SUMMARY

A new continuous cell line derived from a human retinoblastoma cell has been established. This cell line, WERI-Rbl, has been maintained in vitro since December 1974. The purpose of this investigation was to characterize WERI-Rbl on the basis of morphology, growth, tumorigenicity, cyto genetics, and to compare this cell line with Y79, a human retinoblastoma cell line established at another institution. Morphologically, both cell lines were similar; each spontaneously grew as a suspension of small round cells in grape-like clusters. Each exhibited growth of cells in rosettes, as well as unusual chain formations. Growth rates differed: the population doubling times for WERI-Rbl and Y79 were 96 and 33 hr, respectively. When the negative surface charge on a plastic tissue culture flask was changed, each cell line grew as a monolayer. Y79 could be cloned in soft agar; WERI-Rbl could not. An inoculum of 10^7 WERI-Rbl or Y79 cells produced a retinoblastoma in test rabbits. Karyological examination showed each cell line have a stable, near diploid chromosome number. Although large markers were observed in each cell line, they shared no common marker.

INTRODUCTION

Retinoblastoma is the most common intraocular cancer of childhood, occurring with a frequency of about 1 in 20,000 births. The mean age at diagnosis is 24 months (4). The cancer arises in the retina from a rod cell of the visual receptor layer. Rosettes or fleurettes seen in differentiated tumors reveal the histogenesis of retinoblastoma. The tumor cells forming rosettes and fleurettes possess cell processes resembling photoreceptor elements (20-22).

There are 2 types of retinoblastoma, hereditary and nonhereditary (4). About 95% of all cases arise spontaneously, showing no evidence of a manifesting gene carrier parent. The remaining 5% are heritable, showing an affected survivor parent. Studies suggest the latter cases are transmitted as an autosomal dominant trait with 90% penetrance (3). The nonheritable or "spontaneous" classification of retinoblastoma may be misleading, since some may be of a heritable nature. Thus there is a "third type" of retinoblastoma, that is, hereditary but having no family incidence. Knudson (8) and Knudson et al. (9) have suggested that 60% of all retinoblastomas are nonhereditary, whereas 40% are hereditary.

The hereditary form of the disease has been extensively investigated. The association of retinoblastoma with a deletion of the long arm of a No. 13 chromosome has been documented (15, 24).

Tumor cell cultures are a tool for the study of cancer. Recent success in establishing the 1st human retinoblastoma cell line, Y79 (16), and now the establishment of a new human retinoblastoma cell line provide the means of characterizing properties common to this disease.

MATERIALS AND METHODS

Clinical Evaluation. A 1-year-old female Caucasian with no family history of retinoblastoma was admitted to the Wills Eye Hospital for clinical evaluation. Examination revealed a retinoblastoma, and on December 5, 1974, her right eye was enucleated. The globe was sectioned, and a portion of the tumor was sent to the Research Institute for tissue culture studies.

Culture Methods. Tumor tissue was placed in 2 ml of Roswell Park Memorial Institute Medium 1640 and Ham's F10 medium (50:50, v/v), supplemented with 15% heat-inactivated fetal bovine serum and, per ml, 100 IU penicillin, 100 IU streptomycin, and 2.5 μg Fungizone (Grand Island Biological Co., Grand Island, N. Y.). The soft tumor was dissociated by pipetting, and a suspension of single cells and clumps was seeded into a 30-ml Falcon Plastics tissue culture flask (Falcon Plastics, Oxnard, Calif.). The flask was set upright (to maintain the tumor cells at a high density) and incubated at 37°C in a 95% air-5% CO2 humidified atmosphere. After several days, and twice weekly thereafter, 1 ml of expended media was removed from the culture and replaced with 1 ml of the nutrient mixture.

Tumor explants remained in suspension or were adherent to the bottom of the culture flask. Three weeks after initial cultivation, cell migration from the attached explants was observed, and by the 5th week a confluent monolayer was evident. At this time, the tumor cells in suspension were transferred to a new flask which was set upright and incubated. The cells growing in monolayer were treated with 0.25% trypsin in calcium- and magnesium-free phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter; NaHPO4, 1.15 g/liter; and KH2PO4, 0.2 g/liter), resuspended in media,
and transferred to a new flask. This flask was placed in the normal horizontal position and incubated. Subsequent monolayer transfers were made weekly at a 1:2 or 1:4 split ratio in Eagle’s minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics.

The tumor cells in suspension, given the designation WERI-Rb1, were maintained with twice weekly replacement of Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Two months after initial cultivation, WERI-Rb1 was subcultured. Thereafter, transfers were made twice monthly at a 1:2 split ratio, with medium replacement twice weekly.

**Population-Doubling Times.** For estimation of the population-doubling times for cells growing in suspension, replicate 30-ml Falcon Plastics flasks were seeded with $5 \times 10^8$ cells in 5 ml of nutrient medium. Medium was replaced twice during the test period. Cell suspensions from duplicate flasks were removed, pelleted by centrifugation, and resuspended in an appropriate volume of 0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline. Cell preparations were counted using a hemocytometer.

**Growth and Cloning in Agar.** Growth and colony formation in soft agar was demonstrated using the method of MacPherson (11). Base layers of 7 ml of medium containing 0.5% agar (Grand Island Biological Co.), Difco Bacto agar (Difco Laboratories, Detroit, Mich.), and agarose (Gallard Schlesinger Chemical Manufacturing Corp., Carle Place, N. Y.) were set at room temperature in 60-mm tissue culture dishes. A top layer with $10^5$, $10^6$, or $10^7$ cells in 1.5 ml of medium containing 0.3% agar was added to the base layer. The plates were incubated at 37°C in a 95% air-5% CO2 humidified atmosphere. Twenty-four hr later, each plate was examined for cell viability, and single cells were scored. After 10 days of incubation, colonies which grew out from a single cell were removed by pipetting and transferred to a culture flask containing growth medium.

**Attachment and Growth on a Newly Charged Surface.** Alteration of cell growth in vitro was demonstrated using a modification of the BSA polymer technique of Macieira-Coeelho and Avrameas (10).

Falcon Plastics tissue culture flask (30 ml) were treated with 2 ml of filter-sterilized poly-L-ornithine HBr (molecular weight, 122,000; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 1 mg/ml in calcium- and magnesium-free phosphate-buffered saline. After 18 hr at room temperature, the polyamino acid solution was removed, and each flask was washed with 2 changes of 0.1% EDTA in calcium- and magnesium-free phosphate-buffered saline and 2 changes of nutrient medium. Cell suspensions were seeded onto the treated flasks.

For determination of the rate of growth of cells on a polyornithine surface, replicate flasks were seeded with 5 x $10^8$ cells in 5 ml of nutrient medium. Cell “monolayers” from duplicate flasks were treated with 0.25% trypsin in calcium- and magnesium-free phosphate-buffered saline. Cell suspensions were counted using a hemocytometer.

**Tests for Tumorigenicity.** The tumorigenicity of WERI-Rb1 was determined by inoculation into the anterior chamber of the right eye of 5- to 9-pound Dutch Belted or Chinchilla rabbits. The anterior chamber of each contralateral eye was inoculated with Y79. Inoculation was as follows. At 1 mm from the limbal margin, the cornea was penetrated with a 30-gauge needle fitted onto a 1-ml syringe. The anterior chamber was entered, and 0.2 ml of aqueous humor was removed. Using a 2nd syringe, the chamber was reentered at the same site, and $10^6$ or $10^7$ cells in 0.2 ml phosphate-buffered saline were inoculated.

Rabbits received subconjunctival injections of 25 mg cortisone acetate in each eye, 1 day prior to cell inoculation, twice weekly for 2 weeks and once weekly thereafter. At weekly intervals each anterior chamber was examined by direct and by slit lamp observation, and tumor size was recorded for the duration of the experiment. Eyes were enucleated for histopathological evaluation of tumors.

As a control for tumor cell growth, another group of rabbits received anterior chamber inoculations with $10^7$ WI-38 or monolayer cells (from the original tumor).

**Chromosome Studies.** Chromosome preparations were made by the following procedure. After 3 hr exposure to colcemide (4 $\mu$g/ml of growth medium), cells were treated for 16 min in 0.075 N KCl, fixed in 3:1 methanol:glacial acetic acid, and air-dried onto glass slides. For conventional karyotyping, slides were stained in 2% Giemsa in Sorensen buffer, pH 6.8. For banding analysis slides were treated in 0.1% trypsin prior to staining (19).

**RESULTS**

**Pathological Evaluation of Original Tumor.** Microscopic examination of the enucleated eye confirmed the original diagnosis. The retina was shown to be extensively infiltrated by an endophytic retinoblastoma which replaced half of the vitreous cavity. The tumor was composed of small, round cells, having scanty cytoplasm and hyperchromatic nuclei. Mitotic figures were seen in many fields. Large areas of necrosis were present in cords of viable tumor surrounding blood vessels. Well-formed Flexner-Wintersteiner rosettes were absent, indicating an undifferentiated tumor. Portions of the optic nerve head anterior to the lamina cribrosa were invaded by tumor. The distal section (surgical margin) of the optic nerve was free of tumor.

**Tumor Cell Morphology and Growth in Vitro.** Tissue tumors from 20 retinoblastoma patients have been placed in cell culture. Only 1 continuous cell line, WERI-Rb1, has been established. All tumors exhibited identical growth in vitro, growing both as a monolayer of fibroblast- or ganglion-like cells (7) and as a suspension of small round cells in clumps. The monolayer cultures showed degenerative changes with termination by cell death after 20 population doublings. With the exception of WERI-Rb1, all suspension cultures also failed to proliferate.

WERI-Rb1 initially and spontaneously grew in suspension and has been maintained in continuous culture since December 5, 1974. The cells characteristically grew in loose grape-like clusters at the bottom of the culture flask. As the cell proliferated, large clusters resembling retinal tissue were evident. Pipetting the culture yielded a suspension of small clumps (2 to 10 cells), large clumps (>10 cells), single cells, cells in rosette, and unusual chain formations. Histo-
tumor-derived cell lines, neither WERI-Rbl nor Y79 would cells would attach and grow as a monolayer. When ob-
serve with light microscopy, cells growing on this sub-
strate were morphologically identical to those which grow in suspension (Figs. 4 and 5).

The average population-doubling time for WERI-Rb1 and Y79 growing as a monolayer was compared. As indicated in Chart 2, the doubling time of Y79 was 50 hr, with growth as a monolayer reaching a saturation density of 2 x 10⁶ cells/sq cm, at which point, cells detached from the flask and grew as a suspension. Although WERI-Rb1 did attach to the treated flasks, only a limited capacity for growth as a mono-
layer was expressed. The population-doubling time was 7 days.

Test for Tumorigenicity. WERI-Rb1 tumors were ob-
served in 9 of 12 anterior chambers inoculated with 10⁶ cells and in 3 of 8 inoculated with 10⁵ cells.

Y79 tumors were observed in 12 of 12 anterior chambers inoculated with 10⁶ cells and in 8 of 8 inoculated with 10⁴ cells.

Slit lamp observations made during active tumor growth revealed dense clumps and sheets of cells on the iris, the posterior cornea, and the anterior surface of the lens. Vas-
cularization of the cornea and congestion of iris vessels were evident where tumor cells attached and grew. Metas-
tasis into the vitreous chamber was not observed. All tu-
mors reached maximum size between Days 17 and 22 post-
inoculation. Tumor regression was evident by Day 30.

Y79 cells were strongly tumorigenic at an inoculum as low as 10⁵; tumors filled more than one-third of the anterior chamber (Fig. 6). WERI-Rb1 cells were weakly tumorigenic at either inoculum; these tumors reached an average size of only 5 mm.

On Day 14 postinoculation, 4 rabbits were sacrificed for tumor cell recovery and histological specimens. A suspen-
sion of viable Y79 cells was aspirated from 1 chamber and placed in cell culture. In addition, a solid tumor measuring 5 mm x 1 mm was removed. A suspension of viable WERI-Rb1 cells was aspirated from another chamber, and a small solid tumor measuring 2 mm x 0.5 mm which was attached to the iris was also removed. The cells isolated and recultured in suspension were morphologically identical to the respective cell lines.

Histological sections of enucleated eyes confirmed slit lamp observations. Corneal vascularization and edema were evident, especially in the chambers inoculated with Y79 (Fig. 7). Growth of tumor cells along the needle track and in the corneal stroma was observed. All iris tissue showed vascularization and infiltration with mononuclear...
leukocytes. Growth of WERI-Rb1 on the iris was confirmed (Fig. 8). WERI-Rb1 and Y79 tumors were composed of small, round cells with scanty cytoplasm and large nuclei. Large areas of necrosis were observed; all tumors were surrounded with fibrin.

No growth was observed in rabbits inoculated with 10⁷ WI-38 or 10⁸ monolayer cells from the original tumor.

**Chromosome Studies.** Chromosome preparations of WERI-Rb1 were made after 5 months in continuous culture. Characterization was made on the basis of 50 conventionally stained metaphase cells and 12 Giemsa-banded cells.

The modal chromosome number was 46 (Chart 3) with a double-stem-line population of less than 2%. Karyological instabilities were seen in 2% of the cells analyzed, indicating a rather stable cell line. Pulverization and double-minute chromosomes which have been observed by others in retinoblastoma (16), neuroblastoma, and other tumor-derived cell lines (18), were not seen in this culture.

The cells studied contained from 39 to 43 normal human chromosomes and 6 different marker chromosomes (Figs. 9 and 10). Banding studies indicated no abnormalities in the chromosomes classified as normal; each showed the typical banding pattern as revealed by trypsin pretreatment. No cell analyzed contained the normal human complement; cells containing 46 chromosomes always exhibited rearrangements. Each cell contained at least 5 marker chromosomes; some of these were present in duplicate.

The marker chromosomes of WERI-Rb1 are shown in Fig. 11 and are described as follows. MI is a large metacentric of unknown origin. MI is a large metacentric about the size of a normal A1; this may be a variant of MI. MI is a large submetacentric which morphologically resembles an A2; however, the banding pattern indicates that it is a rearranged A3. MI is a large acrocentric which, although larger, resembles a Group D chromosome. It shows some of the major bands of a D13. MI is a medium-sized metacentric. It is larger than an E16; however, the banding pattern indicates it may have been derived from an E17. MI is a small acrocentric. It resembles a Group G chromosome, but having deleted short arms and no satellite, banding studies did not aid in identification of this marker.

Chromosome preparations of Y79 were made after 16 months in continuous culture in our laboratory. The Y79 karyotype is shown in Fig. 12. We found the Y79 to have a near diploid chromosome number with multiple rearrangements. The 2 marker chromosomes of Y79 are also shown in Fig. 11 and are described as follows. MA is a large abnormal chromosome; banding studies indicate it to be derived from an A3 chromosome. However, the banding pattern of MA differed from that of MI of WERI-Rb1. MB is unlike any human chromosome and could not be identified.

**DISCUSSION.**

The cell line, WERI-Rb1, exhibited criteria associated with malignant cells in culture: the capacity for indefinite growth, tumorigenicity, and the tendency to deviate from the normal diploid karyotype. In addition, the cultural characteristics of this cell line were similar to those of Y79, another human retinoblastoma cell line.

The fibroblast-like cells which grew as a monolayer from adherent tumor explants were nontumorigenic and expressed a limited life-span. These cells were probably derived from the “normal” tissue constituents of the tumor.

In vitro growth of WERI-Rb1 and Y79 in rosettes and chains was unique. Pseudorosettes have been described in heterotransplanted neuroblastoma tumors in nude mice (6). It was suggested that a 3-dimensional setting for tumor growth in the host was conducive to pseudorosette formation via activation of cyclic nucleotides. Since WERI-Rb1 and Y79 grew in suspension in 3-dimensional, grape-like clusters, some biochemical mechanism may also be conducive to rosette and chain formation in vitro.

Rosettes and chains were more characteristic to WERI-Rb1 than to the more anaplastic Y79. Therefore, these growth patterns may be an indication of tumor cell differentiation.

When compared to normal cells, tumor cells have a higher net negative membrane charge (13). The greater this negative charge, the more invasive cells are in vivo and the greater the rate of growth in vitro. A difference between the membrane charge on WERI-Rb1 and Y79 was indicated. Y79 grew in soft agar, showed increased growth in vitro, and greater tumorigenicity at an inoculum of 10⁷ and 10⁸ cells.

Other tumor cell lines attach and grow as a monolayer on plastic tissue culture flasks but exhibit reduced adhesiveness to this substrate. WERI-Rb1 and Y79 grew only in suspension and exhibited no adhesiveness or growth as a monolayer. We speculated that this was related to the quantity and distribution of negatively charged membrane components. Growth of WERI-Rb1 and Y79 as a monolayer on polyornithine-treated flasks gave support to the unique negatively charged nature of these cell membranes. Interestingly, all cells attached to the positively charged flask, no change in cell morphology was evident by light microscopic observation (10), monolayer cells could be regrown as a suspension, and monolayer cells were tumorigenic.

WERI-Rb1 showed a stable modal chromosome number of 46, with at least 5 markers present in every cell. With the exception of chronic myelogenous leukemia (17), no human tumor has exhibited a consistent karyotype, although some
correlations have been shown between specific cancers and a specific chromosome abnormality (12). A diploid or near diploid genome has been described in several human tumors and in cell lines derived from them (2, 23); large marker chromosomes often occur in these karyotypes. These large rearranged chromosomes may play a role in the conservation of genetic material in hypodiploid cells. It has been shown that in tumor cells containing less than 46 chromosomes, the genome contains more than the diploid amount of DNA (1).

The importance of all the markers of WERI-Rbl is not known. However, MIII may be significant with regard to the hereditary nature of this particular cancer. This abnormal chromosome is obviously derived from a No. 13 chromosome (by deletion, translocation, or inversion in the long arm). MIII has satellites and, after G-banding, shows some of the major bands of a No. 13 chromosome. The association of retinoblastoma with interstitial deletion of the long arm of chromosome 13 has been established (15, 24). It has been argued that a small mutation in the genome may predispose to oncogenesis; this is illustrated by several genetic diseases which predispose to cancer (5). It is possible that in retinoblastoma a mutant gene sequence involving a locus on the long arm of chromosome 13 may have led to further rearrangement of the genome as a whole and to restructuring of the particular No. 13 chromosome. Thus, assuming a mutation as the first step in the conversion to cancer, this cell line may illustrate the evolution of cancer from an altered genome.

We found the Y79 to have a near diploid chromosome number with 2 marker chromosomes. This finding was in contradiction to that of Reid et al. (16) who reported this cell line to be hypertriploid (at 3 and 27 months in culture). We assume that a karyotypic evolution had occurred and that the more stable near diploid cell line (which we found) had overgrown the more unstable hypertriploid line. While a tumor probably arises from a single mutant cell, further mutations and structural rearrangements can occur as the cancer evolves both in vivo and in vitro. Thus the genetic composition of an established cell line may differ from the aberrant cell from which it originated.

Further cytogenetic analysis of this patient’s lymphocytes and skin biopsies will undoubtedly determine the significance of the MIII of WERI-Rbl. This case may represent a hereditary retinoblastoma of the third type.

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REFERENCES

Figs. 1 and 2. Characteristic grape-like clusters of suspension cultures of WERI-Rbl and Y79, respectively. x 100.
Fig. 3. Rosettes and chain formation, WERI-Rbl monolayer. May-Grünwald-Giemsa preparation. x 250.
Figs. 4 and 5. Monolayer cultures of WERI-Rbl and Y79, respectively. Arrows, rosettes. x 100.
Fig. 6. Rabbit eye showing massive Y79 tumor of the anterior chamber, Day 14 postinoculation.
Fig. 7. Histological section of enucleated rabbit eye. Arrow, anterior chamber with Y79 tumor. H & E, x 100.
Fig. 8. Histological section of enucleated rabbit eye. Arrows, WERI-Rbl tumor on the iris. H & E, x 100.
Figs. 9 and 10. Representative karyotypes of WERI-Rbl. x 1200.
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Fig. 11. Marker chromosomes of WERI-Rb1 (numbered) and Y79 (lettered). Left to right, unbanded, banded, and ideogramatic banded chromosomes. \( \times 120 \).

Fig. 12. Representative karyotype of Y79. \( \times 1200 \).
Characterization of a New Continuous Cell Line Derived from a Human Retinoblastoma

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