Cellular Analysis of Liver Carcinogenesis: the Induction of Large Hyperplastic Nodules in the Liver with 2-Fluorenylacacetamide or Ethionine and Some Aspects of Their Morphology and Glycogen Metabolism

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SUMMARY

A dietary regimen is described whereby large hyperplastic nodules can be obtained in over half the rats fed either of the two hepatocarcinogens, ethionine or 2-fluorenylacacetamide. The nodules are composed predominantly of hepatocytes which show morphologic and biochemical differences from the surrounding nonhyperplastic liver.

The glycogen or its metabolic control are different in the nodule in that a significant amount of glycogen is present even after a 48-hour period of fasting and little breakdown is induced by glucagon administration. The nodules show a consistent and progressive decrease in glucose-6-phosphatase activity as well as in glycogen phosphorylase activity. Evidence implicating the hyperplastic nodule as a step in the carcinogenic process is presented and discussed.

INTRODUCTION

The histogenesis of a neoplastic process, despite its evident importance to the elucidation of the mechanism of carcinogenesis, has yet to be worked out for even a single cancer of any organ or tissue. Although the literature records many studies of the histogenesis of liver cancer in man or of liver cancer induced by hepatic carcinogens in experimental animals, our knowledge about cell transformations and about cells of origin of liver neoplasia is still very meager. The present study was designed with the hope of obtaining further insight into the cellular components of stages of liver carcinogenesis and with the metabolic characterization of such components.

Many studies of liver carcinoma in humans have long suggested an important precursor role for the hyperplastic nodule for the cancer arising in liver cirrhosis (e.g., Refs. 1, 55). Also, the majority of studies of the histogenesis of liver cancer with chemical carcinogens have implicated the hyperplastic nodule as a probable precursor lesion (16, 44, 46, 57). Yet, despite the obvious interest in this lesion, there is almost no solid evidence for the conclusion that hepatocellular neoplasm arises from areas of nodular hyperplasia.

The study of the progression of the carcinogenic process in terms of cellular and molecular events requires the availability of experimental model systems in which a site of origin of cancer (a) can be identified, (b) consists of a uniform or reasonably uniform cell population, and (c) is sufficiently large to enable gross identification for isolation and appropriate biochemical, morphologic, and biologic investigations. It was observed in a previous study that animals fed homocystine or thymine in an ethionine-containing diet developed large hyperplastic nodules in the liver (13, 14). Atypical areas, histologically indistinguishable from well-developed hepatocellular carcinomas, were present in several nodules. These hyperplastic nodules seemed to offer all three of the requirements for a study in depth of hepatocarcinogenesis.

Unfortunately, many experiments presumably performed under experimental conditions similar to those used in the original experiment had failed to give completely reproducible findings. However, repeated efforts over the past several years have led to the development of regimens which, in over half the animals, induce hyperplastic nodules of sufficient size to allow their study with a variety of approaches. The description of the regimens and some morphologic and metabolic characteristics of the nodules induced with either ethionine or 2-fluorenylacacetamide (FAA) form the subject of this communication.

MATERIALS AND METHODS

Male, white Wistar rats (Carworth Farms) weighing from 150 to 200 grams were used. The basal diet was that described previously (12). The animals, three to a cage, were housed in screen-bottomed cages in an air-conditioned room. In all experiments, both FAA and ethionine were added to the basal diet in place of a corresponding amount of sucrose. For induction of hepatocellular nodules with ethionine, the basal diet containing progressively increasing concentrations of DL-ethionine (Calbiochem) was fed as described in Table 1. In the case of FAA, the animals...
were fed alternately the basal diet containing 0.05% FAA (Eastman Chemical) and the basal diet alone as described in Table 1. The melting point (uncorrected) of the FAA was 194–195°C.

All animals used for biochemical study were on the basal diet without added carcinogen for at least ten days prior to sacrifice. The only rats studied were those from which both nodular and non-nodular foci could be obtained from the same liver. Biochemical values for normal animals were obtained from rats fed the basal diet devoid of carcinogen.

All animals used for biochemical studies were killed with a guillotine, following a 24-hour period of fasting, unless otherwise noted. Careful histologic study was performed on all tissues used for biochemical assay. A rim of nodular tissue was routinely left to ensure clean separation of nodular tissue from the surrounding non-nodular tissue. The tissue was fixed in Stieve’s solution for histologic examination after hematoxylin and eosin stain and in formalin for the periodic acid-Schiff (PAS) stain with and without prior incubation with diastase. Frozen sections of some tissue was stained for glucose-6-phosphatase activity by the method of Wachstein and Meisel (58).

Assays for glycogen, glucose-6-phosphatase, and glycogen phosphorylase activities and protein were performed in duplicate or triplicate using methods described previously (15). Values for glucose-6-phosphatase have been corrected by subtracting the activity for nonspecific phosphatase using β-glycerophosphate as substrate (15). The nodular and surrounding non-nodular areas had the same activity for nonspecific phosphatase. Inorganic pyrophosphatase was assayed by the method of Nordlie and Arion (39). Total RNA and DNA were determined by the Logan et al. modification (30) of the Schmidt-Thannhauser method (50). In the experiments in which glucagon was used, the total dose of glucagon (crystalline, Eli Lilly and Co.) was 1.0 mg per kg body weight. One-half the dose was given intraperitoneally at zero time and the remainder fed animals were used. The total dose of glucagon (crystalline, Eli Lilly and Co.) was 1.0 mg per kg body weight. One-half of the dose was given intraperitoneally at zero time and the remainder fed animals were used. The control diet was shortened to 6 instead of 9 weeks.

A total of 96 rats, in two separate experiments, have been used for the initial studies with ethionine reported in this paper. At least 600 rats have been subsequently studied with similar results in 12 further experiments. Six experiments with a total of 237 rats were used in the FAA studies, herein reported. The results with FAA have subsequently been confirmed in at least 12 further experiments with over 600 additional rats.

RESULTS

Mortality

The overall mortality in the FAA-treated animals was 22.4%. The animals died for the most part during the fourth through eighth weeks of the dietary regimen. The percent of animals dying was fairly constant in all six preliminary experiments, as well as in subsequent experiments. Six of the 99 animals on the ethionine regimen died with a mortality of 6.1%. These animals generally died during the 12th week of the experimental diet. This experience has also been confirmed in subsequent experiments.

Number of Animals with Nodules

Nodules of sufficient size for both histologic and biochemical study were obtained from 20 of 42 animals sacrificed during the seventh and eighth weeks of the experimental FAA diet. Tissue for biochemical assay, ranging in weight from 20 to 410 mg, was obtained from these nodules. Of 85 animals on the FAA diet sacrificed 14–17 weeks after beginning the experimental diet, 44 had nodules that were used for both histologic and biochemical study. These ranged in size from 0.7 to 3.0 cm and their mean weight was approximately 100 mg, although some rats had individual nodules weighing over 2 gm. There were 68 animals on the ethionine regimen sacrificed 20–28 weeks after starting on the experimental diet. Of these, 33 had nodules of sufficient size for biochemical assay. These varied in size from 0.6 to 3.5 cm, and has a weight range, usable for biochemical study, of 20 to 1000 mg.

Cancer Induction

The regimen used in this study lead to the appearance of unequivocal hepatocellular carcinoma in a high percentage of animals that are followed for periods up to 8 to 10 months. In several such experiments, each with 10 to 20 animals, the cancer incidence has been 65 to 80 percent with ethionine and 80 to 90 percent with FAA. All the cancers with each carcinogen observed were hepatocellular carcinomas of varying degrees of differentiation, as observed previously with ethionine (15). No cholangiocarcinomas were seen in any of the animals. The glyco- gen content of the neoplasms was uniformly very low (less than 1%).

Gross Appearance

Grossly, the nodules in both the FAA and ethionine groups were sharply demarcated from the surrounding hepatic parenchyma (Figs. 1, 2). Many of the nodules on the liver surface were elevated. They frequently were light gray to yellow-tan and were...
generally more friable in consistency than the investing liver tissue. The larger nodules occasionally would have dull gray, central, soft to friable loci, which upon microscopic examination were seen to be areas of necrosis. These latter portions were not used for biochemical study. Rare golden yellow central areas were present in the hepatocellular nodules of rats ingesting FAA which, upon microscopic examination, proved to be loci of coagulation necrosis. Such areas were not utilized for biochemical study. The number of nodules of sufficient size for simultaneous histologic and biochemical study, obtainable from a single animal on either experimental diet, was somewhat variable but ranged up to about fifteen. In a number of animals of both groups, up to five nodules from a single animal were assayed for glycogen content and glucose-6-phosphatase activity and all were found to be comparable.

Microscopic Findings

Light microscopic examination showed the nodules in all experimental groups to be quite similar (Fig. 3) and to resemble those previously described (13, 14).

The nodules were variably encased in fibroconnective tissue, which in turn was invested by a rim of compressed hepatocytes. Within the nodular areas, bile ducts were scanty or absent and lobular architecture was not preserved. The individual intranodular hepatocytes were large and their cytoplasm was abundant. The cytoplasm most often was loose and irregularly vacuolated, although this was not invariable (Figs. 4, 5). In some nodules, the cytoplasm had an eosinophilic ground-glass appearance. The individual hepatocytes in the non-nodular portion of the liver showed little difference from those in normal liver. There was, however, some variation in the degree of interstitial tissue reaction (12, 57). In the animals fed ethionine, ductular cell proliferation was almost nonexistent at the end of the experimental regimen although isolated islands of cholangiofibrosis were frequently seen. Their white firm and fibrous character and their irregular outline allows easy identification grossly. In the

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TABLE 2
Glycogen Concentration in Hyperplastic Nodules and in the Surrounding Nonhyperplastic Liver in Rats Fed 2-Fluorenylacetamide (FAA) or Ethionine

<table>
<thead>
<tr>
<th>Dietary regimen*</th>
<th>Dietary state</th>
<th>Glycogen concentrationb (gm/100 gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hyperplastic nodule</td>
</tr>
<tr>
<td>Ethionine, 22 weeks</td>
<td>Fed [8]*</td>
<td>8.3 ± 0.9c</td>
</tr>
<tr>
<td></td>
<td>Fasted, 24 hr [6]</td>
<td>1.8 ± 0.3d</td>
</tr>
<tr>
<td></td>
<td>Fasted, 48 hr [5]</td>
<td>0.62 ± 0.01e</td>
</tr>
<tr>
<td>FFA, 15 weeks</td>
<td>Fed [7]</td>
<td>9.1 ± 1.2f</td>
</tr>
<tr>
<td></td>
<td>Fasted, 24 hr [19]</td>
<td>2.0 ± 0.3g</td>
</tr>
<tr>
<td></td>
<td>Fasted, 48 hr [11]</td>
<td>0.6 ± 0.03h</td>
</tr>
</tbody>
</table>

* The time is measured from the beginning of the experiment.

** Mean ± S.E.

* Not significantly different from the non-nodular liver of the same group.

† Significantly different (P < 0.01) from the non-nodular liver of the same group.

FAA groups, thin strands of compressed ductular cells and some fibroblast-like cells were often seen surrounding large islands of intact liver parenchyma. Collections of cysts and areas of cholangiofibrosis were seen occasionally. Many of the hyperplastic liver cells in the nodules obtained from fasted rats stained positively with the PAS stain in contrast to the hepatocytes in the surrounding liver which were rarely positive under the conditions used. The PAS-positive foci failed to react if diastase digestion preceded staining, thus suggesting that the PAS-reacting material was glycogen. The intranodular hepatocytes generally had one or two nuclei which showed few irregularities of size and shape. Occasional prominent nucleoli were present. At times the nodules had areas of cellular and architectural atypia within them (Figs. 6, 7) as has been described earlier (13, 14). Histo-

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TABLE 3
G-6-Pasea PPi-ase Activities of Hyperplastic Nodules and Surrounding Nonhyperplastic Liver in Rats Fed FAA or Ethionine

<table>
<thead>
<tr>
<th>Dietary regimen</th>
<th>Time (weeks)b</th>
<th>G-6-Pase activityc (mg P/hr/gm liver)</th>
<th>PPi-ase activityd (mg P/hr/gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hyperplastic nodule</td>
<td>Nonhyperplastic liver</td>
</tr>
<tr>
<td>Ethionine</td>
<td>20</td>
<td>11.9 ± 0.6 [4]</td>
<td>27.0 ± 1.1</td>
</tr>
<tr>
<td>Ethionine</td>
<td>24</td>
<td>7.3 ± 0.2 [6]</td>
<td>18.2 ± 1.1</td>
</tr>
<tr>
<td>FAA</td>
<td>8</td>
<td>8.3 ± 0.8 [8]</td>
<td>18.2 ± 1.1</td>
</tr>
<tr>
<td>FAA</td>
<td>15</td>
<td>5.6 ± 1.0 [9]</td>
<td>18.2 ± 1.1</td>
</tr>
<tr>
<td>FAA</td>
<td>22-32</td>
<td>6.0 ± 0.3 [4]</td>
<td>20.1 ± 2.8</td>
</tr>
</tbody>
</table>

* G-6-Pase, glucose-6-phosphatase; PPi-ase, inorganic pyrophosphatase; FAA, 2-fluorenylacetic acid.

† The time is measured from the beginning of the experiment.

‡ Mean ± S.E. The number of animals is in brackets. All enzyme activities were assayed in nodular and nonhyperplastic tissue from the same liver. All the differences in both enzyme activities between hyperplastic and nonhyperplastic liver are highly significant (P < 0.01). The G-6-Pase activity was measured in animals which were fasted for 24 hours while the pyrophosphatase activity was measured in fed animals.
chemical examination of the liver for glucose-6-phosphatase by staining showed a consistent decrease in glucose-6-phosphatase activity in the nodules from all animals studied. The histologic findings concerning glycogen and glucose-6-phosphate suggested the presence of reproducible changes in one or more aspects of carbohydrate metabolism and led to the following biochemical studies.

Biochemical Findings

The glycogen content of the nodular hyperplastic liver was the same as in the surrounding liver in fed animals of both the FAA and ethionine groups (Table 2). However, when the animals were fasted, the nodules retained considerably more glycogen than did the surrounding investing liver. This difference was present both 24 and 48 hours after initiating the fast. Although the results in 15- or 22-week periods are recorded in Table 2, similar findings were obtained with longer periods of study up to 32 weeks. In the case of the FAA group, the findings with regard to glycogen varied with the time periods studied. For example, at 8 weeks after initiation of the experimental regimen, the glycogen content of the surrounding nonhyperplastic liver did not decrease following a 24-hour fast period to the extent seen at later time intervals.

The findings concerning glycogen and glucose-6-phosphate suggested an effect most likely due to the direct action of FAA per se on the liver cell (23, 53). However, at all periods studied after 9 weeks, the glycogen in the nonhyperplastic liver responded to fasting as did glycogen in normal liver.

The findings of increased levels of glycogen following fasting in the hyperplastic nodules as compared to the nonhyperplastic liver together with the histochemical findings prompted a systematic biochemical study in the hope of delineating the basis for this altered behavior. The first enzyme studied was glucose-6-phosphatase. This enzyme shows a highly significant decrease in activity in the nodule as compared to the non-nodular liver (Table 3). This quantitative observation confirms the histochemical findings with this enzyme. As observed in Table 3, the activity of this enzyme in the nodule decreases progressively as the length of the experiment increases. In the case of the FAA group, the activity by 22 weeks after the start of the experiment is about 10 percent that of the control value. It has been suggested that glucose-6-phosphatase and inorganic pyrophosphatase activities reside in the same protein (17, 25, 38, 39, 56). The data presented in Table 3 show that the decline in pyrophosphatase activity

![Table 4]

Glycogen Phosphorylase Activity of Hyperplastic Nodules and Surrounding Nonhyperplastic Liver in Rats Fed 2-Fluorenylaceticamide (FAA) or Ethionine

<table>
<thead>
<tr>
<th>Dietary regimen</th>
<th>Time (weeks)</th>
<th>Phosphorylase activity (mg P/hr/gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>15-22 [8]</td>
<td>22.4 ± 1.3°</td>
</tr>
<tr>
<td>FAA</td>
<td>15 [7]</td>
<td>54.1 ± 4.3</td>
</tr>
</tbody>
</table>

* The time is measured from the beginning of the experiment. The number of animals is in brackets.

° Mean ± S.E.

TABLE 5

Glycogen Concentration in Hyperplastic Nodules and in Nonhyperplastic Liver Following Glucagon Administration

<table>
<thead>
<tr>
<th>Dietary regimen</th>
<th>Time (weeks)</th>
<th>Glucagon administration</th>
<th>Glycogen concentration (mg P/gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>Basal</td>
<td>Hyperplastic nodule</td>
<td>12.0 ± 0.2 [5]</td>
</tr>
<tr>
<td>Ethionine</td>
<td>Ethionine</td>
<td>Hyperplastic nodule</td>
<td>8.2 ± 0.9 [8]</td>
</tr>
<tr>
<td>FAA</td>
<td>FAA</td>
<td>Hyperplastic nodule</td>
<td>6.3 ± 0.5 [9]</td>
</tr>
<tr>
<td>2-Fluorenylaceticamide</td>
<td>2-Fluorenylaceticamide</td>
<td>9.1 ± 1.1 [7]</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonhyperplastic liver</td>
<td>8.6 ± 0.3 [8]</td>
</tr>
</tbody>
</table>

* Mean ± S.E. The number of animals is in brackets. All animals were fed throughout the experiment.

° The difference in the glycogen levels in this tissue between the animals with and without glucagon is significant (P < 0.01).

TABLE 6

Concentration of Protein and Nucleic Acids in Hyperplastic Liver Nodules Induced by Ethionine or 2-Fluorenylaceticamide (FAA) and in Livers of Control Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (mg/100 gm liver)</th>
<th>RNA (mg P/gm liver)</th>
<th>DNA (mg P/gm liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>18.1 ± 0.6 [8]</td>
<td>0.96 ± 0.02 [10]</td>
<td>0.27 ± 0.002 [10]</td>
</tr>
<tr>
<td>FAA-hyperplastic nodule</td>
<td>14.5 ± 0.4° [8]</td>
<td>0.15 ± 0.008° [14]</td>
<td></td>
</tr>
<tr>
<td>FAA-non-nodular liver</td>
<td>13.9 ± 1.0° [9]</td>
<td>0.78 ± 0.07° [13]</td>
<td></td>
</tr>
<tr>
<td>Ethionine-hyperplastic nodule</td>
<td>15.0 ± 0.8° [8]</td>
<td>0.18 ± 0.04° [13]</td>
<td></td>
</tr>
<tr>
<td>Ethionine-non-nodular liver</td>
<td>14.6 ± 0.5° [7]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All values are the means ± S.E. The number of animals is in brackets.

° All animals were killed in the fed state. The control animals were on the basal diet, the FAA group were on their dietary regimen for 15 weeks, while the animals in the ethionine group were on their regimen for 22 weeks.

° These values are significantly different (P < 0.01) from the value of the control but not from that of any other group.
parallels that observed with the activity of glucose-6-phosphatase, thus adding further support for the suggested identity of the two enzymatic activities.

Glycogen phosphorylase activity is also decreased in the nodular tissue as compared to the surrounding nonhyperplastic liver (Table 4). The difference between the nodule and non-nodular liver tissue with respect to the enzyme is greater in the FAA group than in the ethionine group. The decrease in phosphorylase activity of the nonhyperplastic liver in the ethionine group as compared to the liver of control animals on the basal diet throughout is noteworthy. No explanation for this finding is evident at present.

The response of the two cell populations in the liver to glucagon administration is quantitatively quite different. As recorded in Table 5, the liver of control animals (basal diet) and the non-hyperplastic liver of animals on FAA or ethionine showed a large decrease in glycogen concentration following glucagon administration. The change in glycogen content in the hyperplastic nodules was very small and was not statistically significant. Thus, a differential response of liver glycogen to glucagon exists between the two cell populations in the same liver.

Basic data concerning the protein, RNA, and DNA concentrations in the hyperplastic nodules and livers from control animals are recorded in Table 6. All three values are significantly less in the nodular tissue than in the control liver. However, the protein content is the same in the nodule and surrounding non-nodular tissue in animals fed either FAA or ethionine. The values for RNA and DNA content of the non-nodular surrounding liver are not included, since they are very variable. This is no doubt due to the variations in the number of proliferating ductular cells in this part of the liver, as indicated above (12, 57).

**DISCUSSION**

It is evident from the results of this study that two different hepatic carcinogens, ethionine and 2-fluorenylacetamide, when fed under conditions leading to the induction of liver cancer, stimulate the formation of large localized collections of new cells (hyperplastic nodules) in the liver. These new cell populations, induced with either of the two carcinogens and appearing before the onset of frank hepatocellular carcinoma, have several morphologic and metabolic similarities and differ in the same general manner from the surrounding nonhyperplastic liver. The known biochemical markers of this cell population, namely altered response of glycogen to fasting and to glucagon administration and a large and progressive decrease in glucose-6-phosphatase activity, persist for at least many weeks after the carcinogen has been removed from the diet and the animals have been returned to a control basal diet. Thus each of the two carcinogens studied, although possessing quite different chemical and metabolic properties (13, 60), induce what appears to be a permanent or relatively permanent change in the metabolic pattern of a group of liver cells.

This property of inducing nodular liver cell hyperplasia prior to the appearance of malignancy, reported previously for both ethionine (12, 27, 42, 43, 59) and 2-fluorenylacetamide (7, 12, 16, 54, 57), is apparently a characteristic of most if not all of the other known liver carcinogens as well (16, 57). Nodular hyperplasia has been described with p-aminoazotoluene (37, 49), 3,4-dimethylaminoazobenzene and derivatives (8, 26, 34, 35, 40, 41, 45), derivatives of aminofluorene other than the 2-acetyl (16, 47, 61), pyrrolizidine alkaloids (6, 51, 52), dimethylnitrosamine and related nitrosamines (33, 48), aramine (44), safrone (24, 31) the glycoside of methylazoxymethanol, cycasin (29), and aflatoxins (4, 36). Significantly, the histologic and histochemical appearances of the nodules, when described in these reports, appear to have similarities to those observed in the present study. For example, the cells of the hyperplastic nodules are frequently reported to be large and vacuolated, and to contain more glycogen (2, 36, 41, 48, 49, 57) than the surrounding nonhyperplastic liver. These findings suggest that the biochemical markers found in the nodules in the present study with ethionine or FAA may also be present in the cells of nodules induced by other carcinogens as well.

An important consideration concerning the hyperplastic nodule is its relationship to carcinogenesis. Is the hyperplastic nodule an essential or obligatory step in the development of a neoplastic liver cell, is it already an early neoplastic cell, or is it merely a coincident change parallel to but not intimately involved in the carcinogenic process? At this time, our knowledge is too fragmentary to discuss these problems with any certainty. In fact, one of the objectives of the study of the hyperplastic nodule is to obtain further insight into just such questions.

However, certain observations make it appear likely that the hyperplastic nodule does participate in the process of liver neoplasia: (a) As already indicated, virtually all hepatic carcinogens investigated induce nodular hyperplasia prior to the appearance of unequivocal liver cancer. (b) Whenever studied, it has been shown that a liver carcinogen can be removed from the diet after a feeding period of from 8 to 12 weeks without decreasing the occurrence of a high incidence of liver cancer weeks or months later. Although individual neoplastic cells would not be identifiable as such and may be overlooked, it is nevertheless clear that no overt neoplasia need be present in the liver at the time of removal of the carcinogen in order to have liver cancer develop. Under these circumstances, the vast majority of histologic and cytologic changes disappear from the liver, with the exception of some hyperplastic nodules. (c) Several compounds such as p-naphthyl isothiocyanate induce many of the histologic and cytologic changes in the liver seen with hepatic carcinogens and yet do not induce liver cancer in the rat (32). It is perhaps significant that nodular hyperplasia is not seen with this compound. (d) One can observe in the interior of hyperplastic nodules cytologic and histologic changes undistinguishable from those seen in metasizing hepatocellular carcinoma without any evidence of malignant neoplasia in the non-nodular portions of the liver (13, 14). This is probably the most direct evidence that the hyperplastic nodule may be at least one site of origin for the advanced malignant neoplastic cell.

Is the hyperplastic nodule already an early neoplasia and should it be so designated? Many investigators using different carcinogens beginning with the earliest study of experimental liver carcinogenesis by Sasaki and Yoshida (49) have already made this judgment and have designated this lesion as a benign neoplasm. Also, the work of Furth (21), Foulds (18-20), Greene (22), and others suggests that the transformation of cells from non-neoplastic to highly malignant neoplastic states is not usually a single step process but rather a series of as yet unde-
 fined sequential changes characterized by the progressive manifestation of properties of increasing malignancy. Accordingly, there might exist cells with many different biologic behavior patterns, and yet all fall within the spectrum of neoplasia. Conceivably, the hyperplastic nodule may fall within this spectrum and may represent an extreme example of a dependent neoplasm (21). It is evident from the results of this as well as previous studies that the hyperplastic nodule persists long after the inciting carcinogen has been removed from the diet, an observation suggesting the acquisition by the nodule of some autonomy. However, it should be emphasized that some hyperplastic nodules, so far indistinguishable morphologically from the irreversible nodules, do disappear from the liver when the carcinogen is removed from the diet. Conceivably, the irreversible ones may represent lesions which are especially susceptible to the earliest neoplastic transformation. Reuber and Firminger (47) have reported the successful transplantation of one hyperplastic nodule induced by 2-fluorenyldiacetamide.

Opie (40) and more recently Daoust (8) have emphasized the importance of the basophilic nodule to the carcinogenic process in the liver. Previous studies have shown islands of basophilic atypical cells within the confines of eosinophilic nodules induced by ethionine (13, 14). Conceivably, the basophilic nodule represents a further step in the progression (18) from the irreversible hyperplastic nodule (earliest stage of neoplasia?) to the autonomous metastasizing hepatocellular carcinoma.

It is evident from this discussion that the hyperplastic nodule offers an interesting and potentially important lesion, the study of which may give new insight into the stages or steps, if any, through which cells pass during the neoplastic process in the liver. To our knowledge, the only other carcinogenic process in which probable intermediates of sufficient size can be obtained for a variety of investigations is the mammary tumor in mice so elegantly studied both biologically and metabolically by DeOme and his coworkers (2, 9, 10, 28). The availability of a reproducible method to obtain large hyperplastic nodules from another neoplastic process, especially when developed in inbred strains of rats, may offer new approaches to the study of some of the basic components of carcinogenesis and to novel ways by which the neoplastic transformation may be regulated or reversed.

REFERENCES


FIG. 1. Rat liver following 22 weeks of the ethionine regimen outlined in the text. Numerous discreet hyperplastic nodules are seen (white arrows).

FIG. 2. Rat liver following 14 weeks of 2-fluorenylacacetamide regimen described in text. The large discreet nodules are present (arrows).

FIG. 3. Photomicrograph of ethionine-induced hyperplastic nodule and surrounding liver. Note that the nodule (arrows) is composed mainly of hepatocytes and lacks portal triads. This and all subsequent photomicrographs are from tissue sections stained with H & E and obtained following either the 2-fluorenylacacetamide or ethionine regimen described in the text. × 85.

FIG. 4. Photomicrograph of hepatocytes within a hyperplastic nodule induced by ethionine. Note that the large hepatocytes generally have an abundant loose, pale, faintly vacuolated cytoplasm. Similar findings were present in some hyperplastic nodules induced by 2-fluorenylacacetamide. × 400.

FIG. 5. Photomicrograph of hepatocytes within a hyperplastic nodule induced by 2-fluorenylacacetamide. Note that the cytoplasm of these intranodular hepatocytes stains quite intensely with eosin and has a "ground-glass" appearance. Similar findings were present in some nodules induced by ethionine. × 450.

FIGS. 6 AND 7. Areas of focal atypie (arrow) within the midst of hyperplastic nodules induced by ethionine. Such atypical areas are quite basophilie when contrasted to the general eosinophilie staining propensity of the investing hepatocytes which form the remainder of the nodule. Note that within the central basophilic areas of atypia, the nuclei are larger and the nucleoli are slightly more prominent than those of the hepatocytes at the periphery of the nodule. × 150.
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Sheldon Epstein, Nobuyuki Ito, Leonard Merkow, et al.


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