Effects of λ-Carrageenan on In Vitro Replication of Feline Herpesvirus and on Experimentally Induced Herpetic Conjunctivitis in Cats

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PURPOSE. To evaluate the inhibitory effect of λ-carrageenan type IV on feline herpesvirus (FHV)-1 in an in vitro model and in experimentally induced conjunctivitis in vaccinated cats.

METHODS. Standard plaque reduction assay, virus titration, and quantitative polymerase chain reaction (qPCR) were used to assess the effect of carrageenan on FHV-1 in vitro. Eighteen adult specific pathogen-free cats, vaccinated against FHV-1 several months earlier, were used to determine the ocular irritative effects of carrageenan, followed by the effect on FHV-1-induced conjunctivitis. Ocular examinations, virus isolation, and partial thromboplastin time (PTT) were evaluated during the study period.

RESULTS. When added before virus adsorption, the 50% inhibitory concentration (IC50) of carrageenan was 5 μg/mL, and the 90% inhibitory concentration (IC90) was 25 μg/mL. When added after virus adsorption, there was no inhibitory effect on plaque formation at any concentration. There was no effect of carrageenan on virus titer. Virus copy numbers assessed by quantitative PCR were significantly but marginally reduced when carrageenan was added before and after virus adsorption. Topical application of carrageenan at 250 μg/mL in cats with FHV-1–induced conjunctivitis resulted in a significant reduction on positive virus isolation samples on day 21 of the study but did not alter clinical signs of disease. There was no adverse effect on PTT values.

CONCLUSIONS. λ-Carrageenan type IV blocked FHV-1 adsorption in the plaque assay. Carrageenan shortened the time period in which infected cats had positive virus isolation from the conjunctiva but did not alter the clinical course of FHV-1 conjunctivitis in cats. (Invest Ophthalmol Vis Sci. 2008;49:1496–1501) DOI:10.1167/iovs.07-1245

Sulfated polysaccharides have been studied extensively over the past two decades for their antiviral effects against a number of different enveloped viruses, including herpes simplex virus (HSV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), vesicular stomatitis virus, togavirus, arena-virus, myxovirus, and rhabdovirus.1,2 The compounds studied have included a variety of red seaweed extracts (carrageenan, xylomannan, galactan sulfate), extracts from brown algae, heparin, dextran sulfate, pentosan sulfate, and dermatan sulfate.3–7 In most in vitro studies to date, the sulfated polysaccharides have inhibited virus replication through blocking adsorption. In one study of HSV, however, λ-carrageenan II (a cyclized derivative) was significantly inhibitory when added any time after viral adsorption.8 λ-Carrageenan has demonstrated potent inhibitory effects against HSV-1 and HSV-2.3

The adsorption blocking mechanism of sulfated polysaccharides in an in vitro study of HSV was found to be shielding of the positively charged sites on the V3 loop of the HIV envelope glycoprotein (gp 120).9 The V3 loop is necessary for virus attachment to cell surface heparan sulfate, a primary binding site, before more specific binding occurs to the CD4 receptor of CD4+ cells. Binding of sulfated polysaccharides to virus glycoproteins was proposed to explain the broad antiviral activity against enveloped viruses.10 Despite numerous studies of the effect of sulfated polysaccharides and their antiviral activity in vitro, few studies have evaluated the effect of these compounds in vivo.

Feline herpesvirus (FHV)-1, a member of the subfamily Alphaherpesvirinae, is an enveloped DNA virus similar in structure and pathogenicity to HSV. The virus causes high morbidity in cats through its effect on the respiratory system and the eye.11 Reactivation of latent virus may occur throughout a cat’s life, with ocular manifestations of conjunctivitis and ulcerative keratitis common. The purpose of the study reported here was to evaluate λ-carrageenan in an in vitro model of FHV-1 replication for antiviral activity and in an experimental model of FHV-1–induced conjunctivitis in cats.

METHODS

The use of animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Purdue University Animal Care and Use Committee. Eighteen healthy 2-year-old specific pathogen-free cats (Harlan Sprague-Dawley, Indianapolis, IN; Liberty Research, Waverly, NY) were used in this study. There were 6 neutered female and 12 neutered male cats. The cats were negative for feline leukemia virus antigen and feline immunodeficiency virus antibody (Snap Combo Test [ELISA], Idexx Diagnostic Laboratories, West Brook, ME). Although the cats were reported by the suppliers to be free of respiratory tract viruses, per institutional requirements all cats were vaccinated with an inactivated four-way vaccine (Fel-O-Vax IV [feline herpesvirus, calicivirus, panleukopenia virus and Chlamyphila felis], Fort Dodge, Overland Park, KS) before the study. Half the cats were vaccinated 12 months before the study, and half were vaccinated 6 months before the study. Each cat received two subcutaneous vaccinations at 2-week intervals. Each of the treatment groups included cats vaccinated both 6 and 12 months before the study. Although vaccination may lessen the severity of FHV-1 when cats are challenged,12 conjunctival FHV-1 inoculation, even in vaccinated cats, produces marked conjunctivitis.13

In Vitro

Crandell Rees feline kidney (CRFK) cells grown in Dulbecco modified Eagle medium (DMEM) with gentamicin, streptomycin, and amphotericin B.
inhibitory concentration 90 (IC90), defined as the concentration of test
4°C, plasma was collected, and the PTT tests were run within 1 hour.
clinically healthy cats. The blood was centrifuged for 15 minutes at
carrageenan (1, 5, 25, 50, 100, and 250 μL) were added either just before virus or after the 1-hour adsorption phase. If added before adsorption, 300 μL carrageenan in DMEM per plate was used. If added after adsorption, carrageenan was in the overlay media. Approximately 100 plaque-forming units (PFUs) of FHV-1 in DMEM were added to the cells. Adsorption was allowed to occur at 37°C with 5% CO2 for 1 hour with gentle continuous rocking of the plates. Excess virus was removed, and the cells were overlaid with a 1.6% carboxymethylcellulose solution containing 2% fetal calf serum. The cultures were incubated for 3 days, at which time the cells were stained with crystal violet and the plaques were counted. Inhibitory concentration 50 (IC50) and inhibitory concentration 90 (IC90), defined as the concentration of test substance that reduced the number of viral plaques by 50% and 90% compared with the untreated control wells, were determined. All concentrations were evaluated in triplicate assays with positive and negative controls included for each test. Positive controls consisted of 100 PFUs in DMEM added to wells, whereas negative controls consisted of an equal volume of DMEM added only to wells.

**Virus Titrations.** The FHV-1 stock titration was 10^7 median tissue culture infective dose (TCID50)/mL. Eight 10-fold dilutions were made for use in the titration assays. Carrageenan, at concentrations of 50, 100, and 250 μg/mL, was used before and after virus adsorption to determine the effect on virus titer. Positive and negative controls were included on each plate. Positive controls consisted of eight dilutions of FHV-1 in DMEM added to separate wells, whereas negative controls consisted of cells only with an equal volume of DMEM added to each well. After a 3-day incubation period, TCID50 values were calculated based on the Karber method, and the plates were then frozen at –80°C and thawed. The supernatant from each well was collected for use in a quantitative polymerase chain reaction (qPCR) assay.

**Quantitative PCR.** A previously published qPCR protocol was used to amplify a conserved 81-bp sequence within the open-reading frame of the glycoprotein B (gB) gene of FHV using a detection system (Stratagene Mx3000 P; Stratagene, Cedar Creek, TX). Supernatants from each of three replicate wells from the virus titration plates were combined. DNA was extracted from each sample using a commercial extraction kit (QIAamp DNA Mini Kit; Qiagen, Valencia, CA). A standard curve was established in each qPCR run using FHV-1 of known titration. Virus copy numbers were then determined for each sample assay.

**Cytotoxicity Assay.** Cytotoxicity assays were performed by determining the number of viable CRFK cells using the trypan blue exclusion method. Cells were evaluated after 24 and 48 hours with concentrations of carrageenan at 250 and 500 μg/mL. The 50% cytotoxic concentration (CC50) was defined as the concentration of carrageenan that reduced cell viability by 50% compared with controls.

**In Vitro Anticoagulation Assay.** Partial thromboplastin time (PTT) was measured using fresh, pooled feline citrated plasma from clinically healthy cats. The blood was centrifuged for 15 minutes at 4°C, plasma was collected, and the PTT tests were run within 1 hour. Each sample consisted of 250 μL plasma plus 50 μL carrageenan dissolved in phosphate-buffered saline (PBS). Concentrations of carrageenan evaluated in the coagulation studies were 1, 2.5, 5, 25, 50, 100, and 250 μg/mL. All samples were run in duplicate. Feline plasma collected in heparinized tubes was used as the negative control, and citrated plasma was used as the positive control. The controls consisted of 50 μL PBS and 250 μL plasma.

**In Vivo**

Ocular examinations by slit lamp and indirect ophthalmoscopy were performed, and results were normal in all cats. Phase 1 of the study was designed to evaluate ocular irritation associated with topical carrageenan administration. Cats were randomly divided into six groups of three cats each, with one female and two male cats per group. Blood was drawn for baseline PTT values. Carrageenan was dissolved in PBS, filter sterilized, and kept refrigerated. Concentrations evaluated were 5, 25, 50, 100, and 250 μg/mL. Each cat within a group received one drop in each eye of saline placebo or one concentration of carrageenan every 3 hours, for a total of four treatments, between 7 AM and 5 PM for 7 days. Blood was drawn for PTT assays at the end of days 1 and 7 of treatment. Slit lamp examination was performed daily by one investigator (JS) who was masked as to treatment groups. Cats were evaluated for blepharospasm, conjunctival hyperemia, chemosis, ocular discharge and corneal ulceration (by fluorescein retention). Scores were assigned for each ophthalmic finding according to the following scale: 0 = negative or normal, 1 = mild, 2 = moderate, 3 = severe. Scores for each ophthalmic finding were combined to get an overall ocular score for each eye at each time point. At the end of 7 days, the cats were allowed a 1-week washout period.

Phase 2 of the study was designed to evaluate the use of topical carrageenan administration on the course of experimentally induced FHV-1 conjunctivitis. The cats were randomly divided into three groups of six cats each, with two females and four males per group. Each group was housed in a different room. Personnel handling the cats changed laboratory coats and gloves between rooms. Within a room, cats were housed in individual cages but were allowed out of the cages to interact with each other for approximately 1 to 2 hours per day. Swabs of both conjunctival sacs were obtained from each cat for virus isolation (VI) before infection. Treatment protocols for the groups were as follows: group 1, saline placebo administered before and after infection; group 2, carrageenan administered before and after infection; group 3, saline administered before infection and carrageenan administered after infection.

The use of carrageenan before infection in one group was performed to evaluate any virus adsorption blocking effect. Treatment before infection consisted of one drop of either saline or carrageenan in each eye of each cat at hourly intervals beginning 2 hours before infection. One hour after the last pretreatment, the conjunctival sac of one eye of each cat was inoculated with 50 μL FHV-1 with a titer of 4.8 × 10^6 TCID50/mL. Infection of the right or left eyes was alternated with each cat. Beginning 24 hours postinoculation (PI), all cats were treated with one drop in each eye of carrageenan or saline every 3 hours, for a total of four treatments, between 7 AM and 5 PM for 21 days. Swabs for VI were obtained from the inoculated conjunctival sac at 24 hours PI and again on days 5, 13, and 21 PI. Slit lamp examinations were performed every third day by one investigator (JS) who was masked as to treatment groups, though some bias might have occurred because treatment groups were housed in different rooms. The scoring system was the same as that used during phase 1 of the study. Scores for each ophthalmic finding were combined to get an overall ocular score for each eye at each time point. Blood was drawn for PTT assays on day 21.

**Statistical Analyses**

Virus titration values were statistically evaluated using ANOVA with Bonferroni comparison. Virus copy numbers were determined for each qPCR assay and compared using ANOVA. Virus isolation results were
analyzed by group and time using the Fisher exact test. Results of the ocular examination scores by group and time were analyzed using repeated-measures ANOVA. Analyses were performed separately for data combined for the two eyes from each cat and scores from the inoculated eye only. Clinical scores were not normally distributed and were transformed using a square root of 1 + score. PTT assay results were analyzed using repeated-measures ANOVA. P < 0.05 was considered statistically significant.

RESULTS

In Vitro

Plaque Reduction Assay. When carrageenan was added before adsorption, the IC$_{50}$ was 5 µg/mL and the IC$_{90}$ was 25 µg/mL. The median number of plaques in control wells was 69 (range, 64–70). The median number of plaques with 5 µg/mL carrageenan added was 11 (range, 10–13), whereas with ≥25 µg/mL carrageenan the median number of plaques was 3 (range, 0–6). When added after adsorption, no concentration of carrageenan evaluated was effective at reducing plaque formation.

Virus Titration. There was no significant difference between viral titers when carrageenan was added before or after adsorption compared with controls (P = 0.160; Fig. 1). The three concentrations of carrageenan did not differ significantly in their effect on viral titers (P = 0.344).

qPCR. When comparing virus copy numbers for samples with carrageenan added before and after adsorption to controls, there was a decrease in virus number but it was only marginally significant (P = 0.048). At 250 µg/mL carrageenan and a stock virus dilution of 10$^{-3}$, the virus copy number for preadsorption was 7.2 × 10$^5$; for postadsorption the number was 1.9 × 10$^5$; for virus only control, the number was 2.6 × 10$^5$. These results are a representative example of the overall qPCR results.

Cytotoxicity Assay. Carrageenan, at the concentrations of 250 and 500 µg/mL, did not affect cell viability compared with control cells at 24 or 48 hours.

Anticoagulation Activity. The PTT value for pooled feline citrated plasma (positive control) was 14 seconds, whereas heparinized feline plasma (negative control) had a PTT value of more than 125 seconds. With carrageenan added to plasma, the PTT values increased as the concentration increased (Fig. 2). PTT values were greater than control values and above the upper limit of normal for the

Purdue University Veterinary Teaching Hospital clinical laboratory (16.9 seconds) at concentrations ≥5 µg/mL.

In Vivo

Phase 1: Ocular Irritation. No cat in any group demonstrated blepharospasm, ocular discharge, or corneal abnormalities. Some cats in each carrageenan concentration group developed mild to moderate conjunctival hyperemia, though this finding was not consistent within the various concentration groups. There was no conjunctival hyperemia in cats in the placebo group. PTT values for all cats were within the normal reference range for the Purdue University Veterinary Teaching Hospital clinical laboratory (10.9–16.9 seconds). Based on the results of the in vitro and phase 1 studies, a carrageenan concentration of 250 µg/mL, 10 times the IC$_{90}$ value, was chosen for phase 2.

Phase 2: FHV-1–Induced Conjunctivitis. One cat in group 1 contracted severe systemic FHV-1–associated illness beginning 2 weeks PI. The cat was withdrawn on day 15 of the study and hospitalized for supportive care, leaving only 5 cats in group 1 for the last week of the study. This cat developed malaise, fever, swelling of the tongue, oral mucosal ulceration, and foot pad and interdigital skin sloughing. After 1 week of supportive care, the cat’s condition continued to deteriorate, and it was humanely killed. Necropsy was performed, and virus isolation and fluorescent antibody staining confirmed FHV-1 in the oral cavity, nasal turbinates, and lung. Virus could not be identified in the skin lesions. These signs were unusual and severe for FHV-1 infection. Feline calicivirus was considered but could not be identified in any tissue by virus isolation or fluorescent antibody staining. Additionally, there was no known exposure to feline calicivirus, and no other cat developed such clinical disease.

All cats had conjunctivitis in the inoculated eyes at the day 3 PI examination. Ocular examination scores peaked at day 9 PI (Fig. 3). The noninoculated eye was included in the data sets for each cat because cross-infection might have occurred. There was no significant difference in the ophthalmic examination scores among groups at any time point in the study (P = 0.535 for inoculated eye only scores; P = 0.486 for combined eye scores).

Baseline (preinoculation) FHV-1 isolation from the conjunctival sac was negative in all cats. A significantly greater proportion of cats in group 1 were positive on VI than in group 2 or
was outside the reference range of 10.9 to 16.9 seconds. Demonstrates the viral adsorption blocking activity of proteins and prevent their binding to heparan sulfate receptors block herpesvirus adsorption are thought to coat viral glyco- surface proteoglycans of target cells. Al- extent, gB, interaction with the heparan sulfate residues involved in attachment to heparan sulfate on the cell surface, it has been shown that HSV can use a variety of glycoproteins to attach to cells, such that no one glycoprotein surface, the exact receptor mechanisms that allow entry of FHV-1 are not fully elucidated. The HSV has 10 known surface glycoproteins. Initial attachment to cells involves viral envelope glycoprotein C (gC) and, to a lesser extent, gB, interaction with the heparan sulfate residues present on the surface proteoglycans of target cells. Although gC plays a major role in viral attachment to the cell surface, it has been shown that HSV can use a variety of glycoproteins to attach to cells, such that no one glycoprotein is used exclusively for cell attachment. Of the seven identified FHV-1 glycoproteins, gC appears to be the major glycoprotein involved in attachment to heparan sulfate on the cell surface, whereas gE and gI are not essential for virus replication in tissue culture. Sulfated polysaccharides evaluated that block herpesvirus adsorption are thought to coat viral glyco- proteins and prevent their binding to heparan sulfate receptors on cell membranes. In one study, carrageenan was added to cultured cells before HSV infection and then was removed before virus was added. This had no effect on reducing virus titer, suggesting that carrageenan failed to interact with the cell receptors. In contrast, when HSV was preincubated with carrageenan and then added to target cells, there was a concentration-dependent reduction in infectivity, confirming the inhibitory interaction between carrageenan and the virus. In the study reported here, carrageenan was added to cells just before FHV-1 addition or 1 hour after adsorption in both the plaque and the virus titration assays. No significant reduction in virus titer compared with controls occurred in either the preadsorption or the postadsorption phase, suggesting carrageenan did not alter FHV-1 replication. Virus copy numbers were only marginally decreased. The most dramatic effect was seen in the plaque assay, in which carrageenan blocked viral adsorption. The difference in the assays is likely explained by the relatively small amount of virus added in the plaque assay compared with the virus titration assay.

For the in vivo portion of the study reported here, a frequency of four times daily application of carrageenan was chosen because other topical antiviral agents, such as trifluridine and idoxuridine, are often used at this frequency in cats. Given the constraints of the laboratory environment, the four times daily treatments were administered between 7 AM and 5 PM. At a concentration of 250 μg/mL, carrageenan appeared to shorten the period of virus shedding but failed to reduce the clinical signs of FHV-1 conjunctivitis in cats. Use of the compound 1 and 2 hours before infection, in an attempt to block virus adsorption, did not alter the course of disease or of virus isolation compared with use of the compound beginning 24 hours after infection. It is possible that little carrageenan remained in the conjunctival sac at the time of inoculation.

Experimental in vivo models of HSV-2 genital infection have demonstrated the efficacy of topically applied carrageenans. In mice, solutions of 0.5% to 1% carrageenan applied 5 minutes before virus prevented vaginal infection in most animals. Similarly, in another study, a 1% solution of λ-carrageenan type IV applied 20 seconds before virus prevented clinically apparent disease from HSV-2 in most mice infected.

It is possible that a higher concentration of carrageenan and an application to the conjunctival sac immediately before inoculation with FHV-1 might have produced more dramatic results in the currently reported study.

Sulfated polysaccharides have potentially negative side effects. They are poorly absorbed by the oral route with a bioavailability of less than 1% and may cause alterations in the virology of the eye. Reducing virus titer in these studies was not dose-dependent; viral titers were reduced regardless of the concentration of carrageenan used. A similar effect was produced by decreasing the time of carrageenan application by 1 hour. This suggested that application of the compound even before virus binding could affect infection. The lack of effect of carrageenan on virus titer was not dose-dependent, indicating that carrageenan did not block virus adsorption. For the plaque assay, carrageenan reduced virus adsorption, but this reduction was not statistically significant. The plaque assay may have demonstrated a lack of effect because the assay was performed on cultured cells, which are not the target cells of FHV-1. The plaque assay may have also been affected by the low concentration of virus used in the assay, which was 100 times lower than that used in the virus titration assay.

The primary objective of this study was to evaluate the effects of a novel compound for use as an antitherpeic ophthalmic preparation. Keratoconjunctivitis caused by FHV-1 in cats is similar to that caused by HSV-1 in humans, and the cat has served as a model for herpetic ocular disease. This study demonstrates the viral adsorption blocking activity of λ-carrageenan, a mixture of sulfated polysaccharides extracted from red seaweeds, against FHV-1 in vitro. The IC50 of 5 μg/mL found in this study for λ-carrageenan against FHV-1 is slightly higher than that found for HSV-1 and HSV-2 (1.6 and 1.5 μg/mL) in another study.

The exact receptor mechanisms that allow entry of FHV-1 into feline cells have not been fully elucidated. The FHV-1 has 10 known surface glycoproteins. Initial attachment to cells involves viral envelope glycoprotein C (gC) and, to a lesser extent, gB, interaction with the heparan sulfate residues present on the surface proteoglycans of target cells. Although gC plays a major role in viral attachment to the cell surface, it has been shown that HSV can use a variety of glycoproteins to attach to cells, such that no one glycoprotein is used exclusively for cell attachment. Of the seven identified FHV-1 glycoproteins, gC appears to be the major glycoprotein involved in attachment to heparan sulfate on the cell surface, whereas gE and gI are not essential for virus replication in tissue culture. Sulfated polysaccharides evaluated that block herpesvirus adsorption are thought to coat viral glycoproteins and prevent their binding to heparan sulfate receptors on cell membranes. In one study, carrageenan was added to cultured cells before HSV infection and then was removed before virus was added. This had no effect on reducing virus titer, suggesting that carrageenan failed to interact with the cell receptors. In contrast, when HSV was preincubated with carrageenan and then added to target cells, there was a concentration-dependent reduction in infectivity, confirming the inhibitory interaction between carrageenan and the virus. In the study reported here, carrageenan was added to cells just before FHV-1 addition or 1 hour after adsorption in both the plaque and the virus titration assays. No significant reduction in virus titer compared with controls occurred in either the preadsorption or the postadsorption phase, suggesting carrageenan did not alter FHV-1 replication. Virus copy numbers were only marginally decreased. The most dramatic effect was seen in the plaque assay, in which carrageenan blocked viral adsorption. The difference in the assays is likely explained by the relatively small amount of virus added in the plaque assay compared with the virus titration assay.

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gastrointestinal microflora and mucosa. Intravenous administration has been associated with profound, but reversible, thrombocytopenia. Another undesirable property of sulfated polysaccharides is their anticoagulant activity. Like heparin, some sulfated polysaccharides prolong thrombin time. In our study, we chose the automated PTT test for its sensitivity and accuracy. Carrageenan caused the PTT to be prolonged compared with control samples at concentrations of ≥5 μg/mL when mixed directly with feline plasma. However, when administered to cats as an ophthalmic preparation for several weeks, there was no alteration of PTT, likely because of the compound’s poor absorption across the conjunctiva and, when swallowed, across the gastrointestinal mucosa. Two studies reported carrageenan to have no anticoagulant properties when mixed directly with human plasma based on an evaluation of thrombin times (TT), though a description of how the time to clot formation was determined was not reported. It is possible that in our study the more sensitive automated PTT test detected anticoagulant activity of carrageenan that was not detected using TT, that the anticoagulant effect was more pronounced in the cat, or that the fractions of carrageenan evaluated in the previously reported studies had less anticoagulant effect than λ-carrageenan type IV.

Despite the potential adverse effects of polysulfated saccharides, carrageenan is termed a GRAS (generally regarded as safe) product by the Food and Drug Administration (www.cfsan.fda.gov/~dms/opascogd.html). It is a common ingredient in many processed foods. The carrageenans have been shown to lack cytotoxic effects when tested in vitro, also a finding in our study. In the study reported here, the lack of cytotoxicity of carrageenan for feline kidney cells at a concentration of 500 μg/mL at 48 hours suggested that topical application of the compound would be safe. However, subconjunctival injection of 500 μg λ-carrageenan was used to induce an experimental model of conjunctivitis in rats. In another study, λ-carrageenan was compounded into microspheres with gelatin and timolol maleate, with a final concentration of 7.5 mg/mL carrageenan. The compound was applied topically to the eyes of rabbits with no ocular irritation noted. Based on the finding of conjunctival hyperemia noted in some of the cats in this study, carrageenan should be considered a mild ocular irritant in this species.

In summary, λ-carrageenan type IV blocked FHV-1 adsorption in vitro. At 250 μg/mL and four times daily application in an experimental model of feline herpetic conjunctivitis, the time period of viral shedding was reduced. Despite the somewhat encouraging results seen in vitro and a shortening of viral shedding time in infected cats, the clinical signs of disease in cats were not altered.

Acknowledgments

The authors thank Suresh Mittal, Anisa Dunham, Pamela Kirby, and Melinda Anderson for technical assistance in this study.

References


