Effects of Varying the Dietary Concentration of Phenobarbital on Its Enhancement of 2-Acetylaminofluorene-induced Hepatic Tumorigenesis

Carl Peraino, Everett F. Staffeldt, David A. Haugen, Louise S. Lombard, Fred J. Stevens, and R. J. Michael Fry

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT

Studies in this laboratory first showed that the incidence of hepatic tumors induced by a brief exposure to dietary 2-acetylaminofluorene (AAF) is substantially increased if the AAF treatment is followed by prolonged phenobarbital feeding. Our subsequent experiments focused in part on an examination of the characteristics of the tumorigenic enhancement process in liver in an effort to determine whether this experimental system constitutes a valid initiation-promotion model that may be useful for mechanistic studies of multistage tumorigenesis. These investigations involved analyses of the effects on hepatic tumorigenesis of changing the duration of AAF feeding, the duration of phenobarbital feeding, and the interval between the termination of AAF feeding and the onset of phenobarbital feeding. The results demonstrated that the characteristics of the hepatocarcinogenic response to the sequential AAF-phenobarbital treatment protocol are consistent with the classic initiation-promotion model originally derived from studies of skin tumorigenesis.

The present investigation further extends the characterization of the liver initiation-promotion model by comparing the tumor promoting effects of different dietary levels of phenobarbital. When fed at dietary concentrations that did not affect body weight gain, phenobarbital elicited dose-dependent increases in the final incidence (plateau level) of hepatic tumors in rats previously fed AAF. However, these phenobarbital treatments did not affect the time to onset or to cessation (attainment of the plateau phase) of tumorigenesis. The highest dietary concentration of phenobarbital (0.25%) retarded tumor formation initially but ultimately had the strongest promoting effect; rats on this regime also showed a reduction in growth rate. None of the phenobarbital treatments affected the growth rates or the morphological characteristics (degree of differentiation) of the tumors that were produced.

On the basis of the evidence presented it is concluded that (a) phenobarbital has no initiating activity and (b) tumor promotion by phenobarbital involves primarily an increase in the probability that initiated hepatocytes will express the neoplastic phenotype and does not influence the character of this phenotype or the kinetics of its expression.

INTRODUCTION

The feeding of phenobarbital to rats previously fed the hepatocarcinogen AAF for a brief interval (sequential treatment protocol) produced a marked increase in the subsequent incidence of hepatic tumors (8-13), although phenobarbital alone did not induce tumor formation (7, 12) and the simultaneous feeding of phenobarbital with AAF reduced subsequent hepatic tumorigenesis (9). Such observations suggest that tumorogenic enhancement by phenobarbital involves neither the modification of carcinogen metabolism nor the independent production of new tumorogenic changes. Instead, phenobarbital apparently facilitates the expression of tumorigenic changes induced by prior carcinogen treatment. These observations suggest that hepatocarcinogenesis proceeds in qualitatively distinct sequential stages analogous to the initiation-promotion sequence that characterizes tumor formation in mouse skin (2). By these criteria, phenobarbital may be considered a promoter of hepatic neoplasia.

In analyzing the characteristics of liver tumor promotion by phenobarbital, we have examined the effects on tumorigenesis of varying (a) the interval between the termination of the AAF treatment and the onset of phenobarbital feeding and (b) the duration of phenobarbital feeding begun immediately after ending the exposure to AAF (11, 13). We observed that the promoting effect of phenobarbital occurred despite the interposition of a 120-day interval between the AAF and phenobarbital treatments (11). These findings indicate that AAF-modified hepatocytes with tumorogenic potential (i.e., “initiated” hepatocytes) persist long after the termination of AAF treatment. Such persistence is in accord with evidence obtained from skin tumorigenesis studies suggesting that the initiation stage of tumorigenesis is irreversible (2). An increase in the duration of the phenobarbital treatment interval following AAF exposure was accompanied by parallel increases in tumor yield (11, 13); prolonged exposure to phenobarbital was required for maximal promoting effectiveness (12).

The present study further defined the characteristics of hepatic tumor promotion by analyzing the relative effects of different dietary phenobarbital concentrations on the kinetics of liver tumorigenesis in rats previously fed AAF. The results suggest that the promoting action of phenobarbital is restricted to an increase in the probability that initiated hepatocytes will express the tumor phenotype and does not involve effects on the time course of tumor expression or the characteristics (growth rate and degree of differentiation) of the tumors that ultimately appear.

MATERIALS AND METHODS

Tumor Incidence. Male CD-1 rats, 22 days of age (Charles River Breeding Laboratories, Wilmington, Mass.), were divided into 5 groups, each containing 252 rats. All groups were fed a diet containing 0.02% AAF (Aldrich Chemical Co., Milwaukee, Wis.) for 14 days followed by a control diet for 7 days. The various groups were then placed on diets containing different percentages of phenobarbital (Sigma Chemical Co., St. Louis, Ill.) for 20 weeks.
Enhancement of AAF-induced Hepatic Tumorigenesis

In Charts 1 to 3, the duration of the AAF treatment is depicted by the black bar along the abscissa. The vertical arrow adjacent to the bar denotes the start of the feeding of the various phenobarbital diets.

Weight Gain

Chart 1 shows the effects of the various phenobarbital treatments on body weight gain throughout the experiment. It is apparent that phenobarbital up to a dietary concentration of 0.05% had virtually no intrinsic effect on the growth of the rats. The slightly lower body weights of the rats receiving 0.05% phenobarbital probably resulted from the fact that these rats had a much higher incidence of hepatic tumors than did the control rats or those given lower phenobarbital dosages (see below). When the concentration of dietary phenobarbital was raised to 0.25%, however, a substantial growth suppression occurred, which was evident even when tumor incidence in this treatment group was less than 10% (Charts 2 and 3). In additional experiments, to be reported in detail elsewhere, we have observed that the growth suppression produced by 0.25% dietary phenobarbital resulted from a phenobarbital-mediated 22% loss in the efficiency of food utilization for growth (g of

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body weight gained per g of diet consumed) rather than from a reduction in food intake. This reduced efficiency may be in part a consequence of the induction of a negative nitrogen balance by dietary phenobarbital (4).

Tumor Incidence

Charts 2 and 3 and Table 1 show the effects of phenobarbital dosage on liver tumor incidence. Rats not given phenobarbital following the initiating AAF treatment showed no tumors until 265 days had elapsed; tumor incidence then rose to a maximum of 20% by 475 days and remained essentially constant through the end of the experiment (610 days). Dietary phenobarbital at a concentration of 0.002% had virtually no effect on the percentage of rats with tumors but caused a slight increase in the average number of tumors per liver (tumor frequency) at the end of the experiment. The latter effect is reflected in the significant increase in overall tumor frequency at this phenobarbital dosage level (Table 1).

Raising the dietary phenobarbital concentration to 0.01% had a significant enhancing effect on tumorigenesis (Table 1), with the attainment of maximal tumor incidences that were approximately twice those in the control animals (Chart 2). A critical feature of this enhancement is the obvious plateau phase in tumor incidence that occurred despite the continued administration of phenobarbital. This plateau was evident with respect to both the percentage of rats bearing tumors and the tumor frequency. When the dietary phenobarbital concentration was raised to 0.05%, tumor incidence showed a further significant increase (Table 1), reaching a plateau at approximately twice the incidence levels attained on the 0.01% phenobarbital diet (Chart 2).

At the highest dietary concentration of phenobarbital (0.25%), the appearance of tumors was considerably delayed in comparison to that in rats given 0.05% dietary phenobarbital. Subsequently, however, a marked increase in tumorigenesis occurred in the 0.25% phenobarbital rats, which led to a nearly 100% tumor incidence by the end of the experiment and a high tumor frequency with no indication of the appearance of a plateau. Data in Table 1 show that the retardation of tumorigenesis at the highest phenobarbital dose level is reflected in the significantly lower overall hepatic tumor incidence, expressed as the percentage of rats with tumors, in rats given this diet as opposed to that in rats given the 0.05% phenobarbital diet. The significantly higher overall tumor frequency in the 0.25% phenobarbital rats compared to that in the 0.05% phenobarbital rats (Table 1), despite the delayed onset of tumorigenesis in the high phenobarbital group, reflects the high actual potency of phenobarbital as a tumor promoter at this dosage level.

The inset of Chart 2 depicts a computer-generated plot of the percentage of tumor incidence data from the 0, 0.01, and 0.05% phenobarbital treatment groups. (The computer program used fits a line to a set of data points by the method of least squares and also detects the point at which the slope of the line changes significantly.) This plot omits the groups receiving the lowest and highest phenobarbital dosages. Omission of the lowest phenobarbital group was based on the absence of a significant increase in the percentage of tumor incidence with this treatment. The highest phenobarbital group was omitted, since it is apparent from the growth curves in Chart 1 and measurements of feed efficiency (see text above) that high dietary phenobarbital causes growth alterations and other metabolic changes that are not apparent at the lower phenobarbital dosages. Such changes could influence tumorigenesis by mechanisms other than those related directly to the promoting action of phenobarbital (19, 22) and might therefore complicate the interpretation of phenobarbital effects on tumor incidence kinetics. The data in the Chart 2 inset show that,

![Chart 3](https://example.com/chart3.png)

**Chart 3.** Tumor frequency (average number of tumors per liver) in the rats described in Chart 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>% of dietary phenobarbital</th>
<th>Elapsed time (days)</th>
<th>Rats examined</th>
<th>Rats with tumors</th>
<th>% of rats with tumors</th>
<th>Total no. of tumors/group</th>
<th>Av. no. of tumors/liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>610</td>
<td>206</td>
<td>25</td>
<td>12</td>
<td>46</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>610</td>
<td>208</td>
<td>28</td>
<td>13</td>
<td>67</td>
<td>0.32 a, c</td>
</tr>
<tr>
<td>3</td>
<td>0.01</td>
<td>610</td>
<td>208</td>
<td>60</td>
<td>29 b, c, d</td>
<td>168</td>
<td>0.83 b, c</td>
</tr>
<tr>
<td>4</td>
<td>0.05</td>
<td>568</td>
<td>190</td>
<td>99</td>
<td>56 b, c, d, e</td>
<td>274</td>
<td>1.5 b, c, d</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>610</td>
<td>192</td>
<td>88</td>
<td>48 b, c, d, e</td>
<td>389</td>
<td>2.03 b, c, d, f</td>
</tr>
</tbody>
</table>

* a Significantly different from Group 1 (p < 0.05).
* b Significantly different from Group 1 (p < 0.001).
* c Significantly different from Group 2 (p < 0.001).
* d Significantly different from Group 3 (p < 0.001).
* e Significantly different from Group 4 (p < 0.05).
* f Significantly different from Group 4 (p < 0.001).
within the specified phenobarbital dose range, neither the time at which AAF-induced tumors began to appear nor the time at which tumorigenesis reached a plateau phase was influenced by the presence of dietary phenobarbital, although the final tumor incidence level was clearly dependent on the dietary phenobarbital concentration.

Chart 4 analyzes the tumor size distribution patterns of the 3 treatment groups shown in the inset of Chart 2. This analysis was carried out by separating the tumors into 4 size classes and then comparing the treatment groups with respect to the total number of tumors in each group that fell into each size class during the designated experimental interval (138 to 568 days after the start of the 14-day AAF treatment). As indicated earlier (see "Materials and Methods"), tumors greater than 40 mm in diameter were not included in this comparison, since many of them contained smaller tumors that had coalesced. Note also that the time interval over which the comparison was made ended at 568 days because data for the rats receiving 0.05% phenobarbital did not extend beyond this point. The data in Chart 4 show that the enhancement of tumorigenesis by dietary phenobarbital was not accompanied by phenobarbital-mediated changes in the distribution of tumor sizes. Therefore, it can be concluded that phenobarbital did not influence the growth rates of those tumors.

**Histological Analysis**

The following describes the spectrum of hepatic lesions observed in this study.

**Neoplastic Nodules.** These lesions were rounded, relatively well circumscribed, and usually occupied more than one liver lobe. The normal liver architecture was absent within the nodule; the cells were arranged in solid or jumbled sheets or in irregular plates which were not contiguous with the hepatocyte plates in adjacent unaltered liver. Intralobular sinusoids were compressed between the enlarged nodular hepatocytes. Portal areas, present infrequently, were generally located at the peripheral margins of the nodules. The nodular hepatocytes were predominantly acidophilic and either granular or with a homogeneous ground glass appearance. A minority of the neoplastic nodules were either basophilic or contained a mixture of acidophilic, basophilic, and clear cells.

Figs. 1 and 2 illustrate the microscopic appearance of acidophilic neoplastic nodules from Group 1 (0% phenobarbital) and Group 5 (0.25% phenobarbital), respectively. The nodule depicted in Fig. 1 shows a trabecular pattern, whereas the lesion in Fig. 2 contains granular and clear cells in sheet formation with compression of sinusoidal epithelium by the enlarged hepatocytes.

Neoplastic nodules were found in livers from all treatment groups, and the nodules showed no morphological differences that could be correlated with the dosage of dietary phenobarbital. In addition, the nodules were invariably seen in association with other lesions, namely foci of cell alteration and frank hepatocellular carcinomas; the latter were generally larger and more irregular than the neoplastic nodules.

**Hepatocellular Carcinoma.** The histological appearance of the hepatocellular carcinomas varied from pure trabecular with well to poorly defined cords of cells, one to several cells in thickness, to trabecular carcinomas that also contained acinar, tubular, and glandular structures. The simplest glandular configuration was associated with the trabecular pattern in the form of an organoid structure that was composed of liver-like plates that terminated in a gland. The glands were separated by trabeculae. Both glands and trabeculae varied in the number of cell layers they contained.

The cells of these carcinomas exhibited varying degrees of structural and cytological divergence from normal hepatocytes. Some tumor cells resembled normal hepatocytes, whereas others either were enlarged, with multiple nuclei and mitotic figures, or were reduced in size and basophilic. In either case, they were periodic acid-Schiff negative. As was the case with the neoplastic nodules described above, the hepatocellular carcinomas observed in all treatment groups were morphologically indistinguishable. Representative carcinomas from Group 1 (Fig. 3) and Group 5 (Fig. 4) are shown to illustrate the absence of an effect of phenobarbital on tumor characteristics.

**DISCUSSION**

Collectively, the tumorigenesis data from this study suggest that hepatic tumor promotion by phenobarbital involves effects on a very limited segment of the sequence of events involved in neoplasia. Thus, the sole manifestation of the promoting action of phenobarbital is an increase in the probability that initiated hepatocytes will express their neoplastic character, as demonstrated by the dose-dependent enhancing effect of phenobarbital on plateau levels of tumor incidence in AAF-treated rats. Other elements of hepatic neoplasia, namely the interval before the first appearance of tumors, tumor growth rates, and the degree of tumor differentiation, are apparently unaffected by phenobarbital.

In a previous communication (13), our data appeared to indicate that the enhancement of hepatic tumorigenesis by phenobarbital did involve decreases in tumor latency and increases in tumor growth rates. We are now convinced that these earlier interpretations should be supplanted by those
derived from the present study, which was considerably more comprehensive in terms of phenobarbital dosage comparisons, duration of the experimental interval, and the numbers of animals and tumors examined. Our current suggestion that the promoting action of phenobarbital does not involve tumor growth stimulation is in accord with recent observations that the prolonged administration of phenobarbital to rats treated previously with diethylnitrosamine increased the number but not the average size of the foci of ATPase-deficient hepatocytes that appear prior to the emergence of liver tumors (6).

In the 3 groups depicted in the Chart 2 inset, it is apparent that each treatment exerted its full effect, but the tumor incidence in each case was not the maximum attainable. This type of graded response suggests that (a) the AAF treatment alone generated a population of initiated cells that either are not capable or have a very low probability of expressing the transformed phenotype without additional stimulation by a promoter and (b) a given dosage of phenobarbital completes the tumorigenic process in a fraction of the AAF-initiated cells.

The inability of phenobarbital to influence those aspects of the tumorigenic process (i.e., time to first tumor appearance and degree of tumor differentiation) that are responsive to carcinogen action (1, 15, 20, 23) constitutes strong evidence that phenobarbital has no initiating activity. Additional evidence in support of this conclusion is provided by the occurrence of plateau phases in tumor incidence (Charts 2 and 3) and in the incidence of diethylnitrosamine-initiated ATPase-deficient hepatocyte foci (6) despite the continued administration of phenobarbital. In view of these findings, it is possible that the recently observed incidence of hepatic tumors in Wistar rats that were fed phenobarbital (14) represents the promotion of tumorigenesis initiated either by inadvertent prior exposure to an exogenous carcinogen of unknown origin or by the action of an endogenous initiating factor. Support for the latter possibility is provided by prior studies showing that phenobarbital enhanced tumor formation in mice subject to spontaneous hepatic tumorigenesis (10, 21) but produced no tumors in mice without this susceptibility (7).

At present, the mechanism by which phenobarbital promotes hepatic tumorigenesis is unknown, but, given the evidence that promoting dosages of phenobarbital are nonhepatotoxic (8, 16), it appears that the promoting action of phenobarbital involves biochemical responses that are well within the physiological limits for normal hepatocellular metabolism. As discussed previously (11, 13), phenobarbital stimulates a wide variety of anabolic processes in the liver in connection with its induction of hepatomegaly (16, 17). These stimulatory effects of phenobarbital undoubtedly involve an enhancement of the expression of appropriate genetic information, as indicated by increases in RNA and protein synthesis (16). It seems probable, therefore, that within this spectrum of hepatic responses to phenobarbital are biochemical events that are common to those involved in liver tumor promotion. Therefore, an examination of the characteristics of the effects of phenobarbital on various aspects of gene expression appears relevant to the elucidation of the mechanism by which phenobarbital promotes hepatic tumorigenesis.

ACKNOWLEDGMENTS
Excellent technical assistance was provided by V. A. Ludeman and K. A. Rettman.

REFERENCES
Enhancement of AAF-induced Hepatic Tumorigenesis

Fig. 1. Edge of neoplastic nodule, Group 1 rat. H & E, × 62.
Fig. 2. Neoplastic nodule consisting of eosinophilic cells. Group 5 rat. H & E, × 62.
Fig. 3. Hepatocellular carcinoma, trabecular type with glands. Group 1 rat. H & E, × 125.
Fig. 4. Hepatocellular carcinoma, trabecular with glands. Group 5 rat. H & E, × 125.
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