A new continuous retinoblastoma cell line (Rb 355-7) derived from a nonfamilial unilaterally-affected child was studied morphologically and with regard to HLA expression. The tumor was compared with two older and widely studied cell lines, the Y-79 and WERI-Rb1 strains. The Rb 355-7 line grew in tissue culture in clusters and chains. Its doubling time was calculated to be 4.4 days. In contrast to the Y-79 and WERI-Rb1 cell lines, the Rb 355-7 showed a paucity of HLA expression. Invest Ophthalmol Vis Sci 28:690-699, 1987

Retinoblastoma is the most common malignant eye tumor of childhood and, after malignant melanoma, is the second most common primary intraocular malignancy of any type. Because of the hereditary pattern and association with the 13q-syndrome, this tumor has been the focus of intensive investigation by oncologists and molecular biologists. Success in establishing two continuous retinoblastoma cell lines, the Y791 and WERI-Rbl,2 has been useful for studying this tumor. The purpose of the current study was to describe HLA antigen expression and electron microscopic studies of an additional retinoblastoma cell line designated Rb355-7.

Materials and Methods

Culture Methods

The cell line Rb 355-7 was established by Dr. Brenda Gallie in Toronto, Canada. The original tumor was obtained from a nonfamilial, unilaterally-affected 11-month-old child, who had an enucleation on May 12, 1979. The tumor was initially placed in vitro on fibroblast feeder layers, and simultaneously injected subcutaneously in the athymic nude mouse. The tumor grew both in vitro and in vivo. Since January, 1980, the retinoblastoma cell line has grown independently of the fibroblast feeder layer. The origin tumor, designated Rb 355, was cloned in methyl cellulose. A clone was picked and designated Rb 355-7. The Rb 355-7 cell line was obtained from Dr. Brenda Gallie in 1983.

Immunofluorescence

Indirect immunofluorescence for detection of HLA A, B, C was performed as previously described3 on a single cell suspension. Briefly, cells were washed in Hanks' (GIBCO, Grand Island, NY) balanced salt solution, fixed with freshly prepared 1% paraformaldehyde at 4°C for 4 min, washed, resuspended in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 0.1M, pH 7.4), and distributed into Linbro U-Bottom (Flow Labs, McLean, VA) microtest wells at a concentration of 200,000 cells per well. After centrifugation, pellets were incubated for 30 min with 50 μl of a 1:100 dilution of appropriate monoclonal antibody or control. Samples were then washed three times, and incubated with 50 μl of a 1:20 dilution of FITC-labelled rabbit antimouse IgG (Cappel, Malvern, PA) for 30 min. Samples were then washed three times, mounted on glass slides with 1% phenylenediamine in carbonate-bicarbonate buffer (pH 8.0), and examined under a Zeiss (Carl Zeiss, West Germany) microscope fitted with epifluorescence illumination and phase optics.

Immunoperoxidase

Immunoperoxidase assays for detection of HLA A, B, C, using the peroxidase-antiperoxidase method as

| W6/32 | 2+ | 3+ | 1-2% cells 1+ to 1+ rest neg | 4+ |
| BBM.1 | 1+ | 2+ | 1-2% cells 1+ rest neg | 8+ |
| BRL antimonocyte | neg | neg | neg | W6/32: anti HLA-A,B,C, heavy chain |
| BBM.1: anti-HLA-A,B,C light chain (BW2Q-microglobulin) |
previously described, were performed. Cytocentrifuge preparations on fresh retinoblastoma cells were made, air dried, and acetone-fixed for 5 min. Slides were washed twice in 1:20 normal swine serum, and then incubated with 50 μl of a 1:100 dilution of monoclonal antibody for 1 hr. Specimens were then washed, and rabbit antimouse IgG at 1:40 for 30 min and washed, followed by incubation with swine anti-rabbit IgG at 1:40 for 30 min. After additional washing, rabbit peroxidase-antiperoxidase was applied for 30 min, and washed. Diaminobenzidine was prepared, 50 mg%, titrated to pH 7.6, with the addition of 100 μl of 30% H2O per 100 ml, filtered, and applied to the slides and washed following appropriate incubation times. After counterstaining with hematoxylin, slides were dehydrated through ethanol and xylene and mounted in

**Table 2. Results of immunoperoxidase assays**

<table>
<thead>
<tr>
<th></th>
<th>Y-79</th>
<th>WERI-Rbl</th>
<th>RB 355-7</th>
<th>LB-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6/32</td>
<td>2+</td>
<td>3+</td>
<td>1-2% 2+</td>
<td>10-12% 1+</td>
</tr>
<tr>
<td>BBM.I</td>
<td>1+</td>
<td>2+</td>
<td>1-2% 2+</td>
<td>10% trace</td>
</tr>
<tr>
<td>BRL</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
</tr>
</tbody>
</table>


Fig. 1. Rb355-7: indirect immunofluorescence assay using W6/32 (anti-HLA heavy chain) monoclonal antibodies. A sub-population of cells is strongly positive for HLA (long arrow). Other cells in the field are negative or trace positive for HLA.

Fig. 2. Rb355-7: immunoperoxidase-PAP techniques with W6/32 confirm findings on indirect immunofluorescence: a sub-population of cells is positive for HLA A, B, C. Other cells (arrows) are negative or trace positive for HLA. (Diamino benzidine as substrate; no counterstain.)

**Table 3. Morphologic characteristics of retinoblastoma cell lines**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Retinoblastoma Y79</th>
<th>Retinoblastoma WERI-Rbl</th>
<th>Retinoblastoma Rb-355-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of morphologically different cell types</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Filopodia</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lamellipodia</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pseudopods</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zeiotic blebs</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tiny holes in surface membrane</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Thick filopodia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lacy pattern of growth</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rosette-like formation</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Indicates this feature was observed in some cells in that category. ++ Indicates this feature was characteristic of the cells of that category.
Permount (Fisher, Springfield, NJ). All immunologic reagents other than the monoclonal antibodies have been absorbed with acetone-fixed human liver and/or kidney power, and with human serum coupled to Sepharose with cyanogen bromide (Pharmacia, Piscataway, NJ).

**Monoclonal Reagents**

Well-characterized monoclonal reagents in ascites form against HLA A, B, C heavy chain (W6/32) were used against the associated B2-microglobulin light chain (BBM.1). An antimonocyte monoclonal antibody (Bethesda Research Laboratories, Bethesda, MD) was used as the negative monoclonal control. In all experiments, a lymphoblastoid cell line (LB-O, gift from Dr. D. Anderson) was used as a tissue control, in addition to the retinoblastoma cell line Y-79 and WERI-Rb1.

**Electron Microscopy**

For SEM studies, following fixation, the cells were left overnight in phosphate buffer (0.1 M, pH = 7.4), and then formed into a soft pellet by centrifugation,
Fig. 4. Triple membrane structures are seen (TMS) and phagolysosomes (P) are present in the cytoplasm (×30,500).

or were allowed to settle by gravity. The cells were washed in buffer, postfixed in 1% osmium tetroxide in buffer for 1 hr, washed again in buffer, and then sprayed directly onto a nuclo pore or millipore filter. The cells were dehydrated through a graded series of alcohols, and dried out in CO₂ in a Samdri (Biodynamics Research Co., Rockville, MD) PVT-3 critical point drying apparatus. After shadowing with gold palladium, the cells were examined with a JEOL (Japanese Electron Optics Co., Ltd., Tokyo, Japan) scanning electron microscope-35.

For TEM studies, material was fixed in 2% phosphate buffered glutaraldehyde for 1 hr, and transferred to the above buffer. After thorough washing in 0.1 M phosphate buffer, the tissues were postfixed in buffered 1% osmium tetroxide for 1 hr. Uranyl acetate staining, dehydration and initial plastic infiltration were carried out in the Reichert EM (Reichert Scientific Instruments, Buffalo, NY) tissue processor, following which the specimens were removed and embedded in Epon 812. In addition, when it was necessary to observe the scanning specimens by TEM, the specimens were removed from the scanning stubs, infiltrated in Epon overnight, and embedded as above. Ultrathin sections were cut with a Dupont diamond knife on an LKB (Bromma, Sweden) ultramicrotome and stained with lead citrate. The sections were examined with the JEM-7 electron microscope.

Results

Pathologic Findings of Original Tumor

The specimen consisted of an enucleated globe with an apparent single large retinal tumor composed of undifferentiated cells with no rosettes or fleurettes identifiable. Areas of the tumor were necrotic and calcified. There were multiple foci of possible seeding of tumor on the surface of the retina. In certain areas, small foci retinoblastoma cells could be identified in the choroid. The optic nerve was invaded by tumor to the level of the lamina cribosa. No extrascleral spread of tumor was noted.

The cornea had mild peripheral neovascularization and anterior stromal scarring. Rubecosis iridis was present, with the chamber angle closed by peripheral anterior synechiae. The lens was adherent to the iris by posterior synechiae. Posterior migration of lens epithelium was noted.

Observation of Cells in Tissue Culture

The cells grew in suspension as clusters and chains. The doubling time was calculated to be 4.4 days.

HLA Antigen Detection

The reactivity of the four cell types with the different monoclonal antibodies is presented in Tables 1 (im-
Electron Microscopic Findings

On TEM examination, the cells appeared small and round. Their nuclei were round to elongated with small, well-formed nucleoli (Fig. 3). Aggregates of dense chromatin were seen throughout the nuclei (Fig. 3), and triple membrane structures (TMS) were found involving the nuclear envelope (Fig. 4). The cytoplasm was scanty with a variable amount of mitochondria, endoplasmic reticulum, free ribosomes, and vacuoles (Figs. 3, 5). Cilia were also present. A relatively large number of phagolysosomes was noted (Fig. 4). Numerous cells were undergoing mitosis (Fig. 6). Cells were attached by desmosomes (Macular adherens), and

Fig. 6. Tumor cells seen in chain formation and undergoing mitosis (M) (x1,785).
some intercellular spaces were filled with a dense material probably representing lipid (Fig. 7). Cells were arranged in straight and branching chains (Fig. 6).

On SEM examination, cells lacked surface characteristics such as lamellipodia and filopodia (Table 3). In some areas, a few cells demonstrated zeiotic blebs (Fig. 8). Numerous cells have a spongy appearance, and possess numerous holes in their surface (Fig. 9). Cells were arranged in multiple clusters (Fig. 8).

**Discussion**

The small, round, undifferentiated cell of the Rb-355-7 cell line showed many of the ultrastructural characteristics previously described in retinoblastoma cells in vitro: small nuclei, small, well-formed nucleoli, scanty cytoplasm, and the characteristic distribution of organelles.6,7

The surface morphology of the Y-79 and WERI-Rbl cell line, in general, is notable for the presence of microvilli, zeiotic blebs, filopodia, and lamellipodia.2,8 WERI-Rbl, as well as fresh retinoblastoma specimens, contain a distinct population of cells characterized by a spherical, smooth shape with no surface morphology.8

The paucity of surface structures seen by SEM in the Rb-355-7 is more consistent with the latter case, and may be a result of cell environment or cell cycle fac-
Fig. 8. A cluster of well formed tumor cells, showing a few ziotic blebs (arrows) (×4,080).

The triple membrane structures and the frequent infoldings of the nuclear membrane of the Rb-355-7 lines have been previously described by Ellsworth. These changes have been observed in a variety of cells undergoing rapid normal or pathological cell division. However, their significance remains unknown.

Rb-355-7 cells were characterized by a spongy appearance, and the progression of tiny holes which penetrated the surface membrane previously identified in the WERI-Rb1 cell line. These cell membrane holes have been interpreted as a sign of necrosis and are similar in appearance to those in cells removed from necrotic areas of fresh retinoblastoma. The presence of accumulated necrotic cells may result from the inability to rid a suspension culture of nonviable cells.

Rosette-like structures characteristic of Y-79 and WERI-Rb1 cells were not seen in the current study. The absence of this growth pattern may reflect the lack of differentiation seen in the original tumor.

Retinoblastoma cells in cell suspension culture are readily studied with immunohistochemical techniques. Both Y-79 and WERI-Rb1 express significant levels of HLA A, B, C that could be demonstrated by indirect immunofluorescence and immunoperoxidase assays with both W6/32 and anti-B2-microglobulin (BBM.1), as has been shown for normal human late fetal and
adult retinal cells (Personal communication from Dr. Delia Sang, February, 1985). By comparison with Y-79 and WERI-Rbl, however, the RB-355-7 cell line showed a distinct paucity of HLA expression. Although it has been shown that, over time, subcultured Y-79 may lose Class I surface HLA expression, this is unlikely in the case of RB-355-7, which is a newly-established cell line. The relative lack of Class I HLA expression may be suggestive of a less differentiated state, as has been noted in early human fetal retinal cells (Personal communication from Dr. Delia Sang, February, 1985) and undifferentiated SF81 (derivative of Y79) retinoblastoma cells. Y-79 and WERI-Rbl cells have been maintained in vitro for 11 and 8 yrs respectively. There is ample evidence that the morphologic and genetic characteristics of cultures grown for extended periods may change. Such variation can lead to misinterpretation when extrapolating the results to fresh tumor. The availability of new cell lines will permit confirmation and additional observation in biochemical, immunologic, and genetic studies regarding retinoblastoma.

Key words: retinoblastoma, HLA antigens, electron microscopy, immunoperoxidase, immunofluorescence
Acknowledgment

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References