Development of Cytomegalovirus-Mediated Sensorineural Hearing Loss in a Guinea Pig Model

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Objective: To develop an animal model for cytomegalovirus (CMV)–induced sensorineural hearing loss.

Design: Guinea pig model.

Setting: University of Utah otolaryngology research labs.

Participants: Thirty-one Hartley guinea pig pups were divided into 4 groups. Group 1 pups were delivered from pregnant dams inoculated with $1 \times 10^5$ plaque-forming units (PFU) of guinea pig CMV (gpCMV). Group 2 and group 3 pups were delivered from pregnant dams inoculated with higher doses of 2 and $4 \times 10^5$ PFU of gpCMV, respectively. Group 4 pups, the control group, were delivered from uninoculated dams.

Main Outcome Measures: All groups underwent weekly auditory brainstem response studies. Six weeks after delivery, the brain, cochlea, salivary glands, lungs, liver, and kidneys were harvested. All tissue except the cochlea was analyzed for histologic evidence of the virus. All tissue underwent polymerase chain reaction (PCR) to detect gpCMV.

Results: Seven of the 19 (37%) inoculated pups developed a 30-dB hearing loss; none of the animals in the control group had a worse click threshold than 20 dB. Group 1 pups demonstrated statistically significant asymmetric hearing loss. All 3 inoculated groups showed evidence of progressive hearing loss over time. The control group did not demonstrate evidence of progressive threshold worsening. The PCR testing detected gpCMV in the cochleas of group 2 and group 3 animals.

Conclusions: We have successfully demonstrated elevated auditory brainstem response click thresholds with characteristics of progressive and asymmetric loss that have been reported in clinical reports of congenital CMV infection. We also detected gpCMV via PCR testing in the cochleas of inoculated pups.

Cytomegalovirus (CMV) is the most common congenital viral infection and an important cause of sensorineural hearing loss (SNHL), with an estimated 40,000 newborns infected annually.\(^1\)\(^-\)\(^3\) It is estimated to account for at least one-third of SNHL in young children.\(^4\) In addition, it is estimated to cause more hearing loss in children than did Haemophilus influenzae meningitis in the pre–H influenzae type b vaccine era.\(^5\)\(^,\)\(^6\) The protean nature of its presentation and its adverse effect on speech and language development contribute to the billions of dollars required for audiologic testing and special services.\(^7\) Active research has concentrated on vaccination or other therapies for the neurologic effects of this condition. Less work has been performed to understand the pathophysiology of CMV-induced SNHL or its potential treatment. A greater understanding of CMV-mediated SNHL using a small animal model, the guinea pig, for congenital CMV, would provide substantial inroads for future diagnosis and treatment.

A guinea pig model has been used by a number of investigators to evaluate the effects of vaccines and passive antibody on congenital CMV infections.\(^8\)\(^-\)\(^10\) In contrast to other rodent CMVs, the guinea pig CMV (gpCMV), like the human CMV, crosses the placenta, infecting the pup in utero.\(^11\) The guinea pig gestational periods are fairly lengthy and can be conveniently divided into trimesters.\(^12\) The guinea pig placenta, like the human placenta, is hemochorial, with a single trophoblast layer separating maternal and fetal circulation.\(^11\)

The goal of this study was to develop CMV-induced congenital SNHL in the guinea pig model. We hypothesized that the inoculation of gpCMV during the sec-
ond trimester in pregnant guinea pig dams would result in transmission of gpCMV and elevated auditory brainstem response (ABR) click thresholds in newborn pups.

**PREPARATION OF VIRUS AND CELLS**

We obtained gpCMV (strain 22122; American Type Culture Collection, Rockville, Maryland) from the laboratory of M.R.S. The strains were propagated on guinea pig fibroblast lung cells (GPL ATCC CCL 138) and maintained in Ham F12 medium (Life Technologies Corporation, Carlsbad, California) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co, St Louis, Missouri), 1% antibiotic-antimycotic (＞100) liquid (Life Technologies Corporation), and 7.5% sodium bicarbonate (Life Technologies Corporation).

**PREGNANCY, BREEDING, AND VIRAL CHALLENGE**

Hartley guinea pigs were used for this experiment. Dams were bred with males purchased from Elm Hill Breeding Laboratories (Chelmsford, Massachusetts). Preinoculation serum samples were obtained to ensure no previous exposure to gpCMV by testing for CMV antibody by enzyme-linked immunosorbent assay. Only seronegative animals were used. Breeding was undertaken at a ratio of 3:1 (female to male), and pregnancy was monitored by palpation and assessment of dates. The animals infected with gpCMV were housed separately from the unin-  

**HEARING ASSESSMENT**

All groups underwent weekly ABR studies for 6 weeks. The ABR studies were recorded using an auditory evoked potential diagnostic system (EP version 3.42 system; Intelligent Hearing Systems Corporation, Miami, Florida). Subdermal needle electrodes (F-E2; Astro-Med, Inc, West Warwick, Rhode Island) were fed by a bioamplifier (Opti-Amp 3000; Intelligent Hearing Systems Corporation) with a 100,000 amplification gain and a 300-Hz to 5-kHz filter setting and placed behind each auricle and at the vertex. Rarefaction clicks (100 microseconds) at a rate of 29.3/second were delivered into insert earphones with the foam earplugs inserted into the external auditory canal. Impedance was maintained at 3 kΩ or less. Electrical activity was amplified 100,000 times and bandpass filtered from 30 to 150 Hz, and up to 512 sweeps were averaged. The averaged waveforms were stored on a personal computer. The averaged click responses were obtained at 10-dB intervals in the range of 0 to 80 dB. Thresholds were defined as the lowest intensity to yield a nonreproducible deflection in the evoked response traces for each animal.

Data were entered into Microsoft Excel 2003 (Microsoft Corporation, Redmond, Washington) and imported into STATA/IC 10.0 for Windows (StataCorp, Chicago, Illinois) for statistical analysis. Treatment was categorized by dose (0, 1, 2, and 4 × 10^5 PFU of gpCMV) and dichotomized by treatment status (treated vs untreated). Cross-sectional time-series regression was used to longitudinally assess differences in averaged click responses across treatment groups with time as a random effect. Analyses used right and left ear measures. Treatment was categorized by dose (0, 1, 2, and 4 × 10^5 PFU of gpCMV) and dichotomized by treatment status (treated vs untreated).

**HISTOLOGIC AND REAL-TIME PCR STUDIES**

Six weeks after delivery, the guinea pig pups were euthanized, and the brain, cochlea, salivary glands, lungs, liver, and kidneys were harvested. The paries labyrinthicus housing the cochlea was exposed after dissection of the temporal bone bulla. Removal of the thin otic capsule under microscopic visualization permitted careful removal of the cochlea. All tissue underwent PCR analysis.

The DNA was extracted from guinea pig tissues using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instruction protocol. The DNA concentrations were determined with a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific Inc, Wilmington, Delaware).

The GP83-specific primer pair, upstream primer UL83F6 (5’CGACGAGGACGATGACGAAAAC-3’), and downstream primer UL83B11 (5’TCTCCTGGTCACAGGAGGTCG3’) were used in this study to detect the presence of CMV DNA in the tissues. The primers were synthesized by the DNA- peptide facility of the Huntsman Cancer Institute, University of Utah. Under optimized conditions, this primer pair amplifies a 225-base pair region, corresponding to Asp402 through Ser473 of the GP83 protein, and generates a single band of expected size on agarose gel. The PCR was performed on a rapid air-heated thermal cycler (LightCycler; Roche Applied Science, Indianapolis, Indiana), in a volume of 20 µL, containing 2 µL of DNA, a 1.5mM concentration of magnesium chloride, a 70mM concentration of TRIS hydrochloride (pH 8.3), 5 µg of bovine serum albumin, a 200µM concentration of each deoxyribonucleotide triphosphate, a 0.5µM concentration of each primer, a 1:30,000 dilution of SYBR Green 1 (Life Technologies Corporation), 0.4 U of Taq DNA polymerase (Roche Applied Science), and 110 ng of TaqStart antibody (Clontech Laboratories Inc, Mountain View, California). The CMV culture at a concentration of 1 × 10^6 PFU/mL was used as the positive control and a no-DNA-added sample was used as the negative control. Four replicates were measured for each sample. The following cycle program was used: 95°C for 2 minutes, followed by 43 four-step cycles, each comprising denaturation at 95°C for 0 seconds, annealing at 64°C for 10 seconds, extension at 72°C for 10 seconds, and detection at 88°C for 2 seconds. Measurement of the PCR amplicon concentration was performed at the last step of each cycle. After amplification, a melting curve was acquired in the range of 55°C to 95°C with a ramp speed of 0.1°C per second. The PCR products underwent electrophoresis through a 2% agarose gel containing 0.5 µg/mL of ethidium bromide.

The brain, salivary glands, liver, lungs, and kidneys were dissected and stored in 5% paraformaldehyde for histopatho-
whose mother was inoculated with 1x10^5 plaque-forming units (PFU) of guinea pig cytomegalovirus (gpCMV). Group 2 consisted of pups inoculated with 2x10^5 PFU of gpCMV. Group 3 consisted of pups inoculated with 4x10^5 PFU of gpCMV. Group 4 was the untreated control group.

### STATUS OF STUDY ANIMALS

Fifteen female and 11 male guinea pig pups were initially procured for this study. Three of the females (F1, P1, and P2) and 4 of the males (M4-M7) were excluded because of seropositive responses on their enzyme-linked immunosorbent assay testing.

Six of the pregnant dams were inoculated with varying doses of gpCMV during the second trimester. Two of each group were injected with 1, 2, and 4x10^5 PFU of virus. Six pregnant dams were not inoculated with the virus (control group 4). One (17%) of the control dams died before delivery. None of the inoculated pregnant dams died before delivery. Thirty-one Hartley guinea pigs were successfully delivered. Four of the inoculated pups (21%) died prematurely between 2 and 5 weeks of life. None of the control pups died prematurely.

### AUDITORY-EVOKED BRAINSTEM RESPONSES

#### Evidence of Asymmetry

An analysis evaluating the degree of hearing threshold symmetry was performed first. As shown in Figure 1, pups whose mother was inoculated with 1x10^5 PFU of gpCMV demonstrated a statistically significant difference between the ears on the basis of a Wilcoxon signed rank test (P = .004). The P values for the 2x10^5 PFU and 4x10^5 PFU groups were 0.64 and 0.08, respectively. The control group did not show a statistically significant difference between the ears.

#### Comparison Among the Groups for Hearing Loss

Representative normal and abnormal ABR thresholds are shown in Figure 2. Five of the 6 pups born to dams that received the highest gpCMV dose (group 3) demonstrated hearing loss of 30 dB. None of the 5 pups who received the next highest dose (group 2) demonstrated hearing loss of 30 dB. Two of the 8 pups in group 1 and none of the animals in group 4 (control) had hearing loss greater than 20 dB. The combination of groups 1 through 3 for each ear and comparison of the thresholds to those of the control group demonstrated a statistically significant difference with respect to dose independent of time and over time for the left ear (Figure 3). The right ear ABR click threshold measurements demonstrated a statistically significant difference between groups 1, 2, and 3 (inoculated) and group 4 (control) over time (P = .001) but not independent of time (P = .07) (Figure 4). When each treated group was compared with the control group individually, the right ear groups were statistically significantly different from the control group for the period but not independent of time (P = .03 for the inoculated vs uninoculated groups over time; P = .20 for groups 1 and 2; P = .08 for group 3) (Figure 5). The left ear ABR click threshold measurements demonstrated a statistically significant difference between groups 1 and 3 (inoculated) and group 4 (control) regardless of the period or over time (P = .02 for group 1, P = .30 for group 2, P = .003 for group 3, P = .001 for the treated vs untreated groups over time) (Figure 6).

Evidence of Progressive Hearing Loss Over Time

The control group did not show evidence of progressive hearing loss over time. Group 1 (right and left ear), group 2 (left ear only), and group 3 (left ear only) showed progressive loss that was statistically significant on the basis of a regression analysis.

#### Real-Time PCR Findings

The number of guinea pigs with detectable gpCMV for each tissue site based on PCR is indicated in the Table. Overall, only a small number of guinea pigs had gpCMV detected in each site. We detected gpCMV in the cochleas of groups 2 and 3 (Table). No gpCMV was detected in the control, uninoculated animals (group 4). Quantitative measures of viral load were not performed given the low number of copies per reaction.

### Histopathologic and Immunohistochemical Analyses

Histologic analysis revealed no evidence of inflammation or virus in the brain, salivary gland, lung, liver, or kidney tissue for all the groups. Routine histologic analysis was not performed on cochlear tissue.

A better understanding of the pathophysiology of this common congenital infection and a useful animal model for antiviral therapy or vaccination would benefit the thousands of newborns and immunocompromised patients with this condition. Evidence from the murine CMV model suggests that SNHL and inner ear infection can be modeled in mice. Davis and Hawriskiak15 inoculated newborn mice...
intracerebrally with murine CMV and found that the infection spreads rapidly into the scala tympani and spiral ganglia along the eighth nerve and cochlear aqueduct. Unfortunately, murine CMV does not cross the placenta or infect the fetus, which makes this a model of limited value for congenital CMV-associated hearing loss.

In contrast to murine CMV, gpCMV crosses the placenta and infects the pup in utero, and this model has

Figure 2. First tracings depict normal responses; second tracings demonstrate hearing loss in group 1 animals (30-dB threshold). Numbers in the tracing labels represent the decibel threshold used.

Figure 3. Left ear auditory brainstem response (ABR) click threshold comparison of combined inoculated vs uninoculated groups. Error bars indicate 95% confidence intervals. \( P < .02 \) for the combined groups vs the control independent of time; \( P < .001 \) for the combined groups vs the control over time.

Figure 4. Right ear auditory brainstem response (ABR) click threshold comparison of combined inoculated vs uninoculated groups. Error bars indicate 95% confidence intervals. \( P = .07 \) for the combined groups vs the control independent of time; \( P = .001 \) for the combined groups vs the control over time.
been used to study viral labyrinthitis. Harris et al\textsuperscript{18} inoculated Hartley guinea pigs with gpCMV via selective perilymphatic perfusion, which resulted in a viral labyrinthitis that inexorably led to profound SNHL within 8 days. These studies were followed by analysis of a transplacental mode of transmission with emphasis on evaluation for labyrinthitis.\textsuperscript{19}

Strauss and Griffith\textsuperscript{20} infected first-trimester, second-trimester, or third-trimester pregnant guinea pigs with salivary gland multiple passaged gpCMV and found histologic immunocytochemical and/or cell culture evidence of CMV infection in 24% of fetal or newborn tissue. Woolf et al\textsuperscript{21} performed higher-dose gpCMV injections of first-trimester and second-trimester pregnant guinea pigs via intracardiac injection and demonstrated temporal bone involvement in 45% of infected neonates and an auditory deficit in 28% of the offspring. We modified the inoculation technique of Woolf et al\textsuperscript{21} and Strauss and Griffith by use of a higher subcutaneous dose to detect labyrinthitis via the more sensitive PCR techniques and to measure neonatal auditory function over time by means of serial ABR studies. We used a subcutaneous delivery method to minimize morbidity yet achieve a high rate of transmission.

The results of our study demonstrated similar characteristics of hearing loss seen in children with congenital CMV-induced SNHL. Pups whose mothers were inoculated with 1 \times 10^5 PFU of gpCMV showed statistically significant asymmetric SNHL. Fowler et al\textsuperscript{22} reported that 50% of 22 children with congenital CMV infection had unilateral hearing loss. The inoculated animals demonstrated statistically significant worsening click thresholds compared with controls over time. Of relevance to congenital CMV infections in infants, we noted statistically significant progressive hearing loss in the inoculated groups. These findings are consistent with the study by Dahle et al\textsuperscript{23} of 860 children with congenital CMV. Polymerase chain reaction testing demonstrated a low number of animals with the virus in the various tissue samples evaluated. We were able to detect the virus in the cochleas of animals inoculated with 2 and 4 \times 10^5 PFU of gpCMV. We were not able to demonstrate signs of an inflammatory response in the brain, salivary glands, lungs, liver, or kidneys by means of histologic studies; however, the cochleas were not examined histologically. These observations may be of great relevance to infants. Most congenitally infected infants who develop SNHL appear healthy at birth and clinically have no evidence of end-organ disease. Despite the subclinical evidence of infection, there is nonetheless a 10% to 15% risk of development of SNHL. The factors that dictate tropism of CMV to the cochlea, even in the absence of systemic or end-organ disease, are unknown. It may be that the blood-labyrinthine barrier\textsuperscript{23} prevents immunocytes from infiltrating the cochlea and clearing viral infection: this theory may also explain the lack of inflammatory cells we observed in PCR-positive cochleas from hearing-impaired guinea pigs. Strauss and Griffith\textsuperscript{20} were also unable to demonstrate evidence of viral labyrinthitis on the basis of routine histologic analysis and an avidin-biotinylated horseradish peroxidase complex method using similar doses.

In conclusion, second-trimester pregnant dams inoculated with gpCMV deliver pups with elevated ABR click thresholds and evidence of gpCMV localization in the cochleas, as assessed by PCR. Our findings of hearing loss with evidence of viral DNA in the cochlea but in

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Abbreviation: gpCMV, guinea pig cytomegalovirus.
the absence of systemic disease or localized inflammation recapitulate the clinical presentation of congenital CMV in many infants. This model may therefore be of value in modeling future vaccine and antiviral studies.

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Author Contributions: Dr Park had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Park. Acquisition of data: Park, Gifford, Chase, McGill, and Li. Analysis and interpretation of data: Park, Gifford, Schleiss, Dahlstrom, and Alder. Drafting of the manuscript: Park, Chase, and McGill. Critical revision of the manuscript for important intellectual content: Park, Gifford, Schleiss, Dahlstrom, Li, and Alder. Statistical analysis: Alder. Obtained funding: Park. Administrative, technical, and material support: Park, Gifford, Schleiss, Dahlstrom, Chase, McGill, and Li. Study supervision: Park.

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