Effects of Varying the Onset and Duration of Exposure to Phenobarbital on Its Enhancement of 2-Acetylaminofluorene-induced Hepatic Tumorigenesis

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SUMMARY

In previous experiments we observed that the short-term feeding of 2-acetylaminofluorene (AAF) at a low dietary concentration resulted in the late appearance of well-differentiated hepatic tumors at low incidence levels. When the AAF treatment was followed by the feeding of a phenobarbital-supplemented diet, the appearance of these tumors was accelerated, and their overall incidence levels were increased. The present study examined the characteristics of this interaction between the effects of AAF and phenobarbital by measuring tumor production after changing either the duration of the interval between AAF and phenobarbital treatments or the duration of the post-AAF exposure to phenobarbital.

When AAF and phenobarbital treatments were separated by increasing intervals (up to 120 days), the resultant levels of tumor enhancement appeared to be influenced mainly by the duration of the post-AAF phenobarbital treatment rather than by the length of the treatment-free interval. Progressive increases in the duration of the phenobarbital treatment (begun within 7 days after the AAF treatment) advanced the time at which tumor enhancement occurred and similarly increased overall tumor incidence levels. The latter enhancement effects occurred and were sustained after the cessation of the phenobarbital treatments. The results of this study suggest that (a) the tumorigenic changes induced by brief AAF treatment are persistent, although the question of their permanence remains open, and (b) phenobarbital treatment produces irreversible changes in AAF-modified cells, which lead to the expression of the tumor phenotype.

INTRODUCTION

Feeding a diet containing phenobarbital to Sprague-Dawley rats, previously fed the hepatocarcinogen AAF for a brief period, markedly increased subsequent hepatic tumor incidence (9-11), although phenobarbital itself did not produce tumors under these conditions (10). These observations support the suggestion (8) that tumorigenesis in rat liver proceeds in stages (i.e., initiation and promotion) analogous to those that occur in mouse skin tumorigenesis (1, 2, 4, 5, 13, 14). According to this model, phenobarbital would be classified in the liver system as a promoter.

The studies in mouse skin have led to the conclusion that the initiation stage is irreversible, whereas the promotion stage is reversible (4). One objective of our work therefore has been to determine the extent to which these characteristics also apply to the analogous stages of rat liver tumorigenesis. Our approach involves measuring the effects on liver tumor incidence of varying (a) the interval between the termination of AAF feeding and the onset of phenobarbital feeding (treatment-free interval) and (b) the duration of the post-AAF exposure to phenobarbital (phenobarbital exposure interval).

The present investigation is an extension of an initial study (11) in which the treatment-free intervals and phenobarbital exposure intervals used were found to be adequate to permit a definitive assessment of the reversibility of either initiation or promotion in rat liver. Therefore, in the present study both the treatment-free intervals and phenobarbital exposure intervals were lengthened considerably. The results obtained engendered a reanalysis of our earlier findings, and this report represents a composite of both sets of data expressed in terms of the kinetics of tumor incidence.

MATERIALS AND METHODS

Male Sprague-Dawley rats at 22 days of age were fed a diet containing 0.02% AAF for 18 days. The rats were then divided into groups containing 120 rats each [except for Group 4 (Table 1), which contained 80 rats] and were fed a control diet or one containing 0.05% phenobarbital, according to the protocols shown in Table 1 and Charts 1 and 2 (see "Results"). The numbers in the tables included in the charts indicate the days during which a designated diet was fed. Additional details regarding diet preparation and animal care were as reported earlier (9, 11).

Rats selected randomly from each group were killed at intervals, and their livers were examined for tumors as described previously (9-11). The numbers of adenomas and...
Table 1
Total incidence of hepatic tumors
Rats were fed the control, AAF, and phenobarbital diets in sequence as designated below and were examined for liver tumors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days on AAF diet</th>
<th>Days on control diet</th>
<th>Days on phenobarbital diet</th>
<th>Days on control diet</th>
<th>Rats examined</th>
<th>Rats with tumors</th>
<th>% of rats with tumors</th>
<th>Total tumors/group</th>
<th>Av. tumors/liver</th>
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<tr>
<td>1</td>
<td>18</td>
<td>414</td>
<td></td>
<td></td>
<td>92</td>
<td>7</td>
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<td>0.12</td>
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<tr>
<td>2</td>
<td>18</td>
<td>7</td>
<td>407</td>
<td></td>
<td>93</td>
<td>58</td>
<td>62*</td>
<td>108</td>
<td>1.21</td>
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<td>3</td>
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<td>60</td>
<td>354</td>
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<td>96</td>
<td>49</td>
<td>51*</td>
<td>77</td>
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<tr>
<td>4</td>
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<td>120</td>
<td>294</td>
<td></td>
<td>64</td>
<td>23</td>
<td>36*</td>
<td>44</td>
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<td>50</td>
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<td>100</td>
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<td>18</td>
<td>7</td>
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<td>257</td>
<td>94</td>
<td>27</td>
<td>29*</td>
<td>41</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Significantly different from Group 1 (p < 0.01).
* Significantly different from Group 2 (p < 0.01).

Chart 1. Effects of a 10-, 30-, 60-, or 120-day interval between the termination of AAF feeding and the onset of phenobarbital (Phe) feeding on the incidence of hepatic tumors as a function of days after termination of the AAF treatment. A and C, percentage of rats with tumors; B and D, average number of tumors per liver. The experimental protocol is tabulated in the body of each panel and indicates the days that each group was fed a given diet. Contr, control; —, animals not fed.
carcinomas (see Ref. 10 for diagnostic criteria) were pooled for all the analyses. In Group 4 (Table 1), 6 to 8 rats were killed at each sacrifice interval, whereas 10 to 12 rats were killed at each interval in the remaining groups. Additional deaths (ranging between 18 and 25% of each group) occurred randomly throughout the experiments and showed no apparent relationship to the experimental treatments. Losses resulting from autolysis and cannibalism precluded accurate tumor counts in these animals, and they were not included in the tabulation of results. Such losses reduce the sample size but should not bias data for tumor prevalence rates. Examination of the lungs when autopsy was possible revealed pneumonia as the predominant cause of death in these animals.

To facilitate comparisons of the tumor incidence patterns among the various treatment groups, each curve in Charts 1 to 3 was smoothed by a moving average procedure (7). This involved averaging the data from 2 successive adjacent points on the curve and plotting the averages at the mean intervals between the original points. Table 1 and Charts 1, C and D, and 2, C and D, represent the results of the new experiments; whereas Charts 1, A and B, and 2, A and B, show the kinetics of tumor incidence calculated from experiments reported previously in summary form (11).

RESULTS AND DISCUSSION

Charts 1 and 2 show the effects on tumorigenic enhancement of lengthening the interval between AAF and phenobarbital treatments. In Chart 1 the tumor incidence data were plotted as a function of the time after termination of AAF feeding; therefore the rats in the groups compared at each sacrifice interval were the same age. This reflects the sacrifice protocol used in the experiment.

Chart 1, A and B, shows that enhancement was not affected by a 10- or 30-day interval between AAF and phenobarbital treatments, but Chart 1, C and D, suggests a decline in enhancement as the treatment-free interval was extended to 60 and 120 days. In terms of cumulative tumor incidence, this decline became statistically significant at the 120-day interval (Table 1). However, in these experiments the corresponding decrease in the duration of exposure to phenobarbital with the lengthening of the treatment-free interval raises the possibility that the observed decline in tumor incidence was a consequence of the reduced phenobarbital exposure. This possibility was examined by replotting the tumor incidence data in Chart 1, C and D, as a function of the duration of exposure to phenobarbital (Chart 2). Although the variability of the individual tumor incidence patterns in Chart 2 precludes a definitive conclusion, the composite pattern (especially as shown in Chart 2A) suggests that tumor incidence was influenced primarily by the duration of exposure to phenobarbital rather than by the length of the interval between AAF and phenobarbital treatments. This interpretation is supported by the observation that the cumulative tumor incidence resulting from 294 days of phenobarbital treatment after the 120-day treatment gap was higher than the incidence in rats receiving 150 days of phenobarbital after a 7-day treatment gap (Table 1). Moreover, a comparison of the times at which the onset of tumorigenic enhancement occurred in rats given phenobarbital, either immediately after AAF (Chart 1, A and B) or after a delay of 120 days (Chart 2, A and B), shows that the enhanced tumorigenesis became evident 120 to 160 days after the beginning of the phenobarbital treatment. This indicates that the 120-day treatment gap did not alter the response of AAF-modified cells to phenobarbital.

Collectively, these data suggest that the tumorigenic changes produced in the liver by brief AAF treatment are persistent, although the question of their permanence remains to be answered. A definitive resolution of this issue will require additional experiments involving the lengthening of both the treatment gap and the interval over which each treatment group is monitored. Such experiments become difficult, however, not only from the standpoint of size but also because of the practical constraints placed on the duration of the experiment by the increasing occurrence of mortality among the experimental animals from competing risks.

Comparison of the tumor incidence patterns in rats fed phenobarbital for increasing intervals (beginning within 7 days after AAF treatment) shows a continual upward trend in tumorigenic enhancement, associated with the lengthening of the exposure to phenobarbital (Chart 3). This trend was expressed both as a progressive advancement in the time of appearance of tumors and as a progressive increase in the tumor incidence level. In terms of cumulative tumor...
incidence, the enhancement became statistically significant after 150 days of phenobarbital treatment (Table 1). From the tumor incidence patterns shown in Chart 3B, however, enhancement was evident after 20-day phenobarbital treatment when the data were expressed in terms of the average number of tumors per liver. The enhancing effects of this brief phenobarbital treatment did not become apparent until approximately 200 days after the treatment had ended. Progressive increases in tumor incidence produced by the longer phenobarbital treatments also appeared and were sustained after the termination of these treatments (Chart 3, C and D).

The manifestation of a detectable although reduced level of tumorigenic enhancement long after the cessation of brief post-AAF exposures to phenobarbital (Chart 3B) suggests that the effects of such phenobarbital exposures are irreversible. Thus, although there is no evidence that phenobarbital causes irreversible changes in normal liver (3, 6, 12), it appears that phenobarbital treatment after exposure to AAF produces irreversible changes in AAF-modified cells,
which lead to the expression of the tumor phenotype. The irreversibility of the phenobarbital effect is also suggested by the maintenance of graded levels of enhanced tumorigenesis long after the cessation of lengthier phenobarbital treatments (Chart 3, C and D).

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REFERENCES

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