Culture of feline corneal epithelial cells and infection with feline herpesvirus-1 as an investigative tool

Lynne S. Sandmeyer, DVM, DVSc; Charlotte B. Keller, DVM; Dorothee Bienzle, DVM, PhD

Objective—To isolate and characterize pure cultures of feline corneal epithelial cells and to assess the extent and nature of feline herpesvirus (FHV-1) infection in these cells.

Sample Population—Healthy eyes from 23 recently euthanatized cats.

Procedure—Stroma and epithelium of the rostral portion of the cornea were surgically isolated, and epithelial cells were detached from the stroma by enzymatic incubation. Epithelial cells were cultured in hormone-supplemented media. Cells were passaged, and cytokeratin expression was assessed. Cells were then infected with FHV-1, and cytopathic effects were determined.

Results—Cell cultures were readily established from samples obtained from each eye and could be maintained through 6 passages. Cultured cells expressed cytokeratins 3 and 12 but not other cytokeratins. Infection with FHV-1 was rapid and caused widespread cytopathic effects.

Conclusions and Clinical Relevance—Feline corneal cells cultured in vitro during multiple passages maintain consistent morphologic characteristics and intermediate filament expression. They are susceptible to infection with FHV-1 and may provide a useful in vitro model for investigation of ocular drugs. (Am J Vet Res 2005;66:205–209)

 Conjunctival and corneal epithelial tissues are the primary targets for ocular infection by feline herpesvirus (FHV)-1.1,2 The most important ocular manifestations of FHV-1 infection are evident during recrudescent infection as a direct result of the cytopathic effect (CPE) of the virus on corneal epithelium.3,4 Unfortunately, few treatments are available for this common condition, and little objective information exists regarding the compounds used. The ability of antiviral agents to limit FHV-1 infection in vivo is avascular and oxygenated by diffusion from the tear film. In addition, corneal cells undergo frequent mitosis, which renders them amenable to in vitro propagation.5

Techniques for the culture of corneal epithelial cells have been described in several species.6–8 Cultures derived from the eyes of humans9 and rabbits10,11 have been useful for the investigation of corneal epithelial growth, cytokine modulation, and wound closure, and cultured bovine corneas have provided information on indicators of toxicity.12 The life cycle of herpes simplex virus (HSV)-1, which includes latent stages similar to those of FHV-1, and the efficacy and cytotoxic effects of anti-HSV-1 drugs have been assessed in cultures of chick13 and human14 corneal epithelial cells. To our knowledge, culture of feline corneal epithelium has not been described. However, the availability of an expanding range of herpesvirus-specific drugs calls for an in vitro assay in which efficacy against FHV-1 may be assessed.15

Thus, the objective of the study reported here was to design a means for evaluation of antiviral drugs in vitro. Our aim was to isolate feline corneal epithelial cells, optimize culture conditions that would result in propagation of pure cultures, immunocytochemically verify corneal origin, and assess the extent and nature of FHV-1 infection of such cells.

Materials and Methods

Sample population—Twenty-three cats were obtained from a local animal shelter within 1 hour after they were euthanatized. Cats were euthanatized for reasons unrelated to the study. Gross examination of eyes was conducted by use of diffuse illumination, and only eyes free of obvious ocular lesions were used.

Culture of corneal epithelial cells—All 46 eyes were enucleated by use of a subconjunctival method. After removal, each eye was irrigated with copious amounts of sterile saline (0.9% NaCl) solution, povidone iodine solution (1:25), and sterile saline solution again. Eyes were allowed to soak for a minimum of 10 minutes in a solution that was a 1:1 combination of Dulbecco modified Eagle medium-Hams F-12 (DMEM-F12)16 with 50 µg of gentamicin sulfate/mL prior to additional processing.

Corneas were prepared in a minimal-traffic surgery suite by use of sterile techniques. Corneas were examined by use of an operating microscope17 prior to additional processing and discarded when epithelial lesions were detected. Corneas were removed by circumferential excision within the limbus. The endothelium and caudal 50% to 75% of the stroma were peeled away to isolate the rostral portion of the cornea. These corneal tissues were covered with a solution of dispase8 (1.2 U/mL) and incubated for 1 hour at 37°C. After incuba-
tion, the dispase was removed and cellular clusters or cohesive sheets of corneal epithelium were gently peeled away from the remaining stroma by use of forceps and placed in a sterile 60-mm culture dish that contained 5 mL of DMEM-F12. Cellular clusters were manually dispersed into individual cells or small clumps by use of gentle repeated pipetting.

Cell suspensions were centrifuged at 500 × g for 5 minutes, and the supernatant was then decanted. The cell pellet was resuspended in supplemented hormonal epithelial medium (SHEM) that consisted of DMEM-F12 containing 10% fetal bovine serum, 100 U of penicillin/mL, 100 µg of streptomycin/mL, 2 mM l-glutamine, 0.1 µg of cholera toxin/mL, 10 ng of epithelial growth factor/mL, 1 µg of hydrocortisone/mL, and 5 µg of insulin/mL. All feline corneal epithelial (FCE) cells from a cornea were seeded into 6 wells of a 12-well plate or 12 wells of a 24-well plate, respectively. Cells were cultured at 37°C and 5% CO2.

Culture medium was initially changed 72 hours after start of culture and every 48 hours thereafter. Subcultures were initiated when the first cultures were 70% to 80% confluent. The SHEM was removed, and the cultures were rinsed with PBS solution and then incubated with 1 mL of 0.05% trypsin plus 0.53 mM EDTA at 37°C and 5% CO2 for 10 to 20 minutes until rounding up and detachment of cells was observed microscopically. Reactions were stopped by addition of 10% fetal bovine serum; cells were then centrifuged at 500 × g for 5 minutes and the supernatant decanted.

Cultures were divided (1:2), and all cells from a cornea were resuspended in SHEM and plated into a 12- or 24-well plate with 2 or 1 mL of SHEM/well, respectively. Cells were cultured at 37°C and 5% CO2. The CRFK cells were grown in Iscove's modified Dulbecco medium with 10% horse serum, 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 2 mM l-glutamine at 37°C and 5% CO2. The CRFK cells were then divided (1:2). The next day, the medium was removed, 1 mL of virus stock was added for 1 hour, and the medium was then replenished. Cells were cultured for 4 days and monitored daily for the development of viral CPE. When 70% to 80% of the cells had evidence of CPE, the culture was frozen overnight at −80°C. The next morning, cultures were thawed and centrifuged at 500 × g for 20 minutes. Supernatant containing the virus was removed and stored in aliquots at −80°C.

**Determination of virus titer**—The titer of virus stock was determined by use of the TCID50 method. The CRFK cells were seeded into 96-well plates and incubated at 37°C 5% CO2 until 70% confluent. Serial 10-fold dilutions of virus and stock (10−1 to 10−7) and media without virus were inoculated into 8 replicate wells. Cultures were incubated for 4 days. The TCID50 was calculated by use of the method of Reed and Muench by determining the dilution of virus causing CPE in 50% of the inoculated cell cultures (50% endpoint dilution). The appearance of any grade of viral CPE in the cell monolayer was considered indicative of infection.

**Infection of corneal cells**—First-passage FCE cells were grown to confluence in 12-well plates. The SHEM was decanted, and 4.98 TCID50 of FHV-1 was added in 0.5 mL of fresh media. The cultures were incubated for 1 hour, supernatant was removed, cells were washed twice with PBS solution, and 2 mL of fresh SHEM was added. Cultures were incubated for another 72 hours and cells examined daily for viral CPE.

**Results**

**Culture of corneal epithelial cells**—Thirty-two of 46 corneas yielded sufficient cells for primary cultures in multiple-well plates. Cells from the remaining corneas did not attach adequately to the culture dish or failed to proliferate. Following attachment, the cells initially appeared stellate and elongate (Figure 1). The adherent cells proliferated, and confluent monolayers were evident 7 to 10 days after initiating the primary culture. At this time, the cells were evenly sized and polygonal with clearly defined margins, giving the confluent monolayer a cobblestone appearance typical of epithelium (Figure 2). When allowed to proliferate past confluence, stratified areas of epithelium became apparent. Primary cultures were passaged at 70% to 80% confluence, and the first subcultures were designated P1. After passage, P1 cultures became confluent in 3 to 5 days. Overall, cultures were passaged 45 times during a period of approximately 32 weeks until a reduction in growth was interpreted as onset of senescence. Between passages 20 and 45, there was a gradual transition from a polygonal shape with a cobble-
stone appearance and ability to stratify to a more elongate shape with cells in single layers. Cultures from various cats had a uniform appearance. Fibroblasts were not identified in the cultures at any time.

Light microscopic examination of Wright-stained, centrifuged slides of primary cells revealed sheets of adherent cells with typical epithelial morphologic characteristics (Figure 3). The cells ranged from those small in size with round edges to those large in size with angular margins. A few mitotic figures were consistently identified.

Immunocytochemical analysis—All cells from primary cell cultures, the sixth passage, and control cells had positive results when stained with the AE5 antibody specific for cytokeratins 3 and 12, which are uniquely expressed in corneal cells. Staining for cytokeratin was localized to the cytoplasm, and a few intercellular adhesions (desmosomes) between cells were outlined by use of this antibody (Figure 4). Larger cells with somewhat angular outlines stained the most intensely. Neither primary FCE cells nor fresh corneal cells were stained with the pancytokeratin antibody AE1/AE3. Faint staining of the sixth passage of FCE cells was seen in the cytoplasm of a few cells after use of antibody AE1/AE3.

Virus infection—Titer of the virus stock was \(10^{4.8}\) TCID\(_{50}\)/mL. This viral concentration was chosen to induce CPE within 12 to 24 hours after inoculation of FCE cultures. The earliest change was the appearance of groups of refractile, rounded, ballooned, or elongated cells. Multinucleation or syncytium formation was frequently detected. Affected cells progressively detached from the culture surface, which left empty plaques (Figure 5). Plaques increased in number and enlarged in size during a period of several days. Between 75% and 100% of cells were affected by CPE within 72 hours after inoculation of FCE cultures.

Discussion

Isolation and culture of FCE cells proved to be relatively uncomplicated. The technique was chosen empirically and is similar to techniques for culture of corneal epithelium in other species. Dispase is a bacterial-neutral protease derived from Bacillus polymyxa that cleaves fibronectin and type IV collagen attachments of epithelial cells to basement membranes, yet causes minimal epithelial-to-epithelial cell disruption. The full-thickness epithelial sheet obtained by use of this method includes intact basal cells, which
presumably mediate attachment of epithelial fragments and provide a mitotically active population, whereas the superficial cells are shed and removed during changes of culture medium. Another commonly used method for isolating epithelial cells is primary explant culture in which epithelial cells migrate from the explant onto the culture substrate. 

Because stromal keratocytes follow later, contamination by stromal keratocytes can be avoided by removing the corneal explant approximately 5 days after explantation. Additional methods of obtaining pure cultures of epithelial cells include establishment of a feeder layer of irradiated 3T3 fibroblasts or use of monoclonal antibodies against fibroblasts to reduce their number in cultures.

Hormone-supplemented medium was used in the study reported here on the basis that it is essential in other species. It is not clear whether all hormones included are essential for growth. Hydrocortisone is a soluble inducer of cellular differentiation in some cell types, whereas insulin and epithelial growth factor are mitogenic, and cholera toxin potentiates the mitogenic effect. Prior to the development of this defined medium, primary cultures of rabbit epithelium could be grown to confluence but could not be passaged. Serially passaged primary cell cultures have a finite life span, and senescence typically develops at 20 to 80 population doublings. Primary feline corneal cells in the study reported here grew exponentially beyond the sixth passage; however, the ultimate time to senescence was not determined.

Criteria used to determine epithelial origin of cultured cells were morphologic characteristics and cytokeratin expression. The cells we cultured had a predilection to adhere to each other and had distinct cell borders and moderately sized nuclei. Primary and early passage cultures of FCE cells maintained a polygonal shape, which gave the confluent cultures a cobblestone appearance similar to that described for corneal epithelial cell cultures of other species. In another study, electron microscopy of corneal epithelial cells of rabbits revealed that cultured cells maintained typical cell polarity and cell-to-cell contact, features that are necessary for normal physiologic processes of the corneal epithelium. Primary cultures of human corneal epithelial cells and human corneal epithelial cell lines retained phenotypic characteristics of corneal epithelium in situ. The corneal epithelium is able to secrete collagen basement membrane-like material in vitro. In another study, cultured corneal epithelial cells continued to synthesize collagenase and express receptors for epithelial growth factor and corneal-specific cytokeratins, although the amount of expression for those culture conditions was less than that observed in situ.

Cytokeratins are intermediate filaments 10 nm in length that form a cytoskeletal network believed to provide mechanical integrity to cells within the context of a tissue environment. Cytokeratins can be divided into acidic and neutral-to-basic subfamilies. They are expressed as pairs consisting of an acidic and a basic member, each having the same size rank within its respective subfamily. The types of keratins synthesized are specific to the developmental stage and phenotype of a cell. There are at least 1 acidic and 1 basic keratin in every epithelium. In general, larger-sized keratin pairs are found mainly in differentiated and stratified epithelia (including skin, cornea, and esophagus), whereas smaller-sized pairs predominate in simple epithelia (such as intestinal epithelium).

Additionally, certain cytokeratins are expressed preferentially in hyperproliferative, neoplastic, or cultured cells. The AE1/AE3 antibody broadly recognizes acidic and basic cytokeratins; therefore, the result is positive staining in many epithelia. The AE5 antibody is most specific for corneal differentiation. Cytokeratin expression in cultured corneal epithelial cells has varied in some reports, but staining for cytokeratins 3 and 12 was consistently identified in the study reported here.

Primary corneal cells were highly susceptible to FHV-1 infection. Cytopathic effects were consistently apparent between 12 and 24 hours after inoculation; infection proceeded to rapidly destroy the cultured cells, with almost all cells affected at 72 hours. The CPE observed in corneal epithelial cells was similar to that described in other tissues, but it appeared more rapidly. Although in vitro systems have inherent limitations for use in mimicking in vivo infections, primary corneal cells appeared to more closely mimic the in vivo replication characteristics of FHV-1 than commonly used cell lines.

In the study reported here, culture of FCE cells was readily accomplished by careful adherence to technique and use of media components specific for enhancing epithelial cell growth. Cells obtained had morphologic and immunocytochemical characteristics of corneal epithelial cells and were highly susceptible to FHV-1 infection. The culture system established here may provide a useful in vitro method for the study of FHV-1 corneal infection and assessment of antiviral or other compounds.

References