Effects of cidofovir on cell death and replication of feline herpesvirus-1 in cultured feline corneal epithelial cells

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Objective—To assess the effect of cidofovir on viability of feline corneal epithelial (FCE) cells, replication of feline herpesvirus (FHV)-1, and virus-induced cytopathic changes.

Sample Population—Healthy eyes from 14 recently euthanized cats.

Procedure—Cidofovir at concentrations ranging from 0.05 to 0.00005 mg/mL was added to primary cultures of FCE cells, and cytopathic changes and effects on cell proliferation and cell viability were determined during the subsequent 48 hours. Efficacy of cidofovir (0.005 and 0.05 mg/mL) to prevent in vitro infection of FCE cells with FHV-1 was determined during 72 hours of culture by assessing viral cytopathic effects and viral titers.

Results—Cidofovir at concentrations of 0.05, 0.005, and 0.0005 mg/mL significantly reduced mean viable cell counts, and cidofovir at a concentration of 0.05 mg/mL significantly reduced the percentage viability of cultured FCE cells. Minimal cytopathic changes were observed at concentrations of 0.02 and 0.05 mg of cidofovir/mL. Cidofovir at concentrations of 0.05 and 0.02 mg/mL abrogated the cytopathic effects attributable to FHV-1 infection and reduced viral titers from ≥ 10^2 TCID_{50}/mL to ≤ 10^0 TCID_{50}/mL.

Conclusions and Clinical Relevance—Cidofovir in vitro was highly efficacious against FHV-1 infection of a primary culture of FCE cells but had cytostatic effects on cultured cells. (Am J Vet Res 2005;66:217–222)

Ocular infection with feline herpesvirus (FHV)-1 is a common problem in cats. Primary infection is seen most often in neonatal and young cats, and ocular manifestations mainly involve the conjunctival and corneal epithelium. Activation of host-defense mechanisms reduces the viral load to latency in sensory ganglia. Reactivation of viral replication is commonly seen in association with immunosuppression as well as circumstances attributable to host factors. During the recrudescent phase of infection, lytic viral replication in corneal epithelial cells results in severe discomfort, corneal ulcers, and potential for complications resulting in loss of vision. Few drugs are currently available to treat cats with ocular diseases associated with FHV-1 infections. Acyclovir, although of proven value against herpesvirus infections in humans, has inadequate efficacy against FHV-1. One possible reason is insufficient drug phosphorylation, which depends on virus-encoded thymidine kinase. Ophthalmic antiviral agents such as trifluridine, vidarabine, and idoxuridine require administration at frequencies that many cats will not tolerate, and some of these agents can be extremely irritating, which further limits their use. There is a need to investigate new agents for efficacy against FHV-1 and practicality for use in feline patients. We have described the use of feline corneal epithelial (FCE) cell culture as a potential in vitro model for investigation of ocular drugs and have used this model to investigate cytoxicity and antiviral activity of interferon-α.

Cidofovir is a nucleoside analogue of deoxycytidine monophosphate that has potent antiviral effects in vitro and in vivo against several herpesviruses of human and veterinary importance. For example, topical application of cidofovir at concentrations of 0.02 to 0.1 mg/mL is effective for treating New Zealand White rabbits with keratitis attributable to infection with herpes simplex virus (HSV)-1, and cidofovir is widely used to treat people with retinitis associated with cytomegalovirus infection. In vitro efficacy of cidofovir against FHV-1 has been documented. Cidofovir is attractive as a potential therapeutic agent for cats with ocular FHV-1 infection because it does not require viral thymidine kinase for activation, has clinical efficacy in humans, and lacks toxic effects when used topically. Cidofovir is converted to an active metabolite (ie, CDVpp) by phosphorylation that uses cellular enzymes rather than viral enzymes. The CDVpp inhibits viral synthesis by competitively inhibiting viral DNA polymerase; CDVpp binds with greater avidity to viral DNA polymerase than it does to host-cell DNA polymerase. The active metabolite CDVpp has an unusually long intracellular half-life of 65 hours, and another metabolite, cidofovir phosphate choline, has an even longer half-life of 87 hours. The latter metabolite may constitute an intracellular reservoir, which would allow for clinical efficacy despite prolonged intervals between doses. Such pharmacokinetic features would be desirable for veterinary applications.

The objectives of the study reported here were to determine the effect of cidofovir on cell viability and morphologic characteristics in a primary culture system of corneal epithelial cells and to assess the in vitro efficacy of cidofovir to reduce cytopathic effects and replication of FHV-1 when tested at concentrations that have been effective in clinical trials of rabbits with keratoconjunctivitis attributable to infection with HSV-1.
Materials and Methods

Sample population—Fourteen cats were obtained from a local animal shelter within 1 hour after they were euthanized. Cats were euthanized for reasons unrelated to the study.

Culturing of corneal epithelial cells—All 28 eyes were cultured by use of a subconjunctival method. Following removal, eyes were soaked for 10 minutes in a solution that was a 1:1 combination of Dulbecco modified Eagle medium-Hams F-12 (DMEM-F12)30 containing 30 µg of gentamicin sulfate/mL. Corneas were examined by use of an operating microscope and discarded when epithelial lesions were detected. Corneal epithelial cells were isolated by use of dispase,5 as described elsewhere.44 Cellular clusters were manually dispersed into individual cells or small clumps by use of gentle repeated pipetting. Cell suspensions were centrifuged at 500 X g for 5 minutes, and supernatant was decanted. The cell pellet was reconstituted in supplemented hormonal epithelial medium (SHEM) consisting of DMEM-F12 containing 10% fetal bovine serum, 100 U of penicillin/mL, 100 µg of streptomycin/mL, 2 mM L-glutamine, 1 µM of chlora toxin/mL, 10 ng of epithelial growth factor/mL, 1 µg of hydrocortisone/mL, and 5 µg of insulin/mL. The FCE cells from a cornea were seeded into 12 wells of a 24-well plate containing 1 mL of SHEM. Cells were cultured at 37°C and 5% CO2. Medium was changed every 48 hours.

Subcultures were initiated when the primary cultures were 70% to 80% confluent. Adherent cells were detached by incubation for 10 to 20 minutes with 1 mL of 0.05% trypsin plus 0.53mM EDTA.25 Reactions were stopped by the addition of 10% fetal bovine serum, cells were centrifuged at 500 X g for 5 minutes, and supernatant was decanted. The cultures were split (1:2); all cells from a cornea were resuspended into wells of a 24-well plate that contained fresh SHEM. Epithelial identity of cultured cells was verified morphologically by use of phase-contrast microscopy and light microscopy of stained sections. Corneal origin of the cells was verified by immunocytochemical staining for cytokeratins 12 and 3, which are markers for cornea-type differentiation,05 by use of the mouse monoclonal IgG, antibody AE5.4

Preparation of cidofovir—Cidofovir (1,000 mg) was reconstituted in 10 mL of sterile water, and pH was adjusted to 7.0 by the addition of 10M NaOH. Additional sterile water was added to achieve a final cidofovir concentration of 83.3 mg/mL. This stock solution of cidofovir was stored at –80°C. For the assays, 60 or 24 µL of cidofovir were added to cultures (50% end-point dilution).

Cytotoxicity assay—Five 10-fold dilutions were prepared in SHEM, resulting in final cidofovir concentrations of 0.05 to 0.000005 mg/mL. First-passage cultures of FCE cells obtained from each eye of each of 2 cats were established. When the cultures were 80% confluent, culture medium was replaced with 1 mL of fresh SHEM or SHEM containing cidofovir. Four replicate cultures from each of these eyes with each of 5 cidofovir concentrations or medium containing no cidofovir were established (4 replicates X 2 replicates/dilution). At 24 and 48 hours, cultures were examined by use of phase-contrast microscopy for cytopathic effect (CPE), which was evaluated on the basis of cell detachment, refractivity, cytoplasmic ballooning, variability of cell size, cell rounding, and death.29,30 Scores for CPE were assigned on a scale of 0 to 4 on the basis of the percentage of cultures that had evidence of toxic effects (0, 0% of cultures; 1, 0% to 25% of cultures; 2, 26% to 50% of cultures; 3, 51% to 75% of cultures; and 4, 76% to 100% of cultures). At 48 hours, the cells were detached as described previously, viable and dead cells were counted by use of the trypan blue dye exclusion method, and percentage viability was calculated.

Preparation of FHV-1 and inoculation of cultures—Crandell-Reese feline kidney (CRFK) cells25 were propagated in Iscove modified Dulbecco medium36 with 10% horse serum, 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 2 mM L-glutamine. Feline herpesvirus-1 strain 605-7 was propagated on CRFK cells to produce a virus stock for all experiments. The viral titer was determined by use of the TCID50 method on CRFK cells, as described elsewhere,5 and the end-point titer was calculated by use of the method of Reed and Muench.28 An FHV-1 stock preparation with a TCID50 of 105.4/mL was used throughout all experiments. Cultured FCE cells were infected with 458 TCID50 of FHV-1 in 250 µL of fresh SHEM/well of a 24-well plate. After incubation for 1 hour at 37°C in 5% CO2, cells were washed twice with PBS solution, and 1 mL of fresh SHEM or SHEM that contained cidofovir was added. Cultures were maintained for 72 hours.

Efficacy of cidofovir against FHV-1—First-passage cultures of FCE cells derived from each of 12 eyes were grown to confluence. For each eye, 3 replicates in each treatment group (uninfected and untreated, infected with 0.02 mg of cidofovir/mL, infected with 0.05 mg of cidofovir/mL, infected with FHV-1 and untreated, infected with FHV-1 and treated with 0.02 mg of cidofovir/mL, infected with FHV-1 and treated with 0.05 mg of cidofovir/mL) were assessed (12 eyes X 3 replicates/eye for each treatment group). Each cell culture was examined by use of phase-contrast microscopy at 24, 48, and 72 hours. A score ranging from 0 to 4 for CPE was assigned.26 An aliquot of supernatant was collected from each cell culture infected with FHV-1 and stored at –80°C for determination of viral titer. An aliquot of cells from each culture was collected for polymerase chain reaction (PCR) assay of FHV-1.

Viral titers—Viral titers were determined in samples of culture supernatant by use of the TCID50 method on CRFK cells25 for serial 10-fold dilutions (from 10–1 to 10–6) of the test sample. End-point titers were calculated on samples collected 4 days after inoculation; end-point titers were the dilution of virus that caused CPE in 50% of the inoculated cell cultures (50% end-point dilution).

PCR assay for FHV-1—Uninfected cultures of FCE cells and cultures of FCE cells infected with FHV-1 and treated by use of cidofovir were detached, lysed in 50mM Tris HCl-0.25% SDS buffer, and incubated for 2 hours at 60°C with 1 µg of proteinase K/µL. The DNA was extracted with phenol-chloroform-isomyl alcohol, precipitated with cold 100% ethanol, and resuspended in 100 µL of 10mM Tris HCl-1mM EDTA. The nested PCR assay targeted a conserved region of the viral polymerase.38 Reaction components consisted of 0.5 to 1.0 µg of DNA, 1 unit of DNA polymerase,28 2.5 µL of 10X buffer,1.5mM MgCl2,1 µL of dimethyl sulfoxide,200mM deoxynucleotide triphosphates,1 and 100mM of each primer (DFA, 5′–GAY TTY GCG AGY TTN TAY CC–3’; ILK,4 5′–TCC TGG ACA AGC AGC ARN YSG CNM TNA A–3; and KG1,4 5′–GTC TTG CTC ACC AGN TCN CAN CCY TT–3). Samples were denatured for 5 minutes at 94°C, and cycled 45 times (30 seconds at 94°C, 60 seconds at 46°C, and 60 seconds at 72°C) with a final extension of 7 minutes at 72°C. An aliquot (2.5 µL) of the initial PCR product was used as a template for the second round of PCR amplification with new primers (TGV, 5′–3′–TGT AAC TCG GTG TAY GGN TTY CAN GGN GT–3’; IYG,4 5′–CAG AGA CTC GGT RTC NCC RTA DAT–3′). The same cycling conditions were used. Control samples consisted of samples lacking DNA, cells infected with FHV-1, and equine herpesvirus-
1 containing DNA. Amplicons were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination. The identity of select PCR products was verified through direct sequencing.

Statistical analysis—Results were analyzed by use of a computer program. Data were initially subjected to comprehensive residual analysis including tests of normality (Anderson-Darling and Shapiro-Wilk tests). An ANOVA was applied to differences in mean viable cell counts and percentage viability for the 5 concentrations of cidofovir, compared with values for untreated cultures. When the overall F test for the ANOVA was significant, adjusted Tukey pairwise tests were performed for multiple comparisons between various cidofovir concentrations. Data were also analyzed by use of regression analysis including quadratic terms to determine whether cidofovir concentration was a significant predictor of percentage viability or viable cell count. Viable cell counts were converted to a natural logarithm to create normally distributed data. Data for percentage viability were logit transformed to accommodate their binomial nature. The following equation was used for logistical regression:

\[
\text{logit} = \log \left( \frac{r + k}{n - (r + k)} \right)
\]

where \( n \) is 100, \( r \) is the percentage viability, and \( k \) is a bias correction of 0.25. Data were reported as means or back-transformed means with 95% confidence intervals. Significance for all analyses was set at a value of \( P \leq 0.05 \).

Results

Cytotoxicity assays—Exposure of FCE cell cultures to 0.05 mg of cidofovir/mL resulted in minimal cell changes consisting of a subjective decrease in the number of cells, compared with results for control cultures, and small numbers of detached cells (data not shown) at 24 and 48 hours that were scored as ≤ 1 (scale of 0 to 4). Exposure to cidofovir concentrations ranging from 0.005 to 0.00005 mg/mL resulted in no cell changes, compared with results for control cultures. Cidofovir concentrations of 0.05, 0.005, and 0.0005 mg/mL significantly reduced viable cell counts; however, the percentage of viable cells was unaffected, except at a concentration of 0.05 mg of cidofovir/mL (Table 1).

Regression analysis applied to mean viable cell counts did not fit a simple linear or quadratic model. Regression analysis of percentage viability revealed a significant (\( P < 0.001 \)) negative linear slope to the curve attributable to increasing cidofovir concentrations (Figure 1). The resulting equation (2.87032 – [12.16216 X cidofovir concentration]) predicted the effect of cidofovir concentration on percentage viability and was used to derive the plotted curve.

Efficacy of cidofovir against FHV-1—Extensive CPE developed in all cultures infected with FHV-1 and not treated with cidofovir. The CPE progressed during the culture period, with 75% to 100% of the cells affected by 72 hours (Table 2). The CPE consisted of cytoplasmic ballooning, refractile and rounded or elongated cells, discontinuous adhesion, and cell death.

<table>
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<th>Cidofovir (mg/mL)</th>
<th>Time (h)*</th>
<th>Mean score†</th>
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<td>0</td>
<td>24</td>
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*Represents time after inoculation of cultures with FHV-1. †Scored on a scale of 0 to 4.
Affected cells progressively detached from the wells, which resulted in floating cell debris and few adherent cell clusters (Figure 2). No CPE was detected in any cultures infected with FHV-1 and treated by use of 0.02 or 0.05 mg of cidofovir/mL.

In this assay, noninfected cultures were also incubated with 2 concentrations of cidofovir to address potential variability among cats. Morphologic changes in corneal epithelial cells cultured for 24, 48, or 72 hours with 0.02 or 0.05 mg of cidofovir/mL were minimal and consisted of small numbers of detached cells and a decrease in the number of cells, compared with results for control cultures. Mean CPE score for these cultures was ≤1 at all time points.

Primary cultures of FCE cells obtained from 12 eyes were inoculated with FHV-1, which resulted in viral titers consistently ≥10^{14} TCID_{50}/mL by 72 hours after initiation of culture. When supernatants from cultures infected with FHV-1 and treated by use of cidofovir were added to CRFK cells for determination of viral titer, no CPE was observed. Therefore, detection of virus in cidofovir-treated primary cultures of corneal cells could not be determined through induction of cytopathic changes in an indicator cell line. Changes observed in CRFK cells exposed to cidofovir-containing supernatant (from uninfected and infected cultures) consisted of a reduction in cell proliferation, an increase in variation of cell size and cytoplasmic granularity, and an increase in cell death (data not shown). Changes in CRFK cells were of greater magnitude than those in primary cultures of corneal cells, were evident only in dilutions of supernatant ranging from 10^{-1} to 10^{-3}, and were identical for supernatants of cultures infected with FHV-1 and treated by use of cidofovir and uninfected cultures treated by use of cidofovir. Therefore, definitive virus titers could not be determined for any cultures infected with FHV-1 and treated by use of cidofovir. Because cytotoxic effects were not detected in dilutions >10^{-3}, actual viral titer of the cidofovir-treated cultures (as determined by use of the method of Reed and Muench for end-point titer calculation) was considered <10^{3.5} TCID_{50}/mL.

PCR assay—Because of a lack of viral CPE in primary cultures of corneal cells treated with cidofovir and lack of viral CPE induced in CRFK cells by supernatants from the same cultures, it was important to determine whether these cultures were infected by FHV-1. A PCR product was obtained from untreated infected cultures and infected cultures treated by use of cidofovir but was not obtained from any of the uninfected cultures (Figure 3).

Figure 2—Phase-contrast photomicrographs of untreated cultured FCE cells (A), FCE cells cultured with 0.02 mg of cidofovir/mL (B), and FCE cells infected with feline herpesvirus (FHV)-1 (C). Notice the polygonal cells typical of epithelium in the untreated FCE cells and the cells cultured with 0.02 mg of cidofovir/mL and severe cytopathic effect in cells infected with FHV-1. Bar = 50 µm for panel A and 100 µm for panels B and C.

Figure 3—Gel electrophoresis of FHV-1 amplicons obtained by use of a polymerase chain reaction (PCR) assay. Samples for lanes (left to right) were as follows: FCE cells infected with FHV-1 and treated by use of 0.05 mg of cidofovir/mL; FCE cells infected with FHV-1 and treated by use of 0.02 mg of cidofovir/mL, uninfected cells, uninfected cells, positive-control sample, and 100-bp DNA ladder. Notice the FHV-specific PCR product at 220 bp (arrow).
Discussion

In the study reported here, an in vitro model of ocular FHV-1 infection was used to assess the ability of cidofovir to protect corneal epithelial cells from infection by FHV-1 and to evaluate the cytotoxic effects of cidofovir on corneal epithelial cells. Concentrations of cidofovir that were chosen for the antiviral assay were those that reportedly were effective in clinical trials of rabbits with keratoconjunctivitis attributable to HSV-1 infection.18

Incubation with cidofovir at concentrations of 0.05 and 0.02 mg/mL caused mild morphologic changes in the FCE cells. Significant reductions in viable cell counts and percentage viability were attributable to effects of cidofovir. Viable cell count was significantly reduced only when results for the 3 highest cidofovir concentrations (0.05, 0.005, and 0.0005 mg/mL) were compared with results for the 2 lowest concentrations (0.00005 and 0.000005 mg/mL) or with results for control cultures. The 2 lowest concentrations did not cause reductions in cell counts, compared with results for control cultures. The cidofovir-associated reduction in viable cell count was likely attributable to a reduction in proliferation through inhibition of host-cell DNA polymerase. Results of a study19 on a human embryonic lung fibroblast cell line revealed that incubation with cidofovir concentrations of 0.02 to 0.1 mg/mL caused a 50% reduction in cellular DNA synthesis and a concentration of 0.1 mg/mL caused a 50% reduction of cell growth. Cidofovir inhibits viral DNA polymerase much more avidly than it does human DNA polymerase because of differing binding affinities.18 Host-cell metabolism or proliferation is affected only at concentrations 100- to 1,000-fold higher than the active concentration for antiviral effects.18,24 Thus, it is possible that concentrations of cidofovir < 0.00005 mg/mL may inhibit FHV-1 proliferation, but additional in vitro studies would be needed to assess this.

Percentage viability was significantly reduced in FCE cell cultures incubated with 0.05 mg of cidofovir/mL. Regression analysis also revealed that an increase in cidofovir concentration was associated with a reduction in percentage viability. A reduction of percentage viability suggests increased cell death because of cidofovir. It is possible that the observed cytotoxic effects were not caused entirely by the cidofovir. Addition of a large volume of drug solution was required to achieve the 0.05 and 0.02 mg/mL concentrations; therefore, nutrients and growth factors in the culture medium may have been diluted sufficiently to contribute to cellular toxicosis and reduced cell proliferation.

Analysis of the results of the study reported here indicates that incubation with cidofovir was mildly cytostatic in cultured FCE cells. Reduced cell numbers, in part because of reduced cellular proliferation, were evident for concentrations of cidofovir ≥ 0.05 mg/mL. Additional in vitro studies would be required to determine the ideal cidofovir concentration, one that was effective in substantially reducing FHV-1 infection while causing minimal inhibition of host-cell proliferation. The antiviral activity of cidofovir at concentrations of 0.00005 or lower should be investigated. In a clinical situation, the 0.02 and 0.05 mg/mL concentrations of cidofovir may reduce cellular proliferation sufficiently to adversely affect the healing of corneal lesions.

Cidofovir at concentrations of 0.02 and 0.05 mg/mL was extremely effective against FHV-1 infection of FCE cells in vitro. Cultures infected with FHV-1 and treated by use of cidofovir that were tested by use of a nested PCR assay had positive results for herpesvirus DNA. Even though FHV-1 was contained in the FCE cell cultures following inoculation, cidofovir completely prevented the virus from causing visible CPE.

It must be emphasized that in contrast to the changes observed in the CRFK cells used to perform the virus titers, the changes observed in primary FCE cell cultures were minimal and did not obscure the ability to evaluate for viral CPE. The difference may have been attributable to an increased sensitivity of CRFK cells, compared with FCE cells, or to a difference in the protocols used for the assay and viral titration procedure. Cidofovir-containing medium was added to CRFK cells at 50% confluence, whereas it was added to FCE cells at 100% confluence. The CRFK cells would have been in a more active phase of growth, which would make them more sensitive to a toxic environment or inhibition of cellular DNA polymerase by cidofovir. A definitive virus titer could not be determined from the culture medium of cells infected with FHV-1 and treated by use of 0.02 or 0.05 mg of cidofovir/mL because of the observed cytotoxic effects on CRFK cells, which may have masked viral CPE; however, these viral titers were deduced to be < 10^3 TCID50/mL. This represents a profound reduction because untreated cultures had titers ≥ 10^4 TCID50/mL. This apparent amplification of viral titer may have been attributable to more efficient infection and replication in primary corneal cells, compared with effects in CRFK cells.

Efficacy of cidofovir against FHV-1 has been documented in an in vitro assay that used CRFK cells.17 Cidofovir had a mean inhibitory concentration 50 of 11.0µM, which was approximately twice that of idoxuridine and gancyclovir.17 Although it was less effective in vitro, compared with results for those other antiviral agents, it is still extremely attractive as a treatment for cats infected with FHV-1, particularly because of the potential for prolonged intervals between subsequent doses. Topical application of cidofovir twice daily at concentrations of 0.02 and 0.05 mg/mL has been effective in treating rabbits with experimentally induced keratitis attributable to HSV-1 infection. Concentrations of 0.02 and 0.05 mg/mL were similarly effective in reducing FHV-1 infection in vitro. The dramatic ability of cidofovir to inhibit FHV-1 in vitro supports prospective clinical studies of topically applied cidofovir for treatment of cats with ocular FHV-1 infection. The potential for cytotoxic effects to corneal cells does exist with these concentrations, and on the basis of results of the study reported here, the 0.02 mg/mL concentration may be safer. Clinical relevance of the observed in vitro cytotoxic effects should be assessed in vivo.
a. DMEM-F12, Sigma Chemical Co, St Louis, Mo.

b. Gentamicin sulphate, Novopharm, Toronto, ON, Canada.

c. Haag Streit HIRE 900, Moller-Wedel, Wedel, Germany.

d. Dispase II, Gibco, Burlington, ON, Canada.

e. Fetal bovine serum, Sigma Chemical Co, St Louis, Mo.

f. Penicillin-streptomycin, Sigma Chemical Co, St Louis, Mo.

g. 1-Glutamine, Sigma Chemical Co, St Louis, Mo.

h. Cholester toxin, Sigma Chemical Co, St Louis, Mo.

i. Epithelial growth factor, Sigma Chemical, St Louis, Mo.

j. Hydorcristosine, Sigma Chemical Co, St Louis, Mo.

k. Insulin, Sigma Chemical Co, St Louis, Mo.

l. Costar tissue culture plates, Corning, Acton, Mass.

m. Trypsin-EDTA, Gibco, Burlington, ON, Canada.

n. Antibody Clone AES, Biosignal Development, Saco, Me.

o. Cidofovir, Gilead Sciences, Foster City, Calif.

p. Provided by S. Carman, Animal Health Laboratory, University of Guelph, Guelph, ON, Canada.

q. Iscove Modified Dulbecco Medium, Sigma Chemical Co, St Louis, Mo.

r. Horse serum, Sigma Chemical Co, St Louis, Mo.

s. Provided by S. Carman, Animal Health Laboratory, University of Guelph, Guelph, ON, Canada.

t. Protease K, Gibco, Burlington, ON, Canada.

u. Taq DNA Polymerase, Gibco, Burlington, ON, Canada.

v. 10% polymerase chain reaction buffer, Gibco, Burlington, ON, Canada.

w. Magnesium chloride, Gibco, Burlington, ON, Canada.

x. Dimethyl sulfoxide, Gibco, Burlington, ON, Canada.

y. Deoxynucleotide triphosphates stock, 10mM, Gibco, Burlington, ON, Canada.

z. Primer DFA synthesized by Gibco, Burlington, ON, Canada.

aa. Primer ILK synthesized by Gibco, Burlington, ON, Canada.

ab. Primer KG1 synthesized by Gibco, Burlington, ON, Canada.

ac. Primer TG1 synthesized by Gibco, Burlington, ON, Canada.

ad. Primer IYG synthesized by Gibco, Burlington, ON, Canada.

ae. London Regional Genomics Centre, Robarts Research Institute, London, ON, Canada.


References


