Ganciclovir-Loaded Polymer Microspheres in Rabbit Eyes Inoculated With Human Cytomegalovirus

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Purpose. To test the antiviral effect of ganciclovir released from biodegradable polymer microspheres in rabbit eyes inoculated with human cytomegalovirus (HCMV).

Methods. Human cytomegalovirus (5 × 10⁵ plaque forming unit in 0.1 ml Hank’s balanced salt solution) was inoculated 4 days after gas compression vitrectomy. Injected after 2 days was 10 mg of 300- to 500-μm ganciclovir-loaded microspheres (89.77 μg ganciclovir/mg) suspended in 0.1 ml of 2% hydroxypropylmethylcellulose. Blank microspheres were injected as control specimens. Vitritis, retinitis, and optic neuritis were graded from 0+ to 4+ for 14 days to separate the early HCMV-induced disease events from later nonspecific host inflammatory responses. Ganciclovir-loaded microspheres also were injected and observed for biodegradation and tissue reaction for 8 weeks.

Results. In eyes injected with ganciclovir-loaded microspheres, vitritis decreased from days 3 to 14, and retinitis and optic neuritis decreased from days 3 to 9. In eyes injected with blank microspheres, vitritis increased from days 3 to 7, retinitis increased from days 3 to 9, and optic neuritis increased from days 3 to 14. Immunofluorescence of HCMV antigens in retinal tissues was shown only in eyes injected with blank microspheres. Histopathologic analysis showed minimal focal disruption of the retinal architecture in eyes injected with ganciclovir-loaded microspheres. Disorganization of the normal retinal architecture was observed in eyes injected with blank microspheres. No adverse tissue reaction was observed clinically and histopathologically in eyes injected with ganciclovir-loaded microspheres after 8 weeks.


Cytomegalovirus (CMV) retinitis is the most common cause of visual loss among patients suffering from acquired immune deficiency syndrome (AIDS), occurring in 20% to 30% of patients. The incidence of CMV retinitis and its severe complications has increased in recent years because of improved patient survival with the introduction of new drugs and treatment methods. The treatment of CMV retinitis has therefore become a major concern.

Ganciclovir sodium (9-[2-hydroxy-1-(hydroxy-methyl)ethoxy)methyl]guanine) is the most widely studied antiviral drug for both intravenous and intravitreous treatments of CMV retinitis. The triphosphate metabolite of ganciclovir is a competitive inhibitor of CMV DNA polymerase. The drug prevents replication of the viral DNA but does not eliminate the virus from the tissue. Thus, long-term therapy with ganciclovir is necessary to control the disease. However, the side effect of long-term intravenous treatment with ganciclovir includes neutropenia and thrombocytopenia, which necessitate immediate cessation of therapy. Also, there is increased risk of sepsis because long-term venous access with an indwelling catheter is required for systemic therapy.

As an alternative to systemic therapy, intravitreous injection of the drug has been studied widely and has shown to be effective. Intravitreous ganciclovir provides higher intraocular drug concentration than does systemic therapy. However, one to two intravitreous
ture injections a week is necessary to maintain therapeu
tic levels, because of the short half-life of the drug.
Frequent intravitreous injections have resulted in cata-
ract, astigmatism, vitreous hemorrhage, retinal detac-
hement, and endophthalmitis. 24–27

An implantable sustained release device is avail-
able that releases 1 to 2 μg/hour of ganciclovir in the
vitreous cavity for several months. This device is made
of poly(ethylene vinyl acetate) and polyvinyl alcohol and
is approximately 9.0-mm long and 3.5-mm wide. The 1 μg/hour device is implanted in the vitreous
cavity through a scleral incision and is immobilized by
a suture. The device releases between 0.5 and 2.88 μg/
hour ganciclovir in vivo and is effective therapeutically
against CMV retinitis for 5 to 8 months. 16, 27–29 How-
ever, this device is not biodegradable. The more com-
mon complications seen in eyes treated with the im-
plant include a temporary reduction in functional vi-
sual acuity, vitreous hemorrhage, and retinal detachments. 39 Investigators also have reported diffi-
culty in replacing this device after all the drug has
been released because of fibrosis of the implant and
scleral thinning and necrosis. 30

Biodegradable polymer microspheres have been
used before for intraocular drug delivery. 31–38 Poly(D,L-lactide-co-glycolide) (PLGA) is biocompatible
and degrades to metabolic products that are elimi-
sated acutely, vitreous hemorrhage, and retinal
complications seen in eyes treated with the im-
sustained release of the drug were developed. The
biodegradable PLGA loaded with ganciclovir to obtain
scleral thinning and necrosis. 39, 40 Recently, microspheres of
biodegradable PLGA loaded with ganciclovir to obtain
sustained release of the drug were developed. The
microspheres release ganciclovir in vitro at concentra-
tions within potential therapeutic range for at least 42
days.

A model of human cytomegalovirus (HCMV)-in-
duced chorioretinal infection in rabbit eyes has been
described by Dunkel et al. 41 However, this model
showed rapid progression of fundus disease only up
to day 9 and resolved by day 21. Also, severe vitritis
obscured comprehensive visualization of the retina
and choroid. Thus, we have modified the procedure
in the current study by doing gas compression vitre-
tomy on the rabbit eyes to improve visualization of the
retina and choroid. In addition, HCMV was sus-
pended in Hank’s balanced salt solution (HBSS)
for inoculation. The separation of early HCMV-induced
disease events from later nonspecific host inflamma-
mary responses in rabbit ocular HCMV infection is es-
sential for characterization of disease and analysis of
therapeutic interventions. We have, therefore, limited
our observation period to 14 days.

The aim of this project is to investigate whether
microspheres of biodegradable PLGA loaded with
ganciclovir can control the progression of disease in
rabbit eyes inoculated with HCMV. The intravitreous
injection of small microspheres would be more practi-
cal than would the implantation of a larger, nonbiode-
gradable sustained release device.

MATERIALS AND METHODS

Poly(D,L-lactic-co-glycolic) acid 50:50, [PLGA, po-
ly(D,L-lactide-co-glycolide)] (Birmingham Polymers,
Birmingham, AL) has an inherent viscosity of 0.39 dl/g:
(9,000 to 12,000 Da) in hexafluoroisopropanol at
30°C. Ganciclovir sodium salt (BW B759 U, DHGP,
dihydroxypropoxymethyl guanine, Cytovene-IV) was
obtained from Syntex Laboratories (Palo Alto, CA).
Methylene chloride and hexane were purchased from
Fisher Scientific (Pittsburgh, PA). Silicone oil (SiO,
polydimethylsiloxane, trimethylsiloxy terminated) with
a viscosity of 500 centistokes, fluorosilicone oil
(FSiO, polymethyl-3,3, trifluoropropyl siloxane) 1000 centistokes, and dimethylsiloxane ethylene ox-
ide-propylene oxide copolymer (DMSiEPO) 1800 cen-
tistokes were obtained from Huls America (Piscataway,
NJ).

Microsphere Preparation

Ganciclovir-loaded microspheres were prepared using
a new oil-in-oil emulsion technique. 32 Briefly, a solu-
tion of 250-mg PLGA in 0.7-ml acetone was added to a
suspension of ganciclovir sodium (equivalent to 25
mg of free ganciclovir) in 100 μl of FSiO. The mixture
was agitated in vortex agitator (Vortex Genie, Spring-
field, MA) for 1 minute and sonicated (50 Sonic Dis-
membrator, Fisher Scientific) for 5 minutes at high
speed with intervals of 1 minute of sonication and
10 seconds of rest periods between sonications. The
copolymer-FSiO-ganciclovir suspension was added to 2
ml of a solution of 50-ml SiO, 5-ml acetone, and 100-
μl DMSiEPO emulgent and sonicated again for 25
seconds at high speed (<80% output power). The
dispersion was added to the remaining SiO-acetone-
emulgent solution and stirred in a magnetic stirrer
(Corning Stirrer/Hot Plate, Corning, NY) under an
aspiring hood overnight, at room temperature, until
the acetone evaporated, leaving solid microspheres.
The microspheres were filtered using sieves with aper-
tures of 500, 300, 212, 106, and 53 μm (Newark Wire
Cloth, Newark, NJ), washed with hexane, and dried
over anhydrous calcium sulfate (W. A. Hammond
Dri-erite, Xenia, OH) in a vacuum desiccator for at least
48 hours before they were used. Blank microspheres
were prepared using the same technique mentioned
above, except that ganciclovir was not added to the
suspension.

Microsphere Evaluation

Ten milligrams ganciclovir-loaded microspheres of
300- to 500-μm were weighed accurately (Electronic
Balance, ER-182 A) and dissolved in 2-ml methylene
The ganciclovir was extracted twice into 3 ml of distilled water. The amount of ganciclovir was determined using a spectrophotometer (Beckman DU-70 Spectrophotometer, Fullerton, CA) at absorbance of 250.5 nm. The absorption was compared with a standard curve. The amount of ganciclovir also was determined in the microspheres sterilized for 15 minutes of ultraviolet (UV) light exposure in a class II safety cabinet (Labconco Purifier; Labconco, Kansas City, MI).

One hundred microliters *Pseudomonas aeruginosa* 6294 (Channing Laboratory, Harvard Medical School, Boston, MA) was grown overnight in a tryptic soy agar plate at 37°C. The *P. aeruginosa* inoculum, obtained from the plates, were adjusted spectrophotometrically to a concentration of 10⁶ colony forming units per milliliter in phosphate-buffered saline (cfu/ml PBS) (optical density, 0.1 at 590 nm). The concentration was diluted to 10⁵ cfu/ml PBS. One milliliter of 10⁵ cfu/ml PBS was mixed with 10 mg of 300- to 500-μm PLGA microspheres. Five sets were exposed to UV light exposure in a class II safety cabinet (Labconco Purifier; Labconco) for 15, 30, 60, 90, and 120 minutes, respectively. One set was not exposed to UV light to serve as a control specimen. All sets were plated in tryptic soy agar. After 36 hours’ incubation at 37°C, *P. aeruginosa* colonies were counted.

**Cell Culture and Human Cytomegalovirus Propagation**

Eagle’s minimum essential medium (Bio Whittaker, Walkersville, MD) supplemented with 10% calf serum, 200-mM L-glutamine, and a mixture of 100 IU/ml penicillin, 210 μg/ml streptomycin, and 0.25 μg/ml fungizone (Bio Whittaker) was used in the cell cultures. Confluent human foreskin cell monolayer (HS68;American Type Culture Collection, Bethesda, MD) was subcultured into 80-cm² flasks (Nunclon, Roskilde, Denmark) after dissociation in 0.2% trypsin ( GibCO, Grand Island, NY). All stock HS68 monolayer and subcultures were maintained in a humid atmosphere containing 5% carbon dioxide at 37°C. HCMV AD169, provided by D. Coen (Harvard Medical School) was grown on HS68 monolayers. An HCMV AD169 supernatant stock was infected directly onto fresh, confluent monolayers of HS68 cells in 80-cm² flasks. Infected cultures were maintained at 37°C in a 5% carbon dioxide atmosphere until <75% of the cells exhibited cytopathogenic effect. The HCMV-infected cells were harvested by scraping and were centrifuged at 1000 rpm for 10 minutes. The cell pellet was suspended in HBSS and was passed five times through a sterile 27-gauge needle to disrupt the HS68 cells and centrifuged at 2000 rpm for 10 minutes to remove cell debris. HCMV in the supernatant was used to inoculate into rabbit eyes.

**Microsphere Injection in Rabbit Eyes**

All surgeries were performed on the rabbit eyes under aseptic conditions and pursuant to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fifty-two (46 pigmented and 6 New Zealand white rabbits) rabbits of both genders weighing 2.1 to 3.4 kg were obtained from Mine Brooks Farm (Pepperell, MA). Animals were housed individually and were held in the vivarium for at least 1 to 2 days before any procedure was performed. All rabbits were examined by slit-lamp biomicroscopy and indirect ophthalmoscopic analysis through dilated pupils (phenylephrine hydrochloride ophthalmic solution 2.5%) to eliminate animals with preexisting anterior segment and posterior segment chorioretinal abnormalities.

All rabbits were anesthetized with chlorpromazine HCl (25 mg/kg body weight) and ketamine HCl (50 mg/kg body weight) intramuscularly. Proparacaine HCl 0.5% eye drops were instilled as a topical anesthetic before surgery. Mydriasis was obtained with 2.5% phenylephrine HCl and 1% cyclopentolate eye drops. Gas compression of the vitreous body with 0.4 ml of 100% perfluoropropane gas (C₃F₈) (Alcon Laboratories, Fort Worth, TX) was performed as described previously.

After 4 days, the rabbits were reanesthetized and the gas was exchanged with balanced salt solution (BSS). A tuberculin syringe with a 30-gauge needle was used to inject the BSS 3 mm posterior to the limbus at the 11 o’clock position, and another 30-gauge needle was inserted at the 2 o’clock position to allow the gas to escape. Sixty-two of 92 pigmented rabbit eyes were inoculated with 0.1 ml of HCMV supernatant (5 × 10⁵ plaque forming units in 0.1-ml HBSS). For inoculation, the HCMV was drawn into a sterile 1-ml tuberculin syringe fitted with a 27-gauge needle. Each eye was propiosed and immobilized, and a scleral site 3 to 4 mm lateral to the limbus was punctured with the needle. The 0.1-ml HCMV inoculum was injected into the vitreous chamber near the vitreoretinal interface over a 15- to 30-second period. The needle was removed slowly to reduce reflux of the inoculum, and the eye was examined to evaluate potential ocular trauma due to the injection. Thirty of 92 pigmented rabbit eyes were sham inoculated with 0.1 ml of HBSS (Bio Whittaker). Two days after HCMV inoculation, rabbits were reanesthetized and topical anesthetic was instilled into the eyes. The 62 pigmented rabbit eyes that were inoculated with HCMV were divided into 4 groups (Table 1). Group 1 eyes (16) were injected with 10 mg of the ganciclovir-loaded microspheres suspended in 0.1-ml 2% hydroxypropylmethylcellulose (HPMC) using an 18-gauge needle 3 mm from the limbus. A 7-0 (Polyglacín...
TABLE 1. Treatment Groups of Rabbit Eyes Inoculated With Human Cytomegalovirus and Sham Inoculated With Hank’s Balanced Salt Solution

<table>
<thead>
<tr>
<th>Group Intravitreal Injection</th>
<th>Details</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>10 mg ganciclovir-loaded microspheres in 0.1 ml 2% hydroxypropylmethylcellulose (HPMC)</td>
</tr>
<tr>
<td>2</td>
<td>10 mg blank microspheres in 0.1 ml 2% HPMC</td>
</tr>
<tr>
<td>3</td>
<td>150 µg ganciclovir in 0.1 ml distilled water and diluted in 0.1 ml 2% HPMC every 4 days</td>
</tr>
<tr>
<td>4</td>
<td>0.1 ml 2% HPMC</td>
</tr>
<tr>
<td>Sham inoculated with Hank’s balanced salt solution</td>
<td>5 0.1 ml 2% HPMC</td>
</tr>
<tr>
<td>6</td>
<td>10 mg ganciclovir-loaded microspheres in 0.1 ml 2% HPMC</td>
</tr>
<tr>
<td>7</td>
<td>10 mg blank microspheres in 0.1 ml 2% HPMC</td>
</tr>
</tbody>
</table>

HPMC = hydroxypropylmethylcellulose.

910) preplaced mattress suture was used to close the wound. Group 2 eyes (16) were injected with 10 mg of the blank microspheres suspended in 0.1-ml 2% HPMC. Group 3 eyes (16) were injected every 4 days with 130-µg ganciclovir dissolved in 0.1-ml distilled water, and this solution was diluted with 0.1 ml of 2% HPMC using a 27-gauge needle. Group 4 eyes (14) were injected with 0.1 ml of 2% HPMC only. The 30 eyes sham inoculated with HBSS were divided into 3 groups (Table 1). Group 5 eyes (10) were injected with 0.1 ml of 2% HPMC. Group 6 eyes (10) were injected with 10 mg of the ganciclovir-loaded microspheres suspended in 0.1 ml of 2% HPMC. Group 7 eyes (10) were injected with 10 mg of the blank microspheres suspended in 0.1 ml of 2% HPMC. All rabbits underwent indirect ophthalmoscopic analysis through dilated pupils on days 1, 3, 5, 7, 9, 11, and 14 after the last intravitreal injection. All examinations were masked as to the treatment received and were performed concurrently by two observers. Fundus disease was graded on a 0+ to 4+ scale of increasing severity and documented by fundus drawing and photography. Vitritis and inflammatory disease scoring was as follows: 0+, no abnormalities; 1+, isolated strands or cells; 2+, mild-to-moderate haze and infiltration with most of the retina visible; 3+, moderate-to-severe haze and infiltration with most of the retina obscured; and 4+, severe haze and infiltration with the retina totally obscured. Posterior hyaloid or retinal and choroidal disease was scored as follows: 0+, no abnormalities; 1+, focal white posterior hyaloid or retinal infiltrates; 2+, focal-to-geographic white posterior hyaloid or retinal infiltrates and vascular engorgement; 3+, white posterior hyaloid or retinal infiltrates, vascular engorgement, and hemorrhages; and 4+, all of the forgoing plus retinal detachment and necrosis. Optic neuritis was scored as 0+ to 4+, ranging from no abnormality to severe vascular engorgement and nerve head swelling. After the 14-day observation period, selected eyes were enucleated and processed for histopathologic analysis and immunofluorescence.

Microsphere Biodegradation and Tissue Reaction

Six New Zealand white rabbits were divided in two sets of three rabbits. Each set received in two eyes each 5 mg, 10 mg, and 15 mg of ganciclovir-loaded PLGA microspheres. The first set of rabbits was observed for 2 and 4 weeks and then these rabbits were killed. The second set of rabbits was observed for 2, 4, 6, and 8 weeks before they were killed. Tissue reaction was monitored clinically by observing changes in the vitreous (vitreous haze) and retina (edema, chorioretinal atrophy, vascular changes, exudative changes, necrosis) adjacent to the microspheres. The presence and appearance of the microspheres also were noted. Histopathologic analysis was performed on all eyes after the rabbits were killed.

Immunofluorescence Detection of Human Cytomegalovirus Antigens in Chorioretinal Tissues

Detection of HCMV antigens in chorioretinal tissue sections was done by indirect immunofluorescence assay. Routinely fixed paraflin tissue sections of selected enucleated rabbit eyes from groups 1 to 7 and from a normal rabbit eye were used for this procedure. Slides were incubated in an oven (Precision Scientific Company, Chicago, IL) at 60°C for 2 hours to melt the paraffin. The slides were dipped in xylene solution for 5 minutes, then in 100% ethanol for 5 minutes. This was done twice. The slides were again dried in the oven at 80°C for 2 hours. Before immunofluorescence, slides were hydrated in PBS for 5 minutes at room temperature. Sections were overlaid with mouse anticytomegalovirus (blend) monoclonal antibody, which reacts with immediate early-, early-, and late-antigen preparations (20 to 25 µl, Chemicon, Temecula, CA) for 30 minutes at room temperature. The slides were washed in two changes of PBS for 5 minutes. The sections then were overlaid with 20 µl of a 1:10 dilution of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (H & L; Pierce, Rockford, IL) at room temperature. Slides were washed two times in PBS for 5 minutes followed by a final 5-minute wash in distilled water before being air dried. A coverslip was placed over the section after the addition of 15 to 20 µl of a glycerol–PBS solution (4:1). Fluorescence was observed on a photo microscope (Nikon Optiphot, Tokyo, Japan).

To confirm the specificity and the sensitivity of
HCMV antigen immunofluorescent detection, in vitro HCMV-inoculated and non-HCMV-inoculated Hs68 monolayers in chamber slides (Labtek; Nunc, Naperville, IL) were processed for immunofluorescence assay as described above. These serve as positive and negative control samples.

RESULTS

Microsphere Evaluation

After filtration of the microsphere suspension through sieves of 500, 300, 212, 106, and 53 μm, only 10% of the microspheres range in size from 300 to 500 μm. Approximately 40% to 50% of the microspheres ranged in size from 106 to 212 μm. By light microscopy, the microspheres were uniformly spherical, and the surface was smooth with occasional small pores and fracture lines on the surface.

The average concentration of ganciclovir in 300- to 500-μm size microspheres was 81.61 ± 3.50 μg/mg. The average concentration of ganciclovir in 300- to 500-μm size microspheres after 15 minutes’ sterilization by exposure to UV light was 89.77 ± 3.47 μg/mg. No statistically significant difference was found between the two groups (chi-square, \( P = 0.935 \)).

After 15 to 120 minutes of UV exposure of the P. aeruginosa and PLGA microspheres mixture, there was <500 cfu/ml. The P. aeruginosa and PLGA microspheres mixture not exposed to UV showed approximately 10³ cfu.

Microsphere Injection in Rabbit Eyes

Immediately after injection, the microspheres were suspended in the anterior to midvitreous cavity, partially obscuring the view of the fundus. One day after injection, the microspheres were noted in the posterior vitreous cavity predominantly in the lower quadrants.

In eyes inoculated with HCMV (groups 1 to 4), 2 days after inoculation, all eyes exhibited grade 2 to 3 vitritis, grade 1 to 2 retinitis, and grade 1 to 2 optic neuritis. In eyes sham inoculated with HBSS (groups 5 to 7), 2 days after inoculation, no vitritis, retinitis, nor optic neuritis was noted in all eyes (Fig. 1). In group 1, there was a gradual decrease in vitritis from day 3 to day 14 after ganciclovir-loaded microsphere injection. There was a decline in retinitis and optic neuritis on days 3 to 9. No optic neuritis was observed after day 9 (Fig. 2). In group 2, there was an increase in vitritis from day 3 and peaked on day 7 after blank microsphere injection. There was a minimal decrease in vitritis on day 9 but this level was maintained up to day 14. Retinitis increased from days 5 to 9 and this level was maintained up to day 14. There was gradual increase in optic neuritis from days 3 to 14 (Fig. 3). In group 3, there was a minimal, slight increase in vitritis and retinitis on day 3, which decreased considerably up to day 14 after ganciclovir solution injection. Optic neuritis also increased slightly on day 3 but decreased considerably to day 9. From days 9 to 14, no optic neuritis was noted (Fig. 4). In group 4, there was an increase in vitritis and retinitis from days 3 to 9 and this level was maintained up to day 14 after HPMC injection. Optic neuritis increased from days 3 to 5 and this stage was maintained up to day 14 (Fig. 5). In group 5, there was no sign of fundus disease after 2% HPMC injection throughout the 14-day observation period. In group 6, mild vitritis was noted on day 3 but was no longer present from days 5 to 14 after ganciclovir-loaded microsphere injection. There were no signs of retinitis or optic neuritis throughout the 14-day observation period. In group 7, fundus findings were the same as those for group 6.

Microsphere Biodegradation and Tissue Reaction

At 2 and 4 weeks after inoculation, the microspheres still were visible by indirect ophthamoscopic analysis in all rabbit eyes (12/12). The microspheres were in close relation with the retina in the lower quadrants. Clinically, we did not observe changes in the vitreous, (vitritis) retina (edema, chorioretinal atrophy, vascular changes, exudative changes, necrosis, traction), and choroid in areas adjacent to the microspheres in the 12 eyes. The microspheres became smaller and their surfaces became irregular with more pores and cracks. At 4 weeks, some of the microspheres seem to coalesce with each other and decreased in number. Histopathologic analysis after 4 weeks showed the microspheres surrounded by mononuclear cells and poorly formed multinucleated giant cells. The retina and choroid adjacent to the microspheres showed no abnormal tissue reaction.

At 6 and 8 weeks after inoculation, the microspheres still were visible with the indirect ophthalmoscope in the six eyes. The microspheres still were in close relation with the retina in the lower quadrants. Clinically, we did not observe changes in the vitreous, retina, and choroid in areas adjacent to the microspheres in all eyes. The amount of microspheres had decreased and they appeared more dispersed in the lower quadrants. The microspheres became smaller, more fragmented, and irregular in shape. Histopathologic analysis after 8 weeks showed mononuclear and multinucleated giant cells. No inflammatory tissue reaction was observed in the adjacent retina and choroid (Fig. 6).

Immunofluorescence

Controls. The Hs68 cell monolayers inoculated with HCMV showed presence of HCMV antigen by
fluorescence. Predominantly granular intranuclear fluorescence was noted adjacent to the cell nucleus. The Hs68 cell monolayers not inoculated with HCMV did not show specific fluorescence staining (Figs. 7A and 7B).

**Tissue Samples.** In rabbit eyes inoculated with HCMV and injected with ganciclovir-loaded microspheres (group 1) and ganciclovir solution (group 3), immunofluorescence was not shown in serial chorioretinal sections (Fig. 7C). Also, in rabbit eyes sham inoculated with HBSS (groups 5 to 7) and in normal rabbit chorioretinal sections, immunofluorescence was not shown in these negative control tissues.

In rabbit eyes inoculated with HCMV and injected with blank microspheres (group 2) and HPMC (group 4), immunofluorescence indicating HCMV antigens were present in serial chorioretinal sections. Focal areas of immunofluorescence were present in the inner and outer layers of the retina and in the epiretinal area (Fig. 7D).

**Histopathologic Analysis**

In the HCMV-inoculated rabbit eyes treated with ganciclovir-loaded microspheres (group 1) and eyes treated with ganciclovir solution (group 3), minimal vitritis composed of mononuclear cells was present. There was minimal focal disruption of the normal inner retinal architecture (Fig. 8A).

In the HCMV-inoculated rabbit eyes treated with blank microspheres (group 2) and in eyes injected with 2% HPMC (group 4), severe vitritis composed of mononuclear cells and polymorphonuclear leuko-

![Figure 1](image1.png)

**FIGURE 1.** Slit-lamp photographs of human cytomegalovirus fundus disease in rabbit eyes. (A) Two days after inoculation with human cytomegalovirus. (B) Two days after sham inoculation with Hank's balanced salt solution.

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![Figure 2](image2.png)

**FIGURE 2.** Clinical disease grading of eyes injected with ganciclovir-loaded microspheres in human cytomegalovirus-inoculated rabbit eyes (group 1).

![Figure 3](image3.png)

**FIGURE 3.** Clinical disease grading of eyes injected with blank microspheres in human cytomegalovirus-inoculated rabbit eyes (group 2).
Ganciclovir From Microspheres in HCMV-Inoculated Rabbit Eyes

4 n

vitritis
retinitis
optic n.

Days After Microsphere Injection

FIGURE 4. Clinical disease grading of eyes injected with ganciclovir solution in human cytomegalovirus-inoculated rabbit eyes (group 3).

...cytes was noted. There was disorganization of the normal retinal architecture in the outer and inner layers. The choroid showed vascular congestion. Monocytic cell infiltrates also were noted (Figs. 8B,C).

In the non-HCMV-inoculated eyes treated with HBSS (group 5), ganciclovir-loaded microspheres (group 6), and blank microspheres (group 7), histologic sections showed normal retinal and choroidal architecture consistent with the clinical findings.

DISCUSSION

Two percent HPMC was used as a suspension media for the microspheres. Our initial experience with injecting microspheres of 300- to 500-μm size suspended in BSS alone resulted in failure to deliver most of the microspheres. The microspheres adhere to the walls of the syringe and to one another and are unable to pass through the 18-gauge needle during injection. HPMC decreases the affinity of the microspheres with each other by separating them into individual units and enables them to flow pass one another through the bore of the needle more easily. Fernandez-Vigo et al.44 proved that intravitreous injection of HPMC showed good tolerance by retinal pigment epithelium cells, absence of cell death or proliferation, maintenance of retinal pigment cell characteristics, and absence of phagocytosis vacuoles in the cytoplasm.

The rabbit eye is a good model for the study of the intravitreous pharmacokinetics of ganciclovir in the diseased human eye.45 Nonbiodegradable-sustained release devices of ganciclovir have therefore been implanted first in rabbits46 and then in patients28 to test their therapeutic effects. Using ganciclovir-loaded microspheres in rabbit eyes to test its therapeutic effect may parallel its pharmacokinetics if the microspheres are injected in human eyes.

In healthy experimental rabbits with an intact immune system, the vitritis and retinitis may be because of an immunologic reaction to viral antigen and not to a disease process. However, our results show a decrease in vitritis, retinitis, and optic neuritis in eyes injected with ganciclovir-loaded microspheres in the early part of the observation period as compared to eyes injected with blank microspheres. Ganciclovir does not contain antiinflammatory properties, which could explain these results. The early vitritis, retinitis, and optic neuritis we observe, therefore, may be caused by HCMV infection, although the contribution of the immune response also should be considered, especially in the second week of observation. From

FIGURE 5. Clinical disease grading of eyes injected with hydroxypropylmethylcellulose in human cytomegalovirus-inoculated rabbit eyes (group 4).

FIGURE 6. Microspheres surrounded by mononuclear cells and multinucleated giant cells 8 weeks after injection. The retina and choroid adjacent to the microspheres show no abnormal tissue reaction (stain, hematoxylin-eosin; magnification, ×435).
this study, however, we cannot conclude the presence of HCMV replication.

Rabbit eyes inoculated with HCMV and treated with ganciclovir-loaded microspheres (group 1) showed an improvement in fundus disease as compared to eyes injected with blank microspheres (group 2), and 2% HPMC only (group 4). Eyes injected with ganciclovir solution every 4 days (group 4) also showed improvement in fundus disease similar to eyes in group 1. The absence of immunofluorescence in group 1 as compared to eyes in group 2 confirms the effectiveness of these microspheres in controlling the HCMV infection. In addition, the histopathologic analysis of eyes in group 1 showed minimal focal disruption of the retinal architecture as compared to severe chorioretinal changes in group 2.

The effectiveness of the microspheres in controlling the progression of fundus disease can be attributed to the new preparation technique. This technique provides the best ganciclovir loading and release profile from 300- to 500-μm microspheres from a relatively low inherent viscosity (0.39 dl/g) PLGA. Also, this technique has shown that 10 mg of microspheres release ganciclovir in vitro at concentrations within therapeutic range for at least 42 days. The rapid solidification of the polymer at the interface with the SiO external phase results in an excellent yield of well-shaped microspheres that contain the entrapped suspension of ganciclovir in FSiO. The entrapped FSiO and the residual SiO make the microspheres more hydrophobic.

Published reports on the biodegradation and clearance time of microspheres in the vitreous cavity have been discordant. Our study shows that 5, 10, and 15 mg of ganciclovir-loaded microspheres still were present in all eyes 8 weeks after injection. Microspheres have small channels or pores in the matrix formed on evaporation of the solvent. Water molecules penetrate these channels or pores and hydrate the polymer, resulting in matrix degradation. Factors that affect biodegradation include the following:

1. Vitrectomized eyes show faster clearance time compared to nonvitrectomized eyes.
FIGURE 8. Histopathologic analysis of human cytomegalovirus-infected retina (stain, hematoxylin–eosin; magnification, X270). (A) Retina from eye injected with ganciclovir-loaded microspheres (group 1) showing minimal focal disruption of the inner retinal architecture. (B) Retina from eye injected with blank microspheres (group 2) showing disorganization of the normal retinal architecture in the outer and inner retinal layers. Vitritis with mononuclear cells is noted. (C) Retina from eye injected with 2% hydroxypropylmethylcellulose (group 4).

2. Low molecular weight polymer and copolymer have faster degradation time with 50:50 PLGA having the shortest half-life.48

3. The presence of drug may affect degradation time.58

4. Smaller size microspheres show faster degradation.49

Clinically, no inflammatory signs were noted in the vitreous, retina, and choroid up to 8 weeks after injection of up to 15 mg of ganciclovir-loaded microspheres. Histologically, at 4 and 8 weeks, a mild, localized foreign body reaction surrounding partially degraded microspheres was noted with no involvement of the retina or other ocular structures. Sutures made from polymers have been shown to cause localized inflammation and foreign body reaction that diminishes over time.50 Similarly, polymer microspheres injected intramuscularly cause mild foreign body giant cell reaction by day 4 with a tendency to decrease over time.51 The ganciclovir-loaded microspheres, therefore, are not toxic to ocular tissue, and the tissue reaction seen histologically is similar to that described for other medical applications of polymers.

In conclusion, 10 mg of 300- to 500-μm ganciclovir-loaded microspheres from 50:50 poly(DL-lactide-co-glycolide) control the progression of fundus disease in HCMV-inoculated rabbit eyes as shown by clinical fundus disease monitoring, histopathologic analysis, and immunofluorescence detection of HCMV antigen in chorioretinal sections. Ganciclovir-loaded microspheres are not toxic to ocular structures within 2 months of injection as confirmed by clinical observation and histopathologic analysis. The biodegradable nature of this system gives it an added advantage over other systems. Results suggest that clinical trials using ganciclovir-loaded microspheres may be effective in controlling the progression of CMV chorioretinitis.

Key Words
acquired immune deficiency syndrome, cytomegalovirus, ganciclovir, microspheres, vitreoretinal disease

Acknowledgment
The authors thank Fee-Lai Leong for her technical assistance.

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