Establishment and Characterization of an Air-Liquid Canine Corneal Organ Culture Model To Study Acute Herpes Keratitis

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ABSTRACT

Despite the clinical importance of herpes simplex virus (HSV)-induced ocular disease, the underlying pathophysiology of the disease remains poorly understood, in part due to the lack of adequate virus–natural-host models in which to study the cellular and viral factors involved in acute corneal infection. We developed an air-liquid canine corneal organ culture model and evaluated its susceptibility to canine herpesvirus type 1 (CHV-1) in order to study ocular herpes in a physiologically relevant natural host model. Canine corneas were maintained in culture at an air-liquid interface for up to 25 days, and no degenerative changes were observed in the corneal epithelium during cultivation using histology for morphometric analyses, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays, and transmission electron microscopy (TEM). Next, canine corneas were inoculated with CHV-1 for 48 h, and at that time point postinfection, viral plaques could be visualized in the corneal epithelium and viral DNA copies were detected in both the infected corneas and culture supernatants. In addition, we found that canine corneas produced proinflammatory cytokines in response to CHV-1 infection similarly to what has been described for HSV-1. This emphasizes the value of our model as a virus–natural-host model to study ocular herpesvirus infections.

IMPORTANCE

This study is the first to describe the establishment of an air-liquid canine corneal organ culture model as a useful model to study ocular herpesvirus infections. The advantages of this physiologically relevant model include the fact that (i) it provides a system in which ocular herpes can be studied in a virus–natural-host setting and (ii) it reduces the number of experimental animals needed. In addition, this long-term explant culture model may also facilitate research in other fields where noninfectious and infectious ocular diseases of dogs and humans are being studied.

Alphaherpesvirus infection is an important cause of disease in humans and many animal species. Characteristics of the members of this Herpesviridae subfamily include short replication cycles, induction of lifelong latency, and a narrow host range (1). Infection occurs via mucosal surfaces of the respiratory and genital tracts or via epithelial surfaces, such as the cornea. In humans, a primary ocular infection with herpes simplex virus 1 (HSV-1) typically results in conjunctivitis, which can then advance to keratitis. A recrudescent keratitis occurs when HSV-1 reactivates from trigeminal ganglia, or possibly other sites, and it is a leading infectious cause of visual impairment and blindness in humans (2, 3). Likewise, it is increasingly recognized that alphaherpesviruses, such as canine herpesvirus type 1 (CHV-1) and feline herpesvirus type 1 (FHV-1), are an important cause of ocular disease in small companion animals. Based on the strong similarities between HSV and CHV-1/FHV-1 regarding ocular pathogenesis and the lesions induced during primary and recurrent infections, dogs and cats represent useful natural-host models for studies on pathogenic mechanisms and viral factors involved in alphaherpesvirus infection in the eye (4, 5). Unfortunately, and despite the clinical importance of herpesvirus-induced ocular disease, the underlying pathophysiology of the disease remains poorly understood, in part because of the lack of adequate in vitro models in which to study the cellular and viral factors involved in acute corneal infection.

In general, knowledge of the pathogenesis of ocular herpes is limited to HSV-1 and, to a lesser extent, HSV-2 (6), and corneal HSV infection is traditionally studied in two types of experimental models. The first is the in vitro model, in which cultured monolayers of corneal epithelial cells are infected with HSV (7). This system offers simplicity and a high level of reproducibility and requires relatively little time and cost. The other model is the in vivo model, in which animals such as rabbits or mice are inoculated directly with HSV by corneal scarification (8). This model provides a more physiologically relevant (in vivo) system but examines disease in nonnatural hosts, is costly and time-consuming, requires animal experimentation, and has a greater degree of variability. More recently, an organotypic corneal model of acute HSV-1 infection using rabbit and human corneas has been reported (9).

For CHV-1, no in vitro corneal epithelial cell culture systems have been developed to date, and only a few in vivo experimental models of primary ocular CHV-1 infections in dogs have been described (10, 11). For FHV-1, a primary corneal cell culture model has been developed, but in general, FHV-1 ocular pathogenesis studies are almost exclusively done in live animals (4, 12). However, the practical use of these animals for in vivo studies is hampered by high animal purchase and maintenance costs, as well as ethical questions on the use of dogs and cats as experimental
animals. Therefore, there is a critical need for the development of physiologically relevant in vitro models that are inexpensive and minimize the number of experimental animals, as outlined in the 3R concept (13). Organ explant models provide a valuable alternative for in vivo systems, since three-dimensional structures and normal cell-cell contacts are maintained (14). Indeed, these models are useful stepping stones bridging in vitro and in vivo models because they allow accurate validation of cell culture results and limit the amount of animal experimentation needed.

Over the years, a variety of human corneal organ culture systems have been established to study corneal wound healing or corneal transplantation and to evaluate ophthalmic drugs (15, 16). More specifically, an air-liquid human corneal organ culture system was shown to be a valuable in vitro system for long-term maintenance of epithelial and endothelial integrity. In this model, corneas are placed epithelial side up in a fixed position, and culture medium is added to a level that exposes the epithelium intermittently to air-liquid environments while rocking on a tilting platform (17, 18). Despite the successful use of this air-liquid model to study corneal wound healing processes, its use for the study of infectious diseases has not been documented so far.

Therefore, the aim of the present study was to establish and characterize an air-liquid model using canine corneas and to evaluate its usefulness to study acute ocular herpesvirus infection in a virus–natural-host setting using CHV-1.

MATERIALS AND METHODS

Collection of canine corneas. Canine corneas were obtained from research beagles euthanized for reasons not related to this study. Ophthalmic examination, including slit lamp biomicroscopy of the anterior segment, was performed prior to euthanasia, and all eyes appeared clinically normal. Both corneas were collected from each dog within 1 h after euthanasia, wrapped in a gauze strip, and soaked in sterile phosphate-buffered saline (PBS) for transport to the laboratory.

Cornea culture medium and virus. The cornea culture medium consisted of Dulbecco’s minimal essential medium (DMEM) (Cell Grow; Corning, Manassas, VA) containing 10% fetal bovine serum (FBS) (Atlanta Biological, Flowery Branch, GA) and supplemented with 1% nonessential amino acids, 1% sodium pyruvate, t-glutamine (300 μg/ml), and penicillin (200 U/ml)/streptomycin (200 μg/ml), all from Life Technologies (Grand Island, NY).

The CHV-1 isolate used in this study was CHV-1 dsk, a strain obtained from a dog diagnosed with dendritic ulcerative keratitis (19).

Air-liquid culture model. The air-liquid culture model for canine corneas is based on previously described human cornea models, with some modifications (9, 17). Briefly, the canine corneas were rinsed thoroughly with PBS and placed epithelial side down in a well of a sterile ceramic spot plate (Avogradro’s Lab Supply). A 1% low-melting-point agarose (Invitrogen, Grand Island, NY) solution in cornea culture medium was prepared and used to fill the endothelial concavity of the cornea. Upon solidification of the agarose solution, the cornea was placed epithelial side up on a Kevlar ring in a cell culture dish containing cornea culture medium and incubated on a rocking platform, creating an air-liquid environment mimicking the natural blinking of the eye. (B) Representation of the quantitative analyses of the corneal epithelium. Shown are gross images of a stained cornea section, with a ruler added for scale, and a magnified image of a stained cornea section, with boxes indicating the 3 measured areas in which nuclei were counted. Scale bar, 100 μm.

Viral inoculation of the canine corneal organ cultures. The canine corneas were infected by placing the corneas epithelial surface down in 6-well culture plates (Corning, Grand Island, NY) in the presence of 2 ml of corneal culture medium containing different concentrations of CHV-1 dsk. After 1 h of incubation at 37°C with 5% CO2, the medium was removed and the corneas were gently rinsed 3 times with PBS to remove any unbound virus. The corneas were then transferred to the air-liquid culture model exactly as described above and incubated at 37°C with 5% CO2 for 48 h.

At 48 h postinfection (p.i.), culture medium was collected and frozen at −80°C for viral titrations and DNA extraction. In addition, the corneas were bisected, with one half embedded in OCT for cryosectioning and the other half snap-frozen at −80°C for DNA extraction.

Histology. The paraffin-embedded corneas were cut into 4-μm sections and mounted on glass slides. The slides were deparaffinized and stained with hematoxylin–eosin (H&E), and changes in the epithelial morphology were evaluated using light microscopy. Quantitative analyses of the epithelial cell layer were performed as follows. Images of tissue sections were captured with a digital camera mounted on an Olympus (Center Valley, PA) BX51 light microscope. Micro Suite Basic Edition software was used to measure and record epithelial thickness at 9 places on each of 4 cornea sections collected per time point. Calculations of the average thickness were based on 36 measurements for each time point. In addition, the average numbers of nuclei per 1 mm2 of epithelium were determined by counting nuclei in 3 areas of 1 × 103 μm2 per time point (Fig. 1B).
TABLE 1 Overview of primers used in qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Function</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Amplicon length (bp)</th>
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<tbody>
<tr>
<td>Ribosomal protein L32</td>
<td>RPL32</td>
<td>Housekeeping gene</td>
<td>TTAGAAGTGTGTAGTGATGCC</td>
<td>GGGATTGTTGACCTGTGAGG</td>
<td>121</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 20</td>
<td>CCL20</td>
<td>Attraction of lymphocytes (strong) and neutrophils (weak)</td>
<td>TCATTGGGCCTCACACACAG</td>
<td>TTGGATCTGCAACACACAG</td>
<td>97</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>IL-8</td>
<td>Attraction of neutrophils; potent promoter of angiogenesis</td>
<td>CACCTCACACCTTTCCATCC</td>
<td>GTCCAGGCACACCTCATTTC</td>
<td>120</td>
</tr>
<tr>
<td>Interleukin 1α</td>
<td>IL-1α</td>
<td>Epidermal cytokine, important for maintenance of the skin barrier</td>
<td>ACCCCACCTATGAGGAAGTC</td>
<td>CATTTGGCTGCCACACTAC</td>
<td>99</td>
</tr>
</tbody>
</table>

IF. OCT-frozen corneas were cut into 6-μm sections, mounted on Superfrost slides (Erie Scientific, Portsmouth, NH), fixed with cold acetone for 10 min, air dried, and stored at −20°C until analyses were performed. The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (VWR, West Chester, PA) was used at 0.5 μM for 5-min incubation at room temperature (RT) to visualize the nuclei. The slides were washed twice with PBS, and coverslips were mounted with a glycerol-based medium (Dako, Carpinteria, CA). The slides were analyzed using a Zeiss (Oberkochen, Germany) LSM confocal microscope, and images were captured with an attached camera controlled by ZEN imaging software.

To analyze epithelial cell viability, the In Situ Cell Death Detection Kit (Fluorescein) (Roche) was used and the TUNEL reaction was performed according to the manufacturer’s instructions. A positive-control cornea, incubated with 1 mg/ml DNase I (Sigma-Aldrich, Saint Louis, MO) in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 1 mg/ml bovine serum albumin (BSA) for 30 min at 37°C, was included.

To analyze CHV-1 infection, slides were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CHV-1 antibodies (VMRD, Pullman, WA) for 1 h at RT. The slides were washed 3 times with FA rinse buffer consisting of 0.09 M Na₂CO₃, 0.4 M NaHCO₃, and 0.15 M NaCl. Cryosections of a confirmed CHV-1-infected liver, a kind gift from E. J. Dubovi, Cornell Diagnostic Center, were included as a positive control.

IHC. Immunohistochemistry (IHC) was performed as described previously (20). To detect apoptosis, polyclonal rabbit anti-caspase 3 Abs or rabbit IgG isotype control Abs (Abcam, Cambridge, MA), diluted 1:100 in PBS, were used as primary Abs, and horseradish peroxidase (HRP)-conjugated goat-anti rabbit IgG (Jackson ImmunoResearch, West Grove, PA), diluted 1:100 in PBS, was used as a secondary Ab. To detect CHV-1-positive cells, hyperimmune serum from an experimentally infected beagle (a kind gift from Leland Carmichael, Baker Institute for Animal Health, Cornell University) was biotinylated using the EZ-link Sulfo-NHS-LC-biotin kit according to the manufacturer’s instructions (Thermo Scientific). A biotin-conjugated control antibody was also included. These primary antibodies were used at a final concentration of 1 mg/ml in PBS, and HRP-conjugated streptavidin (BD Bioscences, San Jose, CA) was diluted 1:100 in PBS. Slides were fixed to cover slips using Glycergel mounting medium (Dako, Carpinteria, CA), and images were captured with a digital camera mounted on an Olympus BX51 light microscope. For each staining, cryosections of CHV-1-infected liver were included as a positive control.

TEM. Corneas for transmission electron microscopy (TEM) were fixed and prepared exactly as described previously (21) and analyzed on a JEOL 1200 EX Twin TEM (FEI, Delmont, PA) operating at 80 kV.

Quantitative PCR (qPCR) and reverse transcriptase PCR (qRT-PCR). Total cellular DNA was extracted from snap-frozen corneas using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). For CHV-1 detection, the following primers targeting glycoprotein E (gE) were used: forward primer, 5′-CACCCCTCATGCAAAACTTGTG-3′; reverse primer 5′-ACCCACTTCATGAGGACTGC CATTGGCTGCCACCACTAC 99; amplicon length, 133 bp. To normalize the quantity of input DNA across cornea samples, beta2-microglobulin (B2M) DNA was amplified using the following primers: forward primer, 5′-CTTGCTCTCTCTCATCTCCTCCT-3′; reverse primer, 5′-GTGGACCTGAGCACTGACG-3′; amplicon length, 133 bp. To normalize the quantity of input DNA across supernatant samples, were spiked with 2 × 10⁶ Marek’s disease virus (MDV) copies before DNA extraction, as previously described (22), and MDV gD was amplified using the following primers: forward primer, 5′-TGGAGCAGCAGAAATGATG-3′; reverse primer, 5′-ATGTTCTATTAGTAGAAGCAGTGGC-3′; amplicon length, 108 bp (23).

Total RNA was extracted from snap-frozen tissues using a Qiagen RNeasy mini plus kit. RNA quality and concentrations were initially quantified using a Nano-Drop spectrophotometer and then assayed on a Fragment Analyzer using ProSize software (Advanced Analytix, Ames, IA). All samples had an RNA quality number (RQN score) above 8.7. One microgram of total RNA from each sample was linearly amplified and Cy3 labeled using the Agilent Low-Input QuickAmp Labeling kit (Agilent, Santa Clara, CA). The labeled samples were hybridized to an 8×60K Canine Gene Expression Microarray (Agilent) containing a total of 43,708 probes, and after hybridization, the slides were scanned on the Agilent DNA Microarray Scanner (G2505B) using a one-color scan. The dye channel was set to red and green, and both the red and green photo multiplier tubes (PMTs) were set to 100%. The scanned images were analyzed with Feature Extraction Software (Agilent) to obtain background-subtracted and spatially detrended processed signal intensities. Samples were then further quantile normalized and filtered using GeneSpring GX.

Canine gene expression array. Total RNA was extracted from snap-frozen tissues using the Qiagen RNeasy mini plus kit. RNA quality and concentrations were initially quantified using a Nano-Drop spectrophotometer and then assayed on a Fragment Analyzer using ProSize software (Advanced Analytix, Ames, IA). All samples had an RNA quality number (RQN score) above 8.7. One microgram of total RNA from each sample was linearly amplified and Cy3 labeled using the Agilent Low-Input QuickAmp Labeling kit (Agilent, Santa Clara, CA). The labeled samples were hybridized to an 8×60K Canine Gene Expression Microarray (Agilent) containing a total of 43,708 probes, and after hybridization, the slides were scanned on the Agilent DNA Microarray Scanner (G2505B) using a one-color scan. The dye channel was set to red and green, and both the red and green photo multiplier tubes (PMTs) were set to 100%. The scanned images were analyzed with Feature Extraction Software (Agilent) to obtain background-subtracted and spatially detrended processed signal intensities. Samples were then further quantile normalized and filtered using GeneSpring GX.

RESULTS
Canine corneas can be maintained in the air-liquid model for at least 3 weeks. Canine corneas were cultured for 25 days in the air-liquid system, as described in Materials and Methods.
Histological analyses of the epithelium showed uniform cell layers (between 5 and 7 layers) horizontal across the corneal surface, and no rounded, sloughed cells were observed over the entire period of cultivation (Fig. 2A). Although there was some variation in epithelial thickness over time, particularly at day 25 of culture (Fig. 2A), no significant differences in epithelial thickness were noted, and the thickness averaged approximately 50 μm at all time points tested (Fig. 2B). This is consistent with the average epithelial thickness of human corneas (24). The slightly thicker epithelium noted at day 25 of culture might be explained by the columnar morphology of the basal epithelial cells, which may be an indication of reduced cell proliferation. With the exception of a drop in the number of nuclei after initiating the culture system, no further significant changes were found in the numbers of nuclei through day 25 of culture (Fig. 2B). TEM was used to further evaluate the integrity of the corneal epithelium, and epithelial cells maintained their cell-to-cell contact up to day 25 of culture, as indicated by the presence of intact desmosomes (Fig. 3).

The viability of the cultured corneas was also assessed, and no TUNEL-positive cells were detected over the period of cultivation (Fig. 2C). TUNEL stainings of DNase I-treated corneas were included as positive controls, and TUNEL-positive cells were readily detected, indicating that our negative results were not due to a technical error (Fig. 2C).

**Canine corneas in the air-liquid model are susceptible to CHV-1 infection.** To evaluate the susceptibility of our air-liquid canine corneal organ culture model to CHV-1 infection, we inoculated canine corneas with different concentrations of the CHV-1 strain CHV-1 duk and analyzed samples at 48 h p.i. At this time (Fig. 1A).
point, CHV-1-positive epithelial cells (Fig. 4A), as well as CHV-1-induced subepithelial plaques (Fig. 4B), were readily observed at all virus concentrations tested using IHC and IF, respectively. For the corneas infected with 1 × 10^6 PFU/ml of CHV-1 duk, 300 consecutive cryosections of 6 µm were analyzed by fluorescence microscopy, and it was calculated that approximately 6 × 10^3 plaques/cornea were present, with an average plaque size of 150 µm². However, since this approach is extremely labor-intensive, we developed a qPCR assay to detect CHV-1 DNA copies by targeting gE. Using qPCR, we were able to detect viral DNA by calculating the fold change compared to mock-infected corneas (Fig. 4C). The observed fold change was dependent on the viral input, indicating that our cornea model is suitable to evaluate and compare different levels of infection (Fig. 4C).

In addition, we collected culture media from infected corneas and performed qPCR. To normalize the quantity of input DNA across samples, supernatants were spiked with MDV copies before DNA extraction, and the numbers of these DNA copies were detected by targeting gD, as described above, and expressed as the fold change compared to uninfected control corneas. In agreement with what we observed in infected cornea tissue, CHV-1 DNA copies could be detected in the collected culture media, and the observed fold change was dependent on the viral input (Fig. 4C). To assess whether the viral DNA detected in the supernatants was infectious, A72 cells, a canine tumoral fibroblast line (ATCC), were inoculated with culture media from CHV-1- or mock-infected corneas, and the cytopathic effect (CPE) was evaluated by light microscopy 2 days later. A72 cells inoculated with supernatants from CHV-1-infected corneas showed morphological changes, including ballooning of cells and the formation of small cytoplasmic tails (Fig. 4D). No such morphological changes were observed in A72 cells inoculated with supernatants from mock-infected cells (Fig. 4D).

CHV-1 does not cause apparent corneal damage at 48 h p.i. To evaluate CHV-1-induced corneal damage in our culture model, histology was performed on mock-infected and CHV-1-infected corneas at 48 h p.i. to study virus-induced changes in the epithelium. Upon CHV-1 infection, no significant changes in the epithelial thickness or number of nuclei were observed at 48 h p.i. (Fig. 5A). Since viral infections, including those with herpesvi-

FIG 4 CHV-1 infection of cultured canine corneas. (A) Representative images of CHV-1-positive epithelial cells at 48 h p.i. visualized with biotin-labeled CHV-1 hyperimmune serum, followed by streptavidin-HRP. The slides were counterstained with Gill’s hematoxylin. Scale bar, 25 µm. (B) Representative confocal images of a subepithelial viral plaque at 48 h p.i. visualized with FITC-conjugated anti-CHV-1 Abs. DAPI labeling was used to visualize nuclei. Scale bar, 50 µm. (C) CHV-1 DNA copies in cultured canine corneas and supernatants of infected canine corneas were determined using qPCR, and the data are represented as the fold change compared to mock-infected canine corneas. The error bars indicate standard deviations. (D) Representative images of A72 cells 2 days postinoculation with supernatants of mock- or CHV-1-infected corneas. The arrows indicate ballooning of cells, indicative of CPE. Scale bar, 25 µm.
expression in the CHV-1-infected corneas (Fig. 6B). Moreover, the fold increase detected by qRT-PCR mirrored the fold increase identified with the microarray perfectly, with CCL20 showing the highest upregulation, followed by IL-8 and finally by IL-1α (Fig. 6B). Although further validation of the other genes identified with the canine microarray is required, our present data on these three cytokines indicate that changes occurring during CHV-1 infection in canine corneas are like those documented during HSV-1 infection of corneal epithelial cells.

**DISCUSSION**

The present study is the first to describe the establishment of an air-liquid canine corneal organ culture model, which mimics the blinking of the eyelids, for studies on the ocular alphaherpesvirus CHV-1. This model system might prove an excellent virus–natural-host model to study ocular herpesvirus infection. The air-liquid corneal organ culture model was originally described in the 1990s by the group of P. Binder to study corneal wound healing in humans (17, 18) but has not been previously used to study ocular pathogens such as alphaherpesviruses.

To date, only one paper has been published describing the use of a rabbit (nonnatural-host) and a human (natural-host) corneal explant model to study acute HSV-1 keratitis (9). In this model, the authors cultured corneas for approximately 7 days by fully immersing them in culture medium, covering the epithelial surface. It has been previously demonstrated that such corneal culture techniques result in a reduction in epithelial cell thickness, increased epithelial edema, and changes in cellular differentiation (29). In contrast, maintenance of natural morphology and an increase in long-term survival have been noted when human corneas are cultured with their epithelial surface intermittently exposed to air and liquid environments (17). Although in the present study we did not directly compare the morphologies and viabilities of canine corneas cultured in the traditional versus the air-liquid system, we did not detect any degenerative changes in the epithelial layers of the corneas cultured for up to 25 days in the air-liquid system. To further mimic the *in vivo* situation, the corneas were filled with agar, allowing them to better maintain their shape. Despite the presence of this supporting gel scaffold, there was an initial drop in the number of nuclei per μm², indicative of cell expansion due to mechanical stress release, but the number of nuclei remained constant for the rest of the culture period and was not associated with cell death. Another advantage of using canine corneas in the air-liquid system instead of human corneas in the traditional system is the quality of the tissue. Corneas from human donors that are available for research are often of poor quality, since these corneas have been found unsuitable for corneal transplantation and are either from older donors or from donors with severe medical disorders. In contrast, canine corneas are often available from young and/or healthy research or privately owned dogs that have been euthanized for reasons unrelated to corneal problems, and these corneas can often be collected shortly after euthanasia. For these reasons, the quality and structural integrity of canine corneas available for experimental work are often superior to those of human corneas.

We confirmed the susceptibility of the air-liquid canine corneal organ culture model for CHV-1 infections and optimized all the tools necessary to study viral replication and virus-induced corneal damage. As proof of principle, we infected canine corneas with different concentrations of CHV-1 duel and analyzed samples...
Our salient findings were that CHV-1 (i) replicates in the corneal epithelial cells, (ii) spreads in a plaquewise manner in the corneal subepithelium, (iii) produces viral DNA copies in a infection concentration-dependent manner, and (iv) does not induce detectable corneal damage and apoptosis of corneal epithelial cells early after infection. Based on the success of our explant model in supporting CHV-1 replication kinetics, future experiments are planned, including (i) examination of infected corneas at various time points p.i. to study CHV-1 replication kinetics and virus-induced corneal damage in more detail, (ii) infecting corneas with clinical isolates of CHV-1 exhibiting different ocular phenotypes to study potential differences in replication kinetics and/or corneal damage, and (iii) infecting corneas with CHV-1 mutants to study the roles of viral ocular virulence factors. We are currently creating a bacterial artificial chromosome (BAC) of CHV-1 duk that we can use to create recombinant viruses in which viral ocular virulence determinants are deleted. We plan to focus first on infected cell protein 0 (ICP0), the immediate-early

![Gene expression array of CHV-1-infected canine corneas. (A) Heat map visualization (i) and box plot representation (ii) of canine genes differentially expressed with at least 10-fold changes. The columns represent the CHV-1- and mock-infected corneas at 48 h p.i., and the rows indicate the genes. The color key indicates gene expression values, with green the lowest and red the highest. (B) (i) Heat map of the cytokines CCL20, IL-8, and IL-1α in CHV-1- versus mock-infected corneas using Excel. The colors range from white (no expression) to red (high expression). (ii) qRT-PCR confirming gene expression of the 3 cytokines. (iii) Fold upregulation of the cytokines in the microarray experiment compared to real-time PCR results. The error bars indicate standard deviations.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Fold change ([0] vs. [1])</th>
<th>Up- or downregulation after CHV-1 infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum amyloid A1</td>
<td>SAA1</td>
<td>1,683.6</td>
<td>Up</td>
</tr>
<tr>
<td>S-antigen, retina and pineal gland</td>
<td>SAG</td>
<td>−900.6</td>
<td>Down</td>
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<td>Interleukin 33</td>
<td>IL-33</td>
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<td>Up</td>
</tr>
<tr>
<td>Synaptobrevin homologue YKT6</td>
<td>YKT6</td>
<td>460.5</td>
<td>Up</td>
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<td>Peripherin 2</td>
<td>PRPH2</td>
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<td>Gamma transducing activity polypeptide 1</td>
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<td>WFDC5</td>
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<td>GABAAR</td>
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</tr>
<tr>
<td>Nucleoporin SEH1</td>
<td>SEH1L</td>
<td>258.6</td>
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</table>

* [0] represents mock-infected corneas, and [1] represents CHV-1-infected corneas.
gene US1, and thymidine kinase (TK), as these viral genes have been confirmed to play important roles during HSV keratitis (30–32).

Using a commercially available canine microarray, we began to decipher alterations in corneal cellular gene expression upon CHV-1 infection. Of the 29 genes found to be up- or downregulated ≥100-fold in CHV-1-infected corneas, 3 genes have been described previously in relation to HSV-1 keratitis. The expression of chemokine ligand 20 (CCL20) was found to be induced in human corneal epithelial cells upon in vitro infection with HSV-1, and an accumulation of this cytokine was found in murine corneas using a murine herpetic stromal keratitis model (27). Using qPCR, we also confirmed in the present study the upregulation of canine CCL20 in CHV-1-infected corneas. Another cytokine, interleukin 8 (IL-8), has been reported previously to be upregulated after HSV-1 infection of a telomerase-immortalized human corneal epithelial cell line (28), and we confirmed canine IL-8 to be upregulated in CHV-1-infected corneas, albeit to a lesser extent than canine CCL20. A third cytokine, IL-1α, has been found to be elevated in the murine model of herpetic stromal keratitis starting at day 2 p.i., with peak levels being reached at day 10 p.i., after which the expression of IL-1α gradually diminished over the next 10 days (26). In our canine cornea explant model, we also confirmed the upregulation of IL-1α at day 2 p.i., and we plan to study the expression kinetics of this cytokine long term using our model. Taken together, our microarray and qRT-PCR data show that canine corneas produce proinflammatory cytokines in response to CHV-1 infection, analogous to what has been described for HSV-1. This emphasizes the value of our system as a virus–natural-host model to study ocular herpesvirus infections. Experiments to study additional innate immune and other genes found to be differentially expressed in CHV-1-infected corneas using the canine gene expression array will soon be initiated. These studies will further improve our knowledge on host responses during acute ocular herpesvirus infection. A more comprehensive understanding of the pathogenesis of the disease will improve the potential for rational development of vaccines and antiviral treatments.

We propose our air–liquid canine corneal organ culture system as a useful model for studies on ocular herpesvirus infections. Our model provides a physiologically relevant system in which ocular herpes can be studied in a virus–natural-host setting and minimizes the number of experimental animals needed. Finally, this long-term explant culture model may also facilitate research in other fields where noninfectious and infectious ocular diseases of dogs and humans are being studied.

ACKNOWLEDGMENTS

This work was supported by a Research Grants Program in Animal Health from the College of Veterinary Medicine, Cornell University. The TEM was performed at the Cornell Center for Materials Research (CCMR), supported by NSF grant DMR-1120296.

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