Populations of Rat Liver Parenchymal Cells with Different Patterns of Ribonucleic Acid Synthesis during Azo-Dye Carcinogenesis

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SUMMARY

"Preneoplastic" parenchyma of liver nodules produced by 3'-methyl-4-dimethylaminoazobenzene could be distinguished as hyperbasophilic and basophilic cells. Ribonucleic acid synthesis in these cells was studied 3 hours after the injection of uridine-3H. Autoradiographic data showed that ribonucleic acid synthesis in the hyperbasophilic cells was 70 percent higher than the basophilic cells and 50 percent greater than the liver parenchyma of the control rats. A similar difference was also evident in the labeling pattern of the cytoplasm, chromatim, and nucleolus. After partial hepatectomy, ribonucleic acid synthesis in the hyperbasophilic cells was increased 60 percent at 12 hours; in the basophilic cells, the same biosynthetic activity was stimulated only up to 29 percent. The hyperbasophilic cells were 50 percent more responsive to the stimulus of partial hepatectomy than the basophilic cells. In the hyperbasophilic cells the increased radioactivity in the chromatin was accompanied by a similar rise of cytoplasmic labeling. The increased uridine-3H uptake in the chromatin of the basophilic cells failed to show an enhancement of the cytoplasmic labeling. In intact liver, one population of the "preneoplastic" parenchyma was hyperactive in ribonucleic acid synthesis while the other was only moderately active. Partial hepatectomy produced different patterns of responsiveness of the ribonucleic acid synthetic processes in these cell populations. Variable basophilic properties of the "preneoplastic" parenchyma thus might be due to the different rate of ribonucleic acid synthesis of these cells. Significance of these findings concerning the proliferative potential of the hyperbasophilic and basophilic cells was discussed.

INTRODUCTION

Prolonged feeding of carcinogenic azo dyes to rats produces liver damage and regeneration, followed by extensive alteration of the histologic architecture of the hepatic tissue (6, 15, 17, 18). Thus it seems that one of the problems in the studies on the origin of hepatomas is to identify the sites of neoplastic transformation in the "preneoplastic" liver. Parenchymal cells of azo-dye-produced "preneoplastic" liver are characterized by variable basophilic properties, and the regions which stain more intensely than the surrounding parenchyma show transformations suggestive of a neoplastic change (17). Recently it was demonstrated by histochemical methods that, in 4-dimethylaminoazobenzene-produced "preneoplastic" liver, different populations of parenchymal cells can be distinguished on the basis of their cytoplasmic RNA staining (5). This finding thus provokes the question concerning the rate of RNA metabolism in the histochemically distinguishable populations of parenchymal cells of the preneoplastic liver.

Partial hepatectomy, used as model system, could reveal critical information concerning the alterations of biochemical and cytologic properties of the "preneoplastic" liver parenchyma; these changes may not be detectable in the intact liver (1, 13). Therefore, the metabolic stimulus of partial hepatectomy was used to determine the pattern of responsiveness of the ribonucleic acid synthetic processes in the parenchymal cells of the remaining lobes of the "preneoplastic" liver. This report presents the results of the study on the rate of RNA synthesis in the different populations of hepatic parenchymal cells of rats fed on the carcinogenic diet, 3'-Me-DAB. High resolution autoradiography permitted the study of a biosynthetic activity in discrete populations of cells distinguishable only in histologic sections.

MATERIALS AND METHODS

Animals and Schedule of Feeding. Male Fischer F344 rats with average body weight of 75 gm were used. The total of 36 rats was divided into two groups: one group of 18 rats was fed, ad libitum, a semisynthetic basal diet containing 0.06 percent 3'-Me-DAB (15), and the second group of 18 rats was fed the basal diet without the carcinogen. The basal diet was composed of: casein, crude, 18%; cereose, 73%; salt mixture, H. M. W. (Hubbel, Mendel and Wakeman), 2%; zinc mixture (20 mg ZnAc2·2H2O), 1%; vitamin powder, 1%. (The vitamin powder consisted of (mg/gm) thiamine HCl, 0.40; riboflavin, 0.80; niacin, 4.0; pyridoxine, 0.50; calcium pantothenate, 4.0; inositol, 20.0; menadione, 0.40; biotin, 0.03; folic acid, 0.40; vitamin B12, 0.02; and choline dehydrogen citrate, 423 in Argo

Received June 27, 1967; accepted October 29, 1967.

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3 Abbreviations used are: 4-DAB, 4-dimethylaminoazobenzene, N,N-dimethyl-p-phenylazoaniline; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene, N,N-dimethyl-p-(m-tolylazo)aniline.
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scored from each sample of liver tissue; the value has been determined. The background grains counted from the adjacent area, nucleolus, and cytoplasm of individual cells was also determined. The number of silver grains on the chromatin (non-nucleolar nuclear area) per gram of body weight, and the uptake of uridine-3H into the parenchymal cells of the liver was used as a measure of RNA synthesis. After 10 weeks of 3'-Me-DAB diet liver lobes of these rats contained numerous hyperplastic nodules; 3-5 of these lesions were carefully dissected out from both the hepatetomized and nonhepatetomized animals. An equal number of tissue samples was also collected from the livers of animals fed basal diet.

Partial Hepatectomy and Labeled RNA Precursor Used. Half of the rats, both from the carcinogen-fed and basal diet-fed groups, were partially hepatetomized (9) and nearly three-fourths of the liver mass was removed under pentobarbital anesthesia; all operations were completed between 9 A.M. and 12 noon. After recovery from the effect of the anesthetics, drinking water for both the operated and intact animals was substituted by 5 percent dextrose solution. At 6, 8, and 12 hours after partial hepatectomy, animals were sacrificed in batches of 3 rats. At each time-point 3 intact rats, both from carcinogen-fed and basal diet-fed groups, were also killed. Three hours prior to sacrifice, each rat was administered by i.p. injection 0.25 μc of uridine-3H (7.2 c/m mole, New England Nuclear Corp.) per gram of body weight, and the uptake of uridine-3H into the parenchymal cells of the liver was used as a measure of RNA synthesis. After 8 weeks exposure of the autoradiographs, a substantial number of silver grains was present over the parenchymal areas of the basal diet-fed rats and nearly 80-90 percent of the cells were labeled (Fig. 4). The frequency of labeled cells in the hyperbasophilic and the basophilic parenchyma of the "preneoplastic" liver was essentially similar to that of the control animals. However, the extent of the labeling in the cells of the different populations of the "preneoplastic" liver was considerably different (Figs. 5, 6). Radioactivity in the hyperbasophilic area was 70 percent higher than in the basophilic cells of the same liver nodule and 50 percent greater than in the parenchymal cells of the control liver (Table 1). The hyperbasophilic regions contained more nuclei per unit area than the basophilic regions of the "preneoplastic" liver (16) and therefore, in addition to scoring the number of silver grains per unit area, the extent of the labeling in the hyperbasophilic and basophilic regions was also determined on a per cell basis (Table 2). Data on the number of silver grains on different structures of individual cells also showed that the labeling in the hyperbasophilic cells was significantly higher than the cells of the basophilic parenchyma. Cytoplasmic labeling in the former was approximately two-fold higher than the same structure of the latter cells. A similar extent of increased labeling was observed in the chromatin of the hyperbasophilic cells. Nucleolar labeling in the same cells also showed a markedly higher value. Data on the labeling pattern per unit area, as well as the labeling in the individual cells, thus clearly show that uridine-3H incorporation.

RESULTS

Histologic Characteristics. In agreement with earlier reports (6, 21) liver of rats fed 3'-Me-DAB showed considerable alteration of the histologic architecture accompanied by a pronounced increase of bile duct cells (Figs. 1, 2). Staining with the basic dye, toluidine blue, showed that sections of the hyperplastic nodules contained parenchymal cells which could be distinguished on the basis of their staining intensity: one cell type stained more intensely than the other (Fig. 3). The intensely stained parenchymal cells also showed the characteristics of altered nucleo-cytoplasmic ratio, prominent nucleoli, and more frequent occurrence of mitosis (16). Intensely stained and faintly stained cells thus appeared to correspond with the "hyperbasophilic" and "basophilic" cells described by Daoust and Molnar (5) in 4-DAB-produced preneoplastic nodules of rat liver. In view of the above similarity, the same terminologies have been used to describe the two types of parenchymal cells observed in the toluidine blue-stained sections of the hyperplastic nodules produced in rat liver by 3'-Me-DAB.

Uridine-3H Uptake in the Parenchyma of Intact Liver. After 8 weeks exposure of the autoradiographs, a substantial number of silver grains was present over the parenchymal areas of the basal diet-fed rats and nearly 80-90 percent of the cells were labeled (Fig. 4). The frequency of labeled cells in the hyperbasophilic and the basophilic parenchyma of the "preneoplastic" liver was essentially similar to that of the control animals. However, the extent of the labeling in the cells of the different populations of the "preneoplastic" liver was considerably different (Figs. 5, 6). Radioactivity in the hyperbasophilic area was 70 percent higher than in the basophilic cells of the same liver nodule and 50 percent greater than in the parenchymal cells of the control liver (Table 1). The hyperbasophilic regions contained more nuclei per unit area than the basophilic regions of the "preneoplastic" liver (16) and therefore, in addition to scoring the number of silver grains per unit area, the extent of the labeling in the hyperbasophilic and basophilic regions was also determined on a per cell basis (Table 2). Data on the number of silver grains on different structures of individual cells also showed that the labeling in the hyperbasophilic cells was significantly higher than the cells of the basophilic parenchyma. Cytoplasmic labeling in the former was approximately two-fold higher than the same structure of the latter cells. A similar extent of increased labeling was observed in the chromatin of the hyperbasophilic cells. Nucleolar labeling in the same cells also showed a markedly higher value. Data on the labeling pattern per unit area, as well as the labeling in the individual cells, thus clearly show that uridine-3H incorpora-

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Cell type</th>
<th>No. of silver grains per unit area ± S.E.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>Control (6)</td>
<td>185 ± 14</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>Hyperbasophilic (9)</td>
<td>287 ± 13</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>Basophilic (9)</td>
<td>168 ± 8</td>
</tr>
</tbody>
</table>

Average number of silver grains per unit area of the different types of parenchymal cells in the livers of intact rats fed on the basal or carcinogen-containing diets. Numbers in parentheses represent the number of rats. 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.

*Three samples of liver tissue were scored from each animal and the data represents the average of the total number of rats in the different groups (see Materials and Methods).
RNA Synthesis during Carcinogenesis

Table 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>Cell type</th>
<th>Average No. of Grains ± S.E. *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chromatin</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Basal</td>
<td>Control (6)</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>Hyperbasophilic (9)</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>3'-Me-DAB</td>
<td>Basophilic (9)</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Average number of silver grains per cell in the liver parenchyma of intact rats fed the basal diet or the carcinogen 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB). The figures in parentheses indicate the number of rats. Three pieces of tissue, collected from the livers of each rat from both the basal- and the carcinogen-fed groups, were scored.

*100-200 cells were counted from the different cell types of each animal; the data represent the average of the total number of rats in the different groups.

Uridine-3H Uptake in the Parenchyma After Partial Hepatectomy. Partial hepatectomy acts as a stimulus for various metabolic activities, including the biosynthesis of RNA in the remaining lobes of rat liver (2, 7). In this experiment attempts were made to determine the post-hepatectomy pattern of responsiveness of the RNA synthetic processes in the hyperbasophilic and basophilic parenchyma of the regenerating "preneoplastic" liver. As determined by unit area of parenchymal cells, removal of three-fourths of the liver mass stimulated the incorporation of uridine-3H in the remaining liver lobes of the control rats (Chart 1). At 12 hours after the operation there was nearly 100 percent increase of the labeling; this result seems to be in general agreement with that of the normal rats (2, 3). A similar pattern of augmented labeling after hepatectomy was also observed in the hyperbasophilic cells of the "preneoplastic" liver and the total increase at 12 hours in these cells was 60 percent over the value of the carcinogen-fed intact rats (Chart 1). On the other hand, responsiveness of the basophilic cells after hepatectomy was distinctly lower than both the hyperbasophilic and parenchymal cells of the control liver. Only 29 percent increase of the labeling of the basophilic cells was observed at 12 hours after the operation and this value was nearly 50 percent less than that of the hyperbasophilic cells (Chart 1).

Frequency of silver grains scored on a per cell basis also showed a similar increased labeling in the hyperbasophilic cells, whereas the basophilic cells perform a near or subbasal level of RNA synthetic activity.

Chart 1. Uridine-3H incorporation, determined by the average number of silver grains per unit area of hyperbasophilic and basophilic cells of the "preneoplastic" liver and the parenchymal cells of the control liver, at various times after partial hepatectomy. Each point represents the average of 3 rats and the vertical lines indicate the standard error of the mean ——○——, hyperbasophilic; —— O ——, basophilic; ...... ▲ ......, control.

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a distinctly low level of radioactivity at 12 hours after the operation. Thus, these data appeared to be consistent with that of the unit area of the same cells. No detectable rise of the labeling in the nucleolus of the basophilic cells was observed up to 12 hours after hepatectomy. Between 8 and 12 hours after the operation there was a 50 percent rise of cytoplasmic as well as chromatin labeling in the hyperbasophilic cells. However, during the same time after the operation the modest increase of the chromatin labeling in the basophilic cells was not accompanied by a perceptible rise of cytoplasmic radioactivity. This seems to suggest that, in addition to the variable RNA synthetic activity among the hyperbasophilic and basophilic cells, a further difference concerning the rate of transport of the newly formed RNA from the nucleus to the cytoplasm may also exist in these cells.

**DISCUSSION**

Consistent with earlier reports (5, 17), the present data showed that the hyperplastic nodules in the liver of 3'-Me-DAB-fed rats contained different populations of parenchymal cells, distinguishable by their basophilic properties. RNA synthesis in the hyperbasophilic cells is significantly higher than in the basophilic cells of the same nodule. This clearly indicates the presence of a variable pattern of RNA synthesis among the parenchymal cells of the "preneoplastic" liver. One population was hyperactive while the other showed a near or subbasal value of RNA synthetic activity. Since activation of RNA synthesis is required for subsequent DNA synthesis (11), augmentation of the former biosynthetic processes in the hyperbasophilic cells thus seems to be consistent with increased DNA synthesis and mitotic activity in these cells (4, 12). Variable basophilic properties of the azo-dye-produced "preneoplastic" liver parenchyma was reported to be due to the difference in cytoplasmic RNA staining of these cells (5, 17). The nucleus is believed to be the cellular site of RNA synthesis and the newly formed RNA is then transported into the cytoplasm. Thus it is possible that an altered rate of RNA synthesis could also change the level of cytoplasmic RNA content. This, in fact, may be the situation as indicated by the data that the increased nuclear RNA synthesis in the hyperbasophilic cells is also accompanied by a similar enhancement of the labeling in the cytoplasm. Both the nucleus and cytoplasm of the basophilic cells, on the other hand, showed a significantly low radioactivity. Evidence thus indicates that the variable basophilic properties of the "preneoplastic" parenchyma may, in fact, be due to the different rate of RNA synthesis in these cells.

It was reported that nuclear RNA content is reduced to a subbasal level in the cells of 3'-Me-DAB-produced hyperplastic nodules of rat liver, and this result was interpreted as due to a decreased RNA synthesis in these cells (24). Present data on the significantly low nuclear RNA synthesis in the basophilic cells seems to support the above view. Unfortunately, however, in the earlier study the increased RNA synthesis in the hyperbasophilic cells apparently remained undetected. This may be because the values obtained by measuring the RNA content of isolated chromatin prepared from the whole liver homogenate failed to show the difference between various types of cells. Such results, therefore, reflect an average value of the total cell population. In this context it may be mentioned that in azo-dye-produced "preneoplastic" liver hyperbasophilic cells constitute a small proportion of the total parenchyma (5).

It is well known that partial hepatectomy acts as a stimulus for RNA synthesis in the hepatocytes of the remaining lobes of the normal liver (2). Present results show that the synthesis of RNA in the parenchymal cells of the "preneoplastic" liver has a different pattern of responsiveness to the stimulus of hepatectomy. The pronounced increase of uridine-3H incorporation in the hyperbasophilic cells after hepatectomy clearly

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**Chart 2. Patterns of uridine-3H labeling in the different cellular structures of the hyperbasophilic and basophilic cells of the "preneoplastic" liver at various times after partial hepatectomy. The points represent the average of 100-200 cells from each of the 3 rats and the vertical lines indicate the standard error of the mean (see Materials and Methods).**
indicates that these cells are highly responsive to the same stimulus. The basophilic cells, on the other hand, demonstrated only a modest increase of uridine-3H labeling after hepatectomy. Thus, it is reasonable to conclude that the ability of the RNA synthetic processes to respond to the stimulus of partial hepatectomy may vary among the different populations of parenchymal cells of these livers. After hepatectomy, an initial activation of RNA synthesis is required for the augmented protein biosynthetic processes to respond to the stimulus of partial hepatectomy. The pronounced increase of RNA synthesis in the hyperbasophilic cells after hepatectomy could delay or block subsequent DNA synthesis and the burst of mitosis (23). Therefore, inadequate or low responsiveness of the RNA synthetic processes in the basophilic cells after hepatectomy may cause a substantial disadvantage for increased DNA synthesis and hence proliferative activity of these cells.

Hyperbasophilic cells are characterized by increased nucleocytoplasmic ratio, prominent nucleoli, augmented DNA synthesis, and mitotic activity, features more conspicuously present in the cells of the hepatoma (4, 5, 12). The present study reveals that in 3′-Me-DAB-produced liver nodules, one population of the parenchyma is hyperactive in RNA synthesis whereas another shows a moderate activity of the same biosynthetic processes. It is also evident that the same characteristic difference of RNA synthesis in this parenchyma persists in the nature of their responsiveness to partial hepatectomy. RNA synthesis is intimately related to the proliferative potential of a cell population (11, 23). The increased activity of the same biosynthetic processes in the hyperbasophilic cells thus seems to substantiate the view (5, 17) that these cells may represent the actual site of neoplastic transformation in the "preneoplastic" liver.

ACKNOWLEDGMENTS

The authors wish to express their sincere thanks to Mrs. Ruby J. Walker, Miss Johanna Zuefle, and Mr. Gerome McDowell for their technical assistance.

REFERENCES

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Fig. 1. Section of liver of rat after 10 weeks feeding of the basal diet. Toluidine blue, × 160.

Fig. 2. Section of liver of rat after 10 weeks feeding of the 3'-methyl-4-dimethylaminoazobenzene, carcinogen-containing diet. Note the altered nature of the histologic architecture and the extensive growth of the bile duct cells. Toluidine blue, × 160.

Fig. 3. Section of liver of a 3'-methyl-4-dimethylaminoazobenzene-fed rat, stained with toluidine blue. Note the distinct hyperbasophilic and basophilic areas of the parenchyma within the same hyperplastic nodule. × 160.

Fig. 4. Autoradiograph of a section of liver of basal diet-fed rat showing the pattern of uridine-3H labeling. Toluidine blue, × 540, focused on silver grains.

Fig. 5. Autoradiograph of a section of liver of 3'-methyl-4-dimethylaminoazobenzene diet-fed rat showing the heavy uridine-3H labeling in the hyperbasophilic area of the nodule. Toluidine blue, × 540, focused on silver grains.

Fig. 6. Autoradiographs of a section of liver of rat fed the 3'-methyl-4-dimethylaminoazobenzene diet. Note the relatively low labeling of uridine-3H in the adjacent basophilic area of the same nodule shown in the previous figure. Toluidine blue, × 540, focused on silver grains.
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