ABSTRACT

The effects of feeding a choline-deficient (CD) diet or a choline-supplemented diet upon the early stages of DL-ethionine carcinogenesis in rat liver were investigated. Low levels of DL-ethionine (0.05 and 0.10%) when fed with a CD diet were found to induce within 4 weeks a massive proliferation of oval cells without significant cell necrosis or presence of inflammatory cell infiltrates. The same levels of ethionine when fed with a choline-supplemented diet caused no significant histological alteration of the liver. In rats fed the CD plus ethionine diets concomitant with the proliferation of oval cells, there was a marked elevation in the content of \( \alpha_f \)-fetoprotein in both liver and plasma. After specific immunofluorescence staining, oval cells stained intensely for albumin and \( \alpha_f \)-fetoprotein. Hepatocytes stained only for albumin, and bile duct cells stained for neither albumin nor \( \alpha_f \)-fetoprotein. These results indicate that a diet deficient in choline markedly alters the response of rat liver to carcinogenetic doses of ethionine. Thus, ethionine hepatocarcinogenesis in rats fed a CD diet may be a useful model for the exploration of the mechanism(s) whereby a dietary factor influences hepatocarcinogenesis.

INTRODUCTION

Although nutritional and dietary factors play a role in the etiology, pathogenesis, or evolution of cancer (36, 37, 41), little is known about the mechanism(s) underlying nutritional modifications of the carcinogenic process. A combined deficiency of lipotropic factors (choline, folic acid, vitamin \( B_12 \), and methionine) has been reported to enhance the induction of tumors in the liver, colon, and esophagus of rats by several chemical carcinogens (25, 26, 28). Furthermore, evidence has been presented indicating that the combined lipotrope deficiency alters the activation of chemical carcinogens by the liver drug-metabolizing enzymes, thus modifying the action of carcinogens on target tissues (26, 29).

For a number of years this laboratory has been studying the structural and functional alterations occurring in the liver of rats fed a diet deficient in choline (18, 19, 32). Recently, we have initiated a series of experiments for the determination of whether a diet deficient in choline is able to modify the process of hepatocarcinogenesis in the rat. DL-Ethionine was selected as the hepatocarcinogen because of its well-known potency in this respect (7) and because it is an antagonist of methionine and, as such, is an inhibitor of choline biosynthesis (7). When fed to rats, low levels of ethionine incorporated into a CD diet were found to induce a massive proliferation of oval cells in the liver and a marked elevation of AFP in plasma. The temporal relationship between oval cell proliferation and elevation of plasma AFP has been analyzed, and a search was made to identify the AFP-producing cells in the liver. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, Pa.) weighing 130 to 150 g were used. Semipurified CD and CS diets were prepared according to basal B diet of Young et al. (42). The composition of these diets is shown in Table 1. The mixture of proteins in these diets was so chosen as to provide an adequate (15%) level of proteins but a limited (0.2%) amount of methionine, in view of the efficient endogenous synthesis of choline from methionine in the rat. In this respect, therefore, the CD diet is, unavoidably, also marginally deficient in methionine. However, the diets contain adequate supplements of folic acid and vitamin \( B_12 \). DL-Ethionine (Sigma Chemical Co., St. Louis, Mo.), at levels of 0.05 or of 0.10%, was incorporated into the CD or CS diet at the expense of sucrose. Fresh batches of the diets were prepared every week. Diet intakes were recorded on 3 consecutive days every 2 weeks.

In 1 experiment, groups of rats were fed ad libitum the plain CD or CS diets or the same diets containing 0.05 or 0.10% ethionine. Three to 6 rats in each group were killed after 1, 2, 4, and 10 weeks. The liver was examined histologically, and serum SGPT and total bilirubin were determined. AFP determinations were made on the liver and plasma of rats fed the various diets for 4 and 10 weeks; sections of the same livers were stained for albumin and AFP by immunofluorescent methods. In a second experiment, a total of 18 rats were fed the 4 different diets (CD, CS, and CD and CS plus 0.05% ethionine) for up to 4 weeks. Blood samples were obtained by cardiac puncture after 1 and 3 days and 1, 2, 3, and 4 weeks, and plasma AFP was determined.

For light microscopic examination, portions of the livers were fixed in Stevie's solution, and sections were stained with hematoxylin-eosin. Mitotic counts were made exclusively on hepatic parenchymal cells. Three or 4 slides from

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2 The abbreviations used are: CD, choline deficient; AFP, \( \alpha_f \)-fetoprotein; CS, choline (0.8%) supplemented; SGPT, alanine aminotransferase.
each liver were examined under a high-power objective. Liver parenchymal cells in 25 to 30 randomly selected fields were counted, and the number of mitoses was scored. For immunofluorescence, small blocks of liver tissue were fixed in cold 95% ethanol, dehydrated, and embedded in paraffin at 56°C. Cut sections were rehydrated by rapid passages through xylene, ethanol at diminishing concentrations, and phosphate-buffered saline. The sections were then stained with a double-antibody method previously described (40). The first antibody was specific goat antisera to AFP or albumin; the second antibody was fluorescein-labeled IgG from specific burro anti-goat IgG serum. AFP was measured in blood plasma and liver by radioimmunoassay (32, 33). Serum SGPT and total bilirubin were measured by the Dupont automatic analyzer based on the modified methods of Bergmeyer and Brent (3) and Van den Bergh and Shapper (39), respectively.

RESULTS

The customary level of DL-ethionine added to the diet for tumor induction experiments is 0.25% (7). However, this level in a CD diet proved to be too toxic for the rat. Levels of 0.10 and 0.05% instead were fairly well tolerated by the animals and still induced reproducible histological and functional changes in the liver.

During the first 2 weeks, the diet intake was similar in the 4 groups of animals and ranged between 10.2 and 14.1 g/day. It increased gradually and similarly thereafter in rats fed the CS, CD, or CS plus ethionine diet to a maximum of 22.2 to 25.1 g/day. However, the diet intake of the rats fed the CD plus ethionine diet dropped by the fourth week to and remained throughout at about 50% that of the other groups of rats.

In the histological analysis of the liver, 4 alterations were particularly scrutinized: fatty infiltration, cell necrosis, oval cell proliferation, and parenchymal cell mitoses. In rats fed the CS diet, the appearance of the liver was normal. The overall alterations in the liver of the other groups of rats are summarized in Table 2 and are illustrated in Figs. 1 to 4. In rats fed the CD diet, the initial reaction was a severe fatty liver that persisted for up to 10 weeks (Fig. 1). Necrosis of isolated fat-engorged hepatocytes was present after 2 weeks of feeding. Oval cells and mitoses of parenchymal cells were not observed. Addition of 0.05 and 0.10% ethionine to the CD diet markedly reduced the severity of the fatty liver within 1 week (Fig. 2). During the first 2 weeks in which these diets were fed, hepatocytes showed a mild degree of swelling and of loss of cytoplasmic basophilia. Necrosis of parenchymal cells was not apparent, and there was no distortion of the lobular architecture. After 4 weeks of feeding, the most striking alteration seen was a massive proliferation of oval cells (Fig. 3). These small, oval cells with elongated, vesicular nuclei and scant cytoplasm were most prominent in the perportal areas but penetrated the liver lobule. Mitosis of oval cells were numerous and conspicuous. All animals fed the CD plus ethionine diets showed this cell reaction, except 1 animal fed the lower level of ethionine. At this stage, the parenchymal cells showed varying degrees of swelling and of cloudy or hyaline degeneration. Although necrosis of single hepatocytes was noted occasionally, there were neither foci of necrosis nor inflammatory cell infiltrates. Mitosis of parenchymal cells were extremely rare (1 to 2 mitoses per 3000 cells counted). Despite the diffuse proliferation of oval cells and the other changes noted, the lobular architecture of the organ was completely disrupted by the proliferating cells, which isolated single hepatocytes or clusters of a few hepatocytes. Necrosis and mitosis of parenchymal cells were negligible. These changes were again relatively uniform in all animals but were absent in 1 rat fed the lower level of ethionine; in this animal the liver showed a mild fatty infiltration without much oval cell proliferation. In rats fed the CS plus ethionine diets, there was no notable histological alteration of the liver during the first 4 weeks. After 10 weeks of feeding, 1 of 6 rats showed a minimal proliferation of oval cells and a mild fatty liver without distortion of the architecture of the organ; the other animals showed no notable alterations.

Immunofluorescent staining for albumin and for AFP was performed on liver sections from rats fed the various diets for 4 and 10 weeks. In rats fed the CS diet, there was random staining of approximately 50% of the hepatocytes for albumin and no staining for AFP. The hepatocytes of rats fed the CD diet stained poorly for albumin due to the extensive accumulation of fat and did not stain at all for AFP. In rats fed the CS plus ethionine diets, the pattern of staining was comparable to that in rats fed the plain CS diet. However, one of the rats had a minimal, periportal proliferation.
of oval cells, and some of these cells stained positively for both albumin and AFP. The hepatocytes of rats fed the CD plus ethionine diets stained weekly for albumin and did not stain at all for AFP. In the same animals, strongly positive staining for both albumin and AFP was displayed by many oval cells, especially those in the periportal areas. In rats fed the CD plus ethionine diets for 10 weeks, ductular cells did not stain for either albumin or AFP; in these animals, newly formed ductules were very conspicuous due to their absence of specific immunofluorescent stainings.

The results of serum SGPT and total bilirubin determinations are shown in Table 3. In rats fed the CS diet, there were minor variations in the levels of both SGPT and total bilirubin with length of treatment. In rats fed the CD diet, the levels of total bilirubin were comparable to those in rats fed the CS diet, but the levels of SGPT were elevated, especially after 2 and 4 weeks of treatment. These elevations are probably accounted for by the necrosis of fat-engorged hepatocytes that was noted histologically at these times. In rats fed the CS plus ethionine diets, there was only a mild elevation of the level of total bilirubin after 4 and 10 weeks of treatment. Significant elevations in the level of total bilirubin occurred in rats fed the CD plus ethionine diets for 2, 4, and 10 weeks. Swelling of parenchymal cells and perportal infiltration of oval cells may cause a disturbance in the normal flow of bile and may be responsible for the increased levels of serum bilirubin. No, or only slight, elevations in the levels of serum SGPT were present in rats fed the CD plus ethionine diets. This result is probably accounted for by the decrease in the severity of the CD fatty liver afforded by ethionine, as noted in the histological examination of the livers.

Chart 1 shows the results of AFP quantification in the plasma and liver of rats fed the various diets. During the first and second weeks of feeding, there was a tendency toward a very slight elevation in the levels of plasma AFP. Thereafter, the levels declined gradually in rats fed the CS and CD diets. They rose sharply in rats fed the ethionine-containing diets, reaching after 4 weeks maximum values of 0.269 ± 0.095 and 25.95 ± 11.10 µg/ml in rats fed the CS plus ethionine and CD plus ethionine diets, respectively. In rats fed the CD plus ethionine diet, the concentrations of AFP in the liver were 1.70 ± 0.76 and 5.31 ± 4.84 µg/g at 4 and 10 weeks of treatment, respectively. In all other groups, the concentration of liver AFP ranged between 0.020 and 0.030 µg/g, except in the group of rats fed the CS diet for 10 weeks in which the mean concentration was 0.256 ± 0.126 µg/g due to an exceptionally high value in 1 of 3 rats.

**DISCUSSION**

The results presented in this paper demonstrate clearly

| Table 2 |

<table>
<thead>
<tr>
<th>Experimental duration (wk)</th>
<th>Type of lesion</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD</td>
</tr>
<tr>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>+++</td>
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<tr>
<td>10</td>
<td>+++</td>
</tr>
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| Table 3 |

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>SGPT (IU/ml)</th>
<th>Bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 wk</td>
<td>2 wk</td>
</tr>
<tr>
<td>CS</td>
<td>14.3 ± 5.9⁣</td>
<td>26.0 ± 5.6</td>
</tr>
<tr>
<td>CD</td>
<td>35.3 ± 5.7</td>
<td>179.7 ± 65.1</td>
</tr>
<tr>
<td>CS + ethionine (0.05%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CS + ethionine (0.1%)</td>
<td>32.3 ± 8.0</td>
<td>20.3 ± 1.8</td>
</tr>
<tr>
<td>CD + ethionine (0.05%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD + ethionine (0.1%)</td>
<td>29.7 ± 1.5</td>
<td>32.0 ± 7.9</td>
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⁣ Mean ± S.E. of 3 to 6 rats.

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that the early response of rat liver to ethionine is markedly influenced by the presence or absence of choline in the diet. Three findings in rats fed ethionine with a CD diet stand out: a massive proliferation of oval cells in the liver; a striking increase in the level of AFP in both serum and liver; and positive immunofluorescent localization of albumin and of AFP in oval cells. These responses are completely absent or much less pronounced when ethionine is fed with a CS diet for a comparable period of time. The responses are elicited by levels of dietary ethionine considerably lower than those customarily used in tumor induction experiments. However, these levels are carcinogenic in rats fed a CD but not a CS diet. Thus the responses elicited thereby are relevant to the process of hepatocarcinoma induction. It is apparent then that besides a combined deficiency of several lipotropic agents (25, 27, 28), a dietary deficiency of choline can alter the response of target tissues to chemical carcinogens. However, the model of choline deficiency and chemical carcinogenesis is a simpler one and as such may offer an advantage in the exploration of the mechanism(s) whereby a dietary factor influences hepatocarcinogenesis.

A dietary deficiency of choline is known to limit the induction of liver drug-metabolizing enzymes (4, 31) and therefore may lead to alterations in the metabolism of ethionine such as to be responsible for the altered response of rat liver to the carcinogen. Structural and functional alterations of the membranous organelles of hepatocytes occur in rats fed a CD diet (18, 31). Furthermore, in the same rats ethionine administration leads to transethylation of phospholipids in the liver with synthesis of significant amounts of the ethyl-choline analogs of lecithins (S. L. Katyal and B. Lombardi, unpublished observation) that become abnormal constituents of hepatocyte membranes. In all probability, this synthesis of lecithin analogs is responsible also for the reduced severity of the choline deficiency fatty liver afforded by ethionine (20, 34). Thus, alterations in the properties of cell membranes could constitute an alternate explanation for the different response of the liver to ethionine in CD or CS rats.

Proliferation of oval cells is induced, with a few notable exceptions (11, 14, 21), by virtually all hepatic carcinogens shortly after they are fed to experimental animals (6, 8). In many instances, however, the proliferation follows the appearance of widely scattered liver cell necrosis and is accompanied by inflammatory cell infiltrates and fibrosis. In these experiments, histological (Table 2) and serum SGPT (Table 3) data indicate that no significant hepatocyte necrosis or inflammatory infiltrates precede or accompany the proliferation of oval cells in the liver of rats fed ethionine with the CD diet. Actually, in these animals the extent of hepatocyte necrosis after 2 weeks of treatment was less than that in the comparable groups of rats fed the plain CD diet and similar to that in the other groups of rats treated for the same length of time. It is unlikely, therefore, that hepatocyte necrosis was the stimulus for the proliferation of oval cells. It is probable, instead, that this stimulus was provided by the carcinogenic regimen.

The magnitude of the increase in the level of plasma AFP, observed in rats fed ethionine with a CD diet, is much greater than that has been observed after administration of ethionine with other modalities (35) or after feeding of other chemical carcinogens to rats (1, 2). Recent studies by several investigators have established clearly that AFP increases in the serum of rats during the early stages of chemical carcinogenesis, well before there is any evidence of hepatocarcinoma (1, 2, 5, 15, 16, 22). However, the genesis and significance of these findings are as yet not completely understood. In particular, conclusive evidence is still lacking concerning the type of cell that synthesizes AFP. Okita et al. (22), using immunofluorescence techniques, showed that hepatocytes of hyperplastic nodules are the major site of localization of AFP during 2-acetylaminofluorene carcinogenesis. Others have reported that oval cells contain AFP. For instance, Dempo et al. (5) and Onda (23) showed that in the early stage of dimethylaminoazobenzene carcinogenesis, AFP-producing cells are localized in clusters of proliferating oval cells and suggested that transitional cells, possible intermediates of oval cells and mature hepatocytes, produce AFP. On the other hand, Becker and Sell (2) observed that extremely low doses of 2-acetylaminofluorene that cause no obvious histological alteration in the liver induce elevation of Serum AFP and postulated that the elevation is due to a direct interaction of the carcinogen with the genome of hepatocytes. In these studies, a striking increase in the level of plasma AFP (Chart 1) occurred in the absence of any significant rate of hepatocyte mitosis (Table 2). The increase occurred very early during the ethionine treatment without any observable regenerative or hyperplastic nodules of hepatocytes. There was a remarkable positive correlation between the time of emergence in the liver of numerous oval cells and the intensity of their proliferation on one hand and the time of onset and magnitude of the AFP increase in the plasma on the other. Moreover, specific immunofluorescent staining of liver sections for AFP showed that only oval cells were positive while no staining of hepatocytes and ductular cells was present. Preliminary results identical with the latter also have been obtained in other studies still in progress in

\[\text{Chart 1. Results of AFP determination in plasma and liver of rats fed various diets. Eth., ethionine. Points, mean ± S.E. (bars) of 3 to 6 rats.}\]
our laboratories. Oval cells have been isolated from the livers of rats fed ethionine with a CD diet for 4 weeks and have been separated from the hepatocytes of the same liver. When preparations of isolated oval cells and of isolated hepatocytes were stained separately forAFP, only the former were positive. Although it is not known what casual relationship, if any, exists between AFP-positive oval cells and subsequent development of hepatocellular carcinoma, it is possible that such oval cells may represent the earliest cell population that eventually progresses to liver cancer. Further functional and cytological studies in a well-defined experimental condition, such as described here, also seem warranted in view of the observation made in these studies of a positive localization of albumin in oval cells. As far as we are aware, this is the first report of such a finding, and it suggests a potential for differentiation of oval cells into mature hepatocytes. Differentiation of oval cells into bile ductular cells was clearly observed in these experiments, as well as in those of others (6, 10, 30). Differentiation of oval cells also into mature hepatocytes has been suggested to occur by Onoe et al. (24), by Inaoka (13), and by Uriel et al. (38) on the basis of their ultrastructural, cytochemical, and radioautographic studies. As to the origin of oval cells, the prevailing view at this time seems to be that they are bile ductular cells (6, 10, 17, 30). However, the view has been presented by Hadjilov (12) and by Onda (23) that oval cells are or originate from a type of primitive blast cell present also in normal liver.

The liver of rats fed low doses of ethionine with a CD diet for 3 to 4 weeks is a very good source of oval cells. From a single liver, a large number of cells can be isolated sufficient not only to establish cell cultures in vitro and for morphological studies but also for direct metabolic studies (unpublished observations). Therefore, it should be possible, in the future, to find satisfactory answers to the many unresolved questions that still exist concerning the properties of these cells. Furthermore, large numbers of bile ductular cells can be isolated from the liver of rats subjected to ligation of the common bile duct (9). By comparing the properties of isolated oval cells from carcinogen-treated animals with those of isolated ductular cells, it may be possible eventually better to define the origin and nature of oval cells that are so ubiquitous in liver carcinogenesis.

REFERENCES

Effects of CD Diet on Rat Liver


Fig. 1. Liver from a rat fed CD diet for 1 week. A severe fatty liver is apparent. H & E, x 110.

Fig. 2. Liver from a rat fed CD diet supplemented with 0.1% dl-ethionine for 1 week. Note the reduction in the severity of the fatty liver and the slight disorganization of the lobular pattern. H & E, x 110.

Fig. 3. Liver from a rat fed a CD diet supplemented with 0.1% dl-ethionine for 4 weeks. Extensive proliferation of oval cells that penetrate into the hepatic lobules. The liver cell cords are disrupted, and the remaining parenchymal cells show swelling of the cytoplasm. H & E, x 110.

Fig. 4. Liver from a rat fed a choline-deficient diet supplemented with 0.1% dl-ethionine for 10 weeks. Oval cell proliferation persists, and the cells tend to form small ductules. H & E, x 110.
Early Histological and Functional Alterations of Ethionine Liver Carcinogenesis in Rats Fed a Choline-deficient Diet

Hishashi Shinozuka, Benito Lombardi, Stewart Sell, et al.


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