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**ARTICLE** *in* HEPATOLOGY · AUGUST 1994

Impact Factor: 11.06 · DOI: 10.1016/0270-9139(94)90203-8



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# Recent Advances in the Isolation of Liver Cells

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The liver consists of two types of epithelial cells: hepatocytes, which represent 60% to 70% of the liver cell population (1), or 90% of total liver mass (2); and intrahepatic bile duct epithelial cells, or cholangiocytes, which make up 2% to 3% of the liver cell population (3-5). Other liver cells include SCs, KCs, FSCs, pit cells, myofibroblasts and other endogenous liver cells (e.g., vascular and lymphatic endothelial cells, dendritic cells), which together account for 25% to 40% of the total hepatic cell population, or 10% of liver mass (1) (Table 1).

For study of the precise functions of various liver cells, isolation techniques are required that provide a sufficient number of cells with a high degree of purity and viability. Justified concerns about the use of isolated cells include (a) the isolation process may alter plasma membrane components, (b) normal cellular functions that require attachment to basement membrane components or cooperative interactions between adjacent cells are lost during cell isolation and (c) isolated cells may exhibit responses that are altered compared with their normal *in situ* functions. Despite these concerns, studies of isolated cells have considerably expanded our knowledge of the biology of individual liver cells in ways not possible with the intact organ (5-17).

Our purpose here is to present an overview of recent advances in the isolation of the distinct cell types found in the liver and to provide technical detail sufficient to permit the experimental hepatologist to select the optimal experimental approach for the purification and characterization of specific liver cells. The first part provides an overview of the general mechanical and enzymatic approaches used for successful liver cell dispersion, together with a description of the morphological, biochemical, histochemical and immunohistochemical criteria commonly used to assess cell yield, viability and purity. After a description of the principles and theory of techniques commonly used for liver cell isolation, the purification and characterization of specific liver cell types are addressed.

## GENERAL CONSIDERATIONS IN DISSOCIATION OF LIVER CELLS

### *Experimental Approach*

Because the rat is the animal model used in our laboratory and in many other laboratories, this review will focus on the purification and characterization of cells from rat liver. Because the basic surgical approach to liver perfusion has already been described (18), only a brief description of the surgery is presented. After anesthesia and a midline abdominal incision, the portal vein is cannulated and perfused with $Ca^{++}$-free, oxygenated preperfusion buffer A (Table 2). After 5 to 10 sec of perfusion, the inferior vena cava is severed distal to the renal veins. A 2- to 3-cm plastic tube (PE 205; Clay Adams, Parsippany, NJ) is secured in the thoracic portion of the inferior vena cava and the abdominal portion of the inferior vena cava is ligated. The liver is perfused with a total of 250 ml of preperfusion buffer A over 5 to 10 min to wash out blood cells and to remove $Ca^{++}$ (19).

When the effluent is clear, the liver is placed in the IPRL apparatus (20) (Fig. 1). The perfusate is changed to a solution containing a combination of proteolytic enzymes (e.g., 0.02% collagenase with 40 to 80 μg/ml DNase I and incubated for 20 min at 37° C. After adequate liver digestion, as determined empirically on the basis of the softness of the organ in response to gentle pressure applied with a wet cotton-tipped applicator, cells are dissociated by means of gentle mechanical combing. Table 3 provides a list of problems that may be encountered during various stages of the liver perfusion stage of cell isolation.

### *Mechanical/enzymatic Dissociation*

For production of a single cell suspension from the liver by means of mechanical (21) and/or enzymatic (5-17, 22, 23) dissociation, the cells must be free of desmosomes and tight junctions (20); in addition, the attachment of cells to the extracellular matrix, a complex network of collagen, proteoglycans and cross-linking proteins including laminin and fibronectin (24, 25), must be abolished. The major advantage of mechanical

Received December 17, 1993; accepted March 29, 1994.

This work was supported by grants DK 24031 and HL 07269 from the National Institutes of Health and by the Mayo Foundation.

*Other abbreviations used in the text:* CFE, counterflow elutriation; DNase I, deoxyribonuclease I; EGTA, ethyleneglycol-*bis*-(β-aminoether)-N,N,N′,N′-tetraacetic acid; FACS, fluorescence-activated cell sorting; FFE, free-flow electrophoresis; FSC, fat-storing cell; GGT, γ-glutamyltranspeptidase; HEPES, N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid; IPRL, isolated perfused rat liver; KC, Kupffer cell; MHC, major histocompatibility complex; NK, natural killer; PEG, polyethylene glycol; SC, sinusoidal endothelial cell; VC, vascular endothelial cell.

HEPATOLOGY 1994;20:494-514.

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0270-9139/94 $3.00 + 0 **31/1/57241**

methods over enzymatic digestion techniques for the dispersion of liver tissue is that membrane proteins are not as likely to be modified as they are with proteolytic enzymes (23, 26). Early reports (21) on liver cell isolation utilized these mechanical techniques; however, because of low cell yield and poor viability (27), mechanical approaches have generally been replaced by more efficient enzymatic cell isolation approaches (5-17, 22, 23, 28). Since its introduction by Howard (7) and Berry and Friend (6), collagenase has become the most widely used protease for the enzymatic digestion of the liver (5, 9, 11, 14, 22, 23, 28). Collagenase perfusion provides a superior cell suspension with reduced alterations in cell membrane composition and greater functional activity (26) than has been observed with mechanical dispersion techniques (21) or with other proteolytic enzymes (22, 23, 26, 28). However, collagenase shows a great deal of variation in enzyme activity and specificity when purchased from different vendors or even among lots from the same vendor (15). Therefore several different lots of collagenase should be tested to determine which lot best preserves the function to be assayed.

The first proteolytic enzymes used for the preparation of nonhepatocyte liver cells were trypsin and pronase (29). These proteases exhibit broad substrate specificity, cause lysis of hepatocytes and alter the quantitative expression of membrane proteins (e.g., receptors) on liver cells (23, 26, 28). Nonetheless, even though various cellular functions are diminished by these enzymes, a brief culture period after isolation may allow reexpression of proteins damaged by these enzymes and restoration of cell function (23, 28, 30). Low-temperature isolation techniques minimize damage to certain cellular membrane proteins and have been successfully used for the isolation of nonhepatocyte cells, including KCs, SCs and FSCs (31).

To enzymatically digest the liver, proteases may be incubated directly with minced liver tissue (32) or perfused through the liver *in situ* through the portal vein (6, 7, 15, 16). However, in some experimental states associated with portal hypertension and fibrosis (4) (e.g., ductular hyperplasia induced by bile duct ligation), access to the portal vein may be technically difficult (Alpini G et al., Unpublished observations, 1993); under these circumstances, enzymatic digestion may be performed by way of a retrograde route, with perfusion of the liver through a cannula inserted into the thoracic portion of the inferior vena cava (33).

Pure preparations of hepatocytes are easily obtained by means of standard collagenase perfusion with mild enzymatic treatment (6, 7, 15, 16). The release of nonhepatocyte liver cells requires additional, "harsher" enzymes, including pronase (34), a collagenase-pronase mixture (5, 9, 14) and trypsin (11, 23). Because hepatocytes are preferentially lysed or damaged by pronase (5, 9, 14), trypsin (11, 23) and endotoxins from *Clostridium perfringens* (35), these harsher enzymatic treatments have been used to isolate nonhepatocyte liver cells and simultaneously remove hepatocytes. Table 4 provides a summary of the enzymes most commonly used for liver cell isolation.

**TABLE 1. Endogenous and exogenous liver cells**

| |
|---|
| Endogenous cells |
| Epithelial cells |
| Hepatocytes |
| Intrahepatic bile duct epithelial cells (cholangiocytes) |
| Cells of the reticular endothelial system |
| SCs |
| KCs |
| Structural and lipid storage cells |
| FSCs/fibroblasts |
| Myofibroblasts |
| Mesothelial cells (Glisson's capsule) |
| VCs |
| Endothelial cells of arteries, veins, and lymphatic vessels |
| Smooth muscle cells of arteries |
| Miscellaneous cells |
| Pit cells (large granular lymphocyte) |
| Dendritic cells |
| Neurons |
| Exogenous cells |
| Leukocytes |
| RBCs |

### *Buffer Composition*

Although ion concentration must be maintained in the physiological range, no single buffer formulation has been demonstrated to possess superior qualities for the isolation of liver cells. Appropriate buffer solutions for liver perfusion include Krebs-Ringer bicarbonate buffer, Hanks' balanced salt solution and Dulbecco's phosphate buffered saline solution (to which glucose, pyruvate, L-glutamine or L-aspartic acid may be added) (36). The formulation provided in Table 2 is a useful buffer that is applicable to the isolation procedures reviewed here. Enzymatic digestion of the liver can be accomplished in either a recirculating (6, 7, 20) or nonrecirculating (37) perfusion apparatus in a temperature-controlled cabinet. Liver perfusion with a nonrecirculating system is not influenced by the release of metabolic products from the liver, which would continuously change the composition of the perfusate; however, this system is both inconvenient and expensive. Therefore rat liver perfusion, as an initial step in the isolation of liver cells, is commonly performed with a recirculating perfusion apparatus (Fig. 1). The recirculating system employs about 150 ml of physiological buffer (pH 7.4) and consists of a peristaltic pump that pushes perfusate from a reservoir to a glass oxygenator. The perfusate is then pumped to the liver at a selected flow rate (e.g., 30 to 40 ml/min) (6, 16, 20). The perfusate finally exits the liver and is returned to the reservoir, thus completing the circuit.

Throughout the isolation procedure, the pH of the solutions should range from 7.4 to 7.5. During perfusion, the metabolic activity of the liver may reduce the pH of the perfusate to 6.8 or less, as a result of the accumulation of acidic metabolic products such as lactic acid (38). Therefore in a closed recirculating system the

TABLE 2. $Ca^{++}/Mg^{++}$-free buffer and additives used in liver cell isolation

| Chemicals | Formula weight | Concentration | |
|---|---|---|---|
| | | gm/L | mmol/L |
| Essential components | | | |
| NaCl[a] | 58.4 | 8.176 | 140 |
| KCl[a] | 74.6 | 0.403 | 5.4 |
| $Na_2HPO_4$[a] | 142.0 | 0.114 | 0.8 |
| HEPES[a] | 238.3 | 5.958 | 25.0 |
| Additives | | | |
| EGTA[b] | 180.2 | 2.253 | 12.5 |
| Sodium pyruvate | 110.0 | 0.253 | 2.3 |
| L-Glutamine | 146.2 | 0.336 | 2.3 |
| $CaCl_2 \cdot 2H_2O$[c, d] | 147.0 | 0.294 | 2.0 |
| $MgSO_4$[d] | 120.7 | 0.097 | 0.8 |
| D-Glucose | 380.4 | 0.192 | 0.5 |

[a]A mixture of compounds with this designation yields buffer A.
[b]The chelating agent EGTA is added to buffer A during preperfusion of the liver.
[c]$Ca^{++}$ is added to buffer A during the collagenase perfusion step.
[d]$Ca^{++}$ and $Mg^{++}$ ions are added to buffer A during digestion with DNase I.

pH should be monitored and adjusted with 0.14N NaOH (14). Alternatively, to avoid continuous pH adjustments during perfusion (15, 16), one may use buffers to maintain a physiological pH (11, 39). The use of organic buffers such as HEPES, not exceeding 25 mmol/L, maintains a stable pH and may be less toxic than other common buffers (e.g., Tris-HCl or $CO_2/HCO_3^-$) (40). If the perfusion solution is buffered with $HCO_3^-$ ions, the pH may be adjusted by means of continuous gassing with 5% $CO_2$ (41).

Some investigators include nutrients such as D-glucose, sodium pyruvate and L-glutamine in cell isolation buffers (36); however, the importance of these components in maintaining cell integrity has not been established. Because the absence of $Ca^{++}$ during the initial 10-min preperfusion step increases cell yield (21, 27), 0.5 mmol/L EGTA, a $Ca^{++}$ chelator, is often included (19). However, after the pre-perfusion step, $Ca^{++}$ should be reintroduced because irreversible damage to hepatocytes may occur when they are incubated in $Ca^{++}$-free buffers for more than 1 hr (39); in addition, $Ca^{++}$ markedly increases the proteolytic activity of collagenase (10). $Mg^{++}$ ions are required for DNase I activity and should also be included; $Ca^{++}$ and $Mg^{++}$ both stabilize this enzyme (11).

### *Assessment of Cell Purity and Viability*

Assessment of cell purity requires morphological (10, 13), biochemical (22), histochemical (9, 11) or immunohistochemical techniques (5, 14) that allow the identification of the specific cells of interest. Whereas hepatocytes are readily identified on the basis of their morphological characteristics (i.e., size and appearance) (15), nonhepatocyte liver cells are not as easily identified without cell-specific assays (5, 10-12, 17). Because the degree of cell purity varies with all isolation procedures, identification of contaminating cells is critical for accurate interpretation of experimental data. A list of markers specific for most liver cells is provided in Table 5.

The simplest and most popular technique for assessing cell viability is the trypan blue exclusion method (42). With this approach, it is easy to differentiate under light microscopy the bright, refractile, nonstaining cells from the blue-staining dead cells. If a hemocytometer counting chamber is used, the cell yield can also be quantified (Fig. 2). Alternatively, cells may be treated with physiologically buffered nitro blue tetrazolium, which living (but not dead) cells reduce to an insoluble blue furazan complex (42). Two fluorescent substrates commonly used to determine cell viability are (a) diacetofluorescein, a fluorescein ester that permeates viable cells and is hydrolyzed by nonspecific intracellular esterases to fluorescein (43); and (b) propidium iodide, a compound commonly used during flow cytometry (44).

Cell viability may also be determined with biochemical assays such as detection of lactate dehydrogenase (6, 45), which is released from leaky or damaged cells with poor membrane integrity. The choice of technique for determining cell yield and viability depends on the time and equipment available. Electronic Coulter counters and Channelyzers (Coulter Electronics, Hialeah, FL) are precise, efficient instruments for determining cell number and size (5, 46), but their use is limited by the cost of the equipment. Cell number, viability, degree of cell clumping and estimation of cell debris are most commonly assessed by means of light microscopic techniques (41).

## GENERAL EXPERIMENTAL APPROACHES FOR LIVER CELL ISOLATION

The heterogeneous nature of cells in the liver, as well as their overlapping physical properties (e.g., size and density, Fig. 3) (1, 5, 8, 9, 11, 46), has led to the

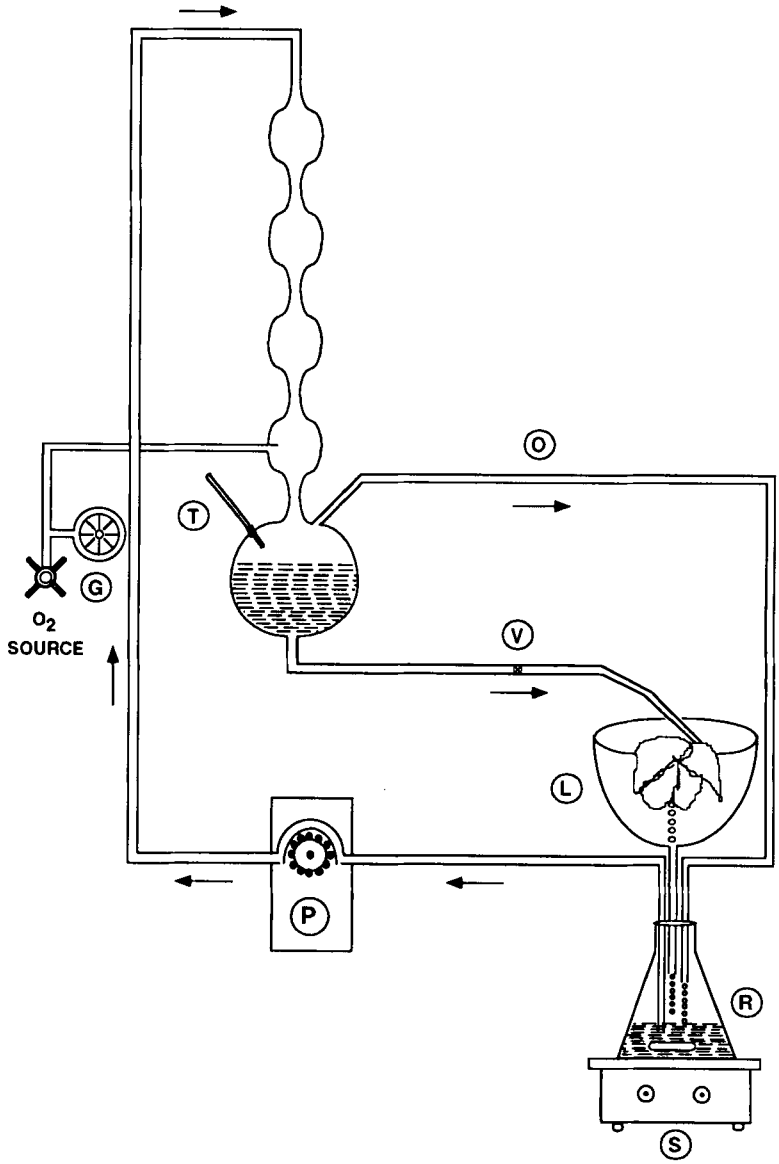


FIG. 1. Diagram of the IPRL apparatus. Entire IPRL is enclosed in a temperature-controlled cabinet (i.e., 37° C). Arrows denote direction of perfusate flow. *S*, stirring plate; *R*, reservoir; *P*, peristaltic pump; *G*, gauge; *T*, thermometer; *V*, valve; *O*, overflow; *L*, liver.

development of many techniques for isolating liver cell subpopulations enriched in one cell type. An understanding of the advantages and disadvantages of these various techniques should simplify the choice of the most appropriate protocol.

### *Isopyknic Centrifugation and CFE*

Centrifugal techniques provide a broad array of approaches to purify cells based on the properties of cell size or density (Table 6). The complexity of these various techniques ranges from simple velocity sedimentation at unit gravity (47) or sedimentation by means of isopyknic centrifugation (8, 11, 13, 14, 35, 48) to more complex computer-generated isokinetic gradient centrifugation (47). Although sedimentation by centrifugation may be accomplished with most clinical centrifuges, methods such as CFE (5, 8, 9, 11, 13, 14, 49), and isopyknic gradient centrifugation (8, 11, 13, 14, 34, 50) require special rotors, modified centrifuges or both (51). With complex mixtures of hepatic cells, no isolation technique based solely on size, density or surface configuration yields a cell preparation of high purity (with the exception of hepatocytes) because of the overlap in size and density of most cells (5, 7, 9, 11) (Fig. 3). Therefore cell separation is best accomplished with a combination of techniques that rely primarily on differences in cell density (11, 13, 14) and size (5, 11, 13, 14, 49).

Isopyknic centrifugation of cell suspensions is generally performed with commercially available gradient materials such as Percoll (sterile colloidal suspension of silica particles coated with polyvinyl pyrrolidine; Pharmacia, Piscataway, NJ) (10, 52), metrizamide

**TABLE 3. Troubleshooting cell isolation schemes**

| Problem | Cause | Solution |
|---|---|---|
| Poor perfusion | Air bubbles in liver | Avoid bubbles during portal vein cannulation and changing cannula from preperfusion step to IPRL; use an air bubble trap in the perfusion apparatus. |
| | Particulates in liver | Filter buffers and enzymes. |
| | Liver blood clots | Use heparin to diminish blood clotting; flush liver rapidly and completely to remove endogenous blood. |
| Incomplete digestion of liver | Poor perfusion | See above. |
| | Insufficient enzyme concentration | Increase amount or specific activity of collagenase. |
| | No $Ca^{++}$ in buffer | Add $Ca^{++}$ to 5 mmol/L. |
| Poor recovery of cells | Large portions of nonperfused liver | See above. |
| | Incomplete digestion | See above. |
| | Low number of viable cells | Try different type or lot of collagenase; decrease amount of collagenase. |
| | | During perfusion, keep liver moist. |
| | Cell clumping | Include DNase I. |
| | Gross contamination by microbes | Clean perfusion apparatus well after each use; prepare fresh buffers or filter buffers before use. |
| | Rough manipulation of Glisson's capsule | Remove Glisson's capsule by means of gentle mechanical dissociation. |
| | Unsatisfactory elutriation | See "General Experimental Approaches for Liver Cell Isolation" |
| | Poor gradient isopyknic centrifugation | See "General Experimental Approaches for Liver Cell Isolation" |

**TABLE 4. Enzymes commonly used for tissue dissociation**

| Enzyme | Specificity | Requirements | pH optimum | Remarks |
|---|---|---|---|---|
| H Collagenase (EC 3.4.24.3) | Highly specific; degrades native helical collagen fibrils | $Ca^{++}$ | 6-8 | Most commonly used enzyme for liver cell dissociation |
| Hyaluronidase (EC 3.2.1.35) | Hydrolyses the 1,4-linkage between D-glucose and 2-acetamido-2-deoxy-β-D-glucose residues in hyaluronate | | 4.5-6 | Conflicting reports for using this enzyme with collagenase |
| DNase I (EC 3.1.21.1) | Digests both single- and double-stranded DNA | $Mg^{++}$ and $Ca^{++}$ | 7.8 | Stabilized against proteolytic digestion by $Ca^{++}$ (5 mmol/L) |
| Pronase | Broad-spectrum | Stabilized by $Ca^{++}$ ions | 7.5 | Causes hepatocyte lysis |
| Trypsin (EC 3.4.21.4) | Highly specificity for proteins with lysine and arginine residues | | 7.9 | Alters cell membrane proteins |
| Dispase (EC 3.4.24.4) | Mixture of broad spectrum proteases | | 8.5 | Alters cell membrane proteins |
| Papain (EC 3.4.22.2) | Wide specificity | Cysteine, β-ME, EDTA | 6-7.2 | Alters cell membrane proteins |
| Chymotrypsin (EC 3.4.21.1) | Broad substrate specificity | | 7.9 | Alters cell membrane proteins |
| Clostripain (EC 3.4.22.8) | Highly specific for carboxyl peptide bond of arginine | $Ca^{++}$, DTT, cysteine | 7.4-7.8 | Alters cell membrane proteins |
| Protease, *S. aureus* (EC 3.4.21.9) | Specifically cleaves carboxyl terminal side of aspartic or glutamic acids | | 7.8 | Alters cell membrane proteins |
| Proteinase K (EC 3.4.21.14) | Hydrolyzes a wide range of denatured and native high molecular weight proteins | Stabilized by $Ca^{++}$ ions | 7.5 | Alters cell membrane proteins |
| Lysozyme (EC 3.2.1.17) | Preferentially hydrolyzes the β-1,4 glucosidic linkage between N-acetyl-muramic acid and N-acetyl-glucosamine | | 9.2 | Conflicting reports for use with collagenase |

*β-ME*, β-mercaptoethanol; *DTT*, dithiothreitol.

13, 34, 50), Ficoll 400 (hydrophobic polymer of sucrose; Pharmacia) (50, 53), Nycodenz (Sigma Chemical Co., St. Louis, MO) (14, 17, 51) or stractan (12). The relatively low viscosities and osmolalities of these gradient materials, in combination with high density properties (15, 30), have led to their frequent use in liver cell isolation (10, 11, 22, 52). Percoll (10, 11, 22, 52, 53) is one of the most commonly used gradient materials and, like Ficoll

**TABLE 5. Marker enzymes/proteins for identifying liver cells**

| Cell | Location | Detection method | Marker | Remarks | Reference |
|---|---|---|---|---|---|
| All hepatocytes | L | E | Glucose-6-$PO_4$ase | | 92 |
| | | M | Appearance | | 15 |
| | | I | Albumin | | 5 |
| Periportal hepatocytes | PP | E | Carbamyl-$PO_4$-synthetase | | 93 |
| Perivenous hepatocytes | PV | E | Glutamine synthetase | | 93 |
| | | I | P-450b | | 93 |
| SCs | S | FM | Acetylated LDL-DIL | Receptor-mediated endocytosis | 126 |
| | | | | Conflicting data as to whether SCs contain vWF | 9 |
| KCs | S | E | Peroxidase | | 112 |
| | | E | Acid Phosphatase | | 120 |
| | | LM or FM | Latex beads, bacteria or yeast | Phagocytosis of probe | 34,112 |
| FSCs | S | E | GGT | | 106 |
| | | I | Vimentin | | 12 |
| | | FM | Vitamin A | | 12,131 |
| | | I | Desmin | | 12 |
| Pit cells | S | I | HNK-1 | | 139 |
| Cholangiocytes | PT | E | GGT | Leukocytes, some hepatocytes and FSC stain for GGT. | 5,11,104-106 |
| | | I | CK-19 | | 5 |
| VCs and lymphatic endothelial cells | PT | I | vWF | | 9 |
| | | I | Factor VIII | | 9,34 |
| Myofibroblasts | PT | I | Myosin, actin, vimentin | | 12 |
| Hepatic neurons | PT | I | NSE | | 147 |
| Lymphocytes | S,PT | I | Panlymphocyte | | 66 |
| Dendritic cells | S,PT | I | MHC class II | | 142,144 |

*L*, lobular; *E*, enzymatic; *M*, morphology; *I*, immunohistochemistry; *PP*, periportal; *PV*, perivenous; *S*, sinusoidal; *FM*, fluorescence microscopy; *LDL-DIL*, low-density lipoprotein–3,3′ dioctadecylindocarbocyanine; *vWF*, von Willebrand factor; *LM*, light microscopy; *HNK-1* monoclonal antibody to human NK cells; *PT*, portal tract; *CK-19*, cytokeratin 19; *NSE*, neuron-specific enolase.

(50, 53), is easily removed from purified cells with washing and subsequent centrifugation (54). Metrizamide, a nonionic derivative of metrizoate, is less viscous than Percoll or Ficoll at high density but may be incorporated into cells (55); it is also less toxic to cells than Percoll (54). Nycodenz—a nonionic, triiodinated derivative of benzoic acid—is commonly used for the purification of various liver cells and does not exhibit any cell toxicity (13, 14, 51). Stractan, an arabinogalactan derivative, is inexpensive, readily prepared and nontoxic and provides highly reproducible results (11). Whatever material is chosen, the gradient should be isosmolar throughout to assure that cell swelling or shrinkage does not occur and alter the centrifugal properties of cells. Density-gradient materials vary considerably in their ease of preparation, storage life, viscosity, osmolarity, range of usable density, cell toxicity and cost; in Table 7, we summarize key features of commonly used gradient materials.

Isopyknic centrifugation separates cells on sedimentation to a point in a gradient equivalent to their own density. An isopyknic gradient is prepared by mixing of cell suspensions with varying amounts of the gradient material to yield distinct regions of gradient media with densities equal to, lower and greater than that of the cells to be purified. While spinning, cells of uniform density partition at a single interface; if necessary, the cells can be purified further according to a technique based on a cell property other than density (e.g., size and immunological affinity) (5, 8, 11).

The condition of the cell suspension placed on a gradient can affect the efficiency of separation; cell clumping or overloading of the gradient with too many cells results in distortion of cell banding and poor cell fractionation. These problems may be minimized with DNase I treatment (5, 9, 11, 13, 14) and light microscopic examination of the cells to assess the degree of cell clumping before centrifugation. Also, only 2.0 to $5.0 \times 10^7$ cells/2 $cm^2$ of cross-sectional area of the centrifuge tube should be placed on the gradient so as not to reduce the resolving capacity of the gradient.

CFE is an efficient and increasingly popular method of separating cells according to their size. It has been used to obtain highly purified liver cell subpopulations including hepatocytes (56), cholangiocytes (5, 11, 13, 14), KCs (8, 13), FSCs (57) and SCs (9). CFE employs a centrifuge equipped with a specially designed rotor that allows fluid to be pumped from the distal end of the rotor chamber to a proximal exit port near the center of revolution during centrifugation (Fig. 4) (3, 49). CFE consists of a peristaltic pump that pushes elutriation medium from a reservoir through a bubble trap, where the cell suspension is loaded into the system with a syringe; from this point, the cell suspension is delivered to the elutriation chamber and then through a bypass

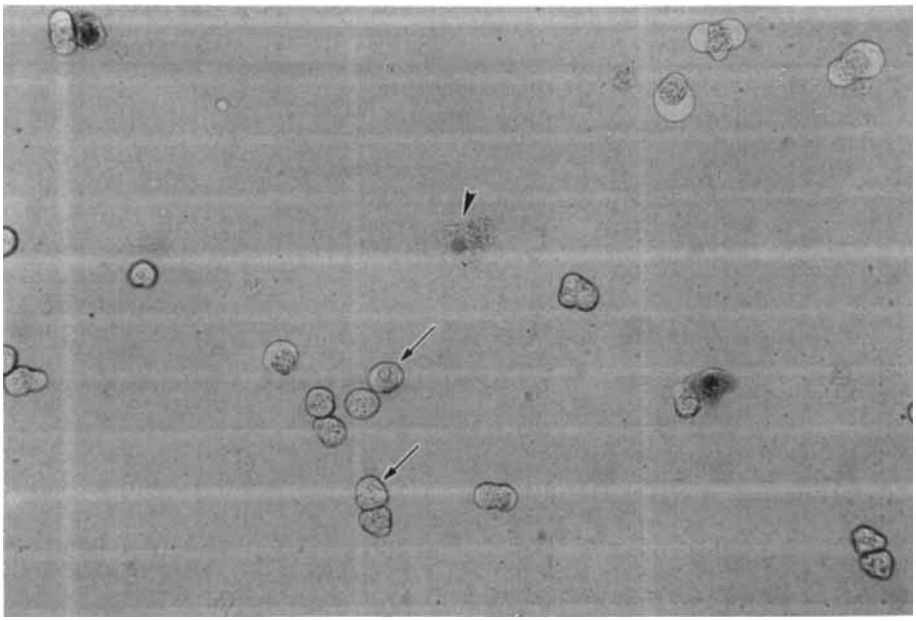

FIG. 2. Assessment of cell viability by means of trypan blue exclusion of a highly purified preparation of hepatocytes isolated from normal rat liver on isopyknic centrifugation through a Percoll gradient. Note that whereas living hepatocytes exclude this dye *(arrows)*, dead cells accommodate it *(arrowhead)* (original magnification ×250).

chamber to the collecting tubes. A pressure gauge downstream from the pump should be included for continuous monitoring of system pressure; irregular liquid flow would increase the back pressure in the system, causing poor cell separation (49). Cells are loaded at a set centripetal flow rate (e.g., 9 to 11 ml/min) into the separation chamber while the rotor is operated at a centrifugal force ranging from 800 to 2,500 rpm; the setting for the centrifuge speed depends on the specific cell type to be isolated (3, 5, 9, 11, 13, 14, 56). While the centrifugal field forces cells outward, the inward flow of buffer counterbalances this centrifugal force and creates a gradient of cells based on cell size (3, 49). If the cells loaded in the elutriation chamber are uniform, they will remain in a stationary phase; however, if there is variation in the size of the cells in suspension, they will sediment at different centripetal forces (5, 9, 11, 13, 14, 56, 57). Thus, by increase of the flow rate of buffer through the elutriation chamber of the rotor, first cell debris, then small cells and, finally, larger cells are sequentially eluted until all cells have been removed from the chamber (3, 5, 8, 9, 11, 13, 14, 56, 57).

Several factors merit consideration for good cell separation during CFE. Significant latitude is available in the choice of buffers; several buffer compositions have been used successfully in CFE (3, 5, 9, 11, 13, 14, 56, 57). However, DNase I (0.004% to 0.006%) should be included during CFE to minimize cell clumping (3, 5, 9, 11, 13, 14). Temperature is an important parameter during CFE (5, 13). Although centrifugation is generally performed at 4° C or less, cell separation with CFE is best achieved at a temperature between 10° C and 22° C (5, 13). The operator should observe several precautions during CFE: (a) proper rotor assembly, as suggested by the vendor's instruction manual; (b) thorough purging of the entire CFE system of air (air bubbles cause an increase in the back pressure and are associated with leaks and poor cell separation); and (c) accurate calibration of the entire CFE system according to the instruction manual to ensure an accurate relationship between centripetal force and cell size.

### *Lectin and Immunoaffinity Techniques*

Affinity cell-separation techniques are relatively new approaches that take advantage of the presence of specific plasma membrane molecules (e.g., proteins, carbohydrates) to yield purified cell populations (10, 11, 17, 30). Several variations of this approach include (a) the selective adherence of cells to various solid materials (10, 17, 30), (b) the ability of plant lectins to bind specific carbohydrate moieties (58) and (c) the use of polyclonal or monoclonal antibodies to bind to specific cell surface proteins (11, 59).

The ability of some cells to adhere to solid materials such as plastic and glass (9, 16, 34) or nylon fibers (60) by some poorly defined "stickiness" is used to purify cells that selectively adhere to these substrates. The selective adherence of KCs to glass or plastic surfaces, described later in this article, is one example of this purification process (10, 17, 30).

Various cell types display a vast array of distinct cell surface carbohydrates. Plant lectins that recognize specific carbohydrate moieties are used to purify cells with unique cell surface carbohydrates. Examples of plant lectins include concanavalin A and peanut agglutinin, which bind α-D-mannose and α-D-galactose, respectively (58, 61). These lectins are immobilized on solid support matrixes such as Sepharose beads (Phar-

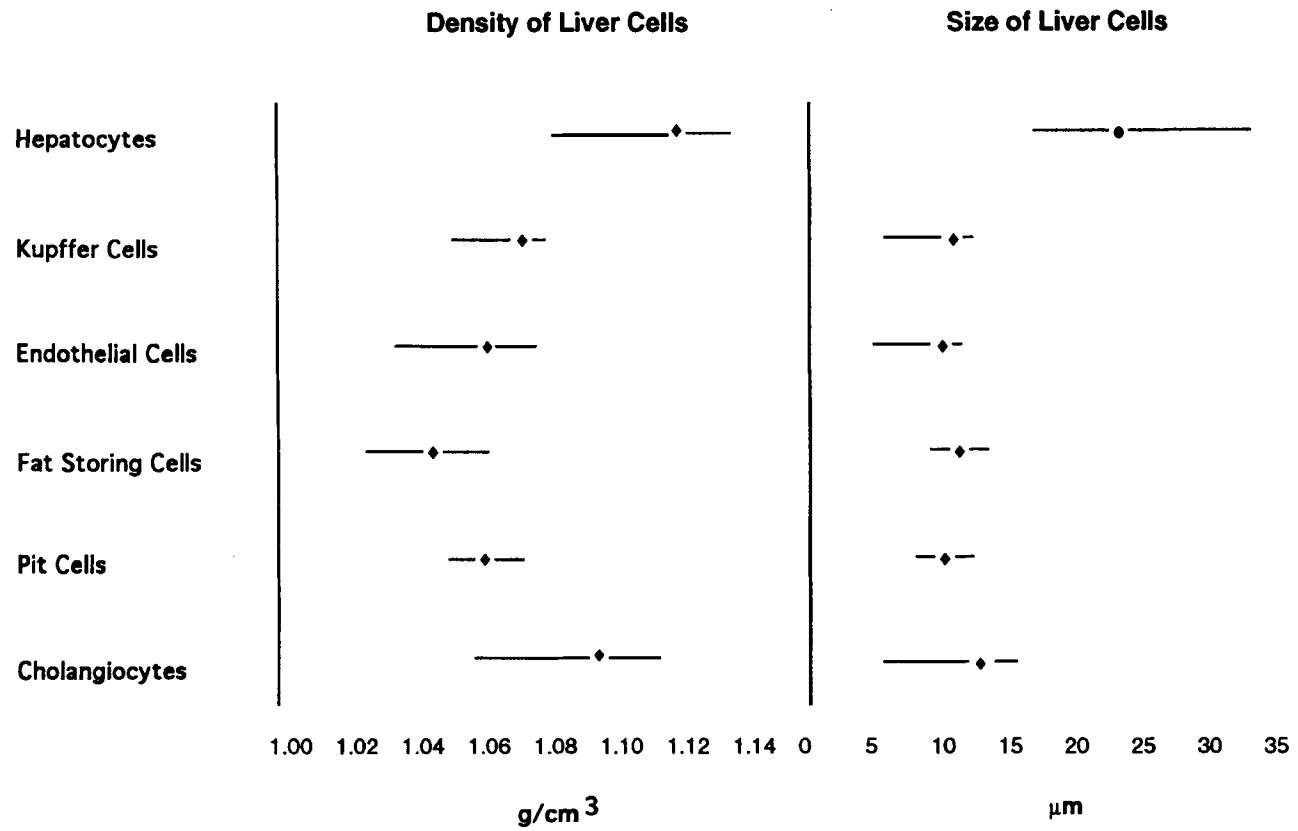


FIG. 3. Range of sizes and densities of various cells found in liver. Mean cell size or density (♦) with the range indicated by horizontal bars.

**TABLE 6. Centrifugal procedures for cell purification**

| Method | Cell separation characteristic(s) | Comments | Reference |
|---|---|---|---|
| Differential centrifugation | Size | Suitable only for hepatocyte isolation. | 5-7,15,16 |
| Isopyknic (gradient) centrifugation | Density | Conventionally used technique | 8,11,14,22 |
| Velocity gradient centrifugation | Size | More difficult to perform than isopyknic gradient; uses low g-forces. | 51 |
| Unit gravity sedimentation | Size | No centrifuge required; used to isolate hepatocytes. | 47 |
| Zonal rotor centrifugation | Size or density | Special rotors, centrifuge or both required; efficient cell separation; high cell capacity. | 51 |
| Counterflow elutriation | Size | Special rotor and modified centrifuge required; efficient cell separation; high cell capacity. | 5,8,9,11,14 |
| Isokinetic gradient centrifugation | Size or density | Optimally uses a computer-generated gradient; more difficult to perform than isopyknic gradient; low g-forces. | 51 |

macia) or plastic Petri dishes, and impure mixtures of cells are added. Unbound cells are removed with thorough washing, and the specifically bound cells are removed with an excess of soluble lectin-specific carbohydrate. An advantage of lectin purification techniques over enzymatic methods is that elution of bound cells with soluble carbohydrate avoids the use of harsh enzymatic treatment (11).

Monoclonal and polyclonal antibodies have revolutionized not only the assessment of the purity of heterogeneous cell mixtures but also the purification of these cells (11). Antibodies may be used in a variety of approaches to purify cells. First, an antibody may be immobilized by means of chemical crosslinking to a solid matrix composed of crosslinked dextran (62), agarose (63), glass (64) or polyacrylamide (63). A cell mixture is then incubated with the immobilized antibody resin; cells with the protein recognized by the antibody are specifically bound to the resin, whereas unbound cells are removed with thorough washing. The specifically bound cells are removed with a change of elution buffer (65). This method may be used to isolate cells that

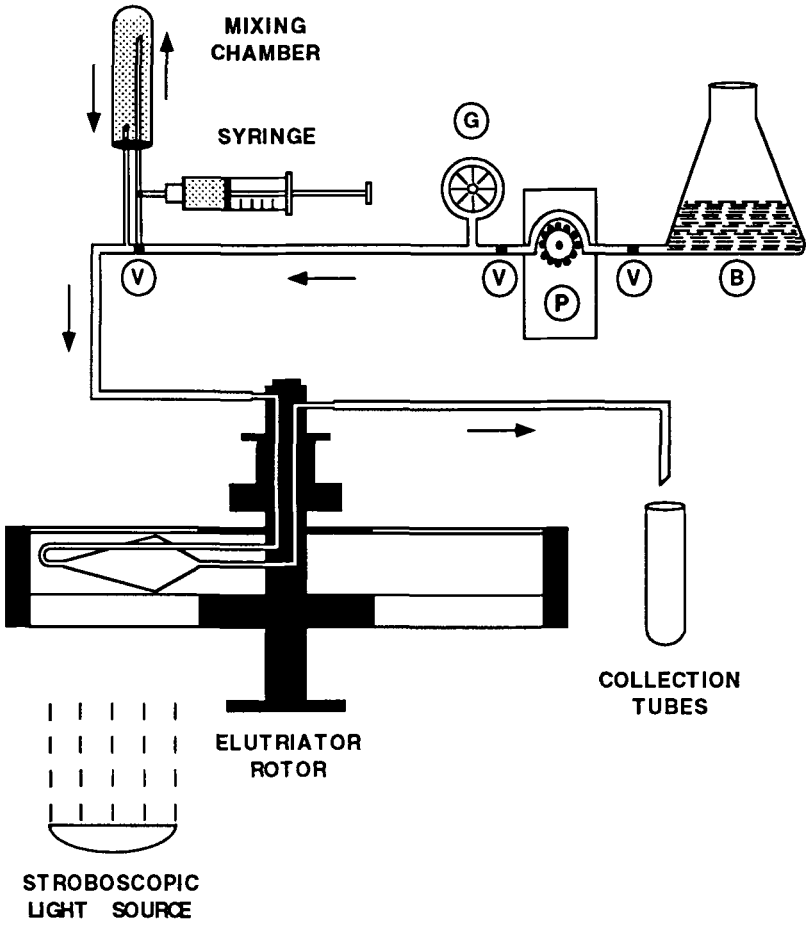


FIG. 4. Diagram of counterflow elutriator, which fractionates cells on the basis of their size. Arrows denote flow direction of elutriation buffer. *B*, elutriation buffer; *P*, peristaltic pump; *G*, pressure gauge; *V*, valve.

**TABLE 7. Properties of commonly used density-gradient materials**

| Gradient material | Molecular weight or size | Physical form | Ease of preparing gradient solutions | Cost/L (density = 1.08 gm/cm³) | Density = 1.04/1.13 gm/cm³ | | Remarks |
|---|---|---|---|---|---|---|---|
| | | | | | Viscosity (centipoise) | Osmolarity (mOsm) | |
| Ficoll-400 (polysaccharide)[a] | 400,000 Da | Powder | Moderate | $227/L (20.4%) | 9.13/72.5 | 21/300 | Unstable |
| Metrizamide (iodinated gradient material)[a] | 789 Da | Powder | Moderate | $459/L (14%) | 1.83/3.09 | 71/250 | Stable SFG, non-AC, absorbs UV |
| Nycodenz (iodinated gradient material)[a] | 821 Da | Powder | Moderate | $195/L (15%) | 1.23/1.64 | 87/252 | Stable SFG, absorbs UV, AC |
| Percoll (polyvinyl pyrrolidine–coated silica)[a] | ≈170 Å | Liquid<br>Liquid | Easy<br>Easy | $101/L (12%) | 1.87/3.30 | 18/32 | Stable SFG, AC |
| Stractum (arabinogalactan)[a] | 30,000 Da | Powder | Most complicated | $219/L (18%) | | | |

*SFG*, self-forming gradient; *non-AC*, nonautoclavable; *AC*, autoclavable.

[a]These gradient materials do not exhibit any significant cell toxicity. For storage life of these gradient materials, refer to the instructions from the vendor.

[b]Gradient materials necessary to obtain density of 1.08 gm/cm³

adhere to the antibody-conjugated resin (11) or, alternatively, to remove unwanted contaminating cells (65). A variation of this technique is "panning" (66), whereby a plastic surface such as a tissue-culture plate is first coated with cell-specific antibody. After blocking of additional unbound protein sites on the plastic with a protein such as albumin, a cell mixture is added to the tissue culture plate, and only cells with the corresponding antigen stick to the dishes. Unbound cells are removed by means of decantation; specifically bound cells remain attached to the antibody-coated plastic surface. This technique has been quite useful for the large-scale purification of lymphoid cells (66); however, results from our laboratory suggest that panning for cholangiocyte purification results in a higher nonspecific binding than does the immunomagnetic bead procedure described below (11).

An alternative approach is an indirect immunoaffinity technique. A cell mixture is first incubated with a soluble cell-specific primary antibody, followed by incubation with a secondary antibody directed to the primary antibody. The secondary antibody is immobilized on a solid support (67) or on magnetic beads (11); antibody-bound cells remain attached to the solid support or magnetic separator. Cells are separated from the solid phase or from immunomagnetic beads by alteration of the buffer conditions or by enzymatic dissociation with trypsin or papain (11). This approach increases the sensitivity of the primary antibody and was recently applied by our laboratory to isolate highly purified cholangiocytes from normal rat liver (11). The optimal concentration of antibodies and the time and temperature of incubation are variables that must be determined empirically. Finally, fluorescently labeled antibodies to specific cell types can be detected with FACS, an approach described below.

### *Selective Attachment*

The selective attachment of cells to a solid surface (e.g., a tissue-culture dish) (10, 17, 30) is a useful tool in the isolation of specific liver cells when it is used in combination with other isolation procedures (e.g., CFE or isopyknic centrifugation). This simple, inexpensive approach is based on the different rates at which cells adhere to plastic or glass surfaces (10, 17, 30) and may reflect different physicochemical and surface charge properties of cells. A frequent application of this technique is the isolation of KCs from other liver cells, including SCs (10). The receptors responsible for macrophage adherence to plastic surfaces have recently been defined with monoclonal antibodies (68). Because KCs adhere to solid surfaces much faster than do SCs (10), exposure of mixed KC and SC populations to plastic cell culture dishes results in the adherence of cells that are nearly totally KCs; the unattached cells in suspension are almost exclusively endothelial cells.

### *Two-phase Aqueous Polymer Extraction*

The aqueous two-phase extraction system consists of two water-soluble, hydrophobic polymers, most commonly dextran and PEG, which when mixed at a specific concentration (commonly 5% wt/wt), form an isosmotic liquid two-phase system (69, 70). The dextran normally partitions to the bottom of the tube, whereas the PEG separates at the top. After mixing with cells, the two phases separate during centrifugation, and cells separate into one of the two phases or at the two-phase interface. Several parameters control the distribution of cells in these two phases; for example, separations have been achieved on the basis of charge density (69), hydrophobicity (70) and immunological properties (71).

In some applications (71), antibodies are conjugated to PEG, and cells containing the specific antigen are recovered in the PEG phase. The physical properties of these two-phase systems may also be modified by change of the polymer concentration or the ionic composition (e.g., by addition of phosphate ions or sodium chloride) or by inversion of the hydrophobicity of the two phases (e.g., by addition of PEG-palmitate) and thus change of the distribution of the cells (71). This experimental approach has recently been used to separate hepatocytes and KCs from SCs (72) and is based on a complex interaction of cell surface charge and weak chemical interactions (e.g., hydrogen bonding, van der Waals forces) of plasma membrane components with a biphasic aqueous system comprising dextran and PEG (71). This technique is extremely sensitive for cell separation but is hindered by its adverse effects on cell viability.

The resolution of this technique may be further increased by performance of multiple continuous extractions with an automated countercurrent distribution apparatus, which fractionates on the basis of subtle changes in surface charges and hydrophobicities of liver cell populations (71, 72). However, because the equipment necessary for this automated biphasic separation is not widely available, this procedure is of limited usefulness.

### *FACS*

FACS is a powerful tool used for both the qualitative analysis and the purification of heterogeneous cell populations (72). Essentially, any cellular property that is detectable with a fluorescent label, whether intracellular or extracellular, can be evaluated with FACS (73). Fluorescent reagents include fluorescein- or rhodamine-conjugated antibodies or other ligands that label individual cells (74) for the study of cell surface immunofluorescence (74, 75), intracellular pH (76) or $Ca^{++}$ concentration (77). Cell mixtures can routinely be stained with several different fluorescent probes (75) and analyzed simultaneously with each fluorochrome evaluated independently (75). Narrow, forward-angle light scatter can also be used to characterize cells (78), as well as to evaluate cell size (79) and to discriminate dead from living cells (80). Propidium iodide, a fluo-

rescent compound that binds to the DNA of dead cells, may also be used to assess cell viability (75).

FACS is an important tool that has had a great impact on the analysis of mixed liver cell populations by allowing assessment of cell purity and physical and immunological properties of specific cell types (73). The utility of FACS for the preparative isolation of specific liver cell populations may be less as a result of (a) relatively low cell yield, (b) slow rates of cell sorting ($1 \times 10^7$/hr), (c) high cost of the instrument and (d) the need for highly trained personnel. A selected review offers comprehensive overviews on flow cytometry and detailed methodology for FACS analysis and cell sorting (81).

### *FFE*

FFE is an extremely sensitive technique that can discriminate among subtle cell differences (e.g., minimal differences in surface charge) in various cell subpopulations (53). It has been used to distinguish intact, viable cells from dead cells, nuclei and debris, which have different electrophoretic mobilities in an electric field (82). The choice of experimental conditions (e.g., composition and flow of the electrophoretic buffer, temperature and voltage) necessary for efficient cell separation depends on the cell type to be isolated. Although employed for the isolation of liver cells, including cholangiocytes (53), this method is limited by the cost of instrument, the long experimental time required and the availability of faster, more satisfactory isolation techniques. Before FFE is more widely utilized, additional experimental work must establish the value of this method for the separation of liver cells.

### *Selective Cytotoxicity*

Selective cytotoxicity is commonly used to remove hepatocytes from a complex mixture of liver cells; appropriate use of proteolytic enzymes such as pronase (5, 9, 13, 14, 29, 34), trypsin (11) and endotoxin from *C. perfringens* (35) results in the selective lysis of hepatocytes with the release of nonhepatocytes from undissociated connective tissue. A variation of this selective cytotoxicity approach is employed in the purification of subpopulations of hepatocytes: The liver is pulse-perfused through either the portal vein or inferior vena cava/hepatic vein with toxins that preferentially damage periportal or perivenous hepatocytes (83-86). Examples of these toxins include digitonin (83), bromobenzene (84), $CCl_4$ (85), ethanol (86) and allyl alcohol (84). With injury and lysis of hepatocytes in a given zone, the remaining hepatocytes are then purified. However, the timing of hepatocyte preparation after toxic injury is a critical factor to consider because "activated" hepatocytes may have different phenotypes than resting hepatocytes (85).

## SPECIFIC APPROACHES FOR ISOLATING SUBPOPULATIONS OF LIVER CELLS

### *Epithelial Cells*

***Hepatocytes.*** The biology and pathobiology of hepatocytes have been reviewed extensively (87, 88). Hepatocytes represent 60% to 70% of the total hepatic cell population (1) and are located in the lobular regions of the liver. Hepatocytes are associated with only small amounts of connecting extracellular matrix (24); therefore they are easily dissociated from the liver by means of perfusion through the portal vein with 0.05% to 0.1% collagenase (5-7, 15, 16).

The flow rate of the perfusate should be carefully monitored; a slow perfusion rate causes liver cell anoxia (89), a fast perfusion rate leads to cell shearing (90). Ideally, a flow rate of 20 to 40 ml/min through an 8- to 10-gm liver should be maintained to yield a perfusion hydrostatic pressure of 20 to 25 cm (13, 15). After enzymatic digestion of the liver, removal of Glisson's capsule not only simplifies cell collection but also increases cell purity. Glisson's capsule is a membranous "skin," rich in extracellular matrix components (91), and is easily removed from the liver with forceps if the liver substance has been adequately digested. A useful indicator of the adequacy of enzymatic digestion is the ease with which the capsule is removed. If the digestion is incomplete, the liver remains firm and the capsule is difficult to remove; in contrast, excessive digestion results in digestion of Glisson's capsule so that it tears into small pieces. Addition of DNase I (40 to 80 μg/ml) (12, 29) and $Mg^{++}$ ions (0.01%) (11) decreases cell clumping caused by the release of DNA from lysed or damaged cells.

Because hepatocyte preparation and its many variables have been recently described by Seglen (15), the general approach for hepatocyte isolation from normal rat liver is not presented here. Hepatocyte purity is assessed with routine hematoxylin-and-eosin cytochemistry; alternatively, hepatocytes may be evaluated with cytochemistry and immunohistochemistry for the hepatocyte-specific protein glucose-6-phosphatase (92) and albumin (5), respectively. If CFE is performed to isolate pure hepatocytes of different sizes, a rotor speed of 840 rpm is used (56). The flow diagram in Figure 5 represents a general isolation scheme to isolate hepatocytes in one fraction and SCs in the other fraction. This flow chart also outlines the steps for the isolation of other nonhepatocyte cells from the portal tract residue. Table 8 provides an estimate of the number of cells present in normal rat liver, as well as their recovery times and purities after isolation.

The heterogenous distribution of various enzymes (e.g., ALT, glutamate dehydrogenase, lactate dehydrogenase, glucose-6-phosphatase and GGT) in periportal and pericentral hepatocytes has been described (93). Close examination of this observation indicates that enzymes involved in oxidative processes—including glycogenolysis, fatty acid oxidation and ureagenesis from amino acids—are found in greater concentrations in periportal hepatocytes, where the oxygen tension is highest (93). Conversely, enzymes that require a lower oxygen tension, such as those involved in glucose uptake, lipogenesis and ureagenesis from ammonia ($NH_3$), are localized mainly in the pericentral zone (93). The different features of hepatocytes isolated from periportal and pericentral lobular zones are listed in Table 9.

For study of the zonal heterogeneity of periportal and pericentral hepatocytes, protocols have been developed to isolate these two cell populations (83, 93). One interesting method uses digitonin infusion through the portal vein for preferential lysis of periportal hepatocytes or digitonin infusion through the inferior vena cava/hepatic veins for lysis of pericentral hepatocytes; this is followed by perfusion with collagenase to limit the amount of cell lysis caused by digitonin and to allow collection of the undamaged cells (83, 93). Obviously only a single parenchymal cell type, either periportal or pericentral, can be isolated from an individual liver with this selective cytotoxic approach.

FACS has recently been applied to separate periportal from pericentral hepatocytes labeled with fluorescent antibodies to specific cell surface antigens (94) or with selective zonal staining with acridine orange (95). FACS has also been applied to isolate cells with mitochondria labeled with varying amounts of rhodamine-conjugated antibodies or cells that differ in size because of glycogen depletion (95).

***Cholangiocytes.*** Intrahepatic bile duct epithelial cells, or cholangiocytes, line bile ducts of different sizes (96), represent 2% to 3% of nucleated liver cells (3, 4) and are located principally in portal tracts (4, 5, 11, 14). Cholangiocytes form a complex network of ramified tubular structures that play an important role in bile formation (4, 97). Evidence suggests that cholangiocytes actively secrete water and electrolytes (4, 97), reabsorb fluid (98) and are involved in protein translocation (99). The intrahepatic biliary duct system extends from the canals of Hering to the porta hepatis and is estimated to be 2.2 km long, thus offering an ideal system for the modification of bile (100). Given the fact that only recently have these cells become an object of serious study, few reviews on cholangiocyte biology have been published (3, 101-103).

Although they overlap in size and density with other nonhepatocyte cells—including endothelial cells, KCs and FSCs (5, 11)—cholangiocytes can be separated from these other cells by means of a combination of techniques based on differences in size (5, 11, 14), density (11, 14, 22) and immunological properties (11). Cholangiocytes are specifically identified by immunocytochemical detection of intermediate filament proteins, including cytokeratins 7 and 19 (Fig. 6A) (5, 14). In rat liver, the enzyme GGT (5, 11) represents in our hands a reliable marker for cholangiocytes (Fig. 6B); however, others have reported GGT activity in normal rat hepatocytes, FSCs and leukocytes (104-106). Although murine cholangiocytes express cytokeratin 19 (107), they do not constitutively express GGT (108). In guinea pig liver, GGT is expressed by both SCs and cholangiocytes (109).

Over the last decade, several laboratories have purified and characterized cholangiocyte-enriched preparations (5, 14, 22, 50, 110). After standard collagenase digestion (6, 7, 15, 16), cholangiocytes are released from the portal tract residue (Fig. 5) by digestion with various proteolytic enzymes, including collagenase, pronase and dispase (5, 11, 14, 50, 110). Many investigators have had moderate success with CFE (5, 14), isopycnic centrifugation or both through discontinuous gradients of metrizamide (22, 50), Ficoll (50) or Percoll (22, 50, 110). The purification of cholangiocytes has also been accomplished with FFE (57); however, the use of FFE for cholangiocyte purification is limited, for reasons described earlier.

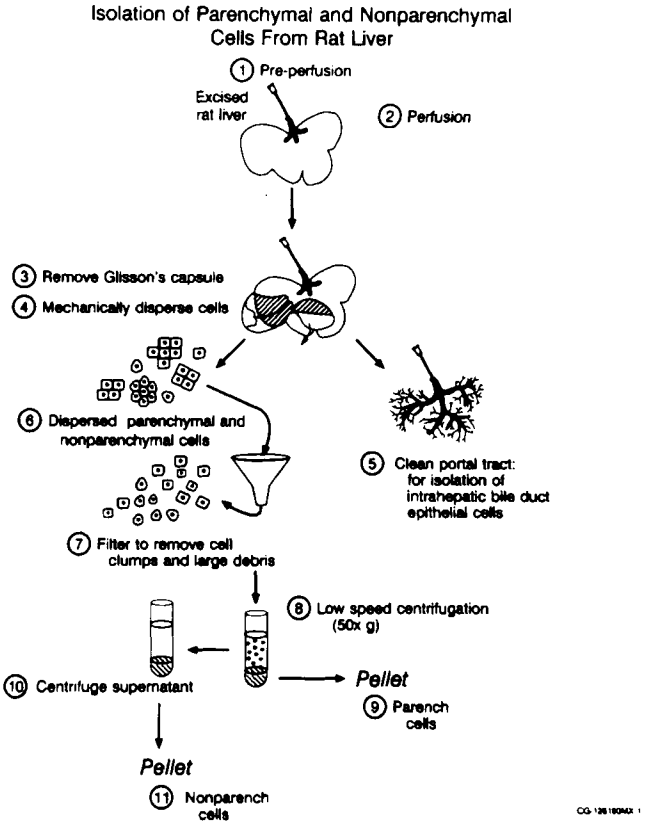


FIG. 5. Collagenase digestion of liver. (1) Preperfusion: 250 ml of solution A (containing 0.5 mmol/L EGTA and saturated with $O_2$ bubbling) is perfused through the liver for 5 to 10 min at 37° C to remove endogenous blood. (2) Perfusion: the excised rat liver is digested for 20 min at 37° C with 150 ml of solution A with 5 mmol/L calcium, 10 to 50 mg collagenase and DNase I (40 to 80 μg/ml) in a recirculating liver perfusion apparatus. (3) Glisson's capsule is removed by means of teasing with curved forceps. (4) Hepatocytes and SCs are carefully dispersed into buffer A by gentle combing of the lobules with curved forceps or a rubber policeman. (5) The intact portal tract residue is digested with a mixture of proteolytic enzymes to release nonparenchymal (i.e., nonhepatocyte) cells, including cholangiocytes. (6) The dispersed cell suspension is (7) filtered through 60-μm nylon mesh to remove cell clumps and tissue debris. (8) The cell suspension is washed three times with buffer A containing $Ca^{++}$ and $Mg^{++}$ at 50 *g* for 2 min. Supernatant is saved for step 10. (9) Pellet contains purified parenchymal cells (hepatocytes). (10) After centrifugation at 50 *g* for 2 min (step 8), supernatants are pooled and centrifuged two times at 300 *g* for 10 min at 4° C to yield the nonparenchymal (nonhepatocyte) cell fraction pellet.

In our laboratory, we developed a novel technique (11) to obtain cholangiocyte preparations from normal rat liver that are at least 95% pure as assessed by means of GGT cytochemistry (11) (Fig. 6B). A mixed cell fraction, obtained by trypsin digestion, is sequentially subjected to CFE to separate cells on the basis of size. The resulting subpopulations of cholangiocytes are further purified with an immunoaffinity approach with a monoclonal antibody not commercially available but ubiqui-

TABLE 8. Recovery and purity of isolated liver cells

| Cell type | Estimated number of cells ($\times 10^6$)/gm liver | Number of cells isolated/gm liver | % Purity | % Recovery | References |
|---|---|---|---|---|---|
| Hepatocytes | 110 ± 20 | 45 ± 15 | ≥95 | ≈48 | 1,153 |
| SCs | 37 ± 4 | 24 ± 6 | ≥95 | ≈65 | 2,8,9 |
| KCs | 27 ± 3 | 7.5 ± 2.5 | ≥95 | ≈28 | 1,10 |
| FSCs | 18 ± 2 | 1.4 ± 0.4 | 70-95 | ≈10 | 2,12,57 |
| Cholangiocytes | 3.4 ± 0.4 | 0.5 ± 0.3 | ≥95 | ≈15 | 5,11,14 |
| Pit cells | 1.5 ± 0.4 | 1.05 ± 0.15 | 65-90 | ≈66 | 136,139 |

Because there is no uniformity in the literature for relating the number of isolated cells to the amount of liver, the authors have normalized the data reported in cells per gram liver.

TABLE 9. Functional heterogeneity of hepatocytes from different lobular zones

| Periportal | Pericentral |
|---|---|
| High oxygen tension | Low oxygen tension |
| Oxidative energy metabolism | Glycolysis |
| Amino acid catabolism | Liponeogenesis |
| Ureagenesis from amino acids | Ureagenesis from ammonia |
| Gluconeogenesis | Biotransformation |
| Bile acid and bilirubin excretion | |

tously expressed on all cholangiocytes. Although this antibody is not commercially available, this general immunological approach has enormous utility for isolating pure preparations of cholangiocytes; a negative-immunoaffinity approach can be used to remove contamination cells (e.g., endothelial cells, KCs) with commercially available antibodies. Most contaminating cells contain vimentin or von Willebrand factor (5, 9, 14), suggesting a mesenchymal or vascular endothelial origin for the contaminating cells. Cholangiocytes are negative for these proteins (5).

An alternative approach for the isolation of large numbers of purified cholangiocytes relies on the selective proliferation of cholangiocytes induced by pathological stimuli including bile duct ligation (4, 5, 14, 22, 97) and α-napthylisothiocyanate feeding (5, 12, 97). This ductular proliferation, which originates from preexisting bile ducts (111), is observed solely in portal areas (3, 4, 13, 111). These hyperplastic cholangiocytes retain the phenotypic traits of normal cholangiocytes and do not express oncofetal proteins suggestive of parenchymal or transitional cell origin (4, 14). Other investigators (22, 53) have used these pathological disturbances to isolate cholangiocytes, but morphological and biochemical characteristics were the only criteria used to assess cell yield and purity. An advancement in the isolation and characterization of cholangiocyte-enriched preparations from bile duct–ligated and α-napthylisothiocyanate–fed rats has been the use of CFE (5, 14).

### *Endogenous Mesenchymal Liver Cells*

The availability of purified nonhepatocyte liver cell populations is a fairly recent achievement. With the advent of the collagenase perfusion technique (6, 7) in combination with other proteolytic enzymes for the selective lysis of hepatocytes (5, 8, 9, 11, 13, 14, 22, 23, 110), methods have become available to isolate pure preparations of several distinct nonhepatocyte liver cells (5, 8-11, 14, 15, 22, 28, 48).

***KCs.*** KCs, which are phagocytic members of the reticuloendothelial cell system (112), possess a typical stellate profile (112) and represent 25% to 40% of the nonhepatocyte cell population of the liver (1, 35). They are located mainly in the periportal region of the liver lining the sinusoids and are in close contact with both SCs and hepatocytes (113). KCs are important for their ability to metabolize bacteria and other foreign materials (112, 114), bacterial endotoxins (114), colloids and macromolecules (114). In addition, KCs produce complement components (114), are important in tumor cell surveillance (115) and clearance of circulating immune complexes (116) and virally infected cells (117) and participate in the metabolism of iron (118) and bilirubin (119). Many reviews on selected aspects of the biology of KCs are available (113, 114, 115-117).

Morphologically, KCs are commonly identified by their ability to phagocytize particles of India ink or colloidal carbon (112) (Fig. 7), or latex (34, 112). Alternatively, KCs may be recognized with cytochemical techniques for endogenous peroxidase (13, 17, 112) or tartrate-resistant acid phosphatase (120).

Nonhepatocyte liver cells, including KCs and SCs, are commonly isolated from the liver by means of one of three different approaches, based on collagenase (9, 10, 17), pronase (8, 48, 57) or combined collagenase-pronase digestion (13). Because KCs bind avidly to most surfaces (10, 17, 30), siliconization of glassware and limited exposure of cells to plastic surfaces greatly increases the cell yield. The isolation of KCs is commonly performed with methods based on cell density or size, such as isopyknic centrifugation through gradient materials including Percoll (10), metrizamide (13, 34), Nycodenz (17) or stractan (121), followed by CFE (17).

Another technique for KC isolation (≈80% purity) uses selective preloading of KC lysosomes with Triton WR 1339 (Sigma Chemical Co.) (122), Jectofer (i.e., iron sorbital) (123), Zymosan (Serva Feinbiochemica, Heidelberg, Germany) (124) or colloidal carbon (112)

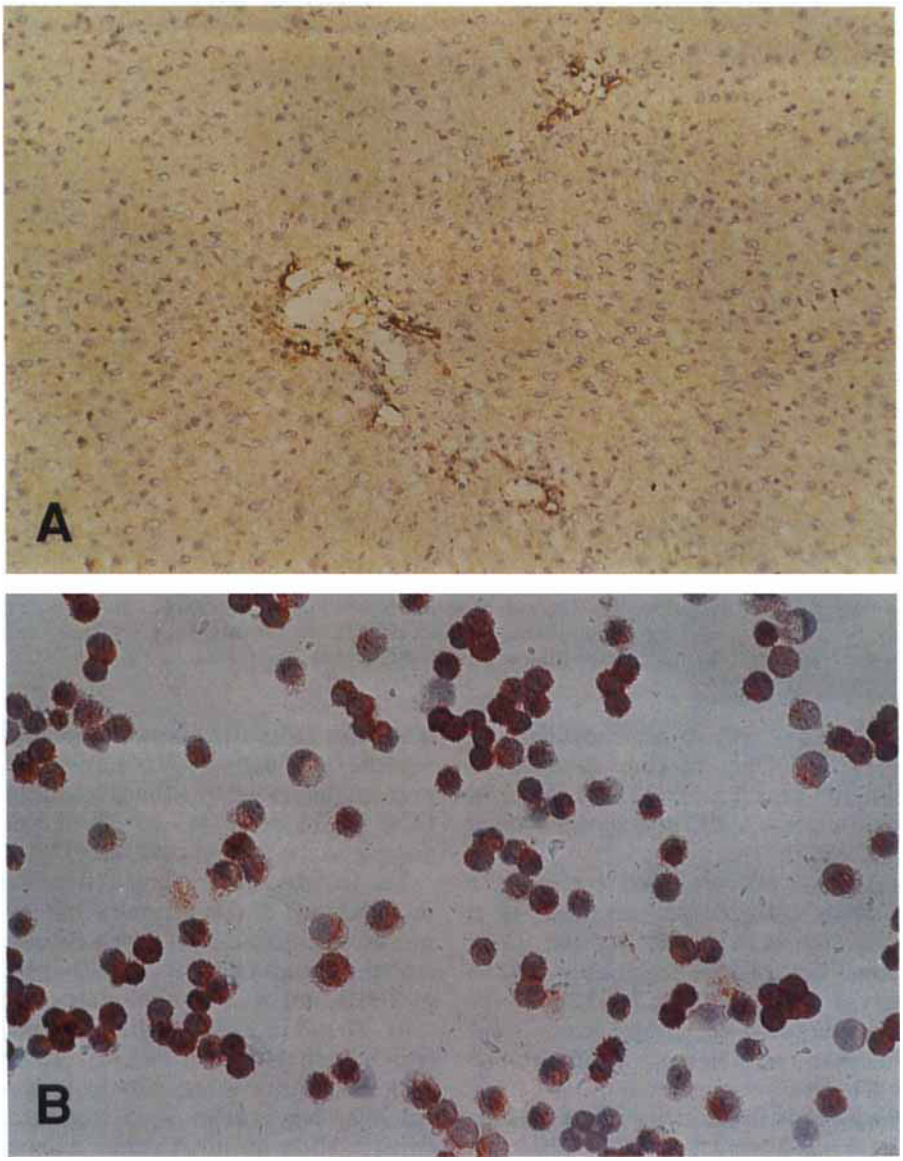


FIG. 6. (A) Immunohistochemistry for cytokeratin 19, a cholangiocyte-specific marker, on a paraffin-embedded tissue section (4 μm) from normal rat liver. The brown reaction product is only visible in bile ducts in portal areas (original magnification ×125). (B) Cytochemistry for GGT in immunoaffinity-purified (≥95%) cholangiocytes from normal rat liver (original magnification ×250).

followed by isopyknic centrifugation. This approach alters the density of KCs (but not other liver cells), thus facilitating KC identification and purification. An alternative selective loading approach is to inject animals with colloidal iron *in vivo* before cell isolation (125); pure preparations of KCs are then separated from SCs with a magnet. Another approach for the isolation of KCs has been described by Blomhoff et al. (17): After collagenase perfusion, the liver cell suspension is treated with an enterotoxin from *C. perfringens* that selectively disrupts hepatocytes. KCs are then isolated from the nonhepatocyte cell fraction by means of centrifugation through a discontinuous Nycodenz gradient followed by CFE. Purified KC preparations may also be obtained by selective adherence of these cells to glass or plastic surfaces (e.g., tissue-culture dishes) (10, 17, 30).

***SCs.*** SCs represent 44% to 60% of the total nonhepatocyte cell population of the liver (1, 17) and form a continuous lining along the hepatic sinusoids, allowing access through their fenestrations to the space of Disse (126). This fenestrated lining forms a barrier that prevents direct contact of blood cells or large chylomicrons with the space of Disse (127). The largest fenestrae range in size from 0.1 μm to several microns, permit the entry of only the smallest chylomicrons into the space of Disse and exclude bacteria and protozoans (127). SCs are active in the clearance of macromolecules from the bloodstream, in the metabolism of lipoproteins and in

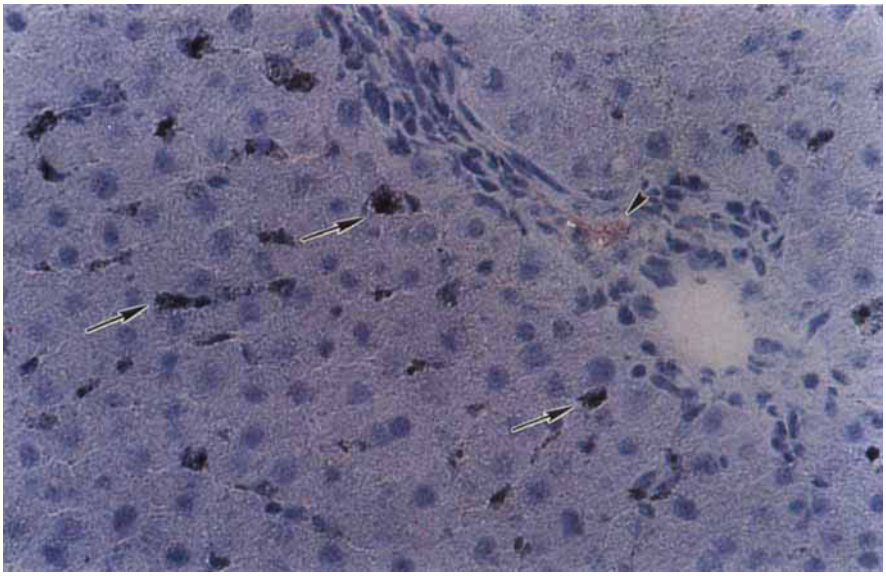

FIG. 7. Frozen tissue section (4 μm) of normal rat liver after intravenous injection of colloidal carbon and counterstained with hematoxylin. Kupffer cells are recognized by their black color, indicating phagocytosis of the injected colloidal carbon *(arrows)*. Arrowhead indicates dendritic cell in the portal tract stained with a monoclonal antidendritic cell antibody (original magnification ×250).

the storage of vitamin A (128). SCs express low-density lipoprotein receptors (126). This marker is used to evaluate the purity of SC preparations (126). Several reviews of more specific aspects of SC biology have been published (31, 32, 128, 129).

SC-enriched preparations are obtained by initially perfusing the liver with collagenase, pronase or a collagenase-pronase combination (8-10, 31, 34, 123). The choice of the proteolytic enzymes does not appear to affect the final purity of SC (8-10, 31, 34, 123). Pure preparations of SC are obtained by isopycnic centrifugation through a discontinuous density gradient followed by CFE (8-10, 31, 34).

One may also separate SCs from other cell contaminants (e.g., KCs) by taking advantage of the different rates at which cells attach to solid surface (e.g., plastic or glass culture dishes); whereas KCs avidly adhere to these surfaces, SCs remain in solution (10, 17, 30). This approach, in combination with other methods based on size (e.g., CFE) (8-10, 31, 34) or density (e.g., gradient centrifugation) (8-10, 31, 123), allows the isolation of pure ($\geq$98%) preparations of SCs.

***FSCs.*** FSCs are associated with no fewer than twenty different designations, including fat-storing cells, lipocytes, Ito cells, myofibroblasts and stellate cells (12). FSCs comprise 5% to 10% of the nonhepatocyte cell fraction (1, 35) and are located in the space of Disse in close contact with hepatocytes (12). FSCs are involved in the storage of lipids (130) and vitamin A (12) and likely play an important role in the production of glycosaminoglycans (131). These cells are also major producers of collagen (132) and thus are implicated in the pathogenesis of liver fibrosis (132). FSC are identified by vitamin A fluorescence if a sufficient level of the vitamin has been stored in these cells (12, 131). FSC may also exhibit GGT activity (106) and contain vimentin (12), a protein displayed by other mesenchymal cells (e.g., KCs, SCs) (4, 8). Selected aspects of FSC biology have been described in several reviews (128, 129).

To isolate FSCs, most investigators (131) use a modification of the pronase/collagenase perfusion technique originally described by Knook et al. (8). The use of collagenase and pronase together provides greater yields of FSCs and KCs but may reduce the recovery of SCs (56). To aid in FSC purification, pronase is employed to lyse hepatocytes (57) and effectively remove one of the major contaminating cells from the preparation. When rat liver has a high lipid content, FSCs have a lower density than do most other liver cells (128); however, when the lipid content is low, the density of FSCs is close to that of KCs and SCs, thus making the separation of FSCs from KCs and SCs more difficult (128). Despite this problem, excellent purity ($\approx$99%) of FSCs has been achieved with enzymatic digestion of the liver followed by isopyknic centrifugation through stractan (12) and Percoll (55) gradients; similar results have also been obtained with isopyknic centrifugation on Nycodenz (51, 131) and metrizamide (57) gradients followed by CFE (57, 131).

The purification of FSCs has been also accomplished with flow cytometry (132), but technical difficulties limit the use of this procedure. Purified preparations of FSCs have also been obtained with the use of calcium immobilized on glass surfaces with covalently coupled iminodiacetic acid (133); this approach allows convenient separation of FSCs from KCs and SCs.

***Myofibroblasts.*** Myofibroblasts morphologically and functionally resemble fibroblasts and smooth muscle

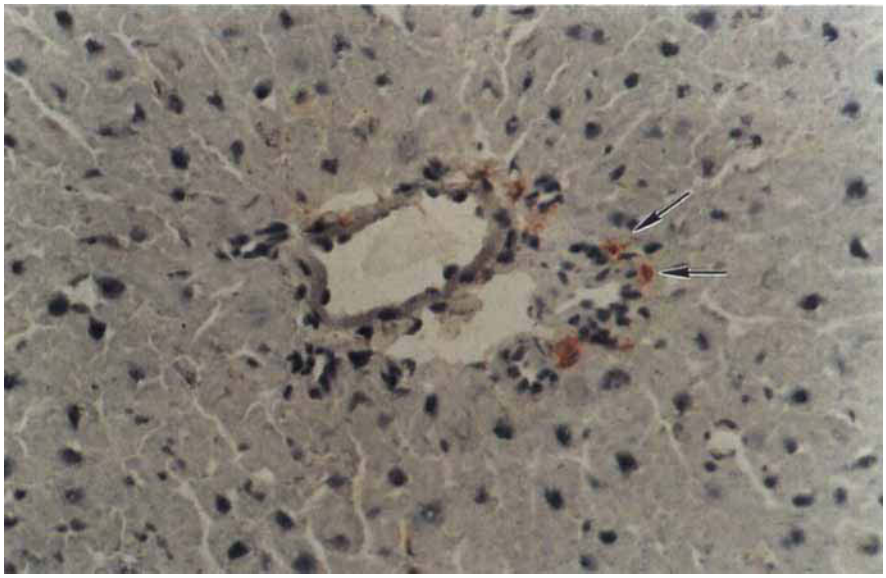

FIG. 8. Immunohistochemistry of MHC class II antigen on frozen tissue section (4 μm) of normal rat liver. Few MHC class II–positive cells *(arrows)* are present in the portal area; they likely represent dendritic cells (original magnification ×250).

cells possessing contractile properties (134). Myofibroblasts, along with FSCs, are implicated as the effector cells of hepatic fibrosis (52). They possess copious amounts of myofibrils with dense bodies and indented nuclei (52) and are major producers of collagen types I, III and IV (52). Highly purified (≈80%) preparations of myofibroblasts are commonly obtained with isopyknic centrifugation through gradient materials (52). Several reviews have been published on the biology of myofibroblasts and their role in liver disease (129, 135).

***Pit Cells, or Large Granular Lymphocytes.*** First described by Wisse in 1970 (136), pit cells, or large granular lymphocytes (137), are resident sinusoidal cells with a frequency of approximately one pit cell for every 10 KCs (138). Pit cells have a pale cytoplasm, chromatin-dense nuclei and characteristic granules (137). Pit cells are identified by their characteristic morphology, as well as by immunocytochemical staining with antibodies to asialo-GM (139) and CD57 (140). There is evidence (139) that pit cells have biological functions similar to NK cells, with a high level of cytotoxicity against the YAC-1 lymphoid cell line. Because pit cells contain surface markers similar to those of NK cells and exhibit cytotoxic activity similar to that of NK cells (139), it has been suggested that pit cells are the hepatic equivalent of NK cells (139). As such, these cells may be important in cancer surveillance (139), as well as in controlling viral infection (139). More information on selected aspects of pit cells is presented in various reviews (139, 141).

Pit cells are isolated from other nonhepatocyte cells by means of conventional techniques that rely on density, size or both and copurify with lymphocytes. Highly purified preparations of pit cells are commonly isolated according to the sinusoidal lavage method described by Bouwens et al. (139).

***Additional Endogenous Liver Cells.*** Other, less well studied, resident cells in the liver include dendritic cells, vascular and lymphatic endothelial cells, nerve cells, smooth muscle cells of arteries and fibroblasts.

Dendritic cells are immune cells localized in portal tracts of the liver (142). They are morphologically characterized by an oval or irregularly shaped nucleus and abundant smooth endoplasmic reticulum (142). In contrast to KCs, dendritic cells are poorly phagocytic and lack both cytoplasmic lysozyme and nonspecific esterase (142).

Although the original description of dendritic cells failed to demonstrate these cells in normal mouse liver (143), subsequent immunocytochemical studies have revealed their presence in normal liver in a variety of species. Thus these cells are frequently recognized as intensely positive MHC class II antigen–bearing cells in the portal tracts (Fig. 8) (142). Dendritic cells are widely distributed throughout the body (142), function as efficient antigen presenting cells (142) and serve multiple immune functions, as described in a number of reviews (142).

Hepatic dendritic cells can be isolated on the basis of their physical properties (142). After collagenase perfusion, dendritic cells are separated from KCs by incubation of the cell suspension in glass Petri dishes at 37° C for 24 hr. The nonadherent cells, which include the dendritic cells, are removed and centrifuged through a discontinuous bovine serum albumin density gradient. Most nucleated cells are pelleted, whereas the low-density dendritic cells (density < 1.082 gm/ml) are harvested from the top of the gradient; the yield of

dendritic cells after this procedure is about $1 \times 10^5$ cells from one rat liver. Approximately 60% of the cells in this enriched population are dendritic cells, with lymphocytes and monocytes as the major contaminants. The recent advent of monoclonal antibodies to rat dendritic cells (Fig. 7) should aid in the purification and characterization of these cells in liver with both immunoaffinity separation techniques and FACS (144).

VCs are important in many physiological liver functions, including regulation of angiogenesis, susceptibility to oxidative injury, inflammation and immune responses (145). These cells also play integral roles in the regulation of coagulation, platelet aggregation, synthesis of stromal components and regulation of vascular tone (145). VCs are identified immunohistochemically by the presence of factor VIII–related antigen (34) and von Willebrand factor (9). Several reviews are available on the biology of VCs (145).

No reports on the isolation of rat VCs exist. A simple and reliable technique, similar to that used for cholangiocyte isolation (11) and based on an immunoaffinity approach with a monoclonal antibody to VCs has recently been developed for the purification of rat VCs (Gores GJ, Personal communication).

In addition to these cell types, the liver also contains smooth muscle cells in the hepatic vasculature. The role of these cells in the regulation of blood and lymphatic flow through the liver is well established (146). Numerous lymphatic vessels also exist in the liver capsule and stroma (146). The role of the nervous system in the innervation of the liver is not well understood; however, it is likely that nerves play a critical role in the hemodynamic regulation of sinusoidal blood flow (147). Unfortunately, little information is available on the isolation and purification of these cell types from the liver.

### *Exogenous Liver Cells*

Leukocytes that migrate into hepatic tissue in response to infection, inflammation or necrosis are an important cell type in liver (148). These cells are also involved in organ transplant rejection, graft-vs.-host disease and autoimmune liver disease. They are responsible for humoral and cell-mediated immune function and act in a complementary fashion to the resident hepatic reticuloendothelial system (148).

***Lymphocytes.*** Lymphomyeloid cells are found in significant numbers in normal liver. They are increased during hepatic infections and chronic cholestatic liver disease (149). Most of these cells are lymphocytes, which are subdivided into B lymphocytes with surface immunoglobulin and T lymphocytes as defined by various monoclonal antibodies (150). Although enzymatic digestion is commonplace for the purification of rat liver cell populations, the isolation and characterization of lymphocytes rely heavily on cell surface markers identified by monoclonal antibodies; therefore proteolytic enzymatic techniques have generally been avoided in the purification of lymphocytes from rodent liver (151).

Another group evaluated the effect of liver perfusion on the yield of lymphocytes from murine liver (152). Perfusion of the liver with phosphate-buffered saline solution before lymphocyte purification does not cause significant differences in the yield or populations of lymphocytes obtained compared with total bleeding or no removal of blood from the liver before lymphocyte purification. However, a decrease in the yield of hepatic mononuclear cells has been noted when collagenase digestion of the liver is used to purify hepatic lymphocytes. During the purification of lymphocytes from the liver, we prefer for adequate perfusion of the liver with an appropriate buffer to be performed to remove contaminating peripheral blood lymphocytes. The use of flow cytometry for the isolation and quantitation of lymphoid cells in liver (151, 152) will enhance our knowledge of the role of these cells in hepatobiliary disease.

## SUMMARY AND CONCLUSIONS

The development of new and refined separation techniques—including FACS, FFE, CFE and isopyknic gradients—has had a profound impact on the ability of investigators to isolate specific cell types from the liver. Although some of these techniques, such as FFE, may be of limited preparative value, they are nonetheless important analytical tools that detect subtle differences among cell subpopulations. The isolation of highly purified preparations of liver cells in large yields requires the use of more conventional purification methods such as CFE and isopyknic centrifugation. Immunological approaches represent a key development for the isolation of specific liver cell types, especially when they are used in combination with other techniques. Excellent, reliable and relatively simple techniques now exist to isolate highly purified preparations of hepatocytes, cholangiocytes, KCs, SCs, FSC, myofibroblasts and pit cells. Additional work is necessary to refine techniques for the isolation of dendritic cells and lymphocytes.

*Acknowledgment:* We thank Ms. Maureen Craft for her secretarial assistance.

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