Antiviral resistance to ganciclovir or foscamet has been reported in 8% of patients taking these medications for several months for the treatment of CMV retinitis. Both are associated with significant systemic toxicities that are dose limiting [5, 6]. Antiviral resistance to ganciclovir or foscarinet has been reported in 8% of patients taking these medications for several months or longer, as determined by virus assay and drug sensitivity testing in vitro [7–10]. Antiviral resistance is even more commonly observed clinically, as manifested by lack of responsiveness to reinduction doses of systemic anti-CMV agents later in the disease process [11, 12]. Because both drugs are virostatic, discontinuation of therapy in patients with poor tolerance or lack of venous access results in progression of retinitis. In addition, patients who develop clinically resistant retinitis show progressive disease and can lose vision even while receiving treatment [11]. As a result, significant interest exists in the use of locally administered antiviral drugs for the treatment of this disease [13–19].

When given intravitreally, both ganciclovir and foscarinet can be effective in the treatment of CMV retinitis [14–17]; however, there are several problems with local intracocular therapies using the two drugs. Intravitreal injections need to be given at least weekly to be effective, and in some instances, twice weekly injections may be indicated. Frequent injections may, however, be poorly tolerated by ill patients [13, 16]. Ganciclovir implants, currently under phase III clinical trials, may be effective for up to 8 months but require a surgical procedure to initiate therapy and may increase the risk of early retinal detachment [18, 19].

In search of a long-acting anti-CMV drug that can be administered by intracocular injection in an office setting, we evaluated a new class of acyclic nucleoside phosphonate analogues that have broad-spectrum anti-DNA virus activity [20–22]. Of this class of drugs, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC; cidofovir) was the most potent and selective inhibitor of CMV in vitro [23–26].

Cidofovir is 10 times more potent and selective than ganciclovir as an inhibitor of human CMV replication in vitro [22]. Cidofovir also has a longer duration of action against human CMV than ganciclovir or foscarinet in vivo and in vitro [26, 27]. This may be due to prolonged intracellular antiviral activity of the parent compound or its metabolites or both [21].

We previously reported that 100-μg intravitreal doses of cidofovir do not produce toxicity in retinas of rabbits and mon-
keys [28]. That intravitreal concentration (100 µg/mL) is 500 times greater than the concentration needed to prevent 90% of the growth of human CMV strains in vitro (0.2 µg/mL) [22]. We subsequently studied the duration of action of 100-µg injections of free cidofovir in preventing retinitis in our rabbit model: The inhibitory effect lasted 21–30 days, which is 10-fold greater than the effect of 200 µg of free ganciclovir [29]. Since cidofovir is more water soluble and polar at neutral pH than either ganciclovir or foscarnet, it is particularly suitable for liposome encapsulation. The purpose of this study was to determine the duration of effect of one 100-µg intravitreal injection of liposome-encapsulated cidofovir in the prevention and treatment of focal nonlethal herpes retinitis in rabbits.

Methods and Materials

Animal methods. Sixty-two pigmented rabbits weighing 2.0–3.5 kg were used. For surgical procedures and selected ocular examinations, the rabbits were anesthetized with intramuscular injections of ketamine (24 mg/kg) and xylazine (12 mg/kg). Topical 1.0% proparacaine HCl was used for anesthetizing corneas. Pupils were dilated with 2.5% phenylephrine hydrochloride and 1% cyclopentolate hydrochloride.

For intravitreal injections, the right eye of each rabbit was prop­­osed, and injection sites were prepared with povidone-iodine ap­­cular microscopes, 0.1 mL of liposome-encapsulated cidofovir (100 µg), ganciclovir (200 µg), or normal saline was injected with a 30-gauge needle into the posterior vitreous cavity 2 mm from the limbus. The optic nerve head was observed to assure retinal perfusion. A cotton applicator was placed over the injection site to prevent efflux of intraocular fluid. Ganciclovir was obtained from Syntex (Palo Alto, CA). Cidofovir was synthesized by I. Rosenberg and A. Holy (Czechoslovak Academy of Sciences, Prague) and encapsulated into liposomes as previously described [30].

Strain PH of herpes simplex virus type 1 (HSV-1; 0.1 mL) was inoculated with a 30-gauge needle viewed through a surgical microscope, using coaxial illumination, onto the retinal surface 2 mm inferior to the optic disk. The virus, which contained a total of 10,000 pfu, was obtained from and titered by J. Oh (University of California, San Francisco, Proctor Foundation). Right and left eyes were examined using a slit lamp and indirect ophthalmoscope on days 1, 3, 5, and 7 after inoculation and weekly thereafter until sacrifice, typically day 21 for experimental animals and between days 7 and 14 for control animals. Careful diagrams of all fundus lesions were made, and in selected cases, photographs were taken. Histopathologic and immunocytochemical studies were done to confirm ophthalmoscopic observations at sacrifice.

Experimental protocols. Immediately after virus inoculation, 9 eyes were treated with balanced salt solution (3 eyes), 200 µg of ganciclovir (3), or 100 µg of liposome-encapsulated cidofovir (3). The remaining experiments involved treatment by inoculation with liposome-encapsulated cidofovir or balanced salt solution at various times between 10 and 120 days before virus inoculation.

For euthanasia, the animals were anesthetized as described above and sacrificed by intracardiac injection of 1.0 mL of pentobarbital sodium (390 mg/mL). Both eyes of each animal were enucleated and placed in cold 4% paraformaldehyde. Whole brains of selected animals were removed intact and placed in the same fixative. Following fixation, the optic nerves, chiasmas, and brains were dissected so that the optic pathways could be isolated and examined histologically.

Histology. Eye and brain tissue was embedded in paraffin for routine sectioning followed by hematoxylin-eosin staining. Brain, optic chiasm, optic nerves, and retinas were evaluated immunocytochemically for herpes simplex viruses. A horseradish peroxidase–conjugated rabbit anti–HSV-1 polyclonal antibody (Dako, Carpenteria, CA) was used.

Following deparaffinization, 5-µm-thick sections of tissue were placed in 1% hydrogen peroxide in absolute methanol solution to neutralize endogenous peroxidase activity. Tissue sections were hydrated in a series of graded alcohols to water and then to a TRIS buffer (0.05 M TRIS, 1.5% NaCl, and 2 mM CaCl₂, pH 7.6) and placed in proteinase K in TRIS buffer (42 µg/mL) at 37°C for 20 min. Next, they were immersed in 2% glycine in PBS for 5 min and subsequently placed in PBS (pH 7.3). The sections were then covered with a 1:40 dilution of antibody in a fish gelatin block, coverslipped, and incubated overnight at 4°C. The next day, coverslips were removed, and the tissue was washed three times with PBS followed by 0.1 M acetate buffer (pH 5.2) and then incubated with aminoethylcarbazole for 30–60 min at room temperature in a humid chamber. The reaction was terminated by placing the developing slides in water. The tissue was then counterstained with hematoxylin.

Statistical analysis. Statistical analysis was done using the Kruskal-Wallis test and SAS software (version 6.3; SAS Institute, Cary, NC). Differences in grades of retinitis in treatment versus control groups were compared. The grading scale is described in Results and in Table 1. To increase the power of the test by increasing the sample size, a worst-case scenario was used by assigning the highest grade present at each time point within each group to any animals that were sacrificed before that time.

Results

Virus-inoculated eyes from 26 control rabbits pretreated at various time points with 0.1 mL of balanced salt solution were examined ophthalmoscopically. All clinical characteristics of control and experimental eyes were compared on the basis of

<table>
<thead>
<tr>
<th>Grade</th>
<th>Day after infection</th>
<th>Optic nerve</th>
<th>Vitreous</th>
<th>Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>1–2+</td>
<td>Normal to 1+</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>3–4+</td>
<td>1–2+</td>
<td>1/4 retina involved</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3–4+</td>
<td>1–2+</td>
<td>1/2 retina involved</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4+</td>
<td>3–4+</td>
<td>&gt;3/4 retina involved</td>
</tr>
</tbody>
</table>
the reproducible natural course of herpes retinitis, as seen in control eyes in our animal model. In such a course, optic nerve congestion typically appears at day 3, mild vitritis and retinitis at day 5, progressive optic nerve congestion, retinitis, and vitritis at day 7, and extensive retinitis with possible retinal detachment at day 10. For statistical analysis purposes, a grading scale was devised. It was based on the date of onset and the extent of optic nerve congestion, retinitis, and vitritis. The scale used grades 0–4, with increasing severity being given a higher score (table 1). Typical control eyes with predictable progressive retinitis were assigned a score of 4 based on the extent of disease at the 10-day time point. Classification was made based on the worst clinical presentation during the course of infection. Treated animals with a clinical presentation similar to the natural course but delayed were graded 0.5 less for statistical purposes.

Histologic specimens from the retinas in cases of early retinitis (day 3–5) demonstrated a leading edge of retinitis characterized by disruption of the retinal architecture. A sharp demarcation zone between the disorganized retina and uninvolved retina was seen in all cases (figure 1). Virus inclusions were present, and moderate-to-severe inflammatory infiltrate was observed in the areas of retinitis. Immunocytochemistry showed multiple areas of HSV-infected cells in all cases of active retinitis (figure 1). By day 7, histologic studies of the inoculated retinas showed evidence of retinitis with retinal necrosis and optic nerve inflammation as well as cellular infiltration. This correlated with medullary ray hemorrhage. Eyes receiving control injections showed no histologic abnormalities.

Of the 9 animals that received treatment immediately following HSV inoculation, the 3 injected with liposome-encapsulated cidofovir showed no evidence of clinical or histologic retinitis, while the 3 injected with saline developed the characteristic intense retinitis described above. The remaining 3 received ganciclovir and did not develop clinical or histologic retinitis.

The eyes of 3 animals were injected with liposome-encapsulated cidofovir 10 days before virus injection. None of them showed clinical or histologic evidence of retinitis; however, a control animal whose eye was injected with saline 10 days before virus inoculation developed retinitis as described above. Of 7 eyes in animals pretreated with liposome-encapsulated cidofovir 20 days before HSV-1 inoculation, 5 had no clinical or histologic evidence of retinitis and 2 developed mild-to-moderate retinitis (grade 1.5–2.5). Immunocytochemical evaluation also revealed no evidence of HSV-1-infected cells in the retinas and brains of animals that did not develop retinitis.

Overall, 22 of 26 eyes pretreated with 100 μg of liposome-encapsulated cidofovir from 10–60 days before HSV-1 inoculation were protected from experimentally induced retinitis, as were 2 of 5 eyes pretreated 120 days before inoculation (figure 2). Two of 3 eyes pretreated 30 days before virus inoculation did not develop retinitis, and 1 had attenuated retinitis (grade 2.5). Seven of 8 eyes pretreated 40 days before virus inoculation did not develop retinitis, and 1 had mild retinitis (grade 1.5). Four of 5 eyes pretreated 60 days before virus inoculation did not develop retinitis, and 1 had mild retinitis (grade 1.5). Two of 5 eyes pretreated 120 days before virus inoculation did not develop retinitis (figure 2): 1 had mild vitritis and retinitis (grade 1.5), and 1 had attenuated retinitis (grade 2.5).

At each time point, all animals without clinical evidence of retinitis also had no histologic evidence of viral retinitis, retinal destruction, or immunocytochemical reactivity using an anti-HSV probe. Conversely, those animals with clinical evidence
of attenuated retinitis also had histologic and immunocytochemical evidence of retinitis with attendant necrosis. At all time points up to 120 days, there was no evidence either clinically or by light microscopy of toxicity from liposomes or drug in eyes without retinitis. It was not possible to determine toxicity in eyes with retinitis because of the necrotic effects of retinitis. At 120 days, both eyes without retinitis showed mild localized retinal pigment epithelial changes with minimal pigment clumping in the inferior retina. Electroretinography was normal. For the time points mentioned above, control injections of saline were administered, and in all cases, severe retinitis occurred (grade 4; table 1).

At all time points, retinitis grades for experimental and control groups were significantly different using the Kruskal-Wallis nonparametric method. Three analyses were done, including the whole-sample worst-case scenario analysis, reverse compounded comparison between control and study groups, and straight comparison between control and study groups (table 2). Study and control groups were statistically different at all time points and by all analyses.

Intravitreal levels of cidofovir were assayed by high-performance liquid chromatography in 3 eyes that received liposome-encapsulated cidofovir 120 days before virus injection. An aliquot of vitreous was removed at a site distant from the injection site where liposomal material could usually be seen by ophthalmoscopy. This was done in an attempt to assay the free, nonliposome-bound drug concentration. The concentrations were 0.8, 0.3, and 1.1 μg/mL. The average concentration (0.73 μg/mL) was 7–15 times the IC₅₀ of the compound for human CMV [23].

The ophthalmoscopic appearance of liposomes was also studied and documented by fundus photography using acid red dye–encapsulated and drug-encapsulated liposomes. The liposomes were intact, with a consistent spherical shape and a minimum of distortion. Liposomes were clumped in the inferior vitreous cavity, away from the visual axis.

Discussion

We previously showed that free (not liposome encapsulated) cidofovir is a potent and relatively long-acting antiviral agent when injected intravitreally at its highest nontoxic dose (100 μg in rabbits and 20 μg in humans) [29, 31]. The rabbit study showed that the compound has a duration of effect that is 10 times greater than that for ganciclovir in the HSV-1 model of focal retinitis. In that setting, an antiviral effect persisted up to 30 days after injection of 100 μg of free cidofovir. By comparison, in humans, single intravitreal, 20-μg injections of cidofovir resulted in control of CMV retinitis for 8–9 weeks [31]. Larger intravitreal doses of unbound drug appear to be toxic in humans [32].

The long-acting (up to 120 days) protective effect of liposome-encapsulated cidofovir against HSV-1 replication in the eye is likely due its slow rate of release in the multivesicular liposome system. A recent study showed that very high doses (1000 μg) of liposome-encapsulated cidofovir prevented development of HIV-1 retinitis in this same model for 170–240 days [33]. However, this higher dose may be of limited relevance because, given the evidence of toxicity in humans and rabbits at doses of >20 and 100 μg, respectively, it might result in toxic levels of drug if even a small percentage of the liposomes prove unstable. Therefore, this study was done to gauge the effect of a more appropriate, nontoxic dose of liposome-encapsulated cidofovir. The liposomal formulation ap-
Table 2. Comparison, using three statistical analyses, of retinitis scores for experimental and control groups at various time points after preretinal inoculation of herpes simplex virus type 1.

<table>
<thead>
<tr>
<th>Pretreatment time point*</th>
<th>Cidofovir group</th>
<th>Control group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Retinitis score</td>
<td>n</td>
</tr>
<tr>
<td>Whole sample using worst-case scenario</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>1.87 ± 0.48</td>
<td>26</td>
</tr>
<tr>
<td>Reverse compounded comparison†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0.40 ± 0.81</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>0.44 ± 0.84</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.48 ± 0.88</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0.45 ± 0.85</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.53 ± 0.90</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.80 ± 1.06</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>&gt;120</td>
<td>5.10 ± 1.02</td>
<td>6</td>
</tr>
<tr>
<td>Straight comparison‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.00 ± 0.00</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.00 ± 0.00</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.00 ± 0.00</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.19 ± 0.53</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>5.50 ± 1.13</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>5.10 ± 1.02</td>
<td>6</td>
</tr>
</tbody>
</table>

NOTE. Retinitis score is mean ± SD and was based on extent of retinitis. Scale used grades 0–4, with increasing severity being given higher score.

* No. of days between liposomal cidofovir injection and virus inoculation.
† Assumes worst-case scenario and assigns maximum grade present at each time point within each group to any animals sacrificed before that time.
‡ Kruskal–Wallis nonparametric test comparing retinitis grades between treatment and control groups.

pears to have prolonged the previously demonstrated antiviral effect of free cidofovir from <1 month to ≤4 months for animals given the same intravitreal dose (100 μg) [29]. Free drug that is released from the liposome may also have a relatively long duration of action because of long-acting intracellular metabolites [34, 35]. Since cidofovir has 50 times more activity against human CMV than against HSV-1, it may be even more promising than suggested by our results using the HSV-1 model [23].

It should be noted that our model uses a prophylaxis paradigm, which would be expected to be effective with lesser amounts of drug than treatment would require. The prophylaxis paradigm was necessary because the retinitis in our model progresses too rapidly to assess the treatment duration effect of antiviral drugs [36]. Nevertheless, the prophylaxis paradigm has been previously tested with both ganciclovir and cidofovir, and cidofovir was far superior [29]. This finding is consistent with the results seen in the pilot study in humans for treatment of CMV retinitis [31]. The average intravitreal concentration of cidofovir seen here (0.73 μg/mL) is lower than the 4 μg/mL administered as a bolus intravitreal injection of unbound drug in humans [31], so this dose may be tolerated by humans in a clinical setting. As such, the use of liposome-encapsulated cidofovir may result in a practical long-acting intravitreal therapy for CMV retinitis that can be administered in an office setting. A dose-ranging multicenter trial of free, unbound, intravitreal cidofovir is underway and is designed to determine the safety and efficacy of intravitreal cidofovir in doses ranging from 5 to 15 μg. In addition, the visual significance of the liposome-drug complex will need to be evaluated, although a human study on liposome-encapsulated ganciclovir suggests that the liposomal formulation may be well tolerated in humans [37]. Intravitreal liposome-encapsulated cidofovir therapy may prove to be a valuable adjunct to systemic therapy, particularly in patients intolerant of systemic treatment or who demonstrate clinically resistant retinitis.

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