The Effect of Cidofovir on Cytomegalovirus-Induced Hearing Loss in a Guinea Pig Model

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Objective: To evaluate the utility of therapy with the cyclic cogener of the anti-cytomegalovirus (CMV) agent cidofovir against CMV-induced hearing loss in a guinea pig model.

Design: Thirty-six guinea pigs were randomly divided into 4 groups of 9. All groups underwent auditory brainstem response testing on days 0, 4, 7, 14, 21, and 28. Group 1 received no intervention. Group 2 underwent sham surgery consisting of unilateral round window injection of 25 µL of sterile viral media on day 0. Groups 3 and 4 underwent round window injection of 1.7 × 10^6 plaque-forming units of guinea pig CMV on day 0. Group 4 received antiviral treatment with intraperitoneal injection of cidofovir (20 mg/kg) on days 1 and 5 after inoculation.

Setting: An animal research facility.

Subjects: Thirty-six weanling Hartley guinea pigs.

Results: Of the animals who received guinea pig CMV and no cidofovir treatment, 4 of 9 (day 4) and 5 of 9 (days 7 and 28) demonstrated a hearing loss of at least 30 dB. In contrast, none of the animals in the untreated, sham surgery, or cidofovir-treated groups had a hearing loss of greater than 20 dB. This difference was statistically significant for day 4 (P = .04, 1-tailed Fisher exact test), day 7 (P = .01), and day 28 (P = .01). Histologic evaluation of hearing-impaired animals revealed inflammatory infiltrates, particularly in the scala tympani. Fibrosis of the basal turn of the cochlea was observed in 7 of 9 untreated animals and 1 of 9 treated animals.

Conclusion: Cidofovir therapy prevents CMV-induced hearing loss and associated histologic changes in guinea pigs.

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infections.\textsuperscript{11,12} Furthermore, in contrast to ganciclovir, GPCMV is fully susceptible to cidofovir, making this an ideal therapy to evaluate in this animal model.\textsuperscript{13} In vitro, cidofovir inhibits viral DNA synthesis and replication for 1 week or longer after a 6-hour exposure,\textsuperscript{13} much longer than the effect of ganciclovir, which is measured in hours. The US Food and Drug Administration has approved cidofovir for the treatment of CMV retinitis with a typical intravenous dosing schedule of weekly injections for 2 weeks (loading doses) followed by maintenance doses every 2 weeks based on studies by Lalezari et al\textsuperscript{14} and Berenguer and Mallolas.\textsuperscript{15} The primary toxic effect of intravenous cidofovir treatment is nephrotoxicity,\textsuperscript{13} which typically resolves with the cessation of therapy. Cyclic cidofovir is metabolized into cidofovir and has been associated with lower nephrotoxicity in both animal\textsuperscript{16} and human\textsuperscript{17} studies.

The pharmacologic profile of cyclic cidofovir makes it a potential candidate for the treatment of neonates with congenital CMV infections. The long intervals between dosing allow the drug to be administered without prolonged intravenous access, and the avoidance of neutropenia is desirable in a population that is already immunocompromised. To date, however, there has been no information available about the potential efficacy of cidofovir for CMV-induced deafness in a relevant animal model. Therefore, the objective of this study was to evaluate the effect of systemic administration of cyclic cidofovir on SNHL and associated cochlear histologic changes in guinea pigs.

METHODS

SUBJECTS

Thirty-six GPCMV-seronegative weanling Hartley guinea pigs (Harlan, Indianapolis, Ind) weighing 250 to 350 g were obtained. Animals were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Once inoculated with GPCMV, animals were separated, with exposed animals isolated from unexposed animals. The Institutional Animal Care and Use Committee approved all procedures.

GUINEA PIG CMV

Guinea pig CMV (strain No. 22122; ATCC VR682) was propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 138) and maintained in F-12 medium supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, Utah), 10,000 IU/L of penicillin G sodium, 10 mg/L of streptomycin sulfate (Gibco-BRL; Rockville, Md), and 7.5% sodium bicarbonate (Gibco-BRL).

EXPERIMENTAL DESIGN

Animals were randomly divided into 4 groups of 9. Hearing was evaluated for all animals by performing auditory evoked brainstem response (ABR) testing on days 0, 4, 7, 14, 21, and 28. Toe clips were performed and blood collection with no other intervention. Group 2, the sham surgery group, received a round window injection of sterile viral media on day 0. Groups 3 and 4 received a round window injection of 1.7 × 10^7 plaque-forming units (PFU) of GPCMV on day 0. Group 3 received sham intraperitoneal injections of sterile saline on days 1, 3, and 16 after inoculation, while group 4 received antiviral treatment with an intraperitoneal injection of cyclic cidofovir (20 mg/kg) on days 1, 3, and 16 after GPCMV inoculation. The cyclic cidofovir used for this study was kindly provided by Gilead Sciences Inc (Foster City, Calif) and was stored refrigerated at 4°C. For administration, cyclic cidofovir was dissolved in sterile distilled water, and the solution was adjusted to pH 7.4 with sodium hydroxide.

ANESTHESIA

Guinea pigs underwent inhalational anesthesia with isoflurane for surgical procedures and ABRs. Animals were placed into an induction chamber with 3.5% isoflurane and supplemental oxygen. After adequate induction of anesthesia, 2% isoflurane was used for maintenance of anesthesia and it was titrated to achieve an effect as needed. Oxygen saturation was monitored with pulse oximetry and maintained at greater than 95% during all procedures.

SURGICAL PROCEDURE

Surgical exposure of the bulla was obtained via a postauricular incision. After dissection of subcutaneous tissue and removal of muscular attachments, several small holes were drilled with a hand drill in the posterosuperior aspect of the bulla. A bone chip was then removed from the bulla, and the hole enlarged to allow adequate exposure of the round window. A micropipette was then placed through the round window, and 1.7 × 10^5 PFU of GPCMV suspended in 25 µL of viral media was injected into the scala tympani. In animals undergoing sham surgery, 25 µL of sterile viral media was injected. After injection, a small piece of fascia from the postauricular wound was placed over the round window defect. The incision was then closed in layers with absorbable suture. In concert with the Institutional Animal Care and Use Committee guidelines, postoperative checks including weights, wound checks, overall appearance, and food intake were monitored daily for 3 days.

REAL-TIME POLYMERASE CHAIN REACTION FOR DNAMemia AND ELISA ASSAY

To ensure that control groups remained unexposed to GPCMV and to examine for systemic infection in treated and control animals inoculated with GPCMV, blood was obtained by toe clip on days 0, 4, 7, 14, 21, 28 and was analyzed for viral DNA using real-time polymerase chain reaction (PCR) analysis.\textsuperscript{18} Heparinized blood (volume, 100 µL) was purified by Qiagen column extraction, using the QIAamp DNA mini kit (Qiagen, Inc, Valencia, Calif), according to the manufacturer’s specifications. Before the samples were subjected to real-time PCR, they were analyzed by spectrophotometry to determine A_{320} DNA concentrations. The GP83-specific primer pair, upstream primer UL83 F6 (5′-CGACGACGAGTACGAAATC-3′), and downstream primer UL83 B11 (5′-TCTCCTGCCTCAACGGAAGGT-3′) amplify a 225-base pair (bp) region, corresponding to Asp402 through the target sequence at a concentration of 10^{-3} genome copies per microliter. For unknown samples, 2 µL of sample at an approximate concentration of 0.1 µg was used. The PCR was performed...
using the Roche LightCycler 1.0 instrument under the following conditions: initial denature at 95°C for 10 minutes, followed by 95°C for 3 seconds, 64°C for 5 seconds, 72°C for 8 seconds for a total of 45 cycles, followed by melting curve analysis starting at 64°C and ending at 95°C, then a final hold at 40°C. The data were analyzed with LightCycler Data Analysis (LCDA) Software 1.0 (Roche Diagnostics). For quantification, standard curves were generated using dilutions of plasmid and viral DNA at known concentrations. The sensitivity of the assay was consistently between 1 and 10 copies per reaction. Viral load was expressed as copy numbers per milliliter of blood. For added confirmation of positive results, gel electrophoresis was performed, on a 2.25% PCR-grade agarose gel.

Seroconversion was determined by ELISA analysis of blood obtained on days 0 and 28 using GPCMV antigen as described by Schleiss et al.19

AUDITORY EVOKED BRAINSTEM RESPONSES

Auditory evoked brainstem responses were obtained under inhalational anesthesia. A microscope was used to examine each animal’s external auditory canal and tympanic membrane prior to each ABR. Cerumen was removed as needed, and all animals remained free of tympanic membrane or middle ear disease for the duration of the study. Needle electrodes were placed at the vertex and in the postauricular region of each animal. A small piece of intravenous tubing was used as an earpiece for the transducer. The earpiece was placed into the lateral portion of the animal’s ear canal. Click stimuli were delivered stepwise in 10-dB increments from 80 to 0 dB. The ABRs were obtained using the SmartEP system (Intelligent Hearing Systems, Miami, Fla), and thresholds were recorded after each session.

Figure 1. Difference in enzyme-linked immunosorbert assay (ELISA) titers in treated vs untreated animals. Error bars indicate standard deviation.

Figure 2. Normal auditory evoked brainstem responses (ABRs) from animals in group 2 (sham surgery) (A) and group 4 (cidofovir-treated guinea pig cytomegalovirus) (B); ABRs depicting hearing loss from animals in group 3 (untreated guinea pig cytomegalovirus) at thresholds of 30 dB (C) and 80 dB (D).
TEMPORAL BONE HISTOPATHOLOGY

After completion of the final ABR, anesthetized animals were given a lethal intracardiac injection of 0.5 mL sodium pentobarbital (390 mg/mL, approximately 500 mg/kg). Next, the chest was opened and an intracardiac perfusion of 10 mL of cold 0.9% saline was delivered, followed by 10 mL of cold 4% paraformaldehyde. Temporal bones were harvested, and the cochlea was gently infused with cold 4% paraformaldehyde and then stored in paraformaldehyde overnight at 4°C. Specimens were then decalcified in 0.1M EDTA and processed into paraffin. The specimens were then sectioned (8 µm thick), mounted, and stained.

STATISTICAL ANALYSIS

Fisher exact test was used for comparison of incidence data between groups. Since the expected change in hearing was unidirectional, a 1-tailed design was used. Antibody titers were compared by the unpaired t-test.

RESULTS

SYSTEMIC EXPOSURE AND IMMUNOLOGIC RESPONSE

All animals were seronegative with no evidence of systemic viral DNA by PCR on day 0. All animals in groups 1 (observation only) and 2 (sham surgery) remained seronegative and virus free for the duration of the study. All animals in groups 3 (untreated) and 4 (treated) seroconverted during the study. Interestingly, there were significant differences in ELISA antibody titer between the treated and untreated groups. The mean ± SD ELISA titer in untreated animals was 3.1 ± 0.1 log10. In contrast, in the cidofovir-treated animals, the mean ± SD titer was 2.8 ± 0.4 log10 (P = .05, t-test) (Figure 1). One animal in group 3 developed high-grade DNAemia during the study (4.8 log10 genome equivalents per milliliter), but no animals in the treatment group had demonstrable DNAemia.

AUDITORY EVOKED BRAINSTEM RESPONSES

Representative normal and abnormal ABRs are demonstrated in Figure 2. Hearing loss (threshold shift) for each animal is depicted in Figure 3. Threshold shifts of 10 dB from baseline, which may be attributed to electrode placement or microphone placement, were considered negligible. All animals had baseline thresholds of 0 to 10-dB sound pressure level. For the purpose of this study, a threshold shift of 30 dB or higher was considered to represent a definite hearing loss.
Animals in groups 1 (observation only, Figure 3A) maintained stable thresholds throughout the study with variations of 10 dB or less. One animal in group 2 (sham surgery, Figure 3B) had an initial postoperative threshold shift of 20 dB, which returned to baseline within 2 weeks. All other animals in group 2 had stable thresholds throughout the study.

Animals in group 3 received intracochlear GPCMV inoculation and intraperitoneal saline injections. Four animals had threshold shifts of 30 dB or more on day 4, significantly more than the other groups (P = .04, 1-tailed Fisher exact test). Five animals had threshold shifts of 30 dB or more on day 7 (P = .01), day 21 (P = .01), and day 28 (P = .01). Three animals had fluctuating hearing loss, one of which returned to baseline by the end of the study. Two animals had progressive hearing loss. These patterns are depicted in Figure 3C. All animals in group 4 (cidofovir treated, Figure 3D) maintained stable hearing throughout the study.

TEMPORAL BONE HISTOPATHOLOGY

Histologic analysis revealed no evidence of cochlear abnormality for any animal in group 1 or group 2. Four animals in group 3 had cellular infiltrates composed of lymphocytes and neutrophils involving the scala tympani (Figure 4) with Reissner membrane thickening and scala vestibuli infiltrate present in 2 animals. All 4 animals with cellular infiltrate present in the cochlea had hearing loss demonstrated by ABR. No animals in groups 1, 2, or 4 had any cellular infiltrates noted.

Seven animals in group 3 and 1 animal in group 4 developed a fibrous infiltrate of the round window and basal turn of the cochlea (Figure 5 and Figure 6). No evidence of round window or basal turn fibrosis was noted in groups 1 or 2. All animals from group 3 who had hearing loss also developed fibrosis of the round window and basal turn of the cochlea.

COMMENT

Cytomegalovirus-associated SNHL affects thousands of neonates per year,1 costing an estimated $1.9 billion annually.20 To date, there is limited information regarding the use of antiviral therapy in the setting of congenital CMV, but ganciclovir therapy has been shown in a phase 2 study to delay progression of hearing loss for 6 months in treated infants. Unfortunately, the use of ganciclovir has been associated with significant toxic effects and was associated with neutropenia in 63% of infants in that study.3 Given the toxicity of
ganciclovir, alternative antivirals for CMV have been considered. One such alternative is the drug cidofovir, which has been used successfully to treat CMV retinitis. Nephrotoxicity is the primary adverse effect of cidofovir. The cyclic cogen of cidofovir is associated with lower nephrotoxicity. Animal models have shown the safety and efficacy of cyclic cidofovir for GPCMV infection when used in an immunosuppression model in which guinea pigs were pretreated with cyclophosphamide prior to viral inoculation. The present study demonstrates that cyclic cidofovir is effective in preventing GPCMV-induced SNHL and associated histologic changes in guinea pigs for 1 month after infection. These results are encouraging and may point to a future role for cidofovir and cyclic cidofovir in the prevention of CMV-associated SNHL in humans.

Studies of antiviral drugs in animal models of CMV must use animal CMVs because CMV is a species-specific virus and infection of animals with human CMV is not possible. Guinea pig CMV crosses the placenta unlike the CMVs of other small mammals, and it mimics the presentation of congenital human CMV with increased pup mortality and end-organ disease that includes the cochlea. These characteristics, as well as the accessibility of the inner ear and availability of guinea pigs, make GPCMV an excellent model for the study of CMV-associated SNHL. Previous studies by Harris et al and Woolf et al have demonstrated cellular infiltrates in the cochlea 8 days after inoculation. Cochlear fibrosis after longer-term CMV infection, as seen in our study, has been previously reported in humans as well as guinea pigs. Such fibrosis often leads to ossification. Both ossification and fibrosis of the membranous cochlea may lead to difficulty in placement of cochlear implant electrodes.

Another limitation of the usefulness of GPCMV for antiviral studies has been its intrinsic resistance to ganciclovir, compared with human CMV. However, GPCMV is fully susceptible to cidofovir, the antiviral agent used in the present study. Thus, further study of this antiviral agent in the guinea pig model should be relevant to treatment and pathogenesis of CMV-induced labyrinthitis and deafness in humans.

It was of interest that GPCMV-induced hearing loss appeared to fluctuate in severity in some of the infected animals. Fowler et al have demonstrated progressive, fluctuating, and delayed SNHL associated with congenital CMV. The median age at first progression was 18 months in this study, and the median age of detection for delayed onset SNHL was 27 months. Although we observed a benefit of antiviral therapy during a short period of evaluation (28 days), it is

Figure 5. Normal basal turn scala tympani with no infiltrate is noted in the sham surgery (A) and cidofovir-treated guinea pig cytomegalovirus (B) groups; fibrosis of the scala tympani (arrows) is noted in the untreated guinea pig cytomegalovirus group (C and D).
unclear whether a beneficial impact against CMV-induced deafness would continue to be observed on long-term follow-up. Future studies will be required in both newborn infants as well as in relevant animal models to ascertain the optimal duration of antiviral treatment required to sustain efficacy against CMV-induced deafness.

An additional limitation of our study was the fact that click stimuli were used to evaluate auditory thresholds rather than tone bursts, which would provide more detailed information regarding frequency-specific SNHL. In designing the study, click stimuli were selected to demonstrate gross hearing loss similar to the studies by Woolf et al. Particularly since histologic changes were most prominent in the basal turn, high-frequency SNHL may be expected as a consequence of cochlear infection in this animal model. Two animals in group 3 of this study had evidence of basal turn fibrosis with no threshold shift in response to click stimuli. This finding was somewhat unexpected; otherwise, frequency-specific testing would have been performed prior to harvesting the temporal bones. High-frequency SNHL is easily missed with click stimuli, which may explain the disagreement between the physiologic and histologic results for these animals. Future studies should incorporate measurement of otoacoustic emissions and tone burst stimuli to assess cochlear injury and frequency-specific hearing.

In conclusion, cyclic cidofovir prevents GPCMV-induced SNHL and associated cochlear histologic changes in guinea pigs. While more study is needed, these results suggest that cidofovir may have a potential role in the treatment and prevention of CMV-related congenital SNHL in humans.

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REFERENCES