ENHANCING EFFECT OF PHENOBARBITAL ON THE DEVELOPMENT OF ENZYME-ALTERED ISLANDS AND HEPATOCELLULAR CARCINOMAS INITIATED BY 3'-METHYL-4-(DIMETHYLAMINO)AZOBENZENE OR DIETHYLNITROSOAMINE

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Effect of dietary phenobarbital on the development of enzyme-altered islands (EAI) and hepatocellular carcinomas initiated by a short-term treatment with 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB) or diethylnitrosamine (DENA) in the rat was studied. Phenobarbital generally enhanced the proliferation of carcinogen-induced (enzyme-altered) cells so that the number and size of EAI were much larger in phenobarbital-fed groups in early stages than in control groups kept on a basal diet. The growth rate of EAI was, however, not uniform and only a small minority progressed to large islands or carcinomas. In groups initially treated with 3'-Me-DAB for 3 weeks, the enhancing effect of phenobarbital on cancer production was remarkable by the 36th week whereas in groups treated with 3'-Me-DAB for 1 week, no cancer developed by the 60th week, although a large number of small EAI appeared. Thus a summation effect of the carcinogen was observed even when the carcinogen was given for a short period as an initiator. The enhancing effect of phenobarbital was also marked in the groups initiated by DENA, in which, however, considerable number of carcinomas developed in control groups also. The carcinogen-induced cells or EAI appeared inherently different, as the population, in the potentiality to progress to carcinoma according to the difference of an initiator.

Phenobarbital enhances hepatocarcinogenesis when given after a short-term treatment with the hepatocarcinogen, acetaminofluorene, diethylnitrosamine (DENA), or 3'-Me-DAB. The enhancing effect of phenobarbital was also noticed in spontaneous hepatocarcinogenesis in the mouse. When given together with the carcinogen, phenobarbital inhibited the hepatocarcinogenesis. Phenobarbital was negative in the mutagenicity test in several systems (Ames' system, Pienta's system, and chromosome aberration test) (Miwa, Takayama, Ishidate, and Kitagawa, unpublished data) and failed to induce enzyme-altered islands (EAI) in the rat when administered alone for 24 weeks. Phenobarbital is considered to act as a "pure" promoter of hepatocarcinogenesis, enhancing the expression of preneoplastic lesions induced by the carcinogen. It has been suggested that phenobarbital may be useful in detecting pure initiators or incomplete carcinogens and also in probing the two-step hypothesis of carcinogenesis which has been largely based on experiments in one system, that of epidermal carcinogenesis in the mouse. Phenobarbital may also be useful for studying the nature of putative preneoplastic lesions appearing in the liver during hepatocarcinogenesis.
Enzyme-altered islands or hyperplastic areas are collection of hepatocytes seen in the liver several weeks after application of the hepatocarcinogen and have been described as putative preneoplastic lesions. The cells of islands show marked enzymic deviation similar to that of hepatocellular carcinomas. EAI are also inducible by a single dose of the carcinogen, DENA, to the hepatectomized rat and, with an additional carcinogenic stimulus, give rise to hepatocellular carcinomas. EAI may be regarded as the immediate progeny of altered hepatocytes induced by the carcinogen during the phase of "initiation."

In the present experiments we have studied how phenobarbital influences the development or growth of EAI induced by two different carcinogens, 3'-Me-DAB and DENA, given for different periods of time or in different ways. One of the objectives of the experiment was to establish a practical system for analyzing the two aspects of "initiation" and "promotion" of hepatocarcinogenesis.

**Materials and Method**

Male weaning Donryu rats at 21 days of age (Nihon Rat Co., Urawa) were divided into 4 groups. They were housed in an air-conditioned room in wire-mesh cages, 5 to a cage. The animals of Groups 1 and 2 were fed a diet (CE-2, CLEA Japan Inc., Tokyo) containing 0.06% 3'-Me-DAB (Tokyo Kasei Co., Tokyo) for 3 or 1 week, respectively. The Group 3 rats were given drinking water containing 0.01% DENA (Tokyo Kasei Co.) for 1 week and the Group 4 rats were injected 2 mg of DENA intraperitoneally once on the first day of the experiment. After treatment with the carcinogen, all the rats were kept on the basal diet for 2 weeks and then the animals of each group were subdivided into phenobarbital group (a) and control (b) groups. The animals of phenobarbital groups were fed a diet containing 0.05% phenobarbital (Iwaki Selyaku Co., Tokyo) throughout the experimental period, according to Peraino's regimen. The control groups were kept on the basal diet. Three rats from Groups 1, 3, and 4 were killed at 8 weeks and several rats from each group were sacrificed at 12 and 24 weeks after the start of feeding the carcinogen. All the rats except those in Group 2 and a part of Groups 1b and 4b were sacrificed at the 36th week. The animals in Group 2 were killed at the 60th week and some in Groups 1b and 4b at the 48th week.

Liver tissues were cut out from median large lobes and frozen on Dry Ice. The portions where macroscopic tumors existed were avoided. Frozen sections were made in a cryostat, dried, fixed in cold formol calcium for several hours, and stained for ATPase by Wachstein-Meisel's method and counterstained with Hematoxylin. The number and size (largest diameter) of ATPase-deficient islands (Photo 1) larger than 50 µm in diameter were scored from several sections. Total area of liver tissue examined for scoring was 5 cm²/rat on average. Islands showing ATPase activity in part or throughout the region were included when they were clearly delineated as islands from the surrounding tissue (Photo 2). The sequential phenotypic change in EAI, especially the tendency for enzymic maturation with time, was previously reported from this laboratory. The number and size (largest diameter) of tumors larger than 5 mm were scored macroscopically. To detect small tumors the liver was sectioned thoroughly at 5-mm intervals. All the tumors, except for some small ones, were examined histologically on conventional paraffin sections.

**Results**

The number of EAI per cm² of liver sections for each group, is shown in Fig. 1 as a function of time. In every group, except in Group 2, many tiny EAI developed by the 8th week, both in phenobarbital (a) and control (b) groups. In phenobarbital groups the number of EAI increased remarkably by the 12th week and remained at the similar level thereafter. In the control groups the number of EAI was roughly constant during the 8th to 36th week in Group 1b or during the 8th to 24th week in Groups 3b and 4b. After these periods, the number of EAI in the control groups increased to the levels of phenobarbital-fed groups at the 12th week. The data on EAI of Groups 1a and 3a at the 36th week were not obtained because of the presence of multiple large tumors in the liver. In Group 2 the number of EAI was very small by the 24th week. At the 60th week, however, there was a large number of small EAI.
Fig. 1. Number of enzyme-altered islands larger than 50 μm/cm² liver section at 8, 12, 24, 36, 48, and 60 weeks after initial day of treatment with carcinogen.

The number on the graph indicates the number of rats sacrificed; vertical lines indicate standard deviation. Groups with (a) and without (b) phenobarbital.

Table I. Number of Tumors at 36, 48, and 60 Weeks

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Weeks checked at</th>
<th>No. of rats</th>
<th>No. of tumors relative to size (mm in diameter)</th>
<th>Total No. of tumors per group</th>
<th>No. of tumors per rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>3'-Me-DAB (3 wk)+PB</td>
<td>36</td>
<td>16</td>
<td>46</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>1b</td>
<td>3'-Me-DAB (3 wk)+BD</td>
<td>36</td>
<td>21</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1b</td>
<td>3'-Me-DAB (3 wk)+BD</td>
<td>48</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2a</td>
<td>3'-Me-DAB (1 wk)+PB</td>
<td>60</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2b</td>
<td>3'-Me-DAB (1 wk)+BD</td>
<td>60</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3a</td>
<td>DENA (1 wk)+PB</td>
<td>36</td>
<td>13</td>
<td>70</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>3b</td>
<td>DENA (1 wk)+BD</td>
<td>36</td>
<td>14</td>
<td>29</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>4a</td>
<td>DENA (ip)+PB</td>
<td>36</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>DENA (ip)+BD</td>
<td>36</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>DENA (ip)+BD</td>
<td>48</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

PB=phenobarbital diet, BD=basal diet
Fig. 2. Size distribution of enzyme-altered islands as a function of time
Vertical lines on the columns indicate standard deviations.
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Group 3 DENA (1 wk)

Group 4 DENA (ip)

12 weeks

24 weeks

36 weeks

No. of islands/cm² liver section

Largest diameter of island (μm)
The size distribution of EAI is shown in Fig. 2 as a function of time. The number of tumors larger than 5 mm for each group, at the experimental weeks indicated, is listed in Table I. In Group 1a there were many relatively large EAI at the 12th week. The number and size distribution did not change during the next 12 weeks. At the 36th week, however, there were many macroscopic tumors, some being very large. In Group 1b the number and size distribution of EAI were similar during the 12th to 36th weeks. At the 36th week the tumor incidence of Group 1a was 3.8 per liver, 13 times larger than that in Group 1b. During the 36th to 48th weeks EAI of small size increased in Group 1b, but the tumor incidence at the 48th week was still very low.

In Group 2 the majority of EAI became detectable only at the 60th week, most of them still remaining in the small classes of size. The 3 small tumors that developed in Group 2a were histologically hyperplastic nodules.

In Groups 3 and 4 EAI were small and relatively uniform in size at the 12th week. During the next 12 or 24 weeks many of them shifted to larger classes. At the 36th week a large number of tumors were noticed both in Groups 3a and 3b. The ratio of tumor incidence of 3a to 3b was 3.0. The tumor incidence in Group 4a at the 36th week was 1.6 while it was nil in Group 4b. By the 48th week many EAI in Group 4 grew larger giving rise to 2 small macroscopic carcinomas. The increment of the number of EAI in Groups 3b and 4b during the 24th to 36th weeks was attributable to the development of EAI of a small size in this phase.

Most of the macroscopic tumors studied histologically were carcinomas, the rest being hyperplastic nodules. Almost all carcinomas were well-differentiated hepatocellular carcinomas. While most of DENA-initiated carcinomas were trabecular hepatocellular carcinomas (Photo 4), about 40% of 3'-Me-DAB-induced carcinomas were adenocarcinomas (hepatocellular carcinoma with glandular formation) (Photo 5), a common and rather characteristic type of azo dye-induced carcinomas.4)

**Discussion**

The enhancing effect of phenobarbital on hepatocarcinogenesis was clearly shown in all experimental groups as an increase in the number and size of EAI in relatively early weeks, as well as those of subsequent carcinomas. Phenobarbital apparently enhances the proliferation of carcinogen-induced (enzyme-altered) cells in general rendering them detectable as EAI. Since a long-term feeding of phenobarbital does not elevate the mitotic activity of normal hepatocytes,9) the promotive action of phenobarbital might be specific on the carcinogen-induced cells. The observation that the growth rate of the individual EAI is not uniform, whether or not phenobarbital was administered, suggests the heterogeneity of the carcinogen-induced cells or EAI. The heterogeneity of EAI has been described in histological20) or histochemical observations,5,13) or in the study on their proliferative property in vivo and in vitro.15)

In every phenobarbital-fed group, except for Group 2, the number of EAI remained relatively constant after the 12th week while in control groups it reached the plateau levels 24 to 36 weeks later. It may be concluded that most, if not all, carcinogen-induced cells not only persisted but also proliferated without the stimulus of a specific promoter, as was previously described by other authors.19)

In Group 2, most EAI became detectable after 24 weeks and remained small in size even at the 60th week, with only a few tiny macroscopic hyperplastic nodules in phenobarbital-fed rats. When this result is compared with that of Group 1, the summation effect of the carcinogen is remarkable even when the carcinogen was given only for 3 or 1 week. Whether this summation effect represents a multi-hit effect of the carcinogen on the
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carcinogen-induced cells during the additional 2 weeks remains to be clarified. Similar dose-dependent or summation effect was observed in experiments inducing liver carcinomas by a single injection of DENA\(^{18}\) or short-term treatment with acetaminofluorene.\(^{9}\) Scherer and Emmelot discussed that this summation effect might largely be attributable to the selective toxicity of the carcinogen on the original hepatocytes,\(^{18}\) a situation known to cause growth promotion on initiated cells.

The behavioral difference between 3'-MeDAB-induced and DEN-induced EAI is of note. The EAI in Group 1 were relatively large by the 12th week but did not grow remarkably during the next 12 weeks and the cancer incidence was high only in the phenobarbital-fed group. In Group 2, most of the EAI became detectable only at the 60th week even in phenobarbital-fed rats. On the other hand, the EAI in Group 3 were small at the 12th week but kept growing during the next 12 weeks and the cancer incidence was high both in phenobarbital-fed and control groups. A similar tendency was observed with the EAI in Group 4. There was also some difference in histological types of carcinoma between those in Group 1 and those in Groups 3 and 4. This is another feature indicating the qualitative difference of carcinogen-induced cells or EAI, as a population, induced by two different carcinogens. The DENA-induced cells appeared generally more potent to progress to carcinoma either spontaneously or under the promotive stimulus of phenobarbital. On the other hand, the promotive effect of phenobarbital was most clearly seen in Group 1 rats. For analyzing the aspect of promotion in terms of timing or duration of the promotive stimulus or reversibility of the promotion effect, the system initiating with 3-week feeding of 3'-Me-DAB would be suitable and the scoring of EAI should be useful.

Initiators of hepatocarcinogenesis may be recognized by detecting EAI rapidly and quantitatively in the system employed in the present work. However, since we obtained only a few small tumors in Group 2 by the 60th week, in the presence of numerous EAI, more than 60 weeks of observation period would be necessary to confirm a putative initiator actually to produce carcinomas with promotion by phenobarbital.

During the preparation of this paper, Rossi and his colleagues reported that they have found neoplastic hepatic nodules in Wistar rats after a very long (78~150 weeks) exposure to phenobarbital.\(^{17}\) Although they have excluded the contamination of aflatoxin, further careful study seems required to exclude the possibility of phenobarbital having acted as a promoter on preexisting potent cells or cells initiated by some unknown environmental carcinogens. In any case, a control study covering such a long period of time in detecting EAI in rats fed phenobarbital alone is now required. An experiment to meet this objective is now under way in our laboratory. During this experiment, no EAI have been detected so far in the control animals fed phenobarbital alone by 24 weeks as was reported previously.\(^{13}\)

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REFERENCES

3) Ishidate, M., Watanabe, M., Odashima, S., Gann, 58, 267~281 (1967).

**EXPLANATION OF PLATE**

Photo 1. EAI in the liver, demonstrated as ATPase-deficient islands of hepatocytes. Staining for ATPase, counterstained with Hematoxylin. ×16.

Photo 2. EAI with partial ATPase activity. Staining for ATPase, counterstained with Hematoxylin. ×16.

Photo 3. EAI comprised of cells with vacuolated cytoplasm. H-E. ×40.


Photo 5. Adenocarcinoma (hepatocellular carcinoma with glandular formation), well-differentiated, that developed in a rat in Group 1. H-E. ×40.

H-E = Hematoxylin-Eosin stain