SUMMARY

Normal rat liver hepatocytes and the hepatocytes of hepatic nodules induced by N-2-fluorenylacetamide respond to 70% hepatectomy with vigorous mitotic activity. The administration of L-asparaginase at the time of operation delays the mitotic cycle of every normal hepatocyte by 10 to 12 hr. When L-asparaginase was administered to rats which had received N-2-fluorenylacetamide, an 80% inhibition of mitotic activity of the hepatocytes of the nodules occurred. However, aggregates of cells resistant to the effect of the enzymes were noted in a number of nodules. These resistant cells represent a metabolic subpopulation within the hyperplastic nodule.

INTRODUCTION

The ingestion of 2-FAA by male rats for long periods results in the appearance of persistent hepatocytic nodules (10, 16, 19). It has been suggested that these nodules bear a lineal relationship to the eventual appearance of hepatomas (11). Those livers in which nodules failed to develop or, once having developed, failed to persist did not develop tumors (18). Many investigators have examined these nodules morphologically or biochemically to determine their relationship to the carcinogenic process. However, there has been no evidence reported that at any time there exist within these nodules premalignant cells, that is, cells altered irrevocably to malignant characteristics and growth.

Both normal hepatocytes and the 2-FAA nodule cells respond to 70% hepatectomy (2, 6, 13). The mitotic response of the cells of the nodules is diffuse and intense, suggesting that most of its cells retain the capacity to respond normally, to this stimulus. It has been demonstrated previously that the mitotic response of the normal hepatocyte is extremely sensitive to low doses of L-asparaginase, which delay preparations for division of every hepatocyte by 10 hr (3, 4). It was the purpose of this study to determine whether the cells of the nodules retained sensitivity to L-asparaginase inhibition or whether subpopulations of cells could be demonstrated which had achieved resistance to this agent.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Carworth Farms, New City, N. Y.), weighing 100 to 110 g upon arrival, were used throughout the experiments. 2-FAA was mixed with regular diet meal (Wayne Meal, Carworth Farms) to a concentration of 0.06% and fed ad libitum for 3 to 4 months. Feeding cycles consisted of 3 weeks of 2-FAA and 1 week of regular meal through 4 cycles (18).

Thereafter, the rats were kept on normal diet pellets. Animals were sacrificed 1 week or 2 months after the 4th feeding cycle, and rats which were fed a normal diet for the same period of time were used as controls.

Details of 70% hepatectomy have been previously published (1). Escherichia coli-derived L-asparaginase was freshly prepared in 0.9% NaCl solution, and treated rats received 100 i.u./kg body weight i.p. at the time of operation. Tdr-3 H (Schwarz Biochemicals, Orangeburg, N. Y.) with a specific activity of 6.0 Ci/m mole was injected i.p. 21.5 hr after operation at a dose of 70 μCi/100 g body weight. At 24 hr, the rats received colchicine at a dose of 1 mg/kg administered i.p. and one-half of that dose s.c. At 30 hr after operation, the rats were exsanguinated under ether anesthesia. The livers were frozen in acetone and Dry Ice after separation of nodular from nonnodular tissue (when this procedure was feasible).

Sections 4 mm thick were taken from each lobe at sacrifice. These were fixed in 10% formalin in 0.01 M phosphate buffer (pH 7.25), embedded in paraffin, sectioned at 4 μ, and stained with hematoxylin and eosin, periodic acid-Schiff, Feulgen (DNA), or methyl green-pyronin (14).

All hepatocyte nuclei of 3 sections stained with hematoxylin and eosin from each rat were counted (approximately 15,000 to 25,000 nuclei), and the mitotic indices were determined. The mitotic count and distribution of mitotic cells of every nodule were performed separately.

DNA was extracted from livers and hepatic nodules by a modification of the method of Schneider (17). The extraction of lipid was performed once with ethanol:chloroform:ether (1:1:1) for 15 min at 50°C. The final supernatant containing DNA nucleotides was then used for determination of radioactivity and, with the colorimetric technique of Burton (7), the concentration of DNA. Radioactivity was counted in a
RESULTS

Liver Alterations. At the end of 4 cycles of 2-FAA ingestion, the livers revealed a 75% conversion to hyperplastic nodules (10, 16, 18, 19). These nodules compressed adjacent parenchyma, from which they were separated by condensed stroma (16, 18). At 7 months, many of these nodules had resorbed (18), and 75% of the parenchyma was histologically normal. Occasional 1- to 2-mm foci of enlarged cells were observable in the parenchyma, as were 2- to 3-mm nodules composed of giant hepatocytes. These huge cells were occasionally detected within hepatic nodules (18, 19).

Poorly to moderately differentiated hepatocarcinomas began to appear at 7 months, with maximal appearance at 9 months (12, 20).

Tdr-3H Uptake (Table 1). The livers of control rats demonstrated an extremely low basal level of Tdr-3H incorporation. The method did not delineate which cell types were synthesizing DNA, and some portion of this minimal incorporation was probably nonhepatocytic. The nonnodular parenchyma of 2-FAA-treated, unoperated rats at 4 and 7 months demonstrated approximately 2 times more incorporation than normal liver, but bile ductular proliferation at these times could have accounted for this alteration. The nodules at 4 and 7 months, made up in the main of hepatocytes, demonstrated a 5-fold greater than normal basal incorporation. This level was commensurate with the somewhat greater basal mitotic rate.

The incorporation of Tdr-3H by normal and 7-month, nonnodular parenchyma was greatly enhanced by 70% hepatectomy [44- and 51-fold, respectively, as related to the basal rate of normal liver (Table 1)]. As judged from the resultant mitotic activity, all of this increase was due to DNA synthesis within hepatocytes. No other liver cell type revealed an increased mitotic activity in response to 70% hepatectomy.

Because of the intimacy of location of nodular and nonnodular tissue at 4 months, in the treated groups, separation was not possible. An overall increase of incorporation of approximately 61-fold was demonstrated by these livers after 70% hepatectomy.

Seventy % hepatectomy increased incorporation of Tdr-3H approximately 100-fold in the 7 month nodules. The specific activity of the DNA of these nodules was twice that of the nonnodular or normal liver tissue following similar stimulation.

L-Asparaginase completely inhibited Tdr-3H incorporation by normal hepatocytes after 70% hepatectomy. A similar inhibition was produced in nonnodular parenchyma at 7 months where a small residual incorporation was accounted for by micronodules revealed in histological sections.

L-Asparaginase decreased the incorporation of Tdr-3H by 7-month nodular hepatocytes by 80%. Although separation of nodular and nonnodular tissue for analysis was not possible at 4 months, histological examination revealed that L-asparaginase only partially inhibited the mitotic activity of the former while totally eliminating that of the latter. The 30% incorporation of Tdr-3H by this tissue after L-asparaginase administration can be accounted for by resistance of nodular hepatocytes.

Mitotic Response (Table 2). Neither the nonnodular

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Procedure</th>
<th>Tissue</th>
<th>cpm/µg DNA</th>
<th>Increase in incorporation (fold)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlb</td>
<td>None</td>
<td>Liver</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Controlb</td>
<td>Hepc</td>
<td>Liver</td>
<td>244.1</td>
<td>44</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>None</td>
<td>Nonnodular hepatocytes</td>
<td>12.3</td>
<td>2</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hepc</td>
<td>Liver</td>
<td>333.2</td>
<td>61</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Hepc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver + nodules</td>
<td>102.3</td>
<td>19</td>
</tr>
<tr>
<td>Controlf</td>
<td>Hepc&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Liver</td>
<td>262.1</td>
<td>48</td>
</tr>
<tr>
<td>Controlf</td>
<td>Hepc&lt;sup&gt;g&lt;/sup&gt; + L-asparaginase&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Liver</td>
<td>8.4</td>
<td>2</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;h&lt;/sup&gt;</td>
<td>None</td>
<td>Nonnodular hepatocytes</td>
<td>13.0</td>
<td>2</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Hepc</td>
<td>Nonnodular hepatocytes</td>
<td>282.3</td>
<td>51</td>
</tr>
<tr>
<td>2-FAA&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Hepc&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Nonnodular hepatocytes</td>
<td>552.5</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Increased incorporation of Tdr-3H as related to control livers.
<sup>b</sup> Five months old; fed normal diet.
<sup>c</sup> Seventy % hepatectomy.
<sup>d</sup> Rat were examined 1 week after termination of 4th feeding cycle.
<sup>e</sup> One hundred i.u./kg administered i.p. at operation.
<sup>f</sup> Eight months old; fed normal diet.
<sup>g</sup> Rats were examined 2 months after termination of 4th feeding cycle.
<sup>h</sup> Several mitotically active nodules were demonstrated histologically.
parenchyma of the control rats nor those of the 2-FAA-fed rats displayed a detectable basal mitotic rate.

It has been demonstrated that, during 2-FAA feeding or for some time afterward, the nonnodular hepatocytes have a diminished mitotic response to 70% hepatectomy (13). This finding was confirmed 1 week after the 4th feeding in the present experiments. At 7 months, however, the mitotic response of nonnodular hepatocytes was equal to that of controls. At 7 months, tiny nodules were occasionally noted histologically in tissue which had been selected as nonnodular for incorporation studies. These nodules demonstrated mitotic activity identical to that of macroscopic nodules.

A very low rate of mitotic activity was evident in nodules without 70% hepatectomy. However, an enormous increase in rate occurred in every nodule examined following 70% hepatectomy at both 4 and 7 months. This mitotic rate far exceeded that achieved in nonnodular or normal parenchyma following 70% hepatectomy. Mitotic activity was diffusely spread throughout each nodule so that every area displayed activity. The huge, polyploid hepatocytes never demonstrated a mitotic response.

Administration of L-asparaginase at the time of 70% hepatectomy completely eliminated the mitotic response of the control rats and that of the nonnodular parenchyma at both 4 and 7 months.

The degree of inhibition of mitosis in nodules at 4 and 7 months was variable. In 50% or more of the nodules at both periods, L-asparaginase completely inhibited all mitotic activity. In 15 to 20% of the nodules, the response was almost completely inhibited, but 1 or 2 foci of 1 to 4 mitotic cells were noted. In 10% of the nodules, inhibition was suggested by a lack of diffuse mitotic response. The mitotic cells were located in clusters of 1 to 6 cells. However, the mitotic rate approached the lowest range achieved by some untreated nodules. All of the mitotic hepatocytes which were apparently resistant to L-asparaginase were present in tight groups in nodules.

DISCUSSION

In response to the ingestion of 2-FAA, rat hepatocytes undergo division and hypertrophy which result in the formation of liver nodules. It has been suggested that these
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nODULES ARE LINEARLY RELATED TO THE EVENTUAL APPEARANCE OF HEPATOCARCINOMAS (11, 18). MOST BIOCHEMICAL OR FUNCTIONAL STUDIES OF NECESSITY HAVE BEEN DONE WITH WHOLE NODULES OR POOLS. THE RESULTS OF THESE EXPERIMENTS HAVE BEEN VARIABLE, WITH SOME DEMONSTRATING BIOCHEMICAL OR FUNCTIONAL ABNORMALITY AND OTHERS DEMONSTRATING GRASSLY NORMAL ACTIVITY (19).

IT IS NOT KNOWN IF THERE EXISTS WITHIN THESE NODULES AT ANY TIME A CELL(S) WHICH CAN BE CONSIDERED PREMALIGNANT, I.E., ONE WHICH WILL Evolve IRCOMBOLY TO A MALIGNANT TUMOR. IN SOME STUDIES, SUBPOPULATIONS OF CELLS HAVE BEEN DISTINGUISHED WITHIN THE NODULES ON MORPHOLOGICAL OR RADIOAUTOGRAPHIC GROUNDS (8, 10). IN OTHERS, CELLS WERE DISTINGUISHED BY THEIR RNA SYNTHETIC RESPONSE TO 70% HEPATECTOMY, WHICH DEMONSTRATED DIFFERENTIAL STIMULATION IN SEVERAL CELL POPULATIONS (9).

THESE EXPERIMENTS ON RNA SYNTHESIS WERE BASED ON THE KNOWLEDGE THAT MANY OF THE CELLS OF THESE HEPATIC NODULES RESPOND MITOTICALLY TO THE STIMULUS OF 70% HEPATECTOMY. THE OBSERVATION THAT THESE CELLS ARE RESPONSIVE TO MITOTIC STIMULATION HAS BEEN CONFIRMED IN THE PRESENT STUDY AND HAS BEEN EXTENDED TO A PERIOD AS LATE AS 7 MONTHS, WHEN HEPATOMAS BEGAN TO APPEAR IN THE 2-FAA-FED RAT POPULATION. EVERY NODULE REVEALED AN INTENSE, DIFFUSE MITOTIC ACTIVITY. IN THIS SENSE, MOST OR ALL OF THE CELLS OF THE NODULE RETAIN THE CAPACITY TO RESPOND TO A MITOTIC STIMULUS IN A MANNER HIGHLY CHARACTERISTIC OF NORMAL HEPATOCYTES.

IT HAS BEEN REPORTED THAT MANY OF THE NODULES WHICH ARE PRESENT AT THE END OF 4 MONTHS OF FEEDING 2-FAA WILL RESORB (19). IT IS THE NODULES WHICH PERsist THAT APPEAR TO BE AT HIGH RISK FOR TUMORIGENESIS. NO DIFFERENCE IN MITOTIC RESPONSE, PATTERN, TIMING, OR INTENSITY WAS DEMONSTRATED BETWEEN EARLY AND LATE NODULES IN THE PRESENT STUDY, SUGGESTING THAT THE MAJORITY OF THEIR CELLS RETAIN THIS NORMAL CHARACTERISTIC.

THE NORMAL HEPATOCYTE DURING ITS RESPONSE TO 70% HEPATECTOMY IS VULNERABLE TO THE ACTION OF THE ENZYME L-ASPARAGINASE. A SUBTUMORICIDAL DOSE OF THE ENZYME INVARIABLY DELAYS THE PREPARATION FOR DIVISION IN EVERY HEPATOCYTE BY 10 TO 12 HR. IT HAS BEEN SUGGESTED THAT THIS DELAY RESULTS FROM A DIMINUTION OF EXOGENOUS ASPARAGINE APPARENTLY REQUIRED BY THE STIMULATED HEPATOCYTE. THE INHIBITION IS EVENTUALLY OVERCOME, POSSIBLY THROUGH THE INDUCTION OF L-ASPARAGINASE SYNTHETASE WITHIN THESE CELLS (5, 15).

THE DNA SYNTHESIS AND MITOTIC RESPONSE OF THE NONNUCLEAR HEPATOCYTES OF 2-FAA-EXPOSED RATS IN THE PRESENT EXPERIMENTS WAS TOTALLY INHIBITED BY L-ASPARAGINASE. THE ENZYME ALSO PRODUCED A SIGNIFICANT INHIBITION (APPROXIMATELY 80% OR MORE OVERALL) OF DNA SYNTHESIS AND OF MITOTIC RESPONSE IN THE CELLS OF THE NODULES. THOSE CELLS WHICH WERE RESISTANT TO ITS EFFECT EXISTED IN FOCAL AGGREGATES IN OCCASIONAL NODULES.

THUS, A COMBINATION OF 70% HEPATECTOMY AND L-ASPARAGINASE TREATMENT HAS REVEALED 3 TO 4 CELL POPULATIONS WITHIN 2-FAA-INDUCED NODULES. THERE ARE ENORMOUS POLYPLOID CELLS WHICH DO NOT RESPOND TO 70% HEPATECTOMY. THE SIGNIFICANCE OF THESE CELLS IS UNCERTAIN, BUT, IN VIEW OF THE DIPLOID OR TETRAPLOID MODE OF HEPATOMAS ARISING IN THESE RATS, IT APPEARED UNLIKELY THAT THEY ARE INVOLVED IN CARCINOGENIC EVOLUTION (20). THERE IS A POPULATION OF CELLS WHICH IS HISTOLOGICALLY IDENTICAL TO THE TYPICAL NODULE HEPATOCYTE BUT WHICH IS NONDIVIDING UNDER OUR CONDITIONS OF EXAMINATION AND WHICH MAY OR MAY NOT BE RESPONSIVE TO 70% HEPATECTOMY. IT APPEARS LIKELY FROM THEIR DIFFUSE LOCATION IN THE NODULE THAT OUR FAILURE TO "CAPTURE" THESE CELLS IN METAPHASE MAY BE RELATED TO PROBLEMS IN TIMING OF A NONSYNCHRONOUS RESPONSE. OF THE CELLS WITHIN THE NODULE WHICH DO RESPOND TO 70% HEPATECTOMY, THE MAJORITY ARE SENSITIVE TO L-ASPARAGINASE. THEY ARE THEREFORE IDENTICAL TO NORMAL HEPATOCYTES IN THESE 2 CHARACTERISTICS.

A SUBPOPULATION OF HEPATOCYTES EXISTS WITHIN THE NODULES; IT IS MITOTICALLY RESPONSIVE BUT L-ASPARAGINASE RESISTANT. THEIR LOCALIZATION IN FOCI, AND THEN ONLY IN OCCASIONAL NODULES, SUGGESTS THAT THEY REPRESENT CLONES OF ALTERED CELLS. THE MOST OBVIOUS CONCLUSION IS THAT THEY NOW POSSESS A SYNTHETIC APPARATUS CAPABLE OF SUPPLYING SUFFICIENT L-ASPARAGINE FOR DIVISION.

THESE L-ASPARAGINASE-RESISTANT CELLS WERE FOUND IN EARLY NODULES AND IN PERSISTENT NODULES. IN OTHER EXPERIMENTS, ANALYSIS OF THE MITOTIC CELLS OF THESE NODULES REVEALED AN EUDIPLOID KARYOTOPE (20), INDICATING THAT L-ASPARAGINASE RESISTANCE WAS NOT ASSOCIATED WITH A GROSS CHROMOSOMAL ALTERATION.

ALTHOUGH THESE DATA DO NOT IN ANY WAY IDENTIFY THE CELLS DISPLAYING AN ALTERED RESPONSE TO L-ASPARAGINASE AS PREMALIGNANT, THEY DO DEMONSTRATE THE VARIETY OF CELLS WHICH EXIST WITHIN THE 2-FAA-REACTIVE NODULE. THE RESULTS SUPPORT THE CONCEPT THAT A SINGLE CELL ALTERATION OR ALTERATION TO SMALL FOCI OF CELLS WITHIN THESE NODULES MIGHT BE RESPONSIBLE FOR THE EVENTUAL APPEARANCE OF CANCER. SUCH RESULTS EMphasize THE NEED FOR CAUTION IN INTERPRETING ANALYSIS OF OVERALL NODULE ALTERATION AS BEING RELATED TO MALIGNANT EVOLUTION.

REFERENCES

Effect of L-Asparaginase on Mitotic Activity


The Effect of L-Asparaginase on Mitotic Activity during *N*-2-Fluorenylacacetamide Hepatocarcinogenesis: Subpopulations of Nodular Cells

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