PERSISTENT CHANGES INDUCED BY SUBCARCINOGENIC DOSES OF 3'-METHYL-4-(DIMETHYLAMINO)AZOBENZENE IN RAT LIVER*1

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Persistence of histological and enzymic alterations induced by subcarcinogenic doses of 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB) was studied in the rat liver. When the animals were fed with a diet containing 0.06% of 3'-Me-DAB for 2 weeks, marked degeneration of hepatocytes occurred and, when dye-diet was given for 4 or 6 weeks, a large number of original hepatocytes were replaced by hepatocytes which seemed to have been transformed from proliferating cholangiolar cells. In accordance with degeneration of the original hepatocytes and proliferation of renewed hepatocytes, activities of glucose-6-phosphatase (G-6-Pase) and acid phosphatase decreased markedly, but cathepsin activity increased and isozyme pattern of aldolase altered. Twenty-four weeks after cessation of the dye feeding for 6 weeks, these biochemical alterations nearly returned to normal, while slight but evident histologically recognizable changes remained. These livers had a focus consisting of altered liver cells which usually showed decreased G-6-Pase activity with occasional decrease in adenosine triphosphatase (ATPase) activity. Such altered cells contained a large amount of glycogen within the cytoplasm which did not disappear even after 12 hr of fasting. When these animals were partially hepatectomized for induction of liver cell regeneration, abnormality in mitosis was found in a considerably high frequency. These persistent alterations were more prominent in rats fed the dye for 4 or 6 weeks than those treated for 2 weeks.

It is generally accepted that hepatocarcinogenesis proceeds through multiple stages. In our previous studies on the early stage of hepatocarcinogenesis by 3'-methyl-4-(dimethylamino)azobenzene (3'-Me-DAB) in the rat liver, rapid proliferation of the cholangiolar cells occurred in the portal area and gradually extended toward the central vein, replacing the degenerated original hepatocytes. It was assumed that, if the dye feeding were continued for more than 4 weeks, the majority of original hepatocytes were replaced by hepatocytes originating from proliferated cholangiolar cells.

When the dye feeding was suspended before 8 weeks and the animals were fed a dye-free basal diet thereafter, carcinoma of the liver was not seen even after a long period of observation in our laboratory. However, it was recently reported that an alteration in the isozyme pattern of aldolase was elicited in the rat liver only by 60-day azo dye feeding, and this was maintained during a 300-day period. Consequently, it seemed quite important to elucidate whether the histological and biochemical changes in the liver completely disappear or persist for a long period when the dye feeding is discontinued in the early stages. The present study was undertaken to examine the persistent alterations in the liver of rats which were administered a subcarcinogenic dose of the azo dye.

MATERIALS AND METHODS

Animals

Male Wistar rats initially weighing 150~180 g were divided into 4 groups, each group consisting of about 40~50 animals. Three groups of animals were fed on a solid diet containing 0.06% of 3'-Me-DAB for 2, 4, and 6 weeks. After cessation of the dye feeding, the animals were fed on a dye-free basal diet throughout the experiment. The animals in each group were subjected to the following examinations at various weeks after cessation of the dye feeding. In some instances, the animals were fasted for 12 hr before killing.

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Light Microscopy. For the histological examination, slices of the liver were fixed in the ice-cold 5% buffered Formalin. In some instances, the whole liver was fixed by perfusion with 1.5% glutaraldehyde through the portal vein. Paraffin sections were routinely stained with Hematoxylin-Eosin or periodic acid-Schiff (PAS) and frozen sections were prepared for Sudan III staining.

Histochemistry. The activities of glucose-6-phosphatase (G-6-Pase) and adenosine triphosphatase (ATPase) were examined by the methods described by Wachstein and Meisel.27,28) Preparation of Homogenates. After cutting off the liver slices for histological examination, the remainder of each liver was washed in an ice-cold 0.25M sucrose and homogenized as described previously.17) Enzyme Assays. The activity of G-6-Pase was measured by essentially the same method as that of Swanson26) as described previously.17) Acid phosphatase activity was determined by the method of Gianetto and De Duve29) with 3-glycerophosphate as a substrate. Both phosphatase activities were determined by measuring the amount of liberated orthophosphate by the method of Fiske and Subbarow.28) Cathepsin activity was measured by the method of Gianetto and De Duve30) using denatured bovine hemoglobin31) as a substrate. The degradation product in the supernatant was measured by the Folin-Ciocalteu reagent, with tyrosine as a standard. To obtain the total enzyme activities of acid phosphatase and cathepsin in the liver, Triton X-100 (0.1%) was added to the reaction medium. Aldolase activity was determined with the liver extract prepared by the method of Masters,19) and assayed by the method of Blostein and Rutter.3) Tris-HCl buffer was used instead of glycylglycine buffer. The reaction mixture was incubated at 37ø for 20 min. The ratio of the activity of fructose 1,6-diphosphate (FDP) to fructose 1-monophosphate (FMP) was obtained. All the reagents for enzyme assay were purchased from the Sigma Chemical Co. (St. Louis).

Estimation of Mitotic Abnormality. Several animals from each group were partially hepatectomized32) at 12 or 24 weeks after cessation of the dye feeding and killed 48 hr after the operation. For control of the aging effect, young male rats, 6 to 8 weeks old, were also partially hepatectomized and killed 31 hr after the operation. Paraffin sections of the liver were cut in 20μ and stained with Feulgen's method. More than 300 mitoses during anaphase and telophase were examined in each liver and percentage of abnormal mitosis (chromosome bridges and acentric fragmentation) was determined.

RESULTS

Histological Findings. After the end of 2 weeks of the dye feeding, parenchymal cells showed swelling and decrease in basophilia of the cytoplasm, with enlargement of nuclei, especially in the periportal and midzonal areas of the liver lobule. Cholangiolar cell proliferation was slight (Photo 1). When the animals were fed on basal diet thereafter, such changes gradually disappeared and the histological appearance returned to normal at 12th week (Photo 2).

After ingestion of the dye for 4 or 6 weeks, diffuse proliferation of cholangiolar cells and small basophilic hepatocytes occurred in the periportal area, and, in some instances, almost entire liver lobule was occupied by these cells (Photo 3). Sometimes, nodular proliferation of original hepatocytes was also observed in the periportal area of the liver lobule (Photo 4).

After cessation of the dye feeding, proliferation of cholangiolar cells became less evident, while the renewed hepatocytes occupied a large part of the liver lobules where the degenerated original hepatocytes disappeared.

At the end of 12th week after cessation of the dye feeding, histological appearance of the liver was restored almost to normal, leaving some cholangiofibrotic lesions (Photo 5) and the foci consisting of various number of altered liver cells (Photo 6). These liver cells showed ample clear cytoplasm which contained a large amount of glycogen and lipid (Photos 9 and 10), but they were not always distinguishable from the surrounding parenchymal cells in the section stained with Hematoxylin and Eosin. Histochemical study of the foci revealed that the altered cells usually showed depressed G-6-Pase activity and, occasionally, depressed ATPase activity (Photos 7 and 8). The mitotic figure was rarely found in the foci and compression of the adjacent parenchyma by the foci was not usually observed.

When the animals were fasted for 12 hr, the PAS reaction in these cells still remained, despite its complete disappearance in the adjacent parenchyma (Photo 10). The incidence of altered liver cell foci and cholangiofibrotic lesions was much higher in the rats given the dye diet for 4 or 6 weeks than those given for 2 weeks. Such changes were also found in high frequency in the liver of rats given the dye diet for 4 or 6 weeks followed by a basal diet for 24 or 56 weeks.
PERSISTENT CHANGES INDUCED BY 3'-ME-DAB

Enzyme Activities

Table I summarizes the enzyme activities in the liver of rats in each group. At the end of 6 weeks of the dye feeding, activities of G-6-Pase and acid phosphatase in the liver were markedly lower than those in the liver of both young and aged normal rats, while the cathepsin activity was two times higher than the normal.

In the liver of rats fed a basal diet for 24 weeks after cessation of the dye feeding for 6 weeks, activities of G-6-Pase and acid phosphatase increased, but they still remained at a lower level than those of the control. On the other hand, the cathepsin activity that increased at the end of 6th week was decreased below the normal value in this period.

As regards the aldolase activity, the total activity which was measured with FDP as a substrate was about 20% lower than the normal throughout the experimental periods, while the activity ratio of aldolase (FDP/FMP) was elevated at the 6th week of dye feeding and it returned nearly to the normal level 24 weeks after withdrawal of the dye.

Frequency of Chromosome Aberrations

The frequency of abnormal mitoses during anaphase and telophase in the 6- to 8-week-old rats was about 2~3%, while it was about 10% in the 6-month-old normal rats. In contrast, it was much higher in the rats fed on the dye-diet for 4 or 6 weeks, followed by basal diet for 12 or 24 weeks. However, it was not significantly high in the rats fed the dye-diet for 2 weeks followed by the basal diet for 12 weeks (Table II).

Table I. Enzyme Activities in Liver of Rats Fed on 3'-Me-DAB Diet and then Basal Diet

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>G-6-Pase (µmol Pi/10 min/g liver)</th>
<th>Acid Pase (µmol/min/g liver)</th>
<th>Cathepsin (µmol tyrosine/10 min/ml liver extract at 37°C)</th>
<th>Aldolase (µmol FDP cleaved/min/ml liver)</th>
<th>Activity ratio of aldolase (FDP/FMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, fed on basal diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>5</td>
<td>56.5±3.93</td>
<td>46.9±4.69</td>
<td>5.57±0.67</td>
<td>1.55±0.10</td>
</tr>
<tr>
<td>Aged</td>
<td>4</td>
<td>61.0±7.72</td>
<td>44.4±2.90</td>
<td>6.25±0.10</td>
<td>—</td>
</tr>
<tr>
<td>Fed on 3'-Me-DAB for 6 weeks</td>
<td>5</td>
<td>24.3±3.84</td>
<td>35.7±6.25</td>
<td>10.45±1.96</td>
<td>1.24±0.10</td>
</tr>
<tr>
<td>3'-Me-DAB for 6 weeks and basal diet for 6 months</td>
<td>7</td>
<td>49.8±4.32</td>
<td>39.5±3.38</td>
<td>4.5±0.66a)</td>
<td>1.29±0.07a)</td>
</tr>
</tbody>
</table>

Values are mean±standard deviation.
a) Five rats were used.

Enzyme Activities

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Table II. Frequency of Mitotic Abnormalities in Liver Cells

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Frequency of abnormal anaphases and telophasesa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, fed on basal diet</td>
<td></td>
</tr>
<tr>
<td>Youngb)</td>
<td>11</td>
</tr>
<tr>
<td>Agedb)</td>
<td>6</td>
</tr>
<tr>
<td>3'-Me-DAB for 2 weeks and basal diet for 12 weeksb)</td>
<td>9</td>
</tr>
<tr>
<td>3'-Me-DAB for 4 weeks and basal diet for 12 weeksb)</td>
<td>6</td>
</tr>
<tr>
<td>3'-Me-DAB for 6 weeks and basal diet for 24 weeksb)</td>
<td>8</td>
</tr>
</tbody>
</table>

a) The rats, 6 to 8 weeks old, were partially hepatectomized and killed 31 hr after operation.
b) The rats were killed 48 hr after hepatectomy.
c) The percentage of abnormal anaphases and telophases (chromosome bridges and acentric fragmentation).
Values are mean±standard deviation.
When the diet containing 0.06% 3'-Me-DAB was given to the rats for more than 4 weeks, degeneration of original hepatocytes and burst of proliferation of cholangiolar cells and small basophilic hepatocytes occurred in the liver. When the dye feeding is discontinued before 6 weeks and animals were fed on the dye-free basal diet thereafter for a long period, such histological alterations in the liver mostly disappeared, and no liver tumor developed. However, the foci consisting of altered liver cells seemed to persist for a considerably long period with cholangiofibrotic changes.

The cells forming the foci had a large amount of glycogen, which remained even after 12 hr of fasting, and many lipid droplets, and showed a decrease in G-6-Pase activity with occasional decrease in ATPase activity. Areas composed of similar cells were observed by several workers in the preneoplastic stage of hepatocarcinogenesis by diethylnitrosoamine. These areas were considered to be one of the precursor lesions of hepatoma by these workers because they showed enzymic changes similar to the hepatoma, appeared to increase gradually in size, and became refractory to some homeostatic regulations in the later stage.

On the other hand, it has been suggested that some changes induced by the subcarcinogenic dose of carcinogen might be irreversible, persisting for a long period, and possibly additive to the next carcinogenic stimuli. Clayton and Baumann reported that, when subcarcinogenic doses of azo dye were given to the rats twice at 1- to 3-month intervals, liver tumors were induced in considerably high frequency. A similar condition is well known in the skin carcinogenesis. It was reported that skin application of subcarcinogenic dose of a carcinogen followed by noncarcinogenic promoting agent led to the development of a skin tumor in a high frequency. Though the exact mechanism of this two-stage carcinogenesis is still obscure, Hennings and Boutwell recently proposed that initial application of a carcinogen in the skin might induce the initiated clone which easily transformed to the neoplastic cell by the promoting agent. It is interesting whether or not the altered cell demonstrated in the present study might correspond to the initiated cell which easily transforms to the neoplastic cell by further carcinogenic stimuli.

The occurrence of these foci was more frequent in the rats given the dye diet for 4 or 6 weeks than those given for 2 weeks. Recently, Hughes reported that, when the rats were fed 0.06% 3'-Me-DAB diet until one week or more after commencement of the dye-induced proliferative response, high incidence of liver tumors was obtained, but cessation of dye feeding immediately after the peak of dye binding to protein resulted in no tumor formation. According to this experiment, it may be assumed that the dye inducing the proliferative response may be somewhat critical for the induction of altered liver cells. In the present study, some differences were observed between the 2 and 4 or 6 weeks of dye feeding, although no liver tumor was formed even by more than one year of observation after the dye feeding, perhaps due to the difference in nutritional condition or genetic property of the animals, etc.

The chromosome damage in liver cells induced by the dye also seemed to persist in the liver of rats fed the dye for 4 or 6 weeks, in accordance with the result of Maini and Stich, while 2 weeks of dye feeding did not result in persistent chromosome damage. Recently, Mironescu reported that the single application of thioacetamide on the regenerating liver, especially during DNA synthetic phase, caused a high incidence of mitotic abnormality. It is assumed that, if the dye feeding were to be continued for more than 4 weeks, the dye might induce chromosome damage in the proliferating renewed hepatocytes with a high incidence, which might remain with incomplete repair for a long period.
Concerning the persistence of biochemical changes, especially of enzyme activities which occurred in the early stage of 3'-Me-DAB feeding, the present results showed that the enzyme activities tended to return to their normal level, but never completely recovered the normal level.

With regard to the aldolase isozyme pattern, Endo et al.\textsuperscript{6)} reported the irreversible fixation of increased level of muscle-type aldolase in the liver of rats fed 3'-Me-DAB diet for 60 days. In addition, they reported that this biochemical change was found in the liver without any appreciable morphological change. However, in the present study, apparent histological alterations with increased abnormalities in mitotic figures and incomplete recovery of enzyme activities seemingly suggest injury in the liver. It was considered that biochemically decreased enzyme activities in the liver might reflect the existence of foci revealing depressed enzyme activities in histochemical examination. It should be necessary to isolate the altered cells from the liver and to determine their biochemical characteristics.

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REFERENCES

15) Inaoka, Y., Gann, 58, 355 (1967).

EXPLANATION OF PLATES

Photo 1. The liver of a rat in the 2nd week of dye feeding. Parenchymal cells in the periportal and midzonal areas of the liver lobule show vacuolation and swelling of the cytoplasm. H-E. ×140.

Photo 2. Twelve weeks after cessation of 2-week dye feeding. Histological appearance of the liver returns almost to normal. H-E. ×140.

Photo 3. In the 4th week of dye feeding. Small basophilic hepatocytes and cholangiolar cells proliferate in the periportal and midzonal areas of the liver lobule. The original hepatocytes remaining in the central area are markedly degenerated and hypertrophied. H-E. ×140.

Photo 4. In the 4th week of dye feeding. The nodular proliferation of hepatocytes with clear cytoplasm in the peripheral area of the liver lobule, compressing the adjacent parenchyma. H-E. ×140.
Photo 5. The cholangiofibrotic lesion, 24 weeks after cessation of 6 weeks of dye feeding. H-E. ×140.

Photo 6. Twenty-four weeks after cessation of 6 weeks of dye feeding. The area composed of altered liver cells with slightly clear and ample cytoplasm. H-E. ×160.

Photo 7. G-6-Pase activity in the altered liver cell focus, 56 weeks after cessation of 6 weeks of dye feeding. ×50.

Photo 8. ATPase activity in the altered liver cell focus, 56 weeks after cessation of 6 weeks of dye feeding. ×140.

Photo 9. PAS reaction of the altered liver cell focus after 12 hr of fasting, 56 weeks after cessation of 6 weeks of dye feeding. ×140.

Photo 10. The altered liver cells show many lipid droplets in the cytoplasm (upper half of the photograph). Sudan III. ×460.

Photos 11 and 12. Chromosome bridges and acentric fragmentation of the mitotic liver cells in the regenerating liver, 24 weeks after cessation of 4 weeks of dye feeding. Feulgen stain. ×1,400.

H-E=Hematoxylin and Eosin stain
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