Bile Acid Involvement in Azo Dye Carcinogenesis

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SUMMARY

3'-Methyl-4-dimethylaminoazobenzene was used to initiate the early stages of liver carcinoma development in Sprague-Dawley rats. Bile acids were analyzed in urine and feces of azo dye-fed and control rats with the use of gas chromatography during the first 66 days of treatment. Seven bile acids were identified in the feces, with deoxycholic acid being the primary excretion product. During the first 36 days of 3'-methyl-4-dimethylaminoazobenzene feeding, the feces showed little variation in bile acid concentration. During the same period, in a total of 112 assays on the urine of control rats, only two showed measurable quantities of bile acids. However, the urine of the dye-fed rats had significant amounts of cholic, hyodeoxycholic, and ursodeoxycholic acids. In three separate experiments, these acids consistently became elevated in the urine in the first 25 days of 3'-methyl-4-dimethylaminoazobenzene feeding. The results obtained were correlated with counts of oval cells in the liver with the use of standard histological procedures. The results showed a close correspondence between maximum urinary bile acid excretions and maximum oval cell proliferation rates, suggesting a relationship between urinary bile acid concentration and "oval cell" proliferation.

INTRODUCTION

When the azo dye 3'MeDAB is fed to rats, it leads to cancer of the liver but of no other organ. One of the most prominent early morphological changes that occurs is perportal oval cell proliferation appearing morphologically like bile duct cells. MacDonald and Pechet (12) studied these cells using tritiated thymidine on common duct-ligated rats. They concluded that the DNA synthesis and mitotic activity of the bile duct epithelium have shown that these cells do proliferate, and new ducts arise from existing bile duct epithelium during this process.

Spain and Griffin (18) studied chemical changes during this same period and observed significant elevations of serum cholesterol in dye-treated rats. Since the degradation of cholesterol to bile acids is probably confined to the liver and has been shown by Suld et al. (19) to take place in the presence of liver mitochondria, there appears to be a link between bile acid concentration and changes in hepatocellular populations.

An early investigation of this relationship was conducted by Mirvish and Gillman (14) with a related dye (4-dimethylaminoazobenzene, butter yellow). Their studies on bile acid composition utilized reversed phase, partition chromatography on bile obtained by cannulation. Although butter yellow did not affect the total production of bile acids in their study, changes in the ratio of di- to trihydroxylated acids and an increase of the bile volume were observed.

Bile duct cell proliferation has now been described in the literature as related to specific bile acids. Hunt et al. (9) and Leveille et al. (10) have shown that lithocholic acid induces ductular cell reaction in a variety of animal species when incorporated in the diet at concentrations as low as 0.1%. Carey and Williams (3) have observed this acid in patients with jaundice, Sandberg et al. (16) have observed cholic acid levels to be significant in bile duct atresia, and chenodeoxycholic acid was found to be elevated in portal cirrhosis.

This study utilizes gas chromatography as an analytical tool in order to observe the extent to which bile acids were involved in the early stages of the 3'MeDAB carcinogenesis process. Primarily, the establishment of the chronological sequence of events that led to oval cell proliferation and its relation to bile acid levels was investigated. Each rat was studied both histologically and chemically to determine what relationship might exist between the bile duct cells and bile acid levels.

MATERIALS AND METHODS

Newly weaned 170- to 190-g male albino rats of the Sprague-Dawley strain were maintained on a purified diet (7) containing 0.06% 3'MeDAB, with control rats on the same diet without the azo dye.

Three series of tests were run. Experiment 1 was designed to quantitatively determine the amount of each bile acid in rat feces and urine as the 3'MeDAB was fed over a period of 36 days. Specimens were collected on alternate days from a pair of controls and 4 azo dye-fed rats. The results obtained from pooled specimens on these animals were reduced to a per-rat-per-24-hr basis. Experiment 2, designed to quantitatively determine the amount of each bile acid in rat urine was run for 66 days on 42 rats. For each day indicated, 2 rats were maintained on 3'MeDAB and 1 was kept on the control diet. Each rat was kept in an individual cage. All rats were placed on the diet at the same time and fed in pairs to maintain good comparison with the control. All tissue specimens were fixed.

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in acetic acid-alcohol-formalin for 2 days. The rats were sacrificed at the same time each day in order to avoid any diurnal variation. Experiment 3 was designed to duplicate the work done in Experiment 2 but was carried only to the 46th day with a total of 33 rats, of which 22 were carried on azo dye. In this experiment, a 1:1000 dilution of mercuric chloride solution was added to the urine collection vessel to ensure that bacterial growth in these samples was not causing any chemical changes in the bile acids during the collection period.

Gas chromatography was done on a F & M Model 500 with an F & M Model 1609 flame ionization attachment, following the basic conditions described by Bloomfield (2). The column was a 6-ft coil, 0.25 inch o.d. packed with 0.75 to 1.0% SE-31 on 80- to 100-mesh Chromosorb W. Temperatures used were: injection port, 290°; detector, 270°; column, 225 to 265°, depending on the most satisfactory response of each column. The carrier gas flow (helium) was maintained at 76 ml/min, the hydrogen for the flame was maintained at 48 ml/min, and the air for the flame was maintained at 360 ml/min.

Reagent grade chemicals were used in all but the following cases: Diazald (N-methyl-N-nitroso-p-toluene sulfonamide) from the Aldrich Chemical Co., Milwaukee, Wis., was used to prepare diazomethane according to the method of Schlenk and Gillerman (17). Bile acid standards were purchased from Calbiochem, Los Angeles, Calif.; Nutritional Biochemicals Corp., Cleveland, Ohio; and K & K Laboratories, Plainview, N.J. 12-Ketolithocholic acid, which proved to be important in any chemical changes in the bile acids during the collection period. The cells were counted with the 43X objective and 10X oculars with a standard counting grid.

The feces were extracted with a 50% (v/v) solution of ethanol-acetone in a blender. The urine samples were measured and filtered. Both the feces and urine samples were hydrolyzed in an autoclave for 3 hr at 20 psi (gauge) in 1.25 N NaOH. Extractions were done with chloroform after the samples were acidified to pH 1 with 6 N HCl. Methyl esters were prepared with diazomethane. The hydroxyl groups were acetylated with acetyl chloride for 30 min at 60°. The samples were dried with a stream of nitrogen and taken up to 0.5 ml with ethylene dichloride.

<table>
<thead>
<tr>
<th>Parent compound</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.88</td>
</tr>
<tr>
<td>3α-Hydroxycholanic acid (lithocholic acid)</td>
<td>1.00</td>
</tr>
<tr>
<td>3α-Hydroxy-12-ketocholecholic acid (12-ketocholecholic acid)</td>
<td>1.12</td>
</tr>
<tr>
<td>3α,12α-Dihydroxycholanic acid (deoxycholic acid)</td>
<td>1.34</td>
</tr>
<tr>
<td>3α,7α-Dihydroxycholanic acid (chenodeoxycholic acid)</td>
<td>1.53</td>
</tr>
<tr>
<td>3α,7α,12α-Trihydroxycholanic acid (cholic acid)</td>
<td>1.72</td>
</tr>
<tr>
<td>3α,6α-Dihydroxycholanic acid (hyodeoxycholic acid)</td>
<td>2.01</td>
</tr>
<tr>
<td>3α,7β-Dihydroxycholanic acid (ursodeoxycholic acid)</td>
<td>2.24</td>
</tr>
</tbody>
</table>

RESULTS

Feces Studies. In Experiment 1, 7 bile acids and cholesterol were demonstrated in the feces. Chart 1 illustrates the chromatographic peaks found in a normal rat after 4 days of synthetic diet. Although 12-ketolithocholic acid was not always observed in rats on a chow (Purina) diet, it soon equaled the concentration of lithocholic acid on the synthetic (basal) diet.

The feces of the Sprague-Dawley rats on a basal diet and azo-basal diet showed deoxycholic acid to be the primary excretion product. Five other bile acids were observed, with cholic and chenodeoxycholic found in the smallest concentrations. These are shown in Table 2, which is arranged in order of the retention times of the bile acids. These results on rat feces were qualitatively consistent with those found by Makita and Wells (13), although quantitatively about 5 times...
Table 2

Experiment 1: mean ± S.D. of fecal bile acids found in 36-day study

<table>
<thead>
<tr>
<th>Acid found</th>
<th>Pooled control basal diet-fed rats (mg/day)</th>
<th>Pooled treated azo diet-fed rats (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithocholic</td>
<td>2.2 ± 0.6</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>12-Ketolithocholic</td>
<td>2.2 ± 0.8</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>Deoxycholic</td>
<td>4.7 ± 1.5</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>Chenodeoxycholic</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Cholic</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Hyodeoxycholic</td>
<td>2.0 ± 0.9</td>
<td>1.5 ± 0.7</td>
</tr>
</tbody>
</table>

as much total bile acids were found in the feces per day as they reported. A typical gas chromatogram of normal rat feces is shown in Chart 1.

As indicated in Table 2, there was little significant variation in the feces assays over the first 36 days of 3'MeDAB feeding. However, the daily output of bile acids did decrease on an overall basis from 12.8 mg/day in the controls to 11.0 mg/day in the azo-fed group at the 36th day. Although production of all acids decreased, the major change came in cholic and hyodeoxycholic acid. Cholic, a trihydroxylated acid, decreased to approximately 50% of its normally observed level. Hyodeoxycholic, a dihydroxylated acid, decreased about 25%. During the same period, these acids appeared in the urine.

Urine Studies. In Experiments 1, 2, and 3, 5 bile acids were identified in the urine of the azo dye- and basal diet-fed rats. These were lithocholic, deoxycholic, cholic, hyodeoxycholic, and ursodeoxycholic. A peak with a retention time of 2.78 (lithocholic taken as 1.00) was also observed but was not identified. Assays have shown that it is not dehydrocholic acid, although it migrating between this acid and ursodeoxycholic acid.

In the basal diet-fed rat, very little was observed in the urine assays. In a total of 112 assays on control diet-fed rats, only 2 showed measurable quantities of bile acids in the urine with the use of the method described in this paper. On the 15th day, a control rat showed 0.02 mg/day of hyodeoxycholic acid, and on Day 66 a control rat showed 0.01 mg/day of lithocholic acid.

The bile acids seen in the azo diet-fed rats were predominantly cholic acid, hyodeoxycholic acid, and ursodeoxycholic acid (Chart 2). The distribution of these acids during the period of greatest elevation is shown in Table 3 for all 3 series of experiments.

<table>
<thead>
<tr>
<th></th>
<th>Cholic (mg/rat/day)</th>
<th>Hyodeoxycholic (mg/rat/day)</th>
<th>Ursodeoxycholic (mg/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.24±</td>
<td>0.41</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>0.29±</td>
<td>0.20</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.21±</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.00±</td>
<td>0.02</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Values are the averages of 2 rats.

Liver Tissue Studies. Observations of oval cell proliferation by standard hematoxylin-and-eosin technique were done on all rats in Experiments 2 and 3 and were comparable to those shown by other authors (2, 6). The bile duct cells increased in numbers of total population from 1% in control animals to as high as 41% in 46 days of 3'MeDAB feeding. Two thousand cells were counted on each slide. In order to obtain a random distribution, the technique of Chalkley (5) was used. Two investigators counted each slide, and the total cells counted represents their combined results.

A graphical representation of the relation between bile dict cell proliferation and total bile acid levels is shown in Chart 3, which presents the data from Experiment 2. The oval cell proliferations seen in the azo dye-fed and control rats of Experiment 3 peaked on the same day as that shown in Experiment 2. The maximum for the bile acids found in that run and in Experiment 1 are shown in Table 3.

Chart 3. Comparison of oval cell proliferation to total bile acids observed in the urine of 3'MeDAB-fed rats. Data from Experiment 2, which was run for 66 days. Experiments 1 and 3 presented the same relationships at the same time periods.
DISCUSSION

The periportal oval cell proliferation seen in a variety of liver abnormalities has been intensively studied. The work of MacDonald and Pachet (12) using tritiated thymidine showed that these cells did apparently arise from a true proliferation of the bile duct epithelium. Electron microscopic observations led Grisham and Hartroft (8) to conclude that these cells resemble cells from bile ductules and to propose that these cells should be called ductular rather than oval cells. Their origin still remains uncertain, however. Although Carruthers and Steiner (4) show evidence, using the electron microscope, that oval cells may arise from bile duct cells, Oneo et al. (15), after an electron microscopic study of azo dye-induced liver cancer, reported that these tumor cells were variable in their ultrastructure and difficult to differentiate. However, they concluded that oval cell proliferation played the most important role in the variability of these tumor cells. Observations of Ma and Webber (11) on 3′MeDAB tumors revealed that electron microscopic examination of the ductal structures formed could be traced partly to the hepatocyte and partly to the ductal cell.

The reactions that are taking place within these cells have been studied to give further insight into the problem. Spain and Griffin (18) first observed elevated bilirubin and cholesterol levels in azo dye-fed rats and reasoned that this might be the result of occlusion of the normal bile passages during azo dye feeding. Although bile acids were not normally detected in any of the control urines reported in this gas chromatographic study, they consistently became elevated in urine after the 25th day of feeding the dye. Histological sections of the liver tissue show a bile duct cell population rising in a logarithmic phase during this same period. The close relationship of these results indicates that the bile acids could be the preliminary stimulus to oval cell proliferation and that the azo dye is acting to stimulate the production of these bile acids.

An alternate explanation of the phenomenon would be that the proliferation of the oval cells is the cause of the bile acid accumulation. However, the work of Hunt et al. (9), Carey and Williams (3), Sandberg et al. (16), and others has indicated that at high levels specific bile acids can induce bile duct cell proliferation and even atresia. Further unpublished work in our laboratory has indicated that hyodeoxycholic acid and ursodeoxycholic acid do cause extensive bile duct cell proliferation in both azo dye-fed rats and those fed only the bile acids with their normal diets. From this evidence, therefore, it would seem reasonable to assume that the elevation of bile acids seen in the urine studies are probably stimulating bile duct cell development.

ACKNOWLEDGMENTS

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