Experimental Ocular Herpesvirus Infection in the Cat

Sites of Virus Replication, Clinical Features and Effects of Corticosteroid Administration

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Experimentally induced ocular feline herpesvirus 1 (FHV-1) infection was studied in 30 specific pathogen-free cats. In ten cats, the ability of five field isolates of FHV-1 to replicate in the epithelium and substantia propria of cornea and conjunctiva was demonstrated by histochemical techniques. Feline herpesvirus 1 was found to preferentially infect and induce necrosis of conjunctival epithelium. Although significant histologic lesions were not induced, all FHV-1 strains were observed to replicate in corneal epithelium; minimal viral antigen was detected in the corneal stroma. The course and clinical features of ocular FHV-1 infection were then studied over a period of 60 days in two groups of ten cats: in one group, infection was preceded by administration of subconjunctival betamethasone. In each of these groups, a distinct clinical syndrome developed. In cats not receiving corticosteroids, a course of epithelial keratitis, characterized by the formation of punctate and dendritic epithelial lesions, persisted for up to 24 days postinfection. In the corticosteroid treated group, a chronic (>60 days) stromal keratitis developed, characterized by geographic epithelial ulceration, interstitial edema and deep vascularization. Other complications observed in corticosteroid-treated animals included decreased tear production, calcific-band keratopathy and a unique stromal disorder of cats termed corneal sequestration. The results of this study indicate that while epithelial keratitis may occur during primary infection, stromal keratitis does not, unless immune responsiveness to FHV-1 is concomitantly suppressed. This feature is similar to naturally occurring HSV-1 keratitis of humans, but contrasts to other animal model systems in which stromal keratitis predictably occurs during primary infection. Study of this animal model, therefore, may allow unique insights into the events preceding the establishment of stromal keratitis. Invest Ophthalmol Vis Sci 30:1758-1768, 1989

Infection by feline herpesvirus 1 (FHV-1, feline rhinotracheitis virus) is one of the most common viral diseases of cats worldwide. Primary infection typically occurs early in life, an estimated 80% of cats will become latently infected, and nearly half will experience spontaneous reactivation and shedding of virus.

During primary infection with FHV-1, upper respiratory lesions predominate, resulting from the diffuse virus replication in epithelium of the nasal mucosa, nasal turbinates, nasopharynx and tonsils. Gingivostomatitis and ulcerative oral lesions occur less commonly, and ocular manifestations are generally limited to the development of conjunctivitis. Although most primary infections are self-limiting, neonatal deaths can occur and have been associated with generalized visceral and central nervous system infection.

The most significant ocular manifestations of FHV-1 infection occur in adult animals. The disease is most often unilateral, and corneal infection is characterized by dendritic and ameboid epithelial lesions. Stromal keratitis develops less commonly; however, persistent and recurrent keratitis may result in sufficient corneal opacification to impair vision. The severity of FHV-1 infection in cats is increased by immunosuppressive systemic diseases, particularly infection by feline retroviruses.

The ocular manifestations of FHV-1 infection have been minimally studied. In the only prior experimental study, the clinical manifestations of ocular FHV-1 infection were evaluated in groups of weanling kittens whose corneas were either sacrificed or left intact, and to which corticosteroids either had or had not been administered subconjunctivally. In all cats, an identical syndrome of upper respiratory infection was induced, but biomicroscopic evaluation failed to

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Resources used to support this work were provided by the State of North Carolina.

Submitted for publication: November 1, 1988; accepted February 3, 1989.

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detect lesions indicative of corneal infection. These observations imply that corneal disease is not a consistent feature of primary ocular FHV-1 infection, and prompt speculation as to the pathogenesis of FHV-1 keratitis.

The domestic cat appears to be the only animal that experiences under natural conditions an ocular herpesvirus infection that in all major respects mimics HSV-1 ocular infection of humans. Features characteristic of both diseases are a primary ocular infection that includes conjunctivitis more consistently than keratitis, the establishment of a latent carrier state, reactivation of latent virus that may cause recrudescence of clinical disease, and the clinical manifestations of epithelial and stromal keratitis. Insights gained in the study of FHV-1 ocular disease of cats, therefore, may potentially contribute to the understanding of human ocular herpesvirus infections. Experiments were designed to determine: (1) if corneal FHV-1 replication is a feature of primary infection; (2) the histologic changes and sites of virus replication during primary infection; and (3) the clinical features of ocular FHV-1 infection in normal cats, and in those whose immunological responsiveness has been suppressed by corticosteroids.

Materials and Methods

Animals

Thirty domestic, short-hair cats varying from 6-8 months of age were used. Minimal disease cats were obtained from several commercial breeders (Liberty Laboratories, Liberty Corner, NJ; New York State College of Veterinary Medicine, Cornell University, Ithaca, NY), and determined to be free of feline leukemia virus infection and serologically negative to FHV-1. Animals were maintained in individual stainless steel cages at least 4 feet apart. All experiments were done in full compliance with the ARVO Resolution on the Use of Animals in Research.

Virus Strains

Five strains of FHV-1 were used. The original Crandell isolate was obtained from the American Type Culture Collection (VR 636) (Rockville, MD). Four FHV-1 strains were isolated from cats with naturally occurring infection; strain 6097 was provided Dr. R. K. Maes, Michigan State University (East Lansing, MI), strain 582 was isolated from a cat at the University of Tennessee (Knoxville, TN), and strains 351 and 727 were isolated from cats treated at the Veterinary Teaching Hospital of the College of Veterinary Medicine, North Carolina State University (Raleigh, NC). All virus strains were verified to be FHV-1 by immunofluorescence with FHV-1-specific antisera. Each virus was plaque-purified by picking one plaque from an infected cell monolayer overlaid with agar. Either the fourth or fifth passage of each virus was used for all experiments. Viruses were grown and titrated on Crandell-Reese feline kidney (CRFK) cells as previously described.

Antisera

Goat origin polyclonal antisera to FHV-1 was provided by Dr. R. K. Maes, Michigan State University. Prior to use, sera was adsorbed repeatedly with CRFK cells to insure specificity for FHV-1. Negative antisera was prepared by repeatedly adsorbing the original antiserum with FHV-1-infected CRFK cells.

Immunohistochemistry

Tissues for fluorescent localization of FHV-1 antigen were stored for up to 18 hr in Michel's solution. Tissues were then snap-frozen in embedding medium (O.C.T. compound, Miles Laboratories, Naperville, IL) using liquid nitrogen and stored at -80°C. Frozen sections were cut at 5 μm, air-dried, and the embedding medium removed by washing in PBSS. Sections were incubated for 15 min at room temperature with anti-FHV sera (1:60 dilution in PBSS), washed in PBSS for 10 min, and incubated 15 min in rabbit anti-goat IgG (1:40) (Miles Laboratories). Sections were washed a second time in PBSS, coverslipped, and examined using a UV-equipped Zeiss microscope.

Tissues for peroxidase-antiperoxidase (PAP) staining were fixed in Bouin’s solution for 18-24 hr, then washed overnight in 50% ethanol in distilled water. Five micron sections were stained by the procedure of Sternberger using a commercially available PAP staining kit (Dako Corporation, Santa Barbara, CA). Positive and negative goat anti-FHV were used at a dilution of 1:50 in PBSS.

Virus Isolation

Samples for virus isolation were obtained from the eye by rolling a moistened dacron-tipped swab in the inferior cul-de-sac. Swabs were then placed in vials containing Dulbecco’s minimal essential media (DMEM) containing the following antibiotics (per ml): gentamicin 50 μg, tetracycline 10 μg, amphotericin B 2.5 μg, and penicillin G 100 μg. Aliquots were allowed to adsorb onto CRFK monolayers in multiwell plates for 1 hr at 37°C, the virus-containing medium was discarded, and fresh DMEM containing 1% bovine fetal serum was added. Monolayers were examined daily for characteristic cytopathic effect. Viral isolates were intermittently verified to be FHV-1 by immunofluorescence.
Serology

Serial 2-fold dilutions of sera were incubated with 100 pfu of FHV-1 for 1 hr at 37°C, added to duplicate wells in 24-well plates containing monolayers of CRFK cells and incubated at 37°C in 5% CO2 for 3 days. Monolayers were observed for cytopathic effect, plaques counted, and the titer expressed as the highest dilution that produced a 50% reduction in plaque number relative to controls.15

Preliminary Studies: Sites of Virus Replication during Acute Infection

Animals were anesthetized by intramuscular ketamine (20 mg/kg) (Ketaset®, Bristol Laboratories, Syracuse, NY). Each of the five FHV-1 strains was inoculated into the eyes of two cats. In the right eye, 100 μl virus solution (106 pfu/ml) was injected intrastromally using a 27-gauge needle and tuberculin syringe. The needle was inserted between collagen lamellae for a distance of at least 5 mm prior to injection to prevent fluid loss during needle withdrawal. In the left eye, virus solution was topically applied after the epithelium was heavily scarified with a 25-gauge needle.

On days 4 and 8 after inoculation, one cat infected with each virus strain was killed by intravenous barbiturate overdose, and the eyes were removed. These intervals were selected based on the prior observation that maximal FHV-1 cytopathic effect in respiratory epithelium occurred between days 4 and 10 after inoculation.7 Conjunctival, corneal and iris specimens from each eye were processed for immunofluorescent and PAP localization of viral antigen as described above. A third specimen was fixed in formalin for routine processing for hematoxylin and eosin staining, and a fourth sample was fixed in McDowell and Trump’s fixative for future electron microscopy studies. To ensure accurate estimation of corneal virus replication, central corneal buttons were evenly tri-sected and step sectioned every 100 μm. In specimens prepared for immunofluorescence studies, virus replication was quantitated by estimating the percent antigen-positive cells. Scores of + to +++ were assigned to represent, respectively, <25, 25–50, 50–75, and 75–100% of the cells being antigen-positive. Peroxidase–antiperoxidase-stained sections of selected tissues were prepared to allow correlation of the pathologic changes with the sites of virus replication.

Features of Primary Ocular Infection in Normal Cats and Cats Receiving Subconjunctival Corticosteroids

Twenty cats were randomly assigned to two groups of ten. Under ketamine anesthesia as previously described, the corneal epithelium of the left eye was heavily scarified with a 25-gauge needle, and 300 μl FHV-1 strain 727 (106 pfu/ml) was topically applied. In the first group, no other treatment was administered. In the second group, FHV-1 inoculation was preceded by three consecutive daily injections of 4 mg betamethasone (Betavet Soluspan; Schering Corporation, Kenilworth, NJ) subconjunctivally in the left eye.

Biomicroscopy was performed bilaterally with a Zeiss slit lamp on all cats daily for the first 14 days following inoculation. Examinations were repeated every other day for the next 14 days, and then weekly for a total of 60 days. Detailed descriptions of ocular discharge, intensity of blepharospasm, degree of conjunctival edema and hyperemia, extent and character of corneal ulceration, intensity of corneal edema, and anterior uveal changes (miosis, aqueous flare and iris hyperemia) were recorded at each evaluation interval, and representative lesions were photographed. Numerical scores were used to record the severity of conjunctivitis and corneal edema: for conjunctivitis 1 = earliest detectable hyperemia, 2 = marked hyperemia, 3 = hyperemia accompanied by mild conjunctival swelling, 4 = conjunctival swelling sufficient to protrude past the eyelid margins; and for corneal edema 1 = superficial stromal edema appreciable only with slit-lamp biomicroscopy, 2 = edema grossly detectable with focal illumination, 3 = diffuse stromal edema of sufficient magnitude to obscure examination of anterior chamber structures, and 4 = complete corneal opacification. Sodium fluorescein and rose bengal stains were used intermittently to facilitate identification of corneal epithelial lesions.

Samples for virus culture were taken from both eyes every third day for the first 2 weeks, every 7 days for the next 6 weeks, and then every 14 days for the remainder of the experiment. Samples for conjunctival cytology were taken at the same intervals, but from the left eye only; the conjunctiva was anesthetized with 1 drop of proparacaine hydrochloride, samples of conjunctival cells were taken from the inferior cul-de-sac using three to four scrapes with a platinum spatula, and smeared on a glass slide. Blood was collected at 7, 30 and 60 days after infection for quantitation of FHV-1-neutralizing antibodies, as previously described.15

Results

Immunofluorescent Localization of FHV Antigen

Table 1 illustrates the distribution of FHV antigen detected by immunofluorescence in conjunctival and corneal tissues at days 4 and 8 of infection. All virus strains tested were found capable of initiating a cor-
Table 1. Immunofluorescent localization of FHV-1 antigen in sections of cornea and conjunctiva from cats inoculated topically (OD) and intrastromally (OS) with different strains of FHV-1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cat</th>
<th>Eye</th>
<th>Epithelium</th>
<th>Substantia propria</th>
<th>Cornea</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Endothelium</th>
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<td>Day 4 postinoculation:</td>
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<td>6097</td>
<td>191</td>
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<td></td>
<td>192</td>
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<td>636</td>
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Day 8 postinoculation: | | | | | | | | |
| 6097  | 194 | OS  | +          | -                 | -      | -          | +      | -          |
| 636  | 195 | OD  | +          | -                 | -      | -          | +      | -          |
| 658  | 197 | OD  | +          | -                 | -      | -          | +      | -          |
| 727  | 198 | OD  | +          | -                 | -      | -          | +      | -          |
| 351  | 200 | OD  | +          | -                 | -      | -          | +      | -          |

+ and − signs indicate relative percentage of FHV-1 antigen-positive cells in tissue sections: − = no antigen detected, + = <25%, ++ = 25–50%, +++ = 50–75%, ++++ = 75–100%.

neal epithelial infection. Conjunctival epithelial cell infection was severe (>75% cells antigen positive) on day 4 of infection. On day 8, <25% of conjunctival epithelial cells were antigen positive; however, by this time the majority of the epithelium had sloughed. Corneal epithelial infection was demonstrated in the corneas of one of five cats on day 4, and in five of five corneas on day 8. Both scarified and nonscarified corneas revealed epithelial infection. Virus antigen was detected in the corneal stroma of three of five cats on day 4, and in one of five cats on day 8. Stromal infection occurred only in eyes receiving intrastromal injections of virus. Virus was not detected in uveal tissue.

Histologic Changes

Differences were not observed between virus strains. Histologic evaluation of hematoxylin and eosin-stained sections of conjunctival tissues at day 4 revealed diffuse necrosis, characterized by partial loss of the epithelial layer, with a moderate neutrophilic inflammatory response in the substantia propria. Eosinophilic intranuclear inclusions were prevalent and readily observed in H&E-stained specimens (Fig. 1). Corneal lesions at the same interval were mild and difficult to identify in H&E-stained sections. Affected areas could be recognized only by subtle changes in epithelial cell morphology and the presence of intranuclear inclusions. Immunohistochemical staining revealed, in both conjunctival and corneal specimens, the diffuse presence of virus antigen in the cytoplasm and nuclei of infected epithelial cells, but not in the substantia propria of either tissue.

On day 8, conjunctival necrosis was severe, with complete loss of epithelium occurring in many areas. Viral inclusions were less prevalent, and massive aggregates of polymorphonuclear inflammatory cells and macrophages were present adjacent to the epithelial basement membrane (Fig. 2). Corneal lesions were more obvious than on day 4, but were still considerably less severe than those of the conjunctiva, and only easily detectable with immunohistochemical techniques. Affected epithelial cells were rounded, with viral antigen observed in both nucleus and cytoplasm, and inflammatory cells were lacking (Fig. 2).

Stromally detected antigen was always located between collagen lamellae; however, it was not possible with the techniques used to determine if antigen was located within keratocytes.

Clinical Features in Normal Cats and Cats Receiving Subconjunctival Corticosteroids

Signs of ocular infection were similar during the first 10 days for cats of both the steroid and nonsteroid groups. Mild blepharospasm, the first sign of ocular infection, was present in most animals by day...
by day 40. Corneal edema was rarely seen and was never severe. Conjunctivitis resolved in all cats of this group by 3-4 weeks of infection.

In the steroid group, mild conjunctivitis developed in the uninoculated right eye of seven of ten cats between days 6 and 9, but at no time were corneal lesions present. In left eyes, epithelialization of scarified sites was nearly complete by day 2. By day 3, dendritic epithelial ulcers developed, but by day 10 these had been replaced by large areas of geographic ulceration. In some eyes, dendrites appeared to coalesce into geographic lesions, and in other eyes, the epithelium appeared to slough. In all eyes, corneal ulceration was associated with moderate to severe stromal edema; superficial stromal scratches resulting from the scarification procedure, visible in none of the nonsteroid-treated cats, became deep and prominent. Epithelial ulceration persisted in nine of ten cats until day 39 of infection, after which ulceration began to gradually resolve. Corneal edema, however, persisted in all cats beyond the time of reepitheli-
alization, and at least until day 53 (Figs. 6, 7). Conjunctivitis was less severe in the steroid-treated group until day 12. Deep corneal vascularization began in four steroid-treated cats between days 26 and 46, and persisted throughout the evaluation period.

In nine of ten cats in the steroid group, changes in addition to corneal ulceration and edema were observed (Table 2). Brown pigmentation of the corneal stroma, corresponding in distribution to the area of ulceration, occurred in five cats. In four cats, mineralization occurred in the medial and temporal superficial corneal stroma, eventually progressing to involve the central cornea in a horizontal band pattern (Fig. 8). The mineralized tissue has subsequently been demonstrated by X-ray probe microanalysis to contain predominantly calcium. In five animals, decreased tear production was also observed (Schirmer tear test results < 4 mm wetting/60 sec [mean normal = 17 mm17 at consecutive observation intervals]). In two of these animals, Schirmer tear test values of 0 mm persisted for longer than 1 week. Aqueous tear production returned to normal in all cats by day 60.

Conjunctival Cytology

At day 3 of infection, cytologic specimens from cats of both groups revealed only normal epithelial...
Serologic Response to FHV-1 Infection

At day 10, antibodies were detected in one cat (titer 1:4) of the nonsteroid group, and in no cats of the steroid group. At day 31, titers varied from 1:4 to 1:16, averaging 1:8 for each group. The mean titer for both steroid- and nonsteroid-treated cats at day 60 was 1:16.

Discussion

These results indicate that during experimental primary FHV-1 infection corneal epithelium supports virus growth, but that replication and subsequent cellular necrosis occur preferentially in epithelial cells of the conjunctiva. The observation that epithelial infection occurred with equal frequency in eyes that were and were not scarified is similar to studies of HSV keratitis in the rabbit demonstrating that scarification is unnecessary if the virus strain is sufficiently virulent.18 Considering the epitheliotropic nature of FHV-1 demonstrated by other studies, the minimal presence of stromal antigen was not surprising. Whether the minimal stromal antigen detected in intrastromally injected eyes indicates active infection or merely virus residual from the injection,
Fig. 7. Photograph of a rose Bengal-stained, steroid-treated cornea on day 54 of infection. The cornea is diffusely edematous and stromal scratches are prominent. Vascularization has reached the margins of the edematous area and adhered mucus is present (arrows).

is not possible to determine with the techniques used. The failure to detect stromal fluorescence, however, in scarified corneas to which virus was topically applied, suggests that FHV-1 does not readily replicate in stromal cells. The detection of viral antigen in endothelial cells of one cat probably resulted from an inadvertent intracameral injection of virus.

Corneal virulence has been demonstrated to vary between strains of HSV-1. Our histochemical studies were primarily done to identify a strain of FHV-1 capable of infecting corneal epithelium during primary infection. This was deemed a necessary preliminary step as corneal lesions were not identified in the prior experimental study of FHV-1-induced ocular disease. Although the number of eyes evaluated was low, our results suggest that the ability of FHV-1 to infect corneal epithelium is not a virus strain-dependent phenomenon, and that minimal strain heterogeneity exists with regards to corneal infectivity. Our findings do not preclude, however, the possibility that FHV-1 strain variations may influence the course and severity of ocular disease.

In animals not receiving corticosteroids, the predominant clinical manifestations were conjunctivitis and a biphasic appearance of punctate and dendritic epithelial ulceration. The first phase (day 3–6) of corneal dendrites undoubtedly represents replication of the topically applied virus. The second phase (day 11–20) of dendritic lesions probably reflects replication of virus released from lysing conjunctival epithelium, shown histologically to have undergone nearly complete necrosis by day 8 of infection. These findings are compatible with the observation in HSV-infected rabbits that peak tear film titers precede corneal lesions.

Of the diverse pharmacologic activities attributable to corticosteroids, those most likely responsible for the exacerbated infections seen in our cats are direct

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<th>Table 2. Time of onset of clinical manifestations associated with FHV-1 stromal keratitis in corticosteroid-treated cats</th>
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connective tissue effects and those mediated by immune alteration. The influence of corticosteroids on connective tissue metabolism include the inhibition of collagen and glycosaminoglycans synthesis.\textsuperscript{21}

![Image](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933379/)

**Fig. 8.** In addition to corneal edema and vascularization, this steroid-treated cat has developed a large area of calcification.

Combined with inhibition of epithelialization and prolonged virus shedding, these effects would allow ample opportunity for virus to infect keratocytes, or diffuse between the damaged corneal lamellae. Progression of corneal lesions in some steroid-treated cats beyond the cessation of virus shedding is putative evidence that viral antigen persisted within the corneal stroma, inciting an inflammatory response.

By inhibiting the accumulation of neutrophils and macrophages at the inflammatory site, corticosteroids suppress the natural resistance mechanisms necessary for recovery from primary viral infection.\textsuperscript{22-24} The increased severity and prolongation of infection in steroid-treated eyes is attributed to this effect. The onset of stromal keratitis by day 6 of infection, a time when cytologic findings continue to indicate corticosteroid suppression of macrophage migration, suggests the initial corneal lesions resulted directly from virus replication. Immunopathologic events, suggested to participate in the development of stromal lesions in other experimental animals,\textsuperscript{25-28} may later have influenced the course of keratitis.

The other changes that developed in corticosteroid-treated eyes were unanticipated. Decreased tear production has been recognized in cats with naturally occurring viral conjunctivitis,\textsuperscript{29} theoretically resulting from either excretory duct occlusion, or from lacrimal adenitis. The recovery of HSV-1 from tears and saliva of asymptomatic humans has prompted speculation that glandular replication may occur, a situation equally plausible in the cat.\textsuperscript{30,31}

**Fig. 9.** Prevalence of virus shedding from the right (a) and left (b) eyes of cats inoculated with FHV-1 in the left eye only. Ocular virus shedding was prolonged in eyes treated with corticosteroids; however, the duration of virus shedding appeared not to be significantly affected in the contralateral eye.

Corneal sequestration is a unique keratopathy of felines that is recognized clinically by chronic epithe-
Corneal ulceration associated with brown discoloration of the underlying stroma. Because the feline cornea rarely develops melanin pigmentation, the clinical appearance is considered pathognomonic. Histologically the affected corneal stroma appears decellularized and necrotic with a surrounding zone of mononuclear inflammatory cells. The source of the brown pigmentation is unknown, but has been suggested to represent absorption of some tear film component. This hypothesis is supported by the observation that the calcium seen in one of our cats developed similar pigmentation. The cause of the condition is unknown, but its association with numerous adnexal conditions that predispose to corneal irritation suggest it may be initiated by a variety of insults. We speculate that the corneal sequestration observed in this study represents not a specific response to FHV infection, but rather a nonspecific sequelae to significant stromal damage.

Conical calcification is rarely observed in cats under natural conditions. Although Bowman's membrane is lacking in cats, the clinical features of the calcific plaques seen in these cats are strikingly similar to those of human band keratopathy. This probably represents a secondary degenerative response related to the severity of corneal stromal damage.

This study has demonstrated that distinct epithelial and stromal syndromes of ocular FHV-1 infection can be induced in cats, and that major similarities exist between feline and human ocular herpesvirus infections. For both cats and humans, naturally occurring, primary ocular infection consistently includes conjunctivitis, with minimal corneal involvement. Primary HSV-1 keratitis may appear as multiple scattered microdendritic figures, similar to those observed in cats following primary exposure to FHV-1. For both FHV-1 and HSV-1, infection of the natural host results in a dendritic keratitis that is usually self-limiting. In contrast to the rabbit and mouse models where stromal keratitis predictably occurs during primary infection, FHV-1 stromal keratitis only occurred in cats immunosuppressed with corticosteroids. FHV-1 keratitis, therefore, may more accurately mimic HSV keratitis of humans in which stromal keratitis is not a feature of primary infection in immunologically competent individuals.

**Key words:** herpesvirus, feline, epithelial keratitis, stromal keratitis, corticosteroid

**References**

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