Isolation, Propagation, and Characterization of Rat Liver Serosal Mesothelial Cells

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Although rat liver epithelial cell (RLEC) lines have been developed by a number of laboratories, the identity of the clonogenic nonparenchymal progenitors is unknown. To provide insight into the derivation of RLEC, we immunosolated serosal liver mesothelial cells (LMC) and bile duct epithelial cells and attempted to propagate each epithelial cell population using culture conditions routinely employed to establish RLEC lines. Briefly, the selective reactivity of LMC with two bile duct cell surface markers, OC2 and BD2, was exploited to develop an immunocytochemical technique to isolate LMC. Livers were collagenase dissociated, the mesothelial capsule was "peeled" and digested with pronase to destroy contaminating hepatocytes, and rare biliary ductal epithelial cells were immunodepleted using OC2. LMC were subsequently isolated by selective binding to magnetic beads adsorbed with BD2 and cultured in supplemented Waymouths 752/1 media containing 10% fetal calf serum. Proliferating BD.2+ LMC rapidly formed epithelial-like monolayers that could be continuously subcultured after trypsinization. In contrast, attempts to establish cell lines from purified OC2+ bile duct epithelial cells were unsuccessful. Results from reverse transcriptase polymerase chain reaction analysis confirmed that LMC expressed Wilms’ tumor transcripts, a lineage marker for mesodermally derived cells. In summary, our findings clearly demonstrate that LMC can be continuously propagated using culture conditions routinely employed to establish RLEC lines, an observation that supports the contention that some RLEC lines may be derived from LMC. (Am J Pathol 1994, 145:1432-1443)

Although methodologies have been developed to routinely establish continuous culture lines of normal rat liver epithelial cells (RLEC) from the nonparenchymal cell (NPC) population,1-7 identification of the clonogenic progenitors of RLEC has been difficult because of the lack of lineage markers that can be used to isolate antigenically defined populations of NPC. Presently, insight into the nature of the RLEC progenitors has been based on the subsequent phenotypic characterization of these cell lines8-13 and from the transplantation of normal and transformed RLEC into syngeneic host.14-18 Of particular interest, Grisham and coworkers17,18 have provided compelling evidence that the WB-F-344 RLEC line developed by their laboratory retains the ability to differentiate along the hepatocyte lineage, suggesting that this RLEC line is derived from facultative liver stem cells. Indeed, recent results obtained from studies of chemically induced hepatic neoplasia in rats has provided strong evidence that the biliary epithelial cell population contains bipotential hepatic precursors.5,8,9,11,19,20 However, most RLEC lines described in the literature do not express hepatocytic characteristics,21 suggesting that there may be more than one progenitor cell type of RLEC. This view is supported by the finding that inoculation of rodents with cultured RLEC transformed either by oncogenes22,23 or carcinogens14,24 produces a histologically diverse spectrum of tumors including carcinomas, sarcomas, mixed epithelial-mesenchymal tumors, and undifferentiated carcinomas. Recent immunocytochemical results from the laboratory of Marceau21,25 suggest that RLEC may be derived from liver mesothelial cells (LMC), a mesodermally derived, simple squamous epithelial cell population that covers the serosal surface of the liver known as Glisson’s capsule. Inasmuch as it is well

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documented that primary tumors derived from transformed mesothelial cells in both rats and humans may exhibit histological differentiation patterns from mesenchymal to epithelial cells, the possibility that some RLEC are derived from LMC warrants further consideration.

The goal of the present study was to determine whether LMC or biliary ductal epithelial cells (BDEC) can be continuously propagated under culture conditions routinely used to establish RLEC. Briefly, a panel of monoclonal antibodies (MAbs) that recognize subpopulations of rat NPC was used to identify and immunosolate pure populations of LMC and BDEC. We found that the antigenically defined LMC population, but not BDEC, gave rise to propagable epithelial cell lines when cultured in media containing 10% fetal calf serum. The mesothelial origin of LMC lines was subsequently confirmed by using the expression of the Wilms' tumor gene as a marker to identify cells of mesodermal origin. A description of our method to isolate LMC and the characterization of propagable LMC lines are presented in this report.

**Materials and Methods**

*Animals*

Adult male F-344 rats (8 to 10 weeks of age) purchased from Charles River (Wilmington, DE) were housed in Thoren cage systems (Thoren Caging Systems, Hazleton, PA) and were fed standard laboratory chow ad libitum. Some rats were euthanized by overdose of methoxyflurane inhalation. Livers were excised and frozen sections were prepared for immunocytochemical analysis. Rats were also used to provide LMC for culture studies. All protocols used in this study were approved by the Rhode Island Hospital Institutional Animal Care and Use Committee and were in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals.

*Cell Lines*

In the present investigation, total RNA was prepared from five RLEC lines designated BDE 1.1, BDE 2, BRL-3A, WB3, and LE/6 and analyzed by the reverse transcriptase polymerase chain reaction (RT-PCR) for the expression of the Wilms' tumor gene (see below). BDE 1.1 and BDE 2 are propagable biliary epithelial cell lines developed by our laboratory as previously described. BRL-3A is a clonal RLEC line obtained from the American Type Culture Collection (Rockville, MD). WB3 and LE/6 two liver-derived cell lines that express hepatocytic characteristics were kindly provided by Dr. Joe Grisham (University of North Carolina, Chapel Hill, NC) and Dr. Nelson Fausto (Brown University, Providence, RI), respectively.

**Isolation and Propagation of LMC**

LMC were selectively isolated from Glisson's capsule after collagenase digestion of normal adult rat livers. Briefly, livers were dissociated by collagenase perfusion as previously described and the intact liver removed and placed in ice-cold calcium-free HBSS containing 50 mmol/L HEPES (pH 7.4, HEPES-HBSS). Glisson's capsule was carefully peeled from the surrounding parenchyma with surgical forceps and digested for 30 minutes with 50 ml of HEPES-HBSS containing 0.1% pronase, 0.1% collagenase, and 0.004% DNAse to destroy contaminating hepatocytes. The enzyme digestion was stopped by the addition of an equal volume of Waymouths 752/1 media containing 10% fetal calf serum. The cell suspension was centrifuged for 1.5 minutes at 50 x g to remove undigested stromal fragments. The resulting supernatant was then centrifuged at 200 x g and the pellet was resuspended and washed once with 50 ml of the above media. The final NPC pellet was resuspended in 15 ml of media plus 10% fetal calf serum. Selective removal of rare contaminating bile ductular cells was accomplished by incubating the cell suspension with OC-2 ascites fluid (1:1000) for 30 minutes at 4 C, pelleting and washing cells three times with media, and binding OC-2+ cells at 4 C with tosyl-activated magnetic Dynabeads (Dynal, Great Neck, NY) adsorbed with goat anti-mouse IgM (Pierce, Rockford, IL) as described in the manufacturer's instructions. OC-2 recognizes a cell surface bile duct antigen not expressed by LMC. The subtraction using tosyl-activated beads was repeated three times to assure that all OC-2+ cells were removed. Subsequently, LMC were isolated by positive selection with Dynal IgG magnetic beads adsorbed with BD.2 as described by the manufacturer. BD.2+ LMCs were resuspended in Waymouths 752/1 media containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 0.1 mmol/L minimum essential media nonessential amino acids, 1.0 mmol/L sodium pyruvate, 2.0 mmol/L L-glutamine, 8.0 μg/ml insulin, 4.0 μg/ml transferrin, and 50 μg/ml gentamicin and plated at approximately 1 x 10^5 to 2 x 10^5 cells per 60-mm tissue culture dish. The plating media is referred to as Waymouth's complete media (WCM). All components except serum were purchased from GibCO BRL, Grand Island, NY. After 24 hours, it was estimated that
the plating efficiency of BD.2+ cells was 30 to 50%. At this time, plates were washed twice with HBSS and cells were refed with WCM. The culture media was changed every 3 to 4 days. Under these culture conditions, BD.2+ LMC proliferated forming confluent monolayers of epithelial-like cells within 14 to 21 days.

Using this approach, we established three parental LMC cell lines designated LMC 1, LMC 7, and LMC 13. In addition, ten clonal cell lines (LMC 13.1 to LMC 13.10) were established by limiting dilution from the LMC 13 parental line. In the present report we have presented a summary of our findings for LMC 13 and LMC 13.1 (passage 3).

**Isolation and Culture of BDEC**

The ability of BDEC to be propagated under standard culture conditions routinely used to establish RLEC lines was also examined in the present investigation. Briefly, the intact biliary tree was isolated and minced as previously described and a single-cell suspension of BDEC was obtained by further dissociating the tree with 50 ml of HEPES-HBSS containing 0.1% pronase, 0.1% collagenase, and 0.004% DNAse for 30 minutes in a shaking water bath (120 rpm). This step was repeated once with fresh enzyme solution to further digest tissue fragments. The enriched BDEC population was pelleted at 200 × g for 5 minutes and then washed twice with an equal volume of Waymouth’s media containing 10% fetal calf serum. OC.2+ BDEC were subsequently isolated by using the magnetic bead methodology as described above, washed twice, resuspended in WCM, and plated on plastic dishes. After 24 hours, cultures were washed twice with HBSS to remove unbound cells and then refed WCM.

**Monoclonal Antibodies**

The production and characterization of MAbs that recognize bile ducts (eg, OC.2, OV-1, OV-6, and BDS7) or hepatocytes (eg, cell CAM 105) has been previously described. MABs OV-1 and OV-6 were kindly provided by Dr. Harold Dunsford (University of Mississippi Medical Center, Jackson, MS) and MAb BDS7 was generously furnished by Dr. Norman Marceau (Laval University Cancer Research Center, Quebec, Canada). MAb against CK-14, a cytokeratin expressed by squamous epithelium was obtained from Biogenix Laboratories (San Ramon, CA) and MAb DP1 which recognizes desmoplakin I was developed in our laboratory. Rabbit anti-rat albumin polyclonal antibody (affinity-purified IgG fraction, Oronon Teknika Corp., Durham, NC) was used in combination with a biotinylated goat anti-rabbit secondary antibody and streptavidin conjugated to fluorescein isothiocyanate. Nonspecific staining was assessed by staining with culture supernatants from P3x63Ag8 myeloma cells or by using the appropriate affinity-purified nonimmune IgG and IgM fractions from mouse or rabbit.

In addition, we recently developed a new MAAb designated BD.2, that recognizes a cell surface antigen expressed by bile ducts and mesothelial cells as well as ethionine- and 2-acetylaminofluorene-induced oval cells in adult rat liver. BD.2 was developed by immunizing a female Balb/c mouse four times at 10-day intervals with 1.2 × 10^7 bile duct cells isolated from a rat 14 weeks after a bile duct ligation as described. Spleen cells were subsequently harvested and fused with 8653 myeloma cells as previously described and one proliferating hybridoma, BD.2, was selected and cloned because of its immunoreactivity with bile ducts and Glisson’s capsule in frozen sections of normal adult rat liver.

**Tissue Section Analysis**

Blocks of liver tissue were snap frozen in hexane chilled by a dry ice acetone bath and stored at −80 C until analyzed. Serial frozen liver sections 5 μ thick were mounted, acetone fixed, air dried, and stained with MAbs as previously described. Double indirect immunofluorescence analysis was carried out with affinity-purified, subclass-specific secondary antibodies (Pierce). Briefly, acetone-fixed frozen sections or cultured cells were incubated with 1% normal goat serum (10 minutes) to block nonspecific binding and then incubated with a mouse IgM primary antibody (30 minutes), washed three times with PBS, reacted with biotinylated goat anti-mouse IgM (μ chain specific) secondary antibody (30 minutes), washed three times with PBS, and then reacted with streptavidin conjugated to Texas Red and washed five times with PBS. The above procedure was repeated with mouse IgG primary antibody and goat anti-mouse IgG (Fc fragment specific) conjugated with fluorescein isothiocyanate for 30 minutes. Nonspecific staining was assessed by examining tissue sections stained with a mixture of purified mouse IgG and IgM and the appropriate secondary antibodies. Coverslips were applied to sections with phosphate-buffered glycerol containing n-propyl gallate, pH 8, and examined with a Nikon Microphot FX microscope equipped with epi-fluorescence condenser and a 35-mm camera. Sections were photographed with Kodak Tmax 100
film (EI 200). Control experiments were carried out to demonstrate the specificity of secondary antibodies and to assure the lack of reactivity when sections were viewed with the different filter sets. For some experiments, intact monolayers of LMC were isolated by the en face imprint method described by Whitaker et al. 42 Briefly, livers were surgically exposed and rinsed with sterile 0.9% saline to remove blood and debris. After excess moisture was allowed to evaporate, intact monolayers of mesothelial cells were obtained by placing a gelatin-coated glass slide against the damped mesothelial surface for 10 to 20 seconds. The slide was carefully lifted allowing the monolayer of mesothelial cells to be peeled onto the slide. These cells were air dried and acetone fixed for indirect immunofluorescence analysis. Phenotypic analysis was also carried out on cytocentrifuge preparations of freshly isolated BD.2+ cells to define the antigenic characteristics of the cells placed in culture.

**Histochemical Analysis**

Histochemical analysis of frozen liver sections, freshly isolated cells, and cultured cells for γ-glutamyltranspeptidase (GGT) activity was carried out according to the method of Rutenburg et al. 43 with γ-glutamyl-4-methoxy-2-naphthylamide (Vega Biochemicals, Tucson, AZ) as the substrate.

**Electron Microscopy**

Ultrastructural analysis of primary cultures of LMC 13, plated on 60-mm LUX dishes (Miles Scientific, Naperville, IL), was carried out on cells that were fixed with 0.1% cacodylate buffer (pH 7.4) containing 1% paraformaldehyde and 2.0% glutaraldehyde for 1 hour at 4 C. Fixed cultures were washed with Dulbecco’s PBS, post-fixed with 1% osmium tetroxide, and embedded in Epox 812 (Ernst F. Fullen, Latham, NY). Ultrathin sections stained with uranyl acetate and lead citrate were examined in a Phillips 301 electron microscope (Philips, Eindhoven, The Netherlands).

**RNA Extraction and Northern Blot Analysis**

Total RNA was prepared from adult rat livers, 17-day fetal rat livers, and subconfluent monolayers of early passage LMC 13.1 cultures by the method of Chirgwin. 44 Aliquots of 10 μg of RNA were separated by agarose/formaldehyde gel electrophoresis and transferred to Nytran (Schleicher and Schull, Keene, NH). The pBAF700 plasmid, which encodes 700 bp (PstI/BamHI fragment) from the 5’ end and central portion of the full length 2.1-kb rat α-fetoprotein (AFP) transcript was kindly provided by Nelson Fausto. 45 A 700-bp fragment of the coding sequence of mouse serum albumin subcloned into pGEM 3 was provided by Stephanie Cascio. 46 The 1.7-kb Hind III fragment of human desmoplakin II clone p23 was kindly provided by Kathleen Green. 47 These fragments were labeled with [32P]dCTP (New England Nuclear, Boston, MA; 3000 Ci/mmol) by using a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Hybridization and washing of membranes was carried out at 65 C according to the procedure of Church and Gilbert 48 and then exposed to Kodak XAR5 film (Kodak, Rochester, NY).

**RT-PCR**

Total RNA prepared from LMC 13.1, BDE 1.1, BDE 2, BRL-3A, WB3, and LE/6 propagable cell lines as described above was analyzed by RT-PCR for the expression of Wilms’ tumor gene (WT1) transcripts in the laboratory of Dr. Cheryl Walker. All samples were randomly coded and analyzed by this double blind study. Briefly, cDNA was synthesized from 1 μg of total RNA by using the random hexamers method as previously described. 49 Subsequently, cDNA was PCR amplified by using two gene-specific primers that amplified a 732-bp fragment at the 5’ end of the WT1 gene (nucleotides 213–944). These primers were 5’-CCA CCC CAC TCC TTC ATC AAA-3’ and 5’-AAG AGT TGG GGC CAC TCC AGA TA-3’ for the sense and

**Table 1. Phenotypic Characterization of Normal Adult Rat Liver Frozen Sections**

<table>
<thead>
<tr>
<th>Marker*</th>
<th>Hepatocytes</th>
<th>Bile Ducts</th>
<th>Mesothelial Cells</th>
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<tbody>
<tr>
<td>BDS7</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>OV-1</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>QC-2</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>GGT</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Vimentin</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CK 14</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>OV-6</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BD.2</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Albumin</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>cCAM 105</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Desmoplakin I</td>
<td>+</td>
<td>+</td>
<td>–</td>
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</table>

* The production and characterization of antibodies is described in references 30, 31, 38, and 39. The reactivity of antibodies on frozen liver sections was determined by indirect immunofluorescence analysis. The expression of GGT enzyme activity was determined histochemically by using the method of Rutenburg et al. 45

†, –, lack of expression of a marker.

+, expression of a specific marker by hepatocytes, biliary epithelial cells, or serosal liver mesothelial cells.
antisense strands, respectively. The PCR product was amplified by 30 reaction cycles: denaturation at 95°C for 1 minute; annealing at 60°C for 1 minute; extension at 72°C for 1 minute; then final extension at 72°C for 5 minutes. The PCR products were visualized in a 1% agarose gel after staining with ethidium bromide. cDNA from rat neonatal kidney and negative control reactions in which cDNA template was omitted were run with each reaction.

Results

Phenotypic Characterization of Rat LMC In Situ

Indirect immunofluorescence analysis of frozen liver sections and en face imprints of Glisson's capsule was carried out by using a panel of MABs that recognize normal hepatocytes, bile duct cells, and mesenchymal cells. As shown in Table 1, LMC that cover the serosal surface of the liver express the ductal markers defined by BD.2 (Figure 1A) and OV-6, the intermediate filaments vimentin and cytokeratin 14 (Figure 2A), and desmoplakin I. In contrast, serosal mesothelial cells lack detectable levels of the ductal antigens designated OC.2, OV-1, BDS7, and GGT as well as albumin and cell CAM 105, two normal hepatocyte antigens.

Isolation and Propagation of LMC

A procedure was developed to obtain a purified suspension of LMC by sequential negative and positive selection of primary isolates with MABs that recognize cell surface biliary epithelial (OC.2) and mesothelial (BD.2) antigens (Figure 1B). In primary culture, proliferating BD.2+ cells formed a confluent monolayer of epithelial-like cells within 14 to 21 days (Figure 1C). It was estimated that >90% of BD.2+ cells that attached to the plastic dishes gave rise to proliferating epithelial colonies. Clonal cell lines of early passage mesothelial cells were routinely established by limit-

Figure 1. Reactivity of BD.2 mouse MAb against rat LMC. A: Indirect immunofluorescence analysis of frozen liver section showing that the serosal mesothelial cells strongly express the BD.2 antigen in vivo (magnification, ×250). B: Immunosolation of LMC with Dynal IgG magnetic beads adsorbed with BD.2. Photomicrograph shows an aggregate of freshly isolated BD.2 positive LMC (×800). C: 5-day primary culture of proliferating BD.2+ LMC showing magnetic beads still attached to cells (×200). D: Indirect immunofluorescence analysis of early passage LMC 13.1 shows that the clonal cell line continues to express BD.2 (×400).
Phenotypic Comparison of Freshly Isolated Primary Cultures and Early Passage BD.2+ LMC 13 Cells

Comparative indirect immunofluorescence analysis of cytocentrifuge preparations of freshly isolated LMC 13 cells and primary and early passage cultures of LMC 13 cells showed that all BD.2+ mesothelial cells continued to express BD.2 (Figure 1D) and vimentin and lacked detectable levels of the hepatocyte (eg, albumin and cell CAM 105) and bile ductal specific markers (eg, OC.2, OV-1, BDS7, and GGT) that were used to phenotype LMC (Table 2). However, indirect immunofluorescence analysis revealed significant differences between the percentage of freshly isolated mesothelial cells and primary and early passage cultures of LMC 13 cells expressing CK-14, OV-6, and desmoplakin I. Although >90% of the freshly isolated BD.2+ LMC expressed OV-6 and desmoplakin I, the percentage of positive cells expressing either of these markers decreased to approximately 30% of BD.2+ cells comprising confluent cultures (Table 2). In contrast, immunocytochemical analysis showed that 25 to 30% of freshly isolated BD.2+ LMC 13 cells expressed detectable levels of CK-14 (Figure 2B) whereas >99% of LMC 13 cells

below. The morphology of early passage LMC 13.1 cells (passage 3) is shown in Figure 3. These small polygonal shaped cells form a cobblestone pattern when grown to confluency on plastic tissue culture dishes.

Figure 2. Indirect immunofluorescence analysis of LMC with a MAb against CK-14. This panel of photomicrographs shows that (A), all LMC comprising en face imprints peeled from the serosal surface of the adult rat liver express CK14 (×500), whereas (B) only a subpopulation (25%) of freshly isolated LMC 13 cells, immunostained with Dynal magnetic beads adsorbed with the BD.2 MAb continue to express CK-14. Long arrows denote some of the CK+ cells; short arrows denote some of the CK-14- cells (×600). C: In contrast, once LMC 13 cells are established in culture (passage 1), all of the mesothelial cells express CK-14 (×120).

Figure 3. Morphology of cultured LMC. Light microscopic examination of LMC 13.1 cells (passage 3) cultured on plastic dishes in WCM for 7 days reveals that proliferating cells produce a confluent monolayer of epithelial-like, polygonally shaped cells that is morphologically indistinguishable from normal rat liver epithelial cell lines developed by other laboratories (×80).
comprising early passage cultures strongly expressed this cytokeratin (Figure 2C). The phenotype of the clonal cell line designated LMC 13.1 was identical to the parent LMC 13 cell line (data not shown).

Phenotypic comparison of LMC 13.1 and WB3 cells revealed several differences in the antigenic profiles of these two RLEC lines (Table 2). In contrast to LMC, the WB3 cell line was comprised of both positive and negative cell populations with regard to the expression of both BD.2 and CK14; however, all cells weakly expressed OV-1, a ductal specific marker. This finding suggests that these two cell lines are derived from different NPC progenitors.

**Northern Blot Analysis**

As shown in Figure 4, Northern blot analysis of total RNA isolated from subconfluent monolayers of early passage LMC 13.1 cells revealed that these mesodermally derived epithelial cells expressed transcripts for desmoplakin but lacked detectable mRNAs for AFP, albumin and cell CAM 105.

**Transmission Electron Microscopy**

Ultrastructural analysis of primary cultures of BD.2+ LMC 13 cells (e.g., the parental cell line) showed that the mesothelial cells readily phagocytized the microscopic paramagnetic particles used to isolate the BD.2+ cells (Figure 5, A–C). The epithelial nature of these mesodermally derived cells was confirmed by the presence of desmosomal junctions between neighboring cells (Figure 5B). The polygonal shaped cells contained elliptically shaped nuclei with one to three prominent nucleoli. The cells contained numerous mitochondria, low amounts of smooth endoplasmic reticulum, and occasional bundles of cytokeratin

<table>
<thead>
<tr>
<th>Marker*</th>
<th>Freshly Isolated BD.2+ LMC 13</th>
<th>Primary Cultured LMC 13</th>
<th>Primary Cultured OC.2+ BDEC</th>
<th>WB3†</th>
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<tbody>
<tr>
<td>BDS7</td>
<td>-†</td>
<td>-</td>
<td>+++++</td>
<td>-</td>
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<tr>
<td>OV-1</td>
<td>-†</td>
<td>-</td>
<td>++++</td>
<td>+++++</td>
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<tr>
<td>OC.2</td>
<td>-†</td>
<td>-</td>
<td>++++</td>
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<td>GGT</td>
<td>-†</td>
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<td>++++</td>
<td>-</td>
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<tr>
<td>Vimentin</td>
<td>++++</td>
<td>++++</td>
<td>R</td>
<td>++++</td>
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<tr>
<td>CK 14</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>OV-6</td>
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<td>BD.2</td>
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<tr>
<td>Albumin</td>
<td>-†</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>cCAM 105</td>
<td>+</td>
<td>+</td>
<td>-</td>
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* The production and characterization of antibodies is described in references 30, 31, 38, and 39. The reactivity of antibodies on acetone-fixed cells was determined by indirect immunofluorescence analysis. The expression of GGT enzyme activity was determined histochemically by using the method of Rutenburg et al.43

† WB3 (passage 7) is a clonal subline of RLEC derived from WB-F344.9

‡ Data represent the percentage of cells expressing a specific marker: -, not detectable; R, <5%; +, 5–25%; ++, 26–50%; ++++, 51–75%; ++++, >95%. Values were determined by averaging the percent of positive cells observed in five randomly selected microscopic fields of view.

**Table 2. Phenotypic Comparison of Freshly Isolated and Primary Culture of BD.2+ LMC 13 cells, Primary Cultures of OC.2+ BDEC and Early Passage WB3 Cells after Plating on Plastic Tissue Culture Dishes and Maintained in WCM for 72 Hours**
fibers (Figure 5C). The cell surface was relatively smooth and there were few apparent gap junctions.

**Characterization of Primary Cultures of OC.2⁺ Biliary Epithelial Cells**

In contrast to LMC, when a single cell suspension of OC.2⁺ bile duct cells was cultured under identical conditions (ie, plated on plastic in WCM), the OC.2⁺ cells attached to the plastic dishes but did not proliferate. Instead, most ductal epithelial cells assumed a squamous-like appearance and sloughed off the dishes within 2 to 3 weeks. Indirect immunofluorescence analysis of short-term primary bile duct cultures maintained in WCM showed that the phenotype of cultured OC.2⁺ bile duct cells was essentially identical to their phenotype in vivo (Tables 1 and 2). However, it should be noted that primary cultures of BDEC contained rare (<1%) OC.2⁺ cells that weakly expressed vimentin or CK-14 (Table 2).

**Expression of WT1 Transcripts**

It was recently reported that mesodermally derived cells that line the body cavities and visceral organs, including heart, lung, intestine, and liver, constitutively expressed transcripts for the tumor suppressor gene WT1 in the adult mouse. In the present investigation, we used RT-PCR to determine whether the clonal BD.2⁺ LMC 13.1 cell line expressed WT1 transcripts. For comparison, we also analyzed RNA samples from BDE 1.1 and BDE2, two rat biliary epithelial cell lines developed in our laboratory, the normal RLEC lines designated BRL-3A and WB3, and LE/6, a cell line derived from a NPC fraction enriched for ethionine-induced oval cells. As shown in Figure 6, LMC 13.1 expressed the 732-bp fragment of WT1. In addition, RT-PCR analysis of BRL-3A, WB3, and LE/6 indicated that these lines also contained cells expressing WT1 mRNA. In contrast, WT1 transcripts were not detected in the BDE 1.1 and BDE 2 cell lines.

**Discussion**

In the present investigation, we have used an immunological approach to identify and isolate an antigenically defined population of LMC to determine whether...
these mesodermally derived epithelial cells can be continuously propagated in vitro under culture conditions routinely used to establish RLEC. Indirect immunofluorescence analysis of frozen liver sections revealed that it was possible to use a combination of immunological markers to unequivocally identify and isolate a pure population of LMC (eg, OC.2+, BD.2+, CK14+, and vimentin+, Table 1). This was accomplished by first depleting the pronase-digested LMC population of contaminating BDEC with the ductal specific marker OC.2 and then isolating OC.2+ LMC with BD.2, which recognizes a cell surface epitope expressed by both BDEC and LMC. Phenotypic analysis of the freshly isolated BD.2+ LMCs confirmed their mesothelial origin and the purity of the cell population (see below). When BD.2+ LMCs were plated on plastic dishes in WCM, we observed that >90% of the bead-bound cells gave rise to proliferating colonies of epithelial-like cells within 10 to 14 days (Figures 1C and 3) that were morphologically indistinguishable from RLEC lines developed by other laboratories (data not shown).

Several observations support the view that LMC and not BDEC are the progenitors of the RLEC-like cell lines in this investigation. First, numerous attempts to establish cell lines from OC.2+ BDEC by using identical culture conditions for establishing the BD.2+ LMC lines were unsuccessful. Although OC.2+ BDEC readily attached to the plastic dishes, the cells rapidly became squamous-like in appearance and sloughed off the dishes within 2 to 3 weeks. In contrast, our laboratory has recently developed selective conditions for the isolation and long-term propagation of well differentiated rat liver BDEC. Unlike LMC, the continuous propagation of BDEC requires that ductal cells be plated on top of collagen gels in media containing forskolin as an essential growth factor. Secondly, phenotypic comparison of primary cultures of BD.2+ LMC and OC.2+ BDEC demonstrated that the in vitro phenotypes of these two epithelial cell populations closely reflected the cell-specific antigenic patterns observed in vivo (Tables 1 and 2). For example, freshly isolated and primary cultures of BD.2+ LMC 13 cells lacked detectable levels of the ductal markers designated OC.2 and GGT but strongly expressed vimentin (Table 2). Although we estimated that only 25% of freshly isolated LMC expressed detectable levels of CK-14 (Figure 2B), this cytokeratin was expressed by all cells comprising primary LMC cultures and propagable BD.2+ LMC lines (Figure 2C).

Inasmuch as all adult serosal mesothelial cells examined by the en face technique expressed CK-14 (Figure 2A), we propose that the expression of the epitope recognized by the CK-14 antibody used in the present study is transiently lost when the serosal mesothelial monolayer is enzymatically dissociated. This view is further supported by the finding that the percentage of cells expressing CK-14 transiently decreases after the brief trypsinization used to subculture the BD.2+ LMC; however, immunoreactive CK-14 protein is constitutively expressed by all LMC after 24 hours in culture (data not shown). Inasmuch as CK-14 expression was not modulated by plating density of LMC, we propose that expression of this cytokeratin is modulated by altering cell shape. In contrast to LMC cultures, indirect immunofluorescence analysis of primary bile duct cultures showed that all BDEC continue to express the biliary specific markers (Table 2). Taken together, our observations are consistent with the indication that the RLEC-like cell lines developed in this study are of mesothelial and not biliary origin.

It should be emphasized that even though primary cultures of BDEC assume a squamous appearance when plated on plastic dishes, CK-14 and vimentin were only expressed by rare OC.2+ ductal cells. Although the biological significance of these antigenically distinct ductal cells is unknown, Marceau has proposed that CK-14 is transiently expressed as bipotential liver precursor cells are committed to the
BDEC lineage.\textsuperscript{25} Thorgeirsson and coworkers\textsuperscript{51,52} suggested that CK-14\textsuperscript{+} RLEC lines may be derived from hepatic stem cells contained in the BDEC population. Inasmuch as these antigenically distinct ductal cells did not proliferate in WCM, it seems highly unlikely that these BDEC are the progenitors of the LMC lines developed in the present investigation. In contrast, virtually all BD.2\textsuperscript{+}, CK-14\textsuperscript{+} LMC give rise to clonogenic colonies of epithelial cells in this study. These findings emphasize the need to use antigenically defined populations of freshly isolated cells when attempting to unequivocally identify the cellular precursors of RLEC.

Inasmuch as it was recently reported that mesodermally derived tissues in the mouse express the Wilms’ tumor gene product, WT1, \textit{in situ}, this tumor suppressor transcription factor may be useful as a lineage marker to confirm the mesothelial origin of our cell lines.\textsuperscript{30} To investigate this possibility, we used RT-PCR to analyze RNA samples obtained from various RLEC lines, including LMC 13.1, BDE 1.1, and BDE 2. We found that the LMC 13.1 cell line but not the bile duct cell lines BDE 1.1 and BDE 2 expressed WT1 transcripts, an observation further substantiating the mesothelial origin of the BD.2\textsuperscript{+} RLEC-like cell lines. In addition, the other RLEC lines examined, BRL-3A, WB3, and LE/6, also expressed messages transcribed from the Wilms’ tumor gene. Although the biological significance of this latter observation remains to be defined, the finding that both the WB-F344 and LE/6 lines express both hepatocytic traits and WT1 transcripts, suggest that 1), these RLEC lines may be derived from a primitive mesodermal precursor, perhaps the nondescript AFP\textsuperscript{+} periductal cells observed in normal liver,\textsuperscript{45,53} and 2), the WB3 and/or LE/6 lines contain a subpopulation of contaminating mesothelial cells. Although there is presently no \textit{in vivo} evidence to suggest that LMC are hepatic precursors, our laboratory is presently conducting extensive \textit{in situ} hybridization studies on normal and carcinogen-treated livers with Wilms’ tumor riboprobes to provide further insight into this question.

The observation that some RLEC lines express hepatocytic markers such as AFP and albumin suggest that these lines may be derived from facultative liver stem cells.\textsuperscript{5,8,9,11,20} Although the identity of the hepatic stem cell is unknown, Fausto and coworkers\textsuperscript{45,54} and Alpini et al\textsuperscript{53} recently demonstrated that the normal adult rat liver contains rare AFP\textsuperscript{+} ductal and periductal cells. Evidence supporting the possibility that these rare AFP\textsuperscript{+} ductal cells may be one of the progenitors of RLEC was recently provided by Grisham and coworkers.\textsuperscript{5,20} These investigators convincingly demonstrated that their AFP\textsuperscript{+} cell line WB-F344 acquires the morphological and phenotypic characteristics of well differentiated hepatocytes when transplanted into the livers of syngeneic host.\textsuperscript{8,20} In contrast, Northern blot analysis of total RNA prepared from early passage LMC 13.1 cells showed that these cells lacked detectable levels of hepatocyte-specific transcripts for AFP, albumin, and cell CAM 105 but expressed desmoplakin transcripts (Figure 4). This finding is consistent with a mesothelial origin of the LMC 13.1 epithelial cell line. Taken together, these results indicate that there are at least two different cellular progenitors of RLEC.

Our laboratory has previously shown that early passage but not late passage BDE 1.1 cells express low levels of AFP 2.1-kb transcripts\textsuperscript{26} suggesting that this propagatable BDEC line may initially contain hepatocytic precursors. Inasmuch as Grisham and coworkers have shown that RLEC culture conditions support the clonogenic growth of WB-F344 cells, it is not clear why the presumptive OC.2\textsuperscript{+}, AFP\textsuperscript{+} ductal cells do not proliferate under the conditions used to establish RLEC in this study. It is possible that LMCs or other mesenchymal cells (eg, Ito cells), which may have been included in Grisham’s NPC fraction, produce essential growth factors that support the clonal proliferation of AFP\textsuperscript{+} NPC in culture. Although there are presently no data available to confirm this hypothesis, it has been shown that Ito cells produce oval cell growth factors during the early stages of the neoplastic process.\textsuperscript{55–57} Ongoing studies in our laboratory are addressing this question.

The elucidation of the cellular progenitors of RLEC will depend on the availability of markers that can be used to define and isolate subpopulations of non-parenchymal liver epithelial cells. In the present investigation, we found that BD.2\textsuperscript{+} LMC isolated from Glisson’s capsule give rise to propagatable epithelial cell lines that uniformly express CK-14 and vimentin. Inasmuch as long-term cultured LMC lines continue to uniformly express each of these three markers and lack the ductal specific markers designated OC.2, OV-1, and GGT (data not shown), this combination of immunological reagents may be useful to identify RLEC lines derived from LMC. In support of this view, immunocytochemical analysis of early passage WB3 cells with these markers revealed that this cell line expresses OV-1 and vimentin (Table 2). In contrast to LMC, a subpopulation of cells comprising the WB3 line (<50%) weakly expressed BD.2 and CK-14 (Table 2). These phenotypic observations suggest that the WB3 cell line is not derived from LMC. We are pres-
ently isolating BD.2+ and BD.2− WB3 cells to further characterize these antigenically distinct subpopulations.

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