Isolation of Preneoplastic Rat Liver Cells by Centrifugal Elutriation and Binding to Asialofetuin

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ABSTRACT

Putative preneoplastic hepatocytes were isolated from male Fischer 344 rats treated with a single dose of diethylnitrosamine, 2-acetylaminofluorene feeding, and partial hepatectomy (Solt-Farber model). The isolation procedure involved, after collagenase dispersion of the liver, separation of the hepatocytes into small- and large-cell fractions by centrifugal elutriation, and subsequent selection of cells deficient in asialoglycoprotein receptor(s) by plating onto asialofetuin (ASF)-coated plates. The number of cell surface binding sites for the asialoglycoprotein receptor was measured with both asialoorosomucoid and ASF as ligands. There was a 50% reduction of binding sites for both ligands in the original cell suspensions obtained from preneoplastic livers. The reduction in receptor binding sites was most pronounced in the large cell fraction (≤30% of control value) after separating the original cell suspension by elutriation into small and large cell fractions. Immunohistochemical studies showed a lack of asialoglycoprotein receptor in preneoplastic (i.e., hyperplastic foci) areas. These areas were entirely superimposable with glucose-6-phosphatase-deficient areas and partially overlapped the γ-glutamyltranspeptidase-positive areas in serial liver sections. The attachment of preneoplastic hepatocytes to ASF-coated tissue culture dishes was greatly impaired, and the number of γ-glutamyltranspeptidase-positive cells on the ASF dishes was reduced to <7% as compared to 45 to 70% on the collagen-coated plates. Thus, the lack of asialoglycoprotein (asialofetuin) surface receptors and the increased size of the early preneoplastic hepatocytes are characteristics that can be used to separate the preneoplastic cell population from normal liver cells.

INTRODUCTION

Rat liver has been extensively used as an experimental model for chemically induced carcinogenesis, and several different treatment protocols are known that cause hepatoma formation (6). It is well established that the process of chemically induced hepatocarcinogenesis in the rat can be separated into both initiation and promotion phases (20). One of the many advantages offered by the rat liver model for experimental studies on chemical carcinogenesis is that it allows identification of preneoplastic cell populations very early in the neoplastic process (9). These preneoplastic hepatocytes have common cytomorphological and cytochemical characteristics in all models of chemically induced rat hepatoma (2, 3).

One of the early morphological changes that characterizes the preneoplastic stage is the appearance of a focus of large (2- to 4-fold the size of a normal hepatocyte) clear or acidophilic hepatocytes (2, 3). The significance of this cell type in the hepatoma formation is unclear, but results from morphometric studies seem to indicate that at least some of these cells may be precursors of the hepatoma cells (4). In addition to the morphological changes in the early preneoplastic hepatocytes, extensive changes in cell surface characteristics take place early in hepatocarcinogenesis (1, 11). One of the surface changes that occurs is a greatly reduced concentration of the surface receptor (hepatic binding protein) for asialoglycoproteins (1, 26).

Isolation of precursors of the hepatoma cells would provide an appropriate cell population in which to begin defining and characterizing factors that determine the sequence of events ultimately resulting in the formation of a hepatoma. In the present paper, we report our results from studies aimed at separating preneoplastic hepatocytes from normal liver cells. This is achieved by using differences in both size and surface receptor properties between normal and preneoplastic hepatocytes.

MATERIALS AND METHODS

Production of Preneoplastic Changes. The Solt-Farber system was used for the induction of preneoplastic changes in Fischer male rats (150 g) (24, 25). This method includes i.p. administration of diethylnitrosamine (200 mg/kg body weight) for initiation, and partial hepatectomy combined with feeding low doses (0.02%) of AAF for selective promotion of the growth of initiated cells. AAF administration was done by stomach tubing 5 times/week for 2 weeks. The amount of AAF administered (~2 mg AAF/day) in corn oil was based on the average food consumption of the animals (approximately 10 to 12 g/day). The animals were used for the experiments 3 to 8 weeks after the administration of AAF. At that time, the weights of animals varied between 250 and 300 g.

Isolation of Hepatocytes. The separation of liver cells was accomplished by the 2-step collagenase perfusion technique described by Seglen (23) as modified by Williams et al. (29). This method includes perfusion of the liver with Hank's balanced salt solution without calcium and magnesium, containing 0.5 mm ethyleneglycol bis(β-aminoethyl ether)-N,N'-N',N''-tetraacetic acid and 10 mm 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid with pH adjusted to 7.3, followed by collagenase (Worthington collagenaseCLS II; 0.07%) in Williams Medium E, pH 7.3. After 20 min of perfusion, the liver capsule was sliced with several cuts, and the cells were suspended in elutriation buffer (see below) containing 0.005% deoxyribonuclease and 0.001% soybean trypsin inhibitor. The cell suspension was filtered through nylon gauze, size 75 μm (Sargent Welch Scientific, Skokie, IL). The resultant cell suspension was sedimented at unit gravity for 10 min at 4°C, and the supernatant was discarded. Cells were resuspended in elutriation buffer and centrifuged at 50 × g for 3 min. The supernatant was discarded, and the cells were resuspended in elutriation buffer containing 0.005% DNase and 0.001% soybean trypsin inhibitor and agitated in a 37°C water bath for 3 min. In some experiments, deoxyribonuclease was replaced by heparin (4 units/ml). Two additional washings with cold elutriation buffer were performed. The final purification of the cell suspension included 2-min sedimentation at unit gravity to exclude the cell aggregates. Viability
of the hepatocytes, as judged by trypan blue exclusion, was 90 to 95%.

Elutriation Centrifugation. The method used for elutriation centrifugation was essentially that of Bernaert et al. (5). The elutriation buffer was Ca^{2+}- and Mg^{2+}-free Waymouth's medium containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH, penicillin-streptomycin (10 IU/ml), 10 mM lactate, 1 mM pyruvate, and 10 mM glucose (pH 7.2). Elutriation was performed in a Beckman centrifuge J2-21M using a Beckman JE-6B elutriator rotor with Sanderson separation chamber. The elutriation rotor was kept at +15°C. The amount of cells loaded into the chamber varied from 5 × 10^6 to 6 × 10^6. Cells were introduced to the chamber at a flow rate of 10 ml/min and at a rotor speed at 840 rpm. The subsequent rates of the counterflow at a constant rotor speed were 15, 20, 25, 30, and 35 ml/min. Samples of 150 ml were collected at each flow rate. Cells were harvested by centrifugation, and an aliquot of each fraction was counted in a hemocytometer. Slides were prepared from the starting sample and from each fraction, and were air-dried. After a short fixation in ice-cold ethanol, slides were stained for GGT activity using the method of Rutenberg et al. (22). The total recovery of the cells introduced to the elutriation chamber varied between 85 and 95%, and the viability was 85 to 90% as judged by trypan blue exclusion.

Hepatocyte Culture. Tissue culture plates were coated with either collagen or ASF. Collagen (bovine type I; Collaborative Research, Waltham, MA) was diluted with phosphate-buffered saline to 100 μg/ml and 2 ml of this solution were added to each tissue culture plate. The tissue culture plates were kept at least 45 min at room temperature in the laminar flow hood. After the appropriate time interval, wells were washed twice to remove excess collagen. After 2 min with phosphate-buffered saline, one final rinse with tissue culture medium was performed.

ASF (enzymatically prepared; Sigma Chemical Co., St. Louis, MO) was diluted with phosphate-buffered saline to 80 μg/ml, and 2 ml of this solution were added per well and allowed to stand in the laminar-flow hood at room temperature overnight. The ASF-coated wells were washed as described for the collagen-coated plates. In all experiments, serum-free medium as described earlier (7) was used; 0.5 × 10^6 cells/ml (1.5 ml/40-mm plate) in complete Waymouth medium, supplemented with CaCl_2 (final concentration, 1.4 mM) and [3H]ASF (1 ng/ml) were added to the wells. After 45 min, medium was removed, and the unattached cells were plated on the collagen-coated plates.

Histological Stainings. The method of Rutenberg et al. (22) was used for histological demonstration of GGT in frozen sections taken 1 week prior to perfusion. Bouin's fixative was used for staining with hematoxylin and eosin. For glycogen, tissue samples were fixed in Carnoy's medium and stained using periodic acid-Schiff. The method for glucose-6-phosphatase was that of Wachstein and Meisel (27). For immunoperoxidase studies, frozen sections of liver were fixed for 5 min in acetone. Avidin-biotin-peroxidase complex method was used as described by Hsu et al. (15) using Vekstra ABC Kit by Vector Laboratories (Burlingame, CA). The primary antibody against rat liver ASF-receptor prepared in a goat was a generous gift by Dr. G. Ashwell, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, NIH, Bethesda, MD. The specificity of this antibody is described in detail by Harford et al. (14). Serial frozen sections were stained for GGT and for glucose-6-phosphatase.

Determination of ASOR and ASF Surface Receptors on Isolated Liver Cells. Tritium-labeled ASOR and ASF were prepared by the method of Means and Feeney (18). Iodine labeling of ASF was accomplished using the chloramine-T method (12). For ASOR receptor assays, liver cells were treated with 20 mM EDTA in calcium-free Waymouth's medium, pH 7.4, for 3 min at 4°C, centrifuged, and washed twice with calcium-free Waymouth's medium. CaCl_2 (final concentration, 1.4 mM) was added to liver cells (1 × 10^6 cells/ml) which were incubated with 4 μg [3H]ASOR for 45 min at 4°C. Control tubes did not contain calcium. ASOR receptor assays were performed in a similar manner, except that CaCl_2 (final concentration, 1.4 mM) and [3H]ASF (1 μg/ml) were added to all tubes. A 200-fold excess of nonlabeled ASF was added to one-half of the tubes to measure nonspecific binding. After 45-min incubation at 4°C, ice-cold Waymouth's medium (5 ml) was added to the cell suspension which was then spun down and washed twice to remove the unbound radioactive asialoglycoprotein. Cells were dissolved in Triton X-100, and radioactivity in 100 and 14C were counted in a liquid scintillation counter (Beckman Model LS9000) and LKB 1270 Rack Gamma II counter, respectively.

RESULTS

Elutriation Studies. The separation of liver parenchymal cells of carcinogen-treated animals in early or late preneoplastic stages was carried out using centrifugal elutriation. One week before elutriation, liver biopsies of carcinogen-treated animals were taken for histological evaluation. Areas of early and late preneoplastic changes according to the definition of Bannasch et al. (4) were identified by hematoxylin and eosin staining and by glycogen staining of liver samples. Only those livers which showed a distinct increase in the size of the cells in the altered liver cell areas were then used for elutriation experiments (Fig. 1). Staining for GGT in frozen liver section varied. Both altered liver cells and bile duct cells in perportal areas showed positive GGT staining.

Cells from the carcinogen-treated animals were separated into 6 fractions using centrifugal elutriation. The average cell size in each fraction was estimated using a gridicule attached to the microscope eyepiece together with stage micrometer. The first eluted fraction was composed mainly of small nonparenchymal cells and dead parenchymal cells. The sizes of the cells in Fractions 2 and 3 varied from 17 to 20 μm (Table 1). These cells constituted 58 to 68% of the total parenchymal cell population. The cells in these fractions ("small cells") were pooled (Fig. 2A). The cells in Fraction 4 were intermediate in size, whereas the cells in Fractions 5 and 6 were considerably larger with sizes varying from 24 to 48 μm (Fig. 2B) ("large cells"). Binucleated cells were common in the large-cell fractions. In general, cells in Fractions 5 and 6 were less uniform in size. These fractions constituted 13 to 25% of the total cell population. Cell smears of each fraction were stained for GGT activity. No significant differences in the number of GGT-positive cells between different cell fractions were observed.

Asialoglycoprotein Receptor. Table 2 shows the binding of ASOR and ASF to the surface receptors on normal and preneoplastic hepatocytes. When either [3H]ASOR or [3H]ASF was used as a ligand for the surface receptor assay, approximately 45% reduction in the binding to the cell surface was recorded in cell suspensions obtained from the early and/or late preneoplastic livers as compared to cell suspensions from normal liver (Table 2). Chart 1 shows the effect of the ligand concentration on the binding of ASF to the cell surface. In this experiment, one μg of ASF in 1 ml of incubation mixture was adequate to saturate the receptor binding sites. Chart 2 shows the dependence of ASF binding on time. The binding sites were saturated after 45 min.

Table 1

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<th>Average size and distribution of liver parenchymal cells separated by centrifugal elutriation</th>
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<tr>
<td>Cell diameter (μm)</td>
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<tr>
<td>Small cells (Fractions 2 and 3)</td>
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<tr>
<td>Intermediate cells (Fraction 4)</td>
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<td>Large cells (Fractions 5 and 6)</td>
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* Values are means of 5 experiments. The cell size was estimated from each fraction on microscopic slides using an ocular grid and a stage micrometer.

* Numbers in parentheses, range.
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Table 2

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<th>Binding of ASOR and ASF to the surface receptors of liver cells from control and carcinogen-treated animals</th>
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<td></td>
<td>([\text{H}])ASOR (specific activity, 1.505 \times 10^{5} \text{ dpm}/\mu\text{g}) was added to one ml of liver cell suspension in Ca^{2+}-free Waymouth’s medium to a final concentration of 4 \mu\text{g}/ml. The values obtained without Ca^{2+} were subtracted from the values obtained with Ca^{2+}. ([\text{H}])ASF (specific activity, 1.150 \times 10^{5} \text{ dpm}/\mu\text{g}) was added to one ml of liver cell suspension in Waymouth’s medium containing 1.4 \text{mM Ca}^{2+} to a final concentration of 1 \mu\text{g}/ml. The nonspecific binding obtained in the presence of 200-fold excess of unlabeled ASF was subtracted from the specific binding.</td>
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<td>ASF</td>
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<td>Preneoplastic liver</td>
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{^a} Avogadro’s number (6 \times 10^{23} \text{ molecules/mol}) was used for calculation of receptor sites per cell.

{^b} Mean \pm S.E. Significant at p > 0.01 (Student’s t test was used for statistical evaluation).

{^c} Histological changes showed clear cell and eosinophilic areas which were either glycogen positive or glycogen negative.

{^d} Significant at p > 0.001.

Chart 1. Effect of (\[\text{H}\])ASF concentration on the specific binding to surface receptors of control liver cells. Cells were incubated in the presence of 1.4 mM Ca^{2+} in Waymouth’s medium at 4\textdegree for 45 min.

For centrifugal elutriation experiments, it was necessary to treat the liver cell suspension with deoxyribonuclease and trypsin inhibitor in order to get a good separation. These treatments did not significantly change the binding of ASF to liver cells.

When albumin was added for preparing the liver cell suspension, it had an inhibitory effect on receptor binding at high concentrations (0.5 to 1%), whereas at low concentration (0.2 to 20 \mu\text{g}/ml), it did not have any effect (data not shown). A comparison between GGT-positive, glucose-6-phosphatase-negative, and asialoglycoprotein-receptor-negative areas (foxi) was performed in preneoplastic livers, and the results are shown in Fig. 3. Frozen serial sections of preneoplastic liver, which were positive for GGT and negative for glucose-6-phosphatase, showed a lack of the asialoglycoprotein-receptor protein in the same areas, as demonstrated by avidin-immunoperoxidase staining (Fig. 3). In these limited series of sections, the areas that were deficient in asialoglycoprotein-receptor were entirely superimposable with those negative for glucose-6-phosphatase, and partially overlapped the GGT-positive areas (Fig. 3).

The observed decrease in binding of ASF to preneoplastic cells and the immunohistochemical demonstration of the lack of ASF-receptor-protein in the preneoplastic areas, led to an attempt to use asialoglycoprotein-coated plates as a selective surface for separating normal liver cells from preneoplastic liver cells. When cells obtained from normal rat liver were plated on ASF-coated plates, maximum attachment was obtained in 45 to 60 min (Chart 2). Chart 3 shows that the attachment to ASF-coated plates was dependent on the cell concentration. The highest number of cells attached when 5 \times 10^5 cells/ml were plated, whereas the highest proportion of the plated cells attached at the concentration of 1 \times 10^6 cells/ml (82% for control cells). When cells from the carcinogen-treated animals were used, the percentage attachment at optimum cell concentration was 30 to 40% less than for the control cells. The attachment of liver cells to the ASF plates was entirely abolished when 5 \text{mM EDTA} was added to the Waymouth’s medium containing 1.4 mM CaCl_2. Receptor assay of supernatant preneoplastic cells from ASF plates gave 1,704 \pm 114 dpm/1 \times 10^6 cells (S.E.) as compared to 13,106 \pm 763 dpm/1 \times 10^6 cells obtained for normal cell suspension.

The percentage of GGT-positive cells has been widely used as a measure for preneoplastic hepatocyte purification (13, 16, 19). Liver cells from carcinogen-treated animals, containing 15 to 25% GGT-positive cells, were plated at different concentrations on ASF-coated plates and after 45-min incubation trans-
ferred to collagen-coated plates. Cells were allowed to attach but not spread on collagen. Both ASF and collagen cells were stained for GGT, and the number of positive cells was counted. GGT-positive cells were practically absent from ASF (<5%) and large-cell fractions obtained by centrifugal elutriation were stained for GGT, and the number of positive cells was counted. Both ASF and collagen cells were not spread on collagen. Both ASF and collagen cells were plated on collagen-coated plates. Both fractions attached rapidly on collagen.

Combined Experiments Using Centrifugal Elutriation and Specific Attachment to ASF-coated Plates. When the small- and large-cell fractions obtained by centrifugal elutriation were plated on ASF-coated plates, the attachment of cells from the large-cell fraction was poor (<10% of control), whereas the cells from the small-cell fraction showed similar attachment (50 to 55%) as was observed with control hepatocytes (0.75 x 10^6 cells were plated/well). After 45 min on ASF plates, media containing unattached cells of both fractions were transferred to collagen-coated plates. Both fractions attached rapidly on collagen.

Receptor assay using [125I]ASF as a ligand was performed on the small- and large-cell fractions. Table 4 shows the results of 2 experiments. The amount of ligand attached in the large-cell fraction was poor (<10% of control), whereas the cells from the small-cell fraction showed similar attachment (50 to 55%) as was observed with control hepatocytes (0.75 x 10^6 cells were plated/well). After 45 min on ASF plates, media containing unattached cells of both fractions were transferred to collagen-coated plates. Both fractions attached rapidly on collagen.

Table 4

| No. of cells plated/ml x 10^5 | % of GGT-positive cells
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<tr>
<td>ASF</td>
<td>Collagen</td>
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<tr>
<td>0.11</td>
<td>2</td>
</tr>
<tr>
<td>0.22</td>
<td>1</td>
</tr>
<tr>
<td>0.44</td>
<td>2</td>
</tr>
<tr>
<td>0.88</td>
<td>3</td>
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</table>

*On both ASF- and collagen-coated plates, 300 cells were counted. Cell concentration of 0.5 x 10^6 cells/ml was routinely used for plating the cells on ASF plates. The average number of GGT-positive cells of 8 separate experiments gave 7 ± 2 and 65 ± 7 on ASF- and collagen-coated plates, respectively. The number of GGT-positive cells in the original cell suspension was 21 ± 4.

Numbers in parentheses, range.

DISCUSSION

One of the early phenotypic changes in chemical hepatocarcinogenesis is the formation of islands or foci of clear or eosinophilic cells. At this early stage of carcinogenesis, the size of the cells in these islands is significantly greater than that of the hepatocytes in general (4–6). These cells are usually glycogen storing cells which then gradually lose their capacity to store glycogen, and simultaneously the size of the cells decreases and staining with hematoxylin-eosin tends to be more basophilic (4).

Concomitant with these cytomorphological changes are extensive modulations of the surface of preneoplastic hepatocytes as well as appearance of histochemical markers such as GGT, that are closely associated with the neoplastic process (1, 9, 11, 20). By using the increased size of the early preneoplastic hepatocyte, and its deficiency of the asialoglycoprotein surface receptor, we have been able to isolate a relatively pure population of these putative precursor cells for rat liver hepatoma.

In order to gain a better understanding of the sequence of cellular alteration that occur during chemically induced hepatocarcinogenesis, it is necessary to characterize the early preneoplastic cell populations. Several methods have been used with different degrees of success, in the search for ways to separate normal hepatocytes and nonparenchymal liver cells from the preneoplastic cells that arise early in chemically induced hepatocarcinogenesis. Following dissociation of the liver by collagenase perfusion, centrifugal elutriation (28, 30), centrifugation in metrizamide and Ficoll gradients (16, 19), and incubation of the liver cell suspension on tissue culture dishes coated with affinity-purified rabbit anti-GGT antibody (13) have been tried.

GGT is the most widely used histochemical marker for the preneoplastic stage in rat liver, and this enzyme is most frequently used to measure enrichment of the preneoplastic cell population during cell separation procedures (10, 13, 16, 19, 28). However, nonparenchymal liver cells such as biliary ductular and ductal cells stain positively for GGT, and the pattern and intensity of staining in the focal islands can vary independently of both liver histological type and the stage of occurrence (17). Consequently, positive staining for GGT cannot unequivocally be regarded as a marker for preneoplastic cells (9, 25).

Recent studies have shown diminished hepatic binding protein(s) for desialylated glycoproteins during chemical hepatocarcinogenesis (26). Our data (Table 2) on the asialoglycoprotein receptor in liver cells from control and carcinogen-treated animals are in agreement with these findings. The results obtained by comparing staining for GGT and glucose-6-phosphatase with the avidin immunoperoxidase staining for the asialoglycoprotein receptor in serial section from livers having numerous clear-cell foci show that areas deficient in the receptor protein and glucose-6-phosphatase overlap entirely, whereas GGT-positive areas are only partially superimposable (Fig. 3). Furthermore, data on the receptor concentrations in the elutriation cell fractions show that...
the "large" cell, presumably containing the clear cells, have much fewer receptor sites than do the "small" cells (Table 4). These results indicate that measurement of ASF-receptor concentration in liver cell suspension obtained by collagenase perfusion reflects the cellular distribution of the receptors in the intact preneoplastic liver.

Adult rat hepatocytes can attach in vitro to tissue culture plates coated with either collagen or asialo-glycoprotein such as asialoglycoplasmin and ASF, in the absence of serum (21). The mechanism of hepatocyte attachment to collagen is clearly different from that involving the attachment to asialoglycoproteins. The effect of Ca++, soluble asialoglycoprotein(s), asialooligosaccharides, and neuraminidase treatment of the cells prior to plating indicate that the attachment to the asialoglycoprotein-coated plates is mediated by the receptor involved in recognition and uptake of asialoglycoprotein (21). We have taken advantage of the difference in asialoglycoprotein-receptor concentration between normal and preneoplastic or neoplastic hepatocytes by selecting the putative preneoplastic cell population on the basis of lack of binding to ASF-coated substratum. This selection can be carried out either directly following the collagenase digestion of the liver or as an additional selection step to centrifugal elutriation. The concentration of ASF-receptor on the cells that did not attach to the ASF-coated plates is less than 10% of that found on control hepatocytes, suggesting that most normal (i.e., not preneoplastic) hepatocytes bind to the plates.

Both normal and preneoplastic hepatocytes attach and spread on collagen substratum without addition of serum. Since the immunoperoxidase staining for the asialoglycoprotein receptor identified focal areas in livers from carcinogen-treated animals that were not GGT-positive (Fig. 3), it was of interest to examine the percentage of cells positive for GGT following the ASF-attachment selection. In the early preneoplastic stage, in which 15 to 25% of the parenchymal cells stain positive for GGT, selection on ASF-substratum, and subsequent plating of the unattached cells onto collagen-coated plates yields 40 to 70% GGT-positive cells (Table 3). Of the cells attached to ASF, only 1 to 3% stain positive for GGT. The present isolation procedure for obtaining preneoplastic hepatocytes selects at least 2 subpopulations of preneoplastic cells, namely, those positive for GGT and negative for the ASF receptor and those negative for both GGT and ASF receptor. Whether phenotypic changes in the expression of ASF receptor and/or the expression of GGT are a direct result of the initiating event or reflect an adaptive response is likely to be elucidated. Nevertheless, we have established that viable and chemically hepatotransformed cell fractions in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (AP) procedure. J. Histochem. Cytochem., 29: 577-580, 1981.


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Fig. 1. Micrograph of the early preneoplastic liver showing the size difference between the normal and altered areas. × 630.

Fig. 2. Cell smears of the elutriated cell fractions. A, small cells; B, large cells (see Table 1). Cell smears were air-dried, fixed with cold ethanol, and stained for GGT activity. × 630.
Fig. 3. Serial sections of preneoplastic liver. A, stained for GGT; B, stained for glucose-6-phosphatase; C, stained for ASF-surface receptor by the avidin-immunoperoxidase method. × 37.
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