Effects of Varying the Exposure to Phénobarbital on Its Enhancement of 2-Acetylaminofluorene-induced Hepatic Tumorigenesis in the Rat

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SUMMARY

In a previous study, phénobarbital feeding enhanced hepatic tumorigenesis in rats previously fed 2-acetylaminofluorene (AAF). The present study analyzed this enhancement by comparing tumor incidences in rats fed phenobarbital for various periods following a fixed exposure to AAF.

A 5- or 20-day treatment with phenobarbital immediately after cessation of AAF feeding produced little tumorigenic enhancement, in comparison with the severalfold enhancement seen in rats receiving phenobarbital for 100 days and longer. On the other hand, the interposition of a 10- or 30-day interval between the cessation of AAF treatment and the beginning of phenobarbital treatment (which was then continued throughout the experiment) produced enhancement comparable to that produced by beginning the phenobarbital treatment immediately after cessation of AAF feeding. These results indicated that (a) prolonged exposure to phenobarbital was required for tumorigenic enhancement and (b) the tumorigenic lesion produced by AAF was relatively stable, and its expression could be enhanced by phenobarbital long after the cessation of AAF treatment.

Observation of the kinetics of tumor incidence throughout the experiment showed that phenobarbital: (a) decreased the latent period between the end of the carcinogen treatment and the appearance of tumors; (b) increased the growth rate of the tumors; and (c) increased the rate of appearance of new tumor foci. No metastases were seen in rats given AAF alone or followed by phenobarbital, and the morphological characteristics of the tumors were similar with both types of treatment. Phenobarbital, therefore, did not appear to alter the degree of differentiation of the tumors.

INTRODUCTION

Previous studies demonstrated that dietary phenobarbital increases the incidence of hepatic tumors in rats previously fed AAF for various periods (23). In those experiments phenobarbital feeding was begun immediately after removal of the rats from the AAF diet and was continued for the duration of the experiment. The objective of the present study was to determine whether tumorigenic enhancement by phenobarbital is altered by varying both the period of exposure to phenobarbital and the time after AAF treatment at which phenobarbital treatment is begun.

MATERIALS AND METHODS

The general experimental conditions (rats, diets, and husbandry) were described previously (23). All diets contained 30% casein; AAF was present in 1 diet at a concentration of 0.02%, while a 2nd diet contained 0.05% phenobarbital; the control diet contained neither agent. Each of 6 experimental groups consisted of 3 replicate groups containing 40 rats each. All rats entered the experiment at 22 days of age. The treatment protocol for the experimental groups was as indicated in Table 1 (see "Results").

Beginning 101 days after the cessation of AAF feeding 12 rats from each experimental group were killed at 3-week intervals and examined for tumors. All livers that did not show superficial evidence of tumors were cut into 5-mm slices with a razor blade to reveal tumors located beneath the surface. Three dimensions of the tumors were measured where possible; otherwise, the greatest diameter was recorded. The greatest diameter was used as the measurement of tumor size for comparative purposes. Samples were taken for both light and electron microscopy. The tissue was fixed in a 20:2:1 mixture of 95% ethyl alcohol:neutral formalin:glacial acetic acid. Sections were stained with hematoxylin and eosin and by the periodic acid-Schiff reaction. At the early sacrifice interval samples of the minute lesions were taken for frozen sectioning and stained for lipid with oil red O. A small number of representative samples were taken from rats after 24-hr fasting. The total tumor incidence was compiled from both the macroscopic and microscopic examinations. The results from the 3 replicate experiments were pooled.

At 288 to 299 days of age rats from Group 3 and 4 and at 415 to 448 days of age rats from Group 1, 2, 4, and 6 were given injections of tritiated thymidine (0.5 uCi/g; specific activity, 0.36 Ci/mmole) i.p. and killed 1 hr later. Autoradiographs were prepared by the dipping technique. In order to monitor tumor growth in situ, 5 rats from each of Groups...
3 and 4 were subjected to laparotomy (under ether anesthesia) at 274 days of age and killed at 359 days of age; the procedure was repeated with rats at 309 days of age, and they were killed at 370 days of age.

RESULTS

Before the appearance of numerous discrete white pinpoint areas of focal degeneration (beginning approximately 100 days after cessation of AAF treatment), the liver showed little or no effect of the low AAF dosage used in this study. In the case of the rats fed phenobarbital, the characteristic changes associated with the drug (cf. Ref. 23) were found in the cytoplasm of the hepatocytes, especially those surrounding the central vein. In a small number of animals (seen chiefly 100 to 120 days after cessation of AAF treatment), the livers showed circumscribed areas of hyperplasia, with the cytoplasmic and nuclear characteristics as well as the marked differential in the positive staining with periodic acid-Schiff after fasting, described by Epstein et al. (5) as features of hyperplastic nodules.

As the experiment progressed, the observed lesions (many of which were liver colored), while continuing to resemble hyperplastic nodules in some respects, exhibited the following characteristics. (a) The cells and nuclei were enlarged and irregular in appearance. (b) Glycogen storage was negligible whether the rats were fed or fasted. (c) Autoradiographs showed evidence of increased proliferation, which varied considerably. (d) All the specimens examined had altered aldehyde dehydrogenase isozyme patterns (6). [No such changes were found during proliferation association with regeneration (R. Feinstein, personal communication).] (e) Examination, by laparotomy, of nodules in situ indicated that slow but persistent growth predominated, with no evidence of regression. This persistence of growth was also evident in the data from the serial killings (see below). The range of lesions that appeared also included adenocarcinomas and trabecular carcinomas, which were the least differentiated tumor types observed. Because the emergence of all these lesions began and progressed long after the cessation of carcinogen treatment, and because phenobarbital appeared to be nonselective in its effects on these lesions (see below), it was decided that for the present analysis of the effects of phenobarbital all nodular lesions observed would be counted as tumors.

Table 1 shows, for each experimental group, the total number of rats with tumors and the total number of tumors by the end of the experiment. Continuous treatment with phenobarbital, beginning immediately after 18 days of AAF feeding, caused a 3-fold increase in the number of rats with tumors of all sizes and an 8-fold increase in those with tumors 10 mm and larger. Phenobarbital treatment increased the overall tumor incidence 7-fold while tumors 10 mm and larger increased 20-fold. Treatment with phenobarbital for only 5 days had no effect on the number of rats with all sizes of tumors and produced a 60% increase in those with larger tumors. Overall tumor incidence increased 50% and larger tumors doubled in number with this treatment. Increasing the phenobarbital exposure to 20 days produced a slightly greater tumor enhancement which, however, was still far below that seen in rats given phenobarbital throughout the experiment. When phenobarbital feeding was delayed for 10 days after cessation of AAF feeding, and then continued for the remainder of the experiment, tumorigenic enhancement was not adversely affected. Increasing the treatment-free interval to 30 days caused only a slight reduction in the enhancing effect of phenobarbital.

Chart 1 shows the effect of prolonged phenobarbital

Table 1
Total incidence of hepatic tumors at the end of 269 days in rats fed a diet containing 0.02% AAF for 18 days followed by the feeding, in various temporal combinations, of a control diet and one containing 0.05% phenobarbital

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Rats/group</th>
<th>Rats with tumors</th>
<th>Total tumors/group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>All sizes</td>
<td>≥ 10 mm</td>
</tr>
<tr>
<td>1</td>
<td>AAF diet 18 days, then control diet</td>
<td>106</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>AAF diet 18 days, then phenobarbital diet</td>
<td>109</td>
<td>73</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>AAF diet 18 days, then phenobarbital diet 5 days, then control diet</td>
<td>106</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>AAF diet 18 days, then phenobarbital diet 20 days, then control diet</td>
<td>108</td>
<td>35</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>AAF diet 18 days, then control diet 10 days, then phenobarbital diet</td>
<td>108</td>
<td>78</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>AAF diet 18 days, then control diet 30 days, then phenobarbital diet</td>
<td>106</td>
<td>64</td>
<td>39</td>
</tr>
</tbody>
</table>
Phenobarbital Effects on AAF Hepatic Tumorigenesis

Chart 1. Tumor incidence at each sacrifice interval in rats fed the AAF diet followed by the control or phenobarbital diet. The numbers in parentheses next to the data points in A represent the numbers of rats used in obtaining the data. These numbers apply to B to D as well. The data for the rats receiving AAF followed by phenobarbital were pooled from Experimental Groups 2, 5, and 6. The term “group” in the ordinate designation in A and B refers to each of the data points. The average number of tumors per rat (ordinates, C and D) were calculated by dividing the total number of tumors per group at each sacrifice interval by the number of animals in that group. The term “days” along the abscissas represents days after cessation of AAF feeding.

Treatment on the time course of liver tumorigenesis in rats previously treated with AAF. Because of their similarity, the results in experimental Groups 2, 5, and 6 were pooled in order to increase the reliability of the observed response patterns. When tumors of all sizes were considered, the rates of increase in the percentage of rats with tumors were roughly parallel (after Day 120) in the AAF and AAF-phenobarbital groups, with the tumor incidence in the AAF-phenobarbital group being approximately 3-fold greater than that in the AAF group throughout the experiment (Chart 1A). Both the parallelism and the overall difference in tumor incidence resemble the results observed...
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after Day 150 in an earlier study of tumorigenic enhancement by phenobarbital (Ref. 23, Chart 3), despite differences in the treatment protocols between the 2 studies. When consideration was restricted to larger tumors, however, the percentage of rats with such tumors increased at a higher rate in the AAF-phenobarbital group than in the AAF group (Chart 1B). The rate at which new tumor foci appeared in each liver was also greater in the AAF-phenobarbital group (Chart 1, C and D) with the largest increment occurring among tumors 10 mm and larger (Chart 1D).

In those tumors examined, the spectra of morphological characteristics (both macroscopic and microscopic) were similar whether the rats were given AAF alone or AAF followed by phenobarbital. No metastases were seen in any of the rats throughout the experiment.

DISCUSSION

The process of tumorigenic enhancement by phenobarbital appears to involve: (a) a decrease in the latent period between the end of the carcinogenic treatment and the appearance of tumors (Chart 1A); (b) an increase in the rate at which new tumor foci appear in each animal (Table I and Chart 1, C and D); (c) an increase in the growth rate of the tumors [incidence of large tumors composed with overall tumor incidence (Table I and Chart 1)]. As yet, however, there is no indication that phenobarbital altered the degree of differentiation of the AAF-induced tumors. This suggests that phenobarbital only accelerates the expression of the neoplastic lesion previously induced by AAF without altering the character of the lesion. The stability of this AAF-induced lesion is indicated by the ability of phenobarbital to enhance tumorigenesis even though its administration was delayed for 30 days after cessation of AAF treatment (Table 1).

In the 1st report of tumorigenic enhancement by phenobarbital (23) several possible explanations were given for this effect. The following is an evaluation of these possibilities in the light of the present results and relevant findings from other laboratories.

1. Phenobarbital may trigger the proliferation of incipient tumor cells as part of a general stimulation of hepatocyte proliferation. This possibility is not supported by our observation that a 5-day exposure to phenobarbital, which is sufficient to produce the transient hepatocyte proliferation characteristic of this compound (23), has little effect on AAF-induced tumorigenesis (Table 1).

2. Phenobarbital may facilitate initiation by AAF previously bound to macromolecules. This possibility also appears unlikely in view of evidence that phenobarbital decreases the binding of AAF to DNA and increases its excretion as the glucuronide (19). This, plus the earlier observation that phenobarbital given simultaneously with AAF decreases AAF-induced tumorigenesis (23), suggests that phenobarbital would antagonize rather than facilitate initiation by bound AAF. In addition the necessity for prolonged phenobarbital treatment in order to observe tumorigenic enhancement (Table 1) is not consistent with the rapidity with which phenobarbital induces enzymes involved in AAF metabolism (19).

3. Phenobarbital may alter the immunological competence of the host in a manner that allows clones of tumors cells, which normally would be destroyed, to survive and multiply. This possibility is raised by the observation that phenobarbital decreases both the responsiveness of human lymphocytes to stimulation by phytohemagglutinin and the delayed hypersensitivity reaction in rabbits treated with dinitrochlorobenzene (22). The relevance of these immunological effects of phenobarbital to tumorigenic enhancement remains to be determined. Evidence exists, however, that indicates that immune suppression is not involved in chemical carcinogenesis in the liver. Thus, suppression of immunity in rats by the administration of hydrocortisone or cyclophosphamide did not enhance either hepatic tumorigenesis induced by diethylnitrosamine or the induction of fibrosarcomas by benzpyrene (25), and the immunosuppressant, azathioprine, did not increase hepatocarcinogenesis induced in rats by N-hydroxy-AAF (7). On the other hand, the stimulation of delayed hypersensitivity in guinea pigs treated with vaccinia virus or oxazolone did not suppress the growth of transplanted hepatoma cells (11). If, as the latter data indicate, this type of immune reaction does not protect against carcinogenesis, then its suppression by phenobarbital (22) is unlikely to be the basis for the enhancing effect of phenobarbital on hepatic tumorigenesis. In addition, preliminary studies in our laboratory have shown that prolonged phenobarbital treatment does not accelerate tumor growth or decrease survival in mice inoculated with leukemia cells and does not reduce the reactivity of mouse spleen immunocytes toward transplanted mastocytoma cells (B. Jaroslow, personal communication).

4. Acting at the molecular level, phenobarbital may enhance the expression of a tumorigenic lesion previously produced by AAF. This possibility is supported by several studies, in addition to those cited earlier (23), describing the effects of phenobarbital on macromolecular synthesis and stabilization in the liver. Such studies have revealed pronounced effects of phenobarbital on the turnover of liver rRNA, involving both a reduction of degradation (1, 2, 20, 26, 27, 31) and a stimulation of synthesis (8, 26, 29). There is some indication that RNA stabilization in phenobarbital-treated rats is related to a reduction in liver and serum RNase activity (18, 26, 27) although certain studies do not support this possibility (9, 31). Other studies of the well-known stimulatory effects of phenobarbital on microsomal enzyme synthesis have shown that phenobarbital both enhances synthesis and reduces degradation of these proteins (4, 9, 10, 15, 16, 28). In keeping with these effects on the turnover of macromolecules, phenobarbital reduces the catabolism of microsomal phospholipids (21, 13) and promotes the accumulation of ATP in the liver (14, 21).

It is apparent from the above considerations that the overall effect of phenobarbital in rat liver is profoundly anabolic, i.e., phenobarbital stimulates a variety of biosynthetic processes and stabilizes the products produced thereby. The possibility that these changes in liver metabolism
are related to tumorigenic enhancement by phenobarbital remains to be assessed. It seems reasonable to speculate, however, that the type of anabolic milieu created in the liver by phenobarbital treatment could magnify the consequences of molecular changes induced by prior exposure to AAF. These AAF-induced molecular changes either may be directly involved in the neoplastic transformation or may lead to the expression of a latent tumor virus (24, 30).

Phenobarbital and other barbiturates have also been shown to influence the output of certain exocrine and endocrine glands, as well as the metabolism of endocrine hormones (3, 17, 32). The relationship between these effects and the enhancement by phenobarbital of liver tumorigenesis is not presently apparent. The possibility must be considered, however, that a disruption of endocrine balance engendered by phenobarbital could enhance tumorigenesis either by accelerating the molecular processes leading to tumor cell formation or by altering the metabolism of the host in a manner favoring the growth of fully transformed cells.

REFERENCES

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