of MC treatment are shown in Fig. 2A.

[8] The terms "2-fold" or "20-fold" to denote magnitude of induction should be used with reservation, if one is dealing with amounts of enzyme that are barely detectable. The limit of sensitivity for our reported (5) hydroxylase assay is about 0.01 unit of enzyme per milligram of protein. If one finds a specific enzyme activity of 0.40 in an MC-treated animal on one day, for example, the magnitude of induction may be "2-fold" or "20-fold," depending on whether the control specific activity for that assay is 0.20 or 0.02.

[9] By paramagnetic determination, the hepatic microsomal cytochrome P-450 content (31) in rats carrying subcutaneously inoculated minimal deviation hepatomas for 8 weeks was about the same as that found in control rats carrying no tumors (32). This finding supports our current data that the hydroxylase activity, a P-450-mediated enzyme system (1, 5, 6, 8, 9, 29), can be nearly normal in livers of animals bearing large tumors.

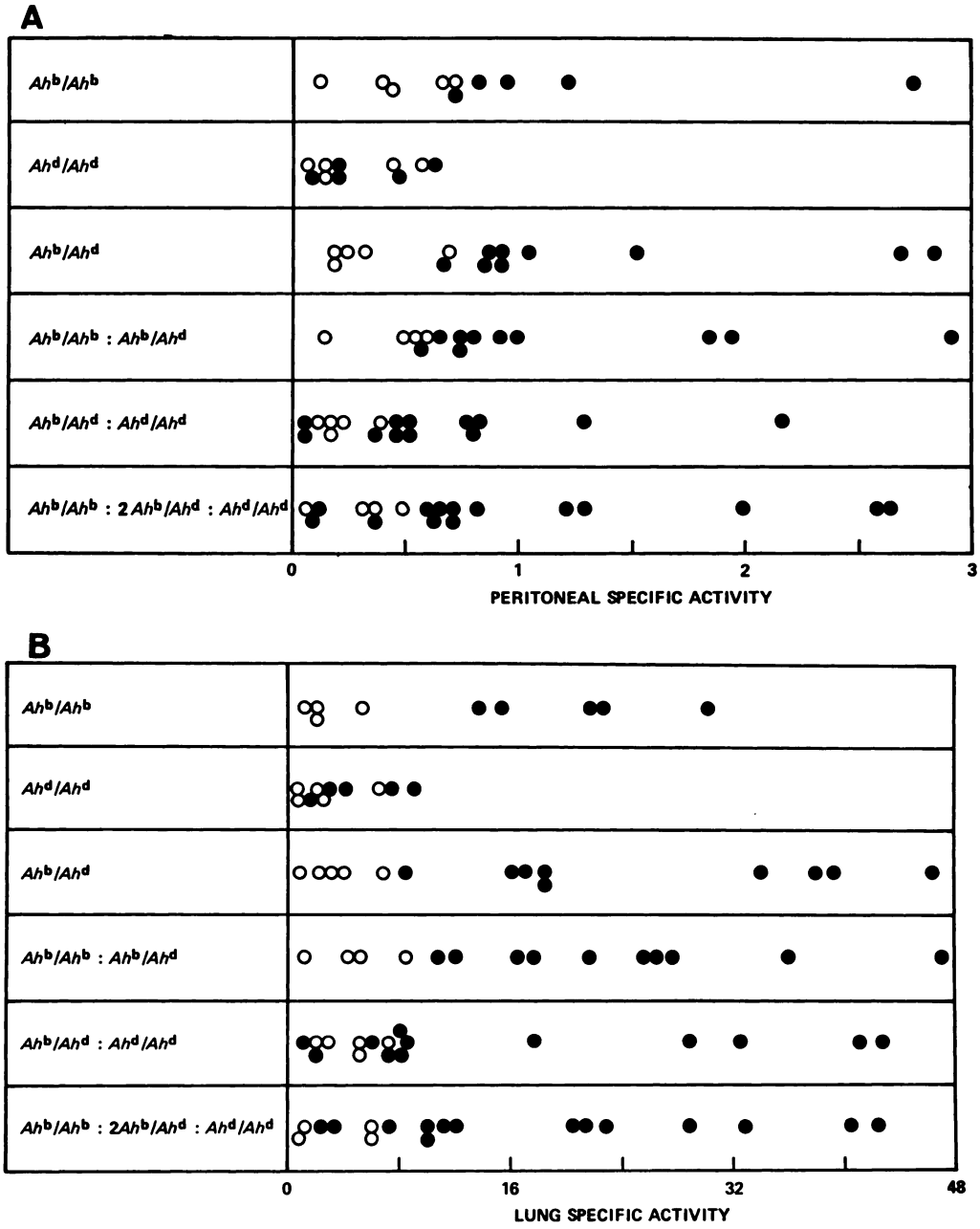

FIG. 3. *Genetic variance of specific aryl hydrocarbon hydroxylase activity in peritoneal lining (A) and lung (B) from control or MC-treated inbred C57BL/6N or DBA/2N mice and their progeny*

The genetic nomenclature is described in footnote 2. ●, a single mouse receiving MC intraperitoneally 24 hr before death; ○, an individual mouse receiving corn oil only (2).

studies. The results of the topical application of 20 μg of DMBA and promotion for about 4 months have been reported previously (3) and are included here only for the sake of comparison. Thus the topical application of BP with promotion produced considerably fewer tumors than did DMBA under similar circumstances. The highest

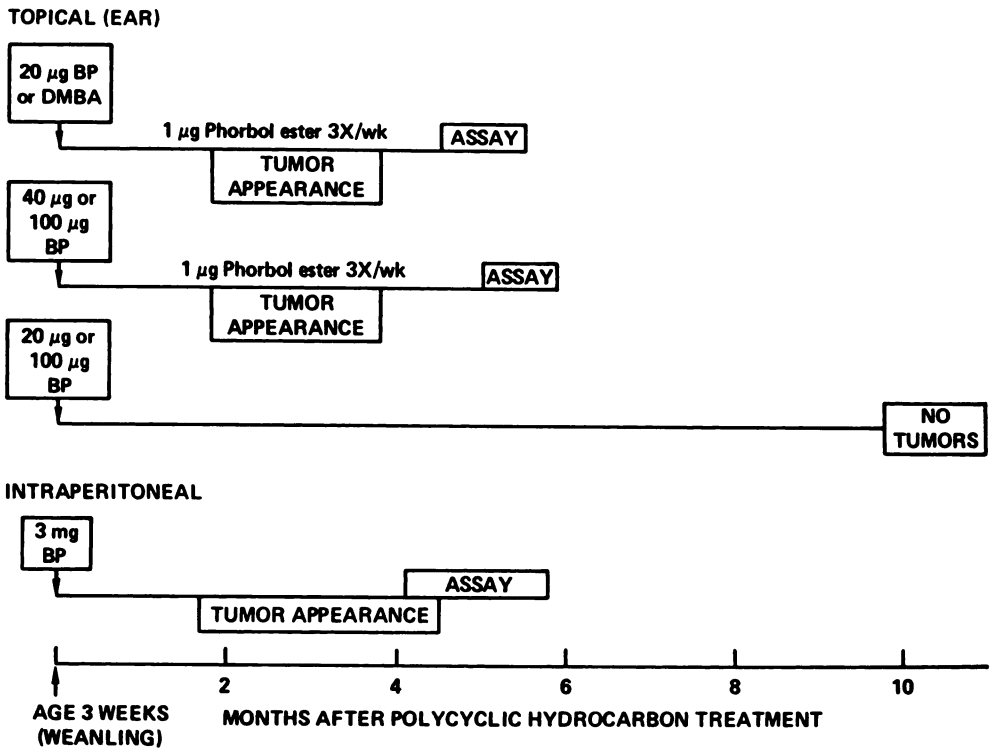

FIG. 4. *Chronological scheme illustrating various ways in which BP or DMBA was administered, with or without phorbol ester promotion, to Ah^b/Ah^b, Ah^b/Ah^d, and Ah^d/Ah^d mice*

When tumors were obvious or when no more tumors were appearing during repeated applications of phorbol ester, inducible aryl hydrocarbon hydroxylase was assessed in order to determine the relationship, if any, between tumor susceptibility and genetic differences in the inducible enzyme activity. The presence or absence of hydroxylase induction in liver microsomes and in kidney was determined 24 hr after the intraperitoneal administration of 80 mg of MC per kilogram of body weight (1, 3, 5).

topical dose of BP (100 μg) actually appeared less tumorigenic than did the lower doses (20 μg and 40 μg). BP at 20 or 100 μg without phorbol ester promotion did not cause any tumors in 10 months.

The most important observation in Table 1 is that either topical application of BP plus the phorbol ester promotion or intraperitoneal administration of BP without promotion produced as many tumors in offspring having the noninducible hydroxylase as in offspring having the inducible enzyme; this finding is similar to observations made with the topical application of DMBA plus promotion (3). The occurrence of more tumors in Ah^d/Ah^d progeny treated with 3 mg of BP intraperitoneally was significantly ($0.02 < p < 0.05$) greater than in mice possessing the inducible hydroxylase.

Intraperitoneal administration of 7,12-dimethylbenz[a]anthracene and topical promotion with phorbol ester. We next wished to determine whether an intraperitoneally administered dose of DMBA, concomitant with phorbol ester promotion of the ear, would produce effects different from a topically administered dose. Table 2 shows that there is a difference. Various amounts of [^{3}H]DMBA in corn oil were injected intraperitoneally, with great care exercised to prevent any leakage of radioactivity through the abdominal wall. When 100 μg of [^{3}H]DMBA had been administered, we found maximal levels of 4 ng of DMBA (or metabolites) per ear, 160 mg/kidney, and 3.2 μg/liver 6 hr after injection; the radioactivity before or after the 6-hr time point was no higher in these tissues. This observation is in accord with a previous

TABLE 1

Effects of topically or intraperitoneally administered BP or DMBA on tumorigenesis in Ah^b/Ah^b, Ah^b/Ah^d, or Ah^d/Ah^d mice

As described in MATERIALS AND METHODS, topical administration always refers to application of the initiator polycyclic hydrocarbon to one ear, and promotion always indicates application of phorbol ester (1 μg in 20 μl of acetone) three times weekly to the ear.

Route and dose of initiator administered	Promotion	Length of time between initiation and evaluation of hydroxylase induction	Tumors per total mice in group			
			Inbred		Progeny from crosses	
			Ah^bAh^b	Ah^dAh^d	Ah^bAh^b or Ah^bAh^d	Ah^dAh^d
		months				
Topical						
20 μg DMBA	Yes	4	0/13	19/21	20/34	16/25[a]
20 μg BP	Yes	4			3/21	3/6
40 μg BP	Yes	5	0/20	2/32[b]	7/29	4/22
100 μg BP	Yes	5	0/20	0/1[c]	2/12	1/10
20 μg BP	No	10	0/20	0/21	0/6	0/4
100 μg BP	No	10	0/10	0/10	0/19	0/16
Intraperitoneal 3 mg BP	No	4–6			3/36[d]	5/19[d]

[a] Data included from previous preliminary report (3).

[b] These values include two tumors in 12 DBA/2N mice and none in 20 NZW/BLN mice.

[c] Nineteen of 20 animals in this group died suddenly for unknown reasons; no tumors were present at the time of death (5 weeks after initiation).

[d] Of the three tumors among the 36 hybrids having the inducible enzyme, there were two pleomorphic fibrosarcomas of the abdominal wall and one lymphosarcoma of the mediastinum. Of the five tumors among the 19 Ah^d/Ah^d progeny, there were two lymphosarcomas of the mediastinum, two reticulum cell sarcomas (one of spleen and one in the thorax), and one pleomorphic fibrosarcoma of the abdominal wall.

TABLE 2

Tumor incidence among DBA/2N mice treated with DMBA intraperitoneally or topically and promoted with phorbol ester on the ear

After various doses of DMBA had been given either intraperitoneally or topically to the ear, the phorbol ester was applied to the ear three times weekly. Four months later the incidence of ear papillomas per total number of mice in each group was evaluated.

Amount of DMBA administered	Method of DMBA administration	Total no. of mice with tumors per total mice in each group
0 μg	Intraperitoneal	0/10
20 μg	Intraperitoneal	0/10
100 μg	Intraperitoneal	9/11
100 ng	Topical	0/10
10 ng	Topical	0/7
1 ng	Topical	0/6

study (33), in which tissue concentrations of [^{3}H]DMBA were found to be maximal 4–6 hr after the intraperitoneal treatment. When DBA/2N mice were given intraperitoneal doses of 100 μg of [^{3}H]DMBA and phorbol ester then was applied three times weekly to one ear of some mice and not to other mice, we found no significant differences in the quantity of radioactivity in the ears of the mice in the two groups during the next 2 months.

Table 2 shows that 9 out of 11 mice which had received 100 μg of DMBA intraperitoneally and then phorbol ester to the ear developed ear papillomas within 4 months. No tumors were found in mice receiving an intraperitoneal dose of 20 μg of DMBA plus the promoter topically. Also, when 100 ng of DMBA were given to the ear and phorbol ester was then applied to the ear three times a week for 4 months,

no tumors were observed. Therefore, intraperitoneal treatment with 100 μg of DMBA—a dose which causes no more than about 4 ng of DMBA (or metabolites) to reach the ear 6 hr after injection—and promotion with phorbol ester on the ear—a procedure which does not influence the accumulation of DMBA (or metabolites) in the ear—produced ear tumors. On the other hand, 25 times more DMBA applied directly to the ear, coupled with phorbol ester promotion, did not produce the ear papillomas.

DISCUSSION

We have shown here that aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons such as MC is probably controlled by a single autosomal dominant trait in skin, peritoneal lining, and lung, as had been previously illustrated in liver, kidney, and bowel (2, 6). However, in the lung and especially in the peritoneal lining and lymph nodes, other factors apparently influence the expression of hydroxylase induction.

We have also shown in this report that, as had been found (3) for DMBA initiation and phorbol ester promotion, the topical application of BP, combined with phorbol ester promotion, produced tumors in the progeny of C57BL/6N, DBA/2N, and NZW/BLN mice that were not related to the genetic differences in hydroxylase induction among these littermates. With this study we have not ruled out the possibility that the nonmetabolized BP is the proximal carcinogen. Or, if metabolic activation of BP by the hydroxylase is necessary, then the basal activities of this enzyme in skin or lymph nodes or peritoneal lining are sufficient to initiate tumorigenesis. In tissues with highly inducible hydroxylase activity, the possible rapid inactivation of BP epoxides (34–36) by epoxide hydrases (12, 37, 38) or by conjugation reactions (3, 10–12) may be important in canceling the effects of the genetically mediated increases in aryl hydrocarbon hydroxylase activity. It is of further interest that sarcomas produced by subcutaneously administered MC among 14 strains of inbred mice have recently been shown (39) to be highly correlated with the genetically regulated presence of inducible hepatic aryl hydrocarbon hydroxylase activity. Kouri and co-workers (39) also found a lack of correlation between DMBA- or BP-evoked sarcomas and the inducible hydroxylase activity among these same inbred strains. These data, together with our previous note (3) and the findings shown in this report, indicate that important differences exist in the specific metabolic activation of each compound—DMBA, BP, and MC—to the proximal carcinogen. These differences might be reflected in the epoxidation reaction[3] of the aryl hydrocarbon hydroxylase system, the formation of dihydrodiols by epoxide hydrases, the rate of conversion of epoxides to glutathione conjugates, or the amount of specific or nonspecific covalent binding of polycyclic hydrocarbons to nucleic acids and proteins. Such differences may explain the seemingly contradictory results from various laboratories (40–49) as to whether activation or detoxification of the parent polycyclic hydrocarbon molecule is important in the etiology of chemically initiated carcinogenesis.

An important finding is the type of tumors produced by the intraperitoneal dose of BP (Table 1). The origin of all the tumors was either connective or reticuloendothelial tissue. These tissues contain very low basal or inducible hydroxylase activities, compared with those in liver especially, but also compared with those in bowel, lung, or kidney. The connective tissue neoplasms were all fibrosarcomas of the abdominal wall, at or near the site of injection, where the largest concentration of parent compound probably remained in nonmetabolized form for the longest period of time. The reticuloendothelial tissues most likely take up foreign material such as polycyclic hydrocarbons, so that these tissues also probably contain large amounts of BP in nonmetabolized form for long periods of time. The possible activation of existing viruses (13–16) by BP and the general knowledge that the lymphatic system acts as a filter for viral particles may be of importance in the etiology of

these tumors. The absence of enzymes which metabolize the epoxides (i.e., epoxide hydrases and glutathione transferases) from skin and lymph nodes is a further possibility to be considered. For example, if a tissue has little or none of these enzymes but has substantially greater amounts of aryl hydrocarbon hydroxylase, perhaps the cells are more subject to attack by the reactive epoxide intermediates.

From Table 2 we conclude that intraperitoneally administered DMBA produced ear tumors, whereas 25 times more DMBA applied directly to the ear did not (when both groups received phorbol ester promotion). Intraperitoneal treatment with DMBA may cause the systemic circulation of an activated proximal carcinogen which effectively damages DNA in the ear and elsewhere. Continual promotion with phorbol ester to that ear may prevent DNA repair (50), thereby leading to tumor formation. This hypothesis would require that the 4 ng of radioactivity found in the ear of mice receiving 100 μg of the parent compound intraperitoneally differ greatly from the 100 ng of radioactivity applied directly to the ear. For example, the 4 ng of radioactivity may represent at least 25 times more hepatic aryl hydrocarbon hydroxylase-generated metabolites of DMBA than the 100 ng of radioactivity applied directly to the ear. This is entirely possible, if one considers that the liver hydroxylase specific activities are at least 500 times greater than skin hydroxylase activities, that the quantity of liver tissue is greater than the quantity of skin tissue from the whole animal, and that reactive intermediates formed in the liver might easily circulate with the blood from the liver to the ear. This hypothesis might be testable. For example, the carcinogenic and toxic effects of polycyclic hydrocarbons are blocked by antioxidants and ethoxyquin (51), and the carcinogenicity of *N*-nitroso compounds is quenched by ascorbic acid (52); these findings are consistent with the idea that unsaturated compounds, or so-called free radical scavengers, are capable of blocking tumorigenesis. Perhaps the administration of such free radical scavengers could prevent phorbol ester-promoted tumorigenesis in the ear after an intraperitoneal dose of DMBA, by preventing sufficient quantities of circulating proximal carcinogens from reaching the ear.

In control DBA/2N mice or in DBA/2N mice treated with either DMBA plus phorbol ester or phorbol ester alone, infectious virus was found[10] in tissue from the ear with the use of the XC test (53). However, existing viruses or subviral particles (13–16) may be activated in some manner by large quantities of DMBA metabolites generated via the hepatic enzyme systems. Such a virus could then initiate tumorigenesis in any area where DNA repair was prevented (50), such as the ear receiving phorbol ester promotion. This possibility might be subject to testing, if interferon (54) or a viral vaccine (16) would prevent the hypothetical circulating viral particle from causing the primary event in carcinogenic initiation.

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