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THE EFFECT OF DIETARY PHENOBARBITAL ON THE INDUCTION OF SKIN TUMORS IN HAIRLESS MICE WITH 7,12-DIMETHYLBENZ[A]ANTHRACENE

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Since phenobarbital is known to induce drug-metabolizing enzymes and to alter the effectiveness of certain carcinogens, its influence on 7,12-dimethylbenz[a]anthracene (DMBA)-induced skin carcinogenesis has been examined. Skin tumor development was studied in groups of hairless mice treated with repeated applications of DMBA and maintained on either a control diet or one supplemented with 0.05% phenobarbital. The feeding of phenobarbital concurrently with the application of DMBA delayed the appearance of papillomas initially, but the suppression of the ultimate tumor yield was variable, and appeared to be dependent on the dose of the carcinogen and the age-dependent toxicity response of the mice. Phenobarbital was ineffective when DMBA was applied in sufficiently large amounts to elicit marked cutaneous damage, or when dietary phenobarbital was started 1 week after the cessation of the DMBA treatment. Although the dietary administration of phenobarbital caused an apparent decrease in the final incidence of papillomas and sarcomas, the feeding did not appear to modify the macroscopic skin response or change the incidence of carcinomas.

Studies have shown that a variety of tissues have inducible microsomal enzyme systems which are implicated in the metabolism of polycyclic hydrocarbons. Although there are apparent species and tissue differences in the expression of these systems, many of the microsomal enzyme inducers, benzo[a]pyrene and flavones, for example, have a generalized effect, inducing microsomal aryl hydrocarbon hydroxylase (AHH) and altering the tumor response in various tissues, including liver, lung, and skin [1–3].

Previous studies have shown that phenobarbital, a known inducer of hepatic microsomal drug-metabolizing enzymes, reduced hepatic tumorigenesis induced by 2-acetylaminofluorene (AAF) when the two agents were fed simultaneously to rats [4]. Pretreatment with injections of phenobarbital also reduced the number of lung tumors induced by urethan in mice [5]. On the other hand, the incidence of AAF-induced liver tumors in rats was significantly increased when dietary phenobarbital was initiated after the cessation of the AAF feeding, and phenobarbital was fed for 100 days or longer [6]. Similarly, dietary phenobarbital enhanced the appearance of spontaneous liver tumors in mice [7]. It is not known whether this enhancing effect is specific to the liver.

The present experiments were designed to determine whether dietary phenobarbital given prior or concurrent to applications of DMBA would reduce the oncogenic effects in the skin and, secondly, whether protracted administration of phenobarbital after the DMBA treatment would enhance the incidence of tumors.

MATERIALS AND METHODS

Female, hairless HRS/J/Anl mice were used in all of the experiments. This inbred strain of mice was caesarean derived and has been maintained at Argonne National Laboratory since 1966. The animals were housed, 10 per cage, at 25°C in fluorescent-lighted rooms (12 hr light; 12 hr dark), and were fed a standard laboratory diet prior to the experiment. While the incidence of lymphoid leukemia is high, 25 to 40% in untreated animals, epidermal tumors are infrequent, with only 2 animals within a control group of 76 mice developing skin lesions, a papilloma and a fibrosarcoma, during a normal lifespan.

The experimental regimens are shown in Figure 1. Thirty mice were assigned to each experimental group on a random but similar age distribution basis. In the initial series of experiments, the animals were 24 to 26 weeks of age at the time of the first of either 6 (Group Ia, Ib) or 12 (Group Ic, Id) weekly applications of 250 μg DMBA. In a second series, the mice were 10 to 12 weeks of age at the time of the first of 12 weekly applications of either 250 μg (Group IIa, IIb) or 100 μg DMBA (Group IIc, IId). At different times with respect to the initial application of DMBA, each group of mice was placed on either a 30% casein pelleted control diet (General Biochemicals, Inc., Chagrin Falls, Ohio) nutritionally adequate in the other dietary components, or one that was additionally supplemented with 0.05% phenobarbital. The mice were maintained on these diets ad libitum throughout the experiment.

7,12-Dimethylbenz[a]anthracene (DMBA, Eastman Organic Chemicals, Rochester, New York) was dissolved

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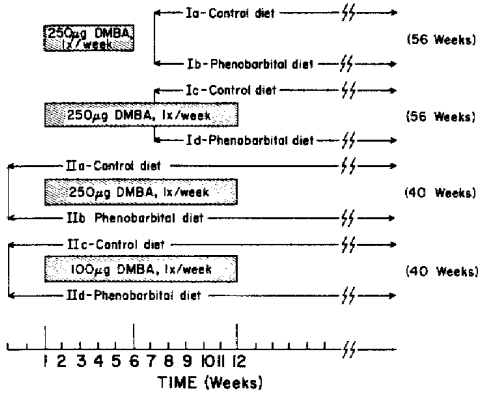

FIG. 1. Experimental procedure.

in acetone, and 0.1 ml of the solution was applied topically by means of a micropipette and distributed over approximately a 10 cm^2 area on the backs of the mice with a No. 3 camel's hair brush. The solutions were made monthly using reagent-grade DMBA and solvent and, during the period of use, the solutions were refrigerated in dark bottles. For all experiments, mice were painted during the same period of the day, with the same stock solutions of the carcinogen.

During the first 20 weeks of the experiment all of the animals were inspected weekly for the appearance of skin tumors. Thereafter, papillomas and other tumors were recorded on a semimonthly basis for a total of either 56 weeks (Group I), or 40 weeks (Group II) from the time of the initial DMBA treatment. Pedunculated, firm masses which projected about 1 to 2 mm were considered papillomas and recorded when they were about 1 mm in size, occurred within the treated area, and persisted for 2 successive weeks. On microscopic examination these masses showed thickened and folded layers of epithelium covering the characteristic elongated papillae which formed the stalk. All mice were necropsied, and tumors as well as about 10% of the papillomas with typical appearance, and all of those which differed macroscopically from the characteristic lesion, were examined histologically. Chi squares were calculated by use of the null hypothesis that the incremental appearance and the frequency distribution of tumors between dietary groups would be the same, with the probability of fit at the 5% level. In addition, differences in the cumulative incidence of tumors between groups were determined by the Student's *t*-test, with log transformation* to approximate a normal distribution.

RESULTS

The percentage of tumor-bearing animals in each treatment group, with respect to the time of the initial application of DMBA, is shown in Figure 2. There were no differences in the survival rates of animals within each of the groups, and the Poisson distribution provided a satisfactory description of the frequency distribution of tumors among animals within groups throughout the period of observation. The cumulative number of epidermal tumors observed in each dietary group, with respect to the time of the initial DMBA treatment, is shown in Figure 3. These values reflect the net number of tumors observed during each of the observation periods.

The phenobarbital-supplemented diet appeared to delay the appearance of papillomas initially, when the diet was started 1 week after a series of 6 weekly applications of DMBA (Group Ib), but not at a statistically significant level when compared

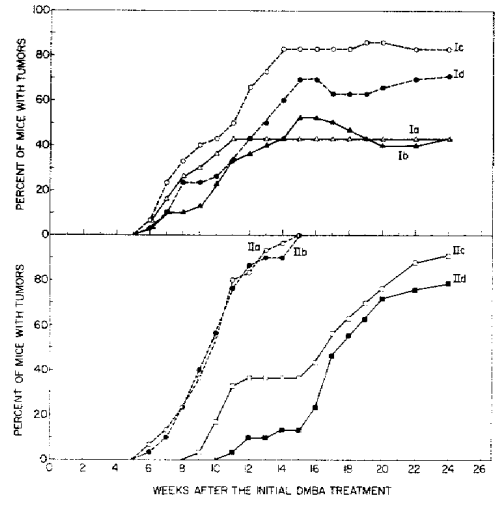

FIG. 2. Effects of dietary phenobarbital on the incidence of DMBA-induced skin tumors. Each group consisted of 30 mice which were treated with either 6 weekly applications of 250 μg DMBA (Δ——Δ), or 12 weekly applications of 250 μg (○- - -○) or 100 μg (□——□) DMBA. Animals in experimental groups Ia, b, c, and d were placed on either a 30% casein diet (*open symbols*), or one that was supplemented with 0.05% phenobarbital (*closed symbols*), from the 7th week after the initial DMBA treatment. Mice in Group II were fed the respective diets beginning 2 weeks before the onset of the DMBA treatment.

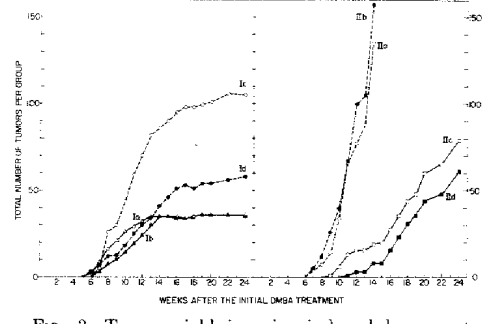

FIG. 3. Tumor yield in mice induced by repeated applications of DMBA and fed a control or phenobarbital-supplemented diet. The conditions of the experiments and the respective symbols are identical to those described in Figure 1. The data for the skin tumors include papillomas, carcinomas, and sarcomas, with papillomas the predominant lesion.

*The values were all increased by an increment of 1 to adjust for zero values.

to the frequency distribution of tumors in animals maintained on the control diet (Group Ia). The regression of papillomatous lesions, and the rate of loss were comparable in the two dietary groups. The final cumulative incidence was 36.4% (Group Ia, 20/55) and 37.7% (Group Ib 20/53). On the other hand, the dietary administration of phenobarbital concurrently with the 7th application of DMBA, in a series of 12 weekly applications, significantly reduced the incremental appearance of skin tumors during the first week of combined treatment (Tab. I). Thereafter, the frequency of tumors in the phenobarbital group (Id) was reduced compared to the incidence in the control group (Ic) during the next 5 successive weekly intervals, i.e., from the 8th week through the 13th week after the initial DMBA treatment, and on an equal probability basis $p = (1/2)^{-5}$. Subsequently, the incremental appearance of papillomas in the two dietary groups was comparable. Statistical analysis of the cumulative incidence of epidermal tumors revealed that the yield of tumors was significantly decreased in mice maintained on the phenobarbital-supplemented diet from the 10th week after the initial DMBA treatment (Tab. II) throughout the duration of the experiment. Likewise the final number of papillomas and sarcomas was significantly lower in the group of animals maintained on phenobarbital, whereas the frequency of carcinomas was comparable in the two dietary groups (Tab. III). Regressions of outgrowths, which were presumed to be papillomas, occurred throughout the period of observation and the frequencies of these lesions were comparable in both dietary groups, namely, 18% (Group Ic, 25/140) and 21% (Group Id, 19/89). These values represent the gross number of papillomas, carcinomas, and sarcomas observed in the treated areas of the epidermis throughout a 40-week interval after the initial DMBA treatment.

The younger, 10- to 12-week-old animals, were markedly more sensitive to the carcinogenic treatment, and 12 weekly applications of 250 μg DMBA caused inflammation and ulceration in all animals irrespective of the diet. Under these conditions phenobarbital had no significant effect on the appearance or development of skin tumors. At the 15th week subsequent to the initial DMBA treatment all of the animals bore multiple papillomas and other epidermal tumors. A major portion of

TABLE I. *Frequency distribution of the increment in the number of skin tumors observed in mice 1 week after the initiation of either a control diet or a phenobarbital-supplemented diet, when the diets were started concurrently with the 7th, in a series of 12 weekly applications of 250 μg DMBA*

		Increment in the number of tumors			
		0	1	2	3
Group Id (Phenobarbital diet)	N = 30 M = 0.40	25	5	—	—
Group Ic (Control diet)	N = 30 M = 0.87	20	5	5	—

N = number of mice.
M = mean number of tumors per mouse.

TABLE II. *Cumulative frequency of distribution of skin tumors in mice maintained on a phenobarbital-supplemented diet as compared to a control diet, 10 weeks after the initial application of DMBA, and a treatment described in Table I*

Although the Poisson distribution provided a satisfactory description of the frequency distribution of tumors, at some weekly intervals deviations at the 0.05 critical limit were noted, for example, at the 10th, 12th, 14th, and 17–20th week in the case of Group Ic, and with respect to Group Id the 10th, 19th, and 20th week. These exceptions were followed by intervals in which the observed frequencies conformed to the Poisson distribution, and have been attributed to a limited sample size.

		Number of tumors						
		0	1	2	3	4	5	≥6
Group Id (Phenobarbital diet)	N = 30 M = 0.60	22	4	1	1	1	1	—
Group Ic (Control diet)	N = 30 M = 1.47	16	3	4	2	2	1	2

N = number of mice.
M = mean number of tumors per mouse.

TABLE III. *The final distribution of papillomas and sarcomas in animals maintained on a control diet (Ic) and a phenobarbital supplemented diet (Id), with treatment as described*

		Number of tumors						
		0	1	2	3	4	5	6
Group Id (Phenobarbital diet)								
Papillomas	N = 28 M = 1.86	4	11	5	5	1	1	1
Sarcomas	N = 28 M = 0.18	23	5	—	—	—	—	—
Group Ic (Control diet)								
Papillomas	N = 27 M = 3.30	2	5	8	4	2	1	5
Sarcomas	N = 27 M = 0.41	17	9	1	—	—	—	—

N = number of mice.
M = mean number of tumors per mouse.

the animals within each group was debilitated with severe ulceration and large tumors, and the experiments were terminated. In the younger animals treated with 12 weekly applications of 100 μg of DMBA, there was a significant decrease in the incidence and frequency distribution of papillomas in the phenobarbital dietary group (IId) compared to that observed in the control group (IIc). These differences were statistically significant during the first 15 weeks after the initial DMBA treatment, with the cumulative tumor yield for the 15th week shown in Table IV. Subsequently, the weekly incremental appearance, and the cumulative number of papillomas, carcinomas, and sarcomas were comparable in both dietary groups. A similar number of outgrowths regressed in the two dietary groups with the total incidence 6.3% (Group IIc, 8/127) and 7.7% (Group IId, 10/130) of the gross number of lesions observed throughout the 40-week interval.

Apart from the differences in the appearance of papillomas, and irrespective of the sequence of the treatment, phenobarbital did not appear to influence the graded skin response, or significantly change the incidence of carcinomas. The initial skin changes and the tumor incidence for the respective experimental groups 40 weeks after the first DMBA treatment, are shown in Table V. The tumors recorded as carcinomas were typical examples of either basal or squamous cell carcinomas, and the sarcomas were fibrosarcomas varying in the degree of differentiation, with all types exhibiting infiltration in the underlying or surrounding tissue.

TABLE IV. *The cumulative frequency distribution of skin tumors observed 15 weeks after the initial application of DMBA, in animals placed on a 0.05% phenobarbital-supplemented diet or a control diet, which were initiated 2 weeks prior to the start of 12 weekly applications of 100 μg DMBA*

		Number of tumors			
		0	1	2	3
Group IId (Phenobarbital diet)	N = 30 M = 0.27	26	3	—	1
Group IIc (Control diet)	N = 30 M = 0.67	19	7	3	1

N = number of mice.
M = mean number of tumors per mouse.

DISCUSSION

The present study showed that the dietary administration of phenobarbital prior to and concurrently with the application of DMBA inhibited initially the appearance of papillomas, but did not completely eliminate the eventual appearance of tumors. Under these conditions the effects phenobarbital exerted on the skin appear to be analgous to the inhibitory effects observed in liver and lung tumorigenesis [4,5]. The mechanism by which phenobarbital reduced the incidence of skin tumors is not clear. It is not known precisely what

TABLE V. *The acute skin response and tumor formation initiated by repeated applications of DMBA in groups of mice fed a control diet or one supplemented by phenobarbital*

Treatment group	Skin response	Final number of papillomas[a]	Survivors[b]	Final incidence of:		Unknown[c]
				Carcinomas	Sarcomas	
Ia	Moderate erythema and slight to moderate desquamation	23 (10)[d]	29	3 (3)	7 (5)	2
Ib	Moderate erythema and slight to moderate desquamation	26 (12)	26	4 (3)	2 (2)	1
Ic	Moderate erythema and desquamation; 10% focal ulceration	89 (25)	27	10 (10)	11 (10)	5
Id	Moderate erythema and desquamation; 10% focal ulceration	52 (24)	28	11 (9)	5 (5)	2
IIa	Marked erythema and desquamation; all animals exhibited general dermatitis and ulceration	All animals bore multiple papillomas and lesions that appeared to be malignant by the 15th week and the experiments were terminated. No histopathologic examination was carried out.				
IIc	Slight to moderate erythema and very slight desquamation	96 (25)	29	15 (13)	6 (5)	2
IId	Slight to moderate erythema and very slight desquamation	101 (25)	29	13 (12)	5 (5)	1

[a] Values shown are the net papilloma count.
[b] Number of mice alive when the first carcinomas were observed in the respective treatment groups; initially 30 mice were assigned to each group.
[c] Malignant-appearing tumors without histologic confirmation, e.g., autolysis or animal missing.
[d] The number of tumor-bearing animals is given in parentheses.

enzyme systems are involved in the inactivation of DMBA or whether phenobarbital is an effective inducer of such enzymes in the skin. Recent studies suggest that hepatic activation of polycyclic hydrocarbons may be a major factor in the production of skin tumors [8]. If this is the case, it is possible that phenobarbital alters the rate of appearance of skin tumors by its effect on hepatic drug-metabolizing enzyme systems and not necessarily by direct effects on the skin.

A decrease in the incidence of tumors, induced by DMBA and 1,2,5,6-dibenzanthracene (DBA) has been found when phenobarbital was applied topically and immediately before the carcinogens [9]. Phenobarbital, which had no apparent effect on AAH activity, caused a significant reduction in the incidence of DBA-induced tumors. This reduction could not be accounted for by any change in the binding of DBA to epithelial cell DNA. In the case of DMBA it was thought that the reduction of binding to DNA did account for the decrease in incidence of tumors. Further studies will be necessary to elucidate the mechanism by which phenobarbital alters the appearance of both DBA-induced and DMBA-induced tumors.

In the current experiments, the dose level of DMBA caused some erythema and desquamation. It is possible therefore, that a saturating level of the carcinogen resulted from repeated applications of DMBA, at the doses used. Irrespective of the mechanism, such an effect of high carcinogenic doses may explain why phenobarbital was ineffective when the topical dose of DMBA caused severe cutaneous damage. With a reduced oncogenic dose, phenobarbital markedly inhibited papillomas at the onset of combined treatment, but the effectiveness was diminished or lost with continued applications of the carcinogen. These results indicate that the dose levels of DMBA used in the fractionated regimens exceeded those levels which could be accommodated by the effects of phenobarbital, and some incremental amount of the carcinogen, or damage, was accrued in the target tissue, which obscured any sustained inhibition.

The difference in the response of mice in the two age groups is consistent with a difference in susceptibility to oncogenesis or to tissue damage, which in turn resulted in tumor production. A difference in susceptibility to tumorigenesis, however, does not appear to be correlated with tissue penetration or binding of the carcinogen [10,11]. It is possible that age-dependent differences in the number of cells in the phases of the cell cycle are important, as it has been shown that tumor susceptibility is correlated with the distribution of cells in cycle [12,13].

An increase in the rate of regression of DMBA-induced papillomas due to vitamin A administration has been reported [14]. In the current experiments the reduction of the incidences of papillomas in the groups given dietary phenobarbital was not due to any apparent increase in the number of papillomas that regressed.

Finally, in contrast to studies in liver tumorigenesis where phenobarbital caused an acceleration in the appearance of hepatic tumors when fed after the treatment with the carcinogen [6], the same dietary level had no enhancing effect on the appearance or frequency of skin tumors. This observation was confirmed in an additional series of experiments (not shown) in which 24-week-old mice received only 2 weekly application of 250 μg DMBA and were then placed on the respective diets which were maintained for 56 weeks

It is clear that phenobarbital given concurrently with the administration of DMBA inhibited the appearance of tumors. The results also suggest that the previously reported tumor-enhancing effect of phenobarbital may be tissue specific.

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