MECHANISMS OF INHIBITION BY SIMULTANEOUSLY ADMINISTERED PHENOBARBITAL OF 3'-METHYL-4-(DIMETHYLAMINO)AZOBENZENE-INDUCED HEPATOCARCINOGENESIS IN THE RAT*1

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The mechanisms of inhibition by simultaneously administered phenobarbital of 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB)-induced hepatocarcinogenesis in the rat were studied. Weanling rats were fed a diet containing 0.06% 3'-Me-DAB or 0.06% 3'-Me-DAB and 0.05% phenobarbital for 3 weeks, followed by either basal diet or a diet containing 0.05% phenobarbital as a promoter. The number and the size of enzyme-altered islands and the number of tumors larger than 5 mm in diameter were scored at week 12 and week 40, respectively. The simultaneous feeding of phenobarbital and 3'-Me-DAB resulted in a significant decrease in the number and size of enzyme-altered islands and in the number of tumors, in comparison with those scored in animals fed 3'-Me-DAB alone. It was concluded that the simultaneous feeding of phenobarbital inhibits both the initiation of carcino genesis and also the promotive action of the carcinogen resulting from its selective toxicity on the liver tissue.

Key words: 3'-Methyl-4-(dimethylamino)azobenzene — Initiator — Promoter — Phenobarbital — Hepatocarcinogenesis

Phenobarbital is known to be a potent promoter of hepatocarcinogenesis when administered after an initial short-term treatment with hepatocarcinogens such as 2-acetylaminofluorene (AAF),11) diethylnitrosamine (DEN),18) and azo dyes.6,7) However, phenobarbital fed simultaneously with a hepatocarcinogen inhibited hepatic tumorigenesis.2,11) The inhibitory effect of phenobarbital is considered to be a result of stimulation of hepatic drug-metabolizing enzymes which detoxify the carcinogen,2,9,10) as is the case with methylcholanthrene.3,13) The question arises, however, as to whether phenobarbital fed simultaneously with a hepatocarcinogen suppresses the initiation of carcinogenesis or inhibits the promotive action of the carcinogen, or both.

Hepatic carcinogens are toxic to normal mature hepatocytes and evoke cellular regeneration after degeneration.1,5) Since carcinogen-induced altered cells are relatively resistant to the toxicity of carcinogens and responsive to regeneration stimuli,4,16) this mechanism of selective toxicity has been pointed out to be promotive for carcinogenesis.1,15) We have already shown that the initiation and promotion of carcinogenesis can be monitored by scoring the number and the size of enzyme-altered islands (EAI), which are thought to be the progenies of carcinogen-induced cells and important precancerous lesions of the liver.7,12,14) Utilizing this technique, we studied the inhibitory effect of phenobarbital fed simultaneously with the hepatocarcinogen 3'-Me-
DAB on the initiation and promotion of carcinogenesis. This paper reports the results, together with the tumor incidence at week 40.

MATERIALS AND METHODS

Male weanling 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 in a cage. The animals of Groups 1 and 3 were fed a diet (CE-2, CLEA Japan Inc., Tokyo) containing 0.06% 3'-methyl-4'-dimethylamino) azobenzene (3'-Me-DAB) (Tokyo Kasei Co., Tokyo) for 3 weeks. The animals of Groups 2 and 4 were fed a diet containing both 0.06% 3'-Me-DAB and 0.05% phenobarbital (Iwaki Seiyaku Co., Tokyo) for 3 weeks. The animals of Groups 1 and 2 were subsequently fed a basal diet throughout the experiment. The animals of Groups 3 and 4 were fed a basal diet during the 4th and 5th weeks of the experiment, then a diet containing 0.05% phenobarbital for the remainder of the experimental period.

In Experiment 1, some animals of each group were sacrificed at weeks 4 and 12 and all the remaining rats were sacrificed at week 40. With animals killed at week 12, liver tissues were cut from the median lobes and frozen on dry ice. Frozen sections were prepared in a cryostat, dried, fixed in cold formol-calcium solution for several hours, and stained for ATPase by Wachstein and Meisel's method, followed by counterstaining with hematoxylin. The number of ATPase-deficient islands larger than 50 \( \mu m \) in diameter was scored from several sections totaling about 5 \( cm^2 \). With animals killed at week 40, the liver was thoroughly sectioned at 5 mm thickness and the numbers of tumors larger than 5 mm in diameter were scored. In Experiment 2, all the animals were sacrificed at week 12, and the liver was processed in the same manner as in Experiment 1. In this experiment, the numbers of EAI in various size ranges were scored (this was not done in Experiment 1).

In every experiment, part of the liver or tumor tissue was fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined histologically.

RESULTS

The number of enzyme-altered islands (EAI) per 1 cm\(^2\) of the liver section at week 12, and the number of tumors larger than 5 mm in diameter per rat at week 40 observed in Experiment 1 are shown in Table I. In the animals fed phenobarbital simultaneously with 3'-Me-DAB (Groups 2 and 4), the numbers of EAI at week 12 are significantly decreased in comparison with those in the animals fed 3'-Me-DAB alone (Groups 1 and 3). The number of EAI in Group 4 is smaller than that in Group 1, even when the animals of Group 4 were additionally given phenobarbital. In the animals additionally treated with phenobarbital as a promoter (Groups 3 and 4), a striking reduction in the number of tumors at week 40 is seen in Group 4 as compared with that in Group 3. Thus, an inhibitory effect of simultaneous feeding of phenobarbital with 3'-Me-DAB on hepatocarcinogenesis is apparent. In Groups

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Initial treatment (3 weeks)</th>
<th>Enhancement by PB</th>
<th>No. of enzyme-altered islands/cm(^2) liver section at week 12</th>
<th>No. of tumors/rat at week 40*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3'-Me-DAB</td>
<td>-</td>
<td>5.53 ± 2.11 (6)</td>
<td>0.1 (19)</td>
</tr>
<tr>
<td>2</td>
<td>3'-Me-DAB + PB</td>
<td>-</td>
<td>0.68 ± 0.31 (6)</td>
<td>0.1 (10)</td>
</tr>
<tr>
<td>3</td>
<td>3'-Me-DAB</td>
<td>+</td>
<td>8.70 ± 3.73 (5)</td>
<td>3.6 (15)</td>
</tr>
<tr>
<td>4</td>
<td>3'-Me-DAB + PB</td>
<td>+</td>
<td>3.86 ± 2.13 (6)</td>
<td>1.2 (13)</td>
</tr>
</tbody>
</table>

No. in parentheses: No. of effective rats
3'-Me-DAB: Diet containing 0.06% 3'-Me-DAB
3'-Me-DAB + PB: Diet containing 0.06% 3'-Me-DAB and 0.05% phenobarbital
* Tumors larger than 5 mm in diameter
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Table II. Number and Size of Enzyme-altered Islands at Week 12 (Experiment 2)

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>No. of EAI in indicated size range (μm)</th>
<th>Total no. of EAI/cm² liver section</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 ~ 250</td>
<td>250 ~ 500</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>2.22 ± 0.47</td>
<td>0.88 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(70.7%)</td>
<td>(28.1%)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.05 ± 0.34</td>
<td>0.2 ± 0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(83.3%)</td>
<td>(16.2%)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1.49 ± 0.85</td>
<td>2.6 ± 0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(34%)</td>
<td>(56.7%)</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>1.95 ± 0.65</td>
<td>1.02 ± 0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(66.4%)</td>
<td>(33.6%)</td>
</tr>
</tbody>
</table>

EAI: Enzyme-altered island

1 and 2, the tumor incidence at week 40 was very low and there was no difference between the groups.

The numbers of EAI in various size ranges per 1 cm² liver section observed in Experiment 2 are shown in Table II. The total numbers of EAI were decreased in Groups 2 and 4 as compared with those of Groups 1 and 3. This result is similar to that of Experiment 1. However, the extents of reduction were not as great as seen in the Experiment 1, for some unknown reason. It is apparent, however, that in the animals fed phenobarbital simultaneously with 3'-Me-DAB (Groups 2 and 4), the EAI are generally smaller in size as compared with those of animals fed 3'-Me-DAB alone (Groups 1 and 3).

Histological observation of the liver at week 4 revealed megalocytic degeneration of hepatocytes and oval cell proliferation, unique to 3'-Me-DAB feeding,5) in Groups 1 and 3. These changes were much less conspicuous in Groups 2 and 4. Most of the tumors larger than 5 mm in diameter were histologically well-differentiated hepatocellular carcinomas, the rest being hyperplastic nodules.

DISCUSSION

Simultaneous feeding of phenobarbital with 3'-Me-DAB decreased tumor incidence of the rat liver at week 40, as compared with the incidence obtained in animals fed 3'-Me-DAB alone. This result confirms previous observations.5,11) Our study of the liver during carcinogenesis also revealed that simultaneous feeding of phenobarbital resulted in a marked reduction of enzyme-altered islands (EAI), both in number and size, during hepatocarcinogenesis.

EAI are considered to be proliferated progenies of initiated cells.7,12,14) Direct proportionality between the number of EAI and the dose of DEN was observed when the carcinogen was injected into partially hepatectomized rats.14) The reduction in the number of EAI indicates, therefore, an inhibitory effect of phenobarbital on the initiation process of carcinogenesis by 3'-Me-DAB. This effect probably results from stimulation of hepatic drug-metabolizing enzymes by phenobarbital, with the resulting detoxification effect being greater than the activation of the carcinogen. It is also conceivable that phenobarbital might competitively inhibit the binding of the carcinogen with cellular DNA; barbiturate forms strong specific hydrogen bonds with derivatives of adenine.8) Pretreatment with phenobarbital reduced the binding of AAF to nuclear DNA.10)

On the other hand, the reduction in the size of EAI in groups given phenobarbital and carcinogen simultaneously may indicate a reduction by phenobarbital of the promo-
tive effect of the carcinogen by the mechanism mentioned in the introduction. The enhanced detoxification of the carcinogen in the presence of phenobarbital was apparent from the histological observations at week 4, which showed much milder toxic change in the livers of animals of Groups 2 and 4 than in their counterparts. Enhanced urinary and biliary excretions of derivatives of carcinogens upon simultaneous feeding of phenobarbital has been reported, although phenobarbital did not enhance the degradation of AAF after its metabolites became bound to DNA.

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REFERENCES