Propagation and Immortalization of Human Lens Epithelial Cells in Culture

Usha P. Andley,* Johng S. Rhim,† Leo T. Chylack, Jr.,‡ and Timothy P. Fleming*

Purpose. To establish primary and immortalized cell cultures of human lens epithelial cells for a model system investigating human lens epithelial physiology and cataract.

Methods. Human lens epithelial cells in culture were grown by isolating epithelium fragments from infant human lenses from patients who underwent treatment for retinopathy of prematurity and by allowing epithelial cells to grow from explants. To immortalize cells, the cultures were infected with an adenovirus 12-SV40 virus (Ad12-SV40).

Results. The primary cells from infant eyes proliferated for three passages before senescence was observed. However, the immortalized cells remained proliferative and retained the morphology of the primary cells. Immunohistochemical analysis demonstrated that these immortalized cells were SV40 large T antigen-positive and ceased to produce infectious virus after a few passages. Immortalized cells passaged to population doubling levels of 76 continued to form confluent cultures within 7 days of subculture. Analysis of proteins by SDS-PAGE and immunoblotting showed that immortalized cells produce a protein with molecular weight of about 25 kD, which reacted with an antibody to βH-crystallin.

Conclusions. This report constitutes the first successful immortalization of human lens epithelial cells. Currently, two cell lines have been created (B-3 and B-4) and passaged to population doubling levels of 76 and 52, respectively. These cells may provide an important human cell line specific to in vivo human lens epithelial cell physiology and would be of interest in establishing a human model to study lens cell differentiation and the etiology of cataract. These cells may also provide a constant and reproducible source of lens epithelial cells for eye-related toxicology studies and to assay inhibitory drugs for the prevention of cataracts and posterior capsular opacification observed after cataract extraction. Invest Ophthalmol Vis Sci. 1994;35:3094-3102.

The study of human lens epithelial cells in culture is of interest because they are the progenitors of the lens fibers in vivo and because information concerning the regulation of their growth and gene expression is limited.1-4 Lens epithelial cells undergo a developmental transition into fiber cells of the lens cortex, a process characterized by distinct biochemical changes such as the synthesis of fiber-specific proteins, β- and γ-crystallins, and morphologic changes such as cell elongation, loss of cellular organelles, and disintegration of the nucleus.3 The lens epithelium is located on the anterior surface of the lens immediately beneath the capsule and is thought to play a pivotal role in the development and progression of human cataracts, particularly those caused by exogenous mutagens.5 Data from experimentally induced cataracts and clinical observations suggest primary damage to the genome of the epithelial cells.5 Damage is mediated by abnormal differentiation of epithelial cells to lens fibers, which is collectively expressed as a cataract.5 Thus, the availability of a cell line that maintains the normal differentiating functions of lens epithelial cells would be of practical importance in the study of cataractogenesis and agents that inhibit cataract.

Several animal lens epithelial cell lines have been described.6-12 An immortalized cell line derived from a newborn rabbit has been well characterized and sup-

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From the *Department of Ophthalmology and Visual Science, Washington University School of Medicine, St. Louis, Missouri; the †Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland; and the Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts. Presented in part at the International Cooperative Cataract Research Group Congress, Bethesda, Maryland, November 1993. Supported in part by National Institutes of Health grant EY05681 and core grant EY02687 for vision research, and by an unrestricted grant to the Department of Ophthalmology and Visual Science from Research to Prevent Blindness, Inc., New York, New York. Submitted for publication October 6, 1993; revised December 17, 1993; accepted January 5, 1994. Proprietary interest category: N. Reprint requests: Usha P. Andley, PhD, Department of Ophthalmology and Visual Science, Washington University School of Medicine, Campus Box 8096, 660 S. Euclid Avenue, St. Louis, MO 63110.
ports the expression of exogenous α- and γ-crystallin gene promoters. Tumor viruses and growth genes from viruses have been used to produce long-lived or immortal lens epithelial cell lines. Transgenic mice carrying the αA-crystallin gene promoter fused to the early region genes of the simian virus 40 (SV40) or polyoma virus large T antigen have been studied extensively. Lines derived from the lenses of transgenic mice expressing the SV40 or polyoma T gene produce α-, β-, and γ-crystallins. One of these lines (αTN4-1) synthesizes αA and αB crystallins. A temperature-sensitive mutant of the SV40 virus has been used conditionally to immortalize bovine lens epithelial cells. Rat lens epithelial cells transformed using Rous sarcoma virus retained partially differentiating function as shown by β-crystallin synthesis.

Early attempts to culture human lens epithelial cells were moderately successful because of their low proliferative ability in vitro, which decreased with tissue age. More recently, human lens epithelial cells have been cultured by several investigators using fetal or infant lens epithelial explants. Primary cultures of infant and fetal cells can be successfully grown and cultured through at least three passages with the consistent formation of a monolayer with population doublings of up to 12 in culture. These cells express lens crystallins, synthesize the capsule in vitro, and can undergo cell differentiation into fiber-like cells of lentoids on low protein-binding surfaces or in cocultures of lens epithelial cells and ciliary fibroblasts. Despite these advances, the usefulness of human lens epithelial cells is hindered by the limited availability of infant and fetal lenses, diminished in vitro growth after four to six population doublings (pdis) in cell culture, the lack of cell lines, and the long proliferation times required to obtain a sufficient number of cells for use as a model for studying cell differentiation and the etiology of cataract.

Currently, no immortalized cell lines exist for the human lens epithelium. However, the availability of such a cell line would greatly enhance our understanding of the human lens epithelial physiology and may aid in the design of drugs that inhibit both primary and secondary human cataracts. In the current study, we attempted to immortalize human lens epithelial cells using an adenovirus12-SV40 hybrid virus (Ad12-SV40) to maintain propagation of the cells in vitro. This hybrid virus infects epithelial cells efficiently. Our results show that immortalized human lens epithelial cells can be maintained in culture for 30 passages over 76 population doublings, with no diminution in proliferative capability. These cells still synthesize β-crystallin as monitored by immunoblot assay, indicating that the immortalizing event has not altered their normal differentiating function. These cells may be an important model system to study human lens-specific physiology and to investigate the role of the lens epithelium in cataract formation.

**MATERIALS AND METHODS**

**Primary Cultures**

Human lenses were obtained within 24 hours from 5- to 12-month-old patients who underwent treatment for retinopathy of prematurity. A small cut was made in the posterior capsule of the lens, the free edge was grasped with forceps, and the capsule with attached epithelium was placed in a 60 mm tissue culture dish (Corning, Corning, NY). The epithelium was cut into two or three fragments, and each fragment was placed in a separate dish. Three milliliters of Eagle’s minimum essential medium (EMEM) (Sigma, St. Louis, MO) containing 20% fetal calf serum (Sigma) and 50 μg/ml gentamicin were added to the culture. The cultures were maintained at 37°C in a water-saturated air atmosphere containing 5% CO2. Medium was changed twice weekly. Cultures were routinely examined using a Nikon Diaphot phase-contrast microscope and photographed with a 35 mm Nikon N2020 camera (Nikon, Tokyo, Japan).

**Viral Infection**

After the primary cultures achieved confluence, the cells were subcultured using trypsin EDTA as described previously. The secondary cultures were passed into 100 mm plates. At 60% confluence, cells in the tertiary cultures were infected with a 1:10 dilution of Ad12-SV40 virus and incubated for 24 hours. At this time, the culture medium was removed, and 10 ml fresh medium was added to the cultures. Cells were allowed to grow for 1 week and subcultured at densities between 5 to 10 × 10^4 per 100 mm tissue culture dish. Cells were routinely counted using a Coulter (Hialeah, FL) counter (Model Zr).

To screen for production of infectious virus in the culture medium of lens epithelial cells, a cytopathic effect assay was used. Vero cells in a six-well tissue culture plate were exposed for 24 hours to 0.1 ml of lens epithelial cell culture medium removed from a plate after 3 days in culture and were filtered to remove cell debris (0.22 μ, Millipore, Bedford, MA). At 21 days, if the Vero cells died, the culture was called a producer. Early passages (up to 10) of lens epithelial cells produced cell lines that released virus into the culture medium. This medium was cytopathic to Vero cells. With prolonged passage, these lens epithelial cells became viral nonproducers. Control tests with a 1:1000 dilution of Ad12-SV40 virus demonstrated that the virus killed Vero cells under identical conditions.
Cryopreservation

Cells at each passage were enzymatically removed from culture dishes, resuspended in freezing medium (EMEM containing 20% fetal calf serum and 10% DMSO [Sigma], or fetal calf serum containing 5% DMSO), aliquoted (1 to 2 × 10^6 cells per cryovial), frozen at a rate of 1°C/min, and preserved in liquid N2.

Proliferation of Immortalized Cells

To determine the proliferative ability of the infected cells, 50,000 cells were plated in 60 mm plates (three replicates for each time point), and the number of cells was counted using a Coulter counter as described previously. The growth assay was performed over a period of 10 days, at which time the cultures were confluent.

Population Doubling Levels

To calculate pdl, equation (1) was used:

\[ N_f/N_0 = 2^x \]  

(1)

where \( x \) = number of population doublings, \( N_f \) = final number of cells, and \( N_0 \) = number of cells seeded into the plate initially. \( x = \log (N_f/N_0)/\log 2 \). After each passage, \( x \) was added to the previous population doubling level to get the new pdl.

Immunohistochemistry

Cells were plated in 24 well plates, grown to 60% confluence, and fixed with 1 ml of 70% ethanol for 15 minutes. After the addition of 200 μl of 0.05 M Tris-Cl, pH 7.4 (wash buffer), cells were incubated with the primary antibody (monoclonal mouse IgG for SV40 T antigen, Oncogene Science [Uniondale, NY]) at a dilution of 1:20 for 1 hour. After three washes with wash buffer, cells were incubated with a biotin-labeled secondary antibody (Dako, Carpenteria, CA) for 15 minutes at room temperature. Cells were washed as before, incubated with avidin-peroxidase for 1.5 minutes, washed three times, and treated with freshly prepared substrate (diaminobenzidine) for 15 minutes. After extensive washing, cells were hydrated in wash buffer and observed in a phase-contrast microscope.

SDS-PAGE and Western Blotting

Cells were lysed at 4°C, centrifuged at 10,000g, and the supernatants were treated with the primary antibody. The cells were immunoprecipitated using agarose-coupled Protein G (Gibco-BRL, Grand Island, NY), and the immune complexes were collected by centrifugation, washed with lysis buffer, and suspended in 200 μl of SDS-PAGE buffer containing 50 mM Tris-Cl, pH 6.8, 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue. Electrophoresis was performed using 10% acrylamide gels according to Laemmli. After immunoblotting on nitrocellulose membranes, the blots were probed with the same antibody and 125I-labeled protein A (Amersham, Arlington Heights, IL), and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) for 1 to 7 days. Polyclonal antibodies for β-crystallins were prepared by immunizing rabbits with purified calf lens βH-crystallin (Cocalico Biologicals, Reamstown, PA). Antibody to human γ-crystallin was obtained by immunizing rabbits with the monomeric protein fraction from infant human lenses. Antibody to calf γ-crystallin was obtained by immunizing rabbits with calf lens γ-crystallin.

Isozyme Phenotype and Karyotype Analysis

Isozyme and karyotype analysis were performed by Dr. Joseph Kaplan, Children’s Hospital of Michigan, Detroit, Michigan. Enzymes used for species identification and calculation of phenotypic frequency were lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH), phosphoglucomutase-1 (PGM1), esterase D (ESD), malic enzyme, mitochondrial (Me-2), adenylate kinase (AK-1), and glyoxalase-1 (GLO-1). Chromosome counts were determined in 100 metaphases, and nine Giemsa-banded karyotypes were examined.

This research was conducted in accordance with the tenets of Declaration of Helsinki. Institutional human studies committee approval was obtained at Washington University. Human lenses used in this study contained no personal identification.

RESULTS

Capsule epithelium fragments obtained from infant human lenses attached to the culture dish, and cell outgrowth was evident within 3 days of the initiation of the primary cultures (Fig. 1A). The primary cultures were allowed to grow to confluence (Fig. 1B) and subcultured (1:2) for two passages. Figure 1C shows that the cells maintain an epithelial morphology after the first passage. Cells were infected with the Ad12-SV40 hybrid virus typically after a second passage achieved 60% confluence. The passage at which cultures were infected did not appear to affect their immortalization. One week after virus infection, the infected cells continued to propagate to confluence (Fig. 2A), but the noninfected cultures showed diminished growth and failed to achieve confluence. Virus-infected cells ceased to produce intact virus in culture supernatants after 10 passages, as shown by cytopathic effects on Vero cells. Infected cells maintained their epithelial morphology and continued to grow to confluence when subcultured (Fig. 2B). Cells in passage 19 maintained an epithelial morphology (Fig. 2C) and formed...
confluent cultures within a week (Fig. 2D). Freezing the cells using 5% to 10% DMSO using standard techniques did not alter cell viability. Cells were routinely subcultured after they reached confluence (about 7 days).

The growth potential of the virus-infected cells was studied next; 5 x 10^4 cells were plated in 60 mm culture dishes, and the number of cells on days 1 to 10 was determined. Figure 3 shows the growth curve for the virus-infected cells in passage 7. The cells achieved four population doublings on day 7 and did not exhibit a decrease in growth potential at passage 22. Figure 4 shows the population doubling levels as a function of passage number for two cell lines and control uninfected cells. The infected cultures have achieved population doubling levels of 76 and do not show a diminution of proliferation at these population doubling levels. In control (uninfected) cultures, most of the proliferation occurred between passages 1 and 3.

The effect of long-term culture on virus-infected cells was studied. Some areas of the cultures showed extensive aggregation of cells that were translucent, notably in cultures older than 1 week. After about 30 days in culture, the monolayer of cells began to detach.
PASSAGE NUMBER

FIGURE 4. Propagation of infant human lens epithelial cell lines, expressed as passage number versus population doubling level (pdl). (•), cell line B-3; (○), cell line B-4; (●) primary (uninfected) B-4 cells.

from the edges of the culture dish. These sheets of cells were viable, and, when removed from the plates and placed in fresh medium, they showed renewed attachment to the plate (Fig. 5). The attachment to the plates was somewhat enhanced when the plate was coated with fibronectin (2 μg/cm²). The reattached cells continued to propagate into confluent cultures.

Figure 6 shows that the virus-infected cells reacted positively with the monoclonal antibody to the SV40 large T antigen. Nuclear staining was observed only in the virus-infected cells, whereas uninfected cells gave a faint background staining in the cytoplasm. Nearly 100% of the proliferative cells in the confluent monolayer are T antigen positive, indicating that the entire surviving population of cells contains the T antigen gene responsible for proliferation.

Analyses of proteins by immunoprecipitation, SDS-PAGE, and Western blotting showed that the immortalized cells produce proteins of molecular weights from 20 to 30 kD, which react with the antibodies to βH- and γ-crystallins (Fig. 7), proteins that are markers for cell differentiation in the lens epithelium.30,31 The antibody to βH-crystallin immunoprecipitated a protein of apparent molecular weight of ~25 kD. This observation is consistent with the results of Reddy et al.28 who observed the expression of a β-crystallin in infant lens epithelial cultures using a monoclonal antibody to β5. The production of β-crystallin continued in cells that had been passaged 17 times (at a population doubling level of 38.6). The band corresponding to β-crystallin was not detected in human corneal fibroblasts by this technique. The expression of a ~21 kD protein was observed in cells in passage 4 using a polyclonal antibody to calf lens γ-crystallin. The calf γ-crystallin antibody showed some cross-reactivity with human corneal fibroblasts at longer autoradiographic exposures (not shown). The antibody to human monomeric crystallins also detects a protein of ~21 kD in the cell line at passage 3. This antibody appears to cross-react with other proteins of 24 to 31 kD, which are also present in the corneal fibroblasts, indicating that the latter bands are not specific to the lens epithelial cells.

The human origin of the line was confirmed by analysis of seven enzymes, all of which were found to be human (Table 1). Phenotypic frequency was calculated to be 0.0336, indicating that less than 4% of the cell lines might be expected to have an isozyme phenotypic profile identical to this line. Analysis of the karyotypes of cell line B-3 showed that the cell line is aneuploid female (XX/XXX/XXXX) with most chromosomes in the tetraploid range (Table 2), as is typical of virally immortalized lines.35

DISCUSSION

The human lens is a unique system for examining the relationship between gene expression and differentiation because populations of quiescent, dividing, differentiating, and terminally differentiated cells are

FIGURE 5. Phase-contrast micrographs of virus-infected lens epithelial cells after 30 days in culture. (A) The monolayer begins to detach from the edge of the culture dish at right (x100). (B) Upon subculture, cells come off the plate as a sheet and outgrow the sheet to form a monolayer on plates coated with 2 μg/cm² fibronectin (x200). (C) A region of the culture exhibiting areas that are translucent and may be lentoids (x40). (D) A confluent monolayer of cells in passage 20 (x200).
spatially segregated. Recent studies suggest that damage to the genome of lens epithelial cells by exogenous agents, such as ionizing radiation or other forms of oxidative stress, may initiate or potentiate cataract formation. Our recent studies show that ultraviolet radiation interferes with the synthesis of specific proteins and causes the release of prostaglandins in cultured rabbit lens epithelial cells. It is, therefore, important to investigate the physiology of the normal human lens epithelium and study its response to cataractogenic agents. These studies have been hindered by the lack of human lens epithelial cell lines that provide a homogeneous population of cells for the study of such human lens specific functions as expression of crystallins genes.

Tumor viruses (e.g., human adenovirus) and oncogenes (e.g., the Harvey murine sarcoma virus) can be used to transform human cells. In the current study, we have infected the cells with Ad12-SV40, a hybrid of adenovirus 12 and simian virus 40 (SV40), which induces mRNA from the transforming region of SV40 (and not the early 1 region of adenovirus 12) during the transformation events. This increases the cell growth potential of the infant human lens epithelial cells to propagate in long-term culture. The cell lines described in the current study have a stable epithelial morphology and continue to produce lens β-crystallin, a protein characteristic of lens cell differentiation in vivo. Currently, two cell lines (B-3 and B-4) have been passaged through 30 and 19 passages, respectively, to population doubling levels of 76 and 52. It is unknown at present whether the lines are truly immortal, but their extended life is significant to generate a large number of cells for research.

Human lens epithelial cells expressing crystallins have been described recently by Reddy et al and Nagineni and Bhat using primary infant and fetal human lens epithelial cells, respectively, for up to three passages. Reddy et al reported the expression of αA, β5, and β6 in cell cultures using monoclonal antibodies. The expression of these crystallins increased with passage number. Lentoid bodies derived from the cells expressed γ-crystallin and MP26, as well as αA-, β2-, β5- and β6-crystallin, similar to differentiated lens fiber cells in vivo. Nagineni and Bhat described a system in which human lens epithelial cells from fetal tissue were cocultured with ciliary body fibroblasts. In this system, morphologic and biochemical evidence for differentiation was reported. Differentiating lentoid structures were predominantly associated with fibroblasts and expressed (in secondary cultures) fiber cell-specific proteins, αA, β2, and γS. None of these crystallins was found in the surrounding undifferentiated lentoid epithelial cells. β-Crystallins isolated from the human lens predominantly contain polypeptides of molecular weights between 20 and 30 kD, and the human lens monomeric fraction contains three polypeptides of molecular weights 24, 21, and 19 kD, corresponding to γS, γC, and γD, respectively. The Western blot data (Fig. 7) using the calf γH antibody suggests that the ~25 kD protein visualized in the immortalized human lens epithelial cells may be βB2-crystallin. The 21 kD protein detected using the γ-crystallin antibody may represent γC-crystallin. However, the reported cross-reactivity associated with these antibodies makes the interpretation of the data difficult. Further studies are in progress to confirm the identity of these polypeptides.

The present study also shows that cultures older than 4 weeks exhibit tight cell-to-cell relationships with the generation of high cell density. These conditions may be similar to that found in vivo and could be important for the expression of crystallins. This obser-
FIGURE 7. Autoradiograms of SDS-PAGE analyses of proteins from an infant human lens epithelial cell line by immunoprecipitation and immunoblotting. (A) β-crystallin antibody; (B) γ-crystallin antibody. Lane 1, cells in the third passage after virus infection (pdl = 6.4) immunoblotted with an antibody to calf lens BH-crystallin. Lane 2, human corneal fibroblasts immunoblotted with the antibody to βH-crystallin. Lane 3, human lens epithelial cells in passage 17 (pdl = 38.6) immunoblotted with the antibody to calf lens BH-crystallin. Lane 4, human corneal fibroblasts immunoblotted with an antibody to calf lens BH-crystallin. Lane 5, human lens epithelial cells in passage 4 (pdl = 9.9) immunoblotted with an antibody to calf lens γ-crystallin. Lane 6, human corneal fibroblasts immunoblotted with an antibody to calf lens γ-crystallin. Lane 7, human lens epithelial cells in passage 3 (pdl = 6.4) immunoblotted with an antibody to human lens monomeric crystallins. Lane 8, human corneal fibroblasts immunoblotted with an antibody to human lens monomeric crystallins.

vation is consistent with the reports that lens differentiation and expression of crystallins is promoted on culture dish substrates that enhance cell-to-cell interactions. Interellular attachment in the sheets of cells (Fig. 5) appears to be strong, with areas having spherules of cellular structures similar to lentoid bodies. Electron microscopic studies are in progress to evaluate the ultrastructure of these translucent regions or lentoids. The sheets of cells formed in long-term cultures remained undispersed by trypsinization but were dispersed into single cell suspensions by preincubation with collagenase (not shown).

The availability of human lens epithelial cell lines may offer a convenient system for the expression of cloned crystallin genes. These cells would be useful for studying species differences between animal and human lens epithelial cell physiology. It may be possible to extend the present approach to create human lens epithelial cell lines from cataractous lens cells. Immortal or long-lived animal lens epithelial cell lines have been created using viruses and growth genes from viruses. Bovine lens epithelial cells transformed with SV40 continue to synthesize α- and γ-crystallin. Transgenic mice carrying the hybrid gene containing αA-crystallin gene promoter fused to the coding region of the SV40 T antigens have been studied extensively. Two distinct lines of transgenic animals have been described. One of these, αT1, expressed the transgene very early in lens development and showed no primary fiber differentiation, whereas the other, αT2, expressed the SV40 gene late in development and was well differentiated. Lines derived from the lenses of αT2 transgenic animals produced significant amounts of α- and β-crystallins and reduced levels of γ-crystallin, indicating that some features of differentiated lens cells were maintained in vitro for several generations. The cell line αTN4-1 continues to synthesize αA- and αB-crystallins. The transgenic ap-

TABLE 1. Isozyme Phenotypes of Infant Human Lens Epithelial Cell Line B-3

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<tr>
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<th>LDH</th>
<th>G6PDH</th>
<th>PGM1</th>
<th>ESQ</th>
<th>Mε2</th>
<th>AK-1</th>
<th>GLO-1</th>
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<tr>
<td>Human</td>
<td>B</td>
<td>1</td>
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proach cannot be extended to the human system, but it allows one to target specific proteins in animal lens epithelial cells and to study the effect of oncogene expression on differentiation.16-20

The human lens epithelial cell lines reported in the current study are capable of expressing endogenous crystallin genes while retaining the capability to propagate in short population doubling times by immortalization with a virus. Further studies are in progress to characterize their transport functions, antioxidant levels, response to ultraviolet light, and other risk factors for cataract development. These cell lines could present an important practical model for the study of human lens epithelial cell differentiation and its pathologic manifestations in cataract. These cells would also be useful for the assay of inhibitory drugs to prevent primary and secondary cataract formation, eye-related toxicology studies, and development of specific cell markers and immunodiagnostic tests useful for the clinical assessment of cataract development.

Key Words
lens epithelium, human, tissue culture, immortalized cells, virus, crystallins

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References

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TABLE 2. Chromosome Count/Ploidy Distribution per 100 Metaphases (2N = 46) of Infant Human Lens Epithelial Cell Line B-3

<table>
<thead>
<tr>
<th>Chromosome Count</th>
<th>Number of Metaphases</th>
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<tr>
<td>45-46</td>
<td>25</td>
</tr>
<tr>
<td>82-90</td>
<td>88</td>
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<td>2X (82-90)</td>
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Human Lens Epithelial Cell Lines


